Selective Transgene Expression for Detection and Elimination of Contaminating Carcinoma Cells in Hematopoietic Stem Cell Sources

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Abstract

Tumor contamination of bone marrow (BM) and peripheral blood (PB) may affect the outcome of patients receiving high dose chemotherapy with autologous transplantation of hematopoietic stem cell products. In this report, we demonstrate that replication defective adenoviral vectors containing the cytomegalovirus (CMV) or DF3/MUC1 carcinoma-selective promoter can be used to selectively transduce contaminating carcinoma cells. Adenoviral-mediated reporter gene expression in breast cancer cells was five orders of magnitude higher than that found in BM, PB, and CD34$^+$ cells. Our results demonstrate that CD34$^+$ cells have low to undetectable levels of integrins responsible for adenoviral internalization. We show that adenoviral-mediated transduction of a reporter gene can detect one breast cancer cell in 5 $\times$ 10^5 BM or PB cells with a vector containing the DF3/MUC1 promoter. We also show that transduction of the *HSV-tk* gene for selective killing by ganciclovir can be exploited for purging cancer cells from hematopoietic stem cell populations. The selective expression of TK followed by ganciclovir treatment resulted in the elimination of 6-logs of contaminating cancer cells. By contrast, there was little effect on CFU-GM and BFU-E formulation or on long term culture initiating cells. These results indicate that adenoviral vectors with a tumor-selective promoter provide a highly efficient and effective approach for the detection and purging of carcinoma cells in hematopoietic stem cell preparations. (*J. Clin. Invest.* 1996. 98:2539–2548.) Key words: adenovirus • bone marrow • breast cancer • thymidine kinase • gene therapy

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1. *Abbreviations used in this paper:* Ad, adenovirus; β-gal, β-galactosidase; BFU-E, erythroid burst-forming unit; BM, bone marrow; CFU-GM, granulocyte-macrophage colony-forming unit; CMV, cytomegalovirus; FDG, fluorescein di-β-D-galactopyranoside; GCV, ganciclovir; HSV-tk, herpes simplex virus thymidine kinase; LTC-ICs, long-term culture-initiating cells; MOI, multiplicity of infection; PB, peripheral blood; RLU, relative luminescent units; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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Introduction

High dose chemotherapy followed by autologous transplantation of bone marrow (BM)[1] or peripheral blood (PB) as sources of hematopoietic stem cells is being used as a treatment option for patients with breast cancer (1–4). While this approach results in a proportion of patients with prolonged disease-free survival, most patients eventually relapse. One potential explanation for relapse is reinfusion of tumor cells that contaminate the hematopoietic cell preparations (5–7). Immunocytochemistry (5, 8), flow cytometry (8, 9), and PCR analysis (10, 11) have been used to detect contaminating breast cancer cells in BM and PB preparations. Although the significance of breast cancer cell contamination to relapse remains unclear, tumor-free hematopoietic stem cell products for autologous transplantation are nonetheless desirable. In this context, various approaches using mAbs or cytotoxic drugs have been developed for purging of carcinoma cells from BM or PB collections (12–17). These approaches have resulted in the elimination of two to five logs of clonogenic breast cancer cells and varying degrees of toxicity to hematopoietic progenitor and stem cells.

Gene therapy is a potentially novel approach for the purging of carcinoma cells from hematopoietic stem cell preparations. However, efficacy of purging cancer cells will require gene delivery systems which possess a high gene transduction efficiency and target cell specificity. Human adenoviruses are nonenveloped double-stranded DNA viruses which when deleted at the E1 region are replication defective (18). Adenovirus-mediated gene transfer is a highly efficient means of delivering genetic material into a wide spectrum of cells in vitro and in animals. However, in the setting of bone marrow purging, one goal is the selective transduction of exogenous genes into contaminating cancer cells. A potential strategy to achieve such selectivity would be to use a tumor cell specific/selective promoter to direct the expression of a therapeutic gene in the desired target cell. In this context, recent studies have demonstrated that the promoter of the DF3/MUC1 gene can be used to confer selective expression of heterologous genes in breast cancer cells (19, 20). DF3/MUC1 antigen is a member of a family of high molecular weight glycoproteins which are aberrantly overexpressed in breast and other carcinomas (21–23). Adenoviral vectors containing the β-galactosidase or the herpes simplex virus thymidine kinase (*HSV-tk*) gene under control of the DF3 promoter have thus been developed to confer efficient and selective expression of these genes in cancer cells (20).

In the present work, we demonstrate that adenoviral vectors containing the DF3/MUC1 promoter can be used for detection of carcinoma cells in preparations of hematopoietic stem cell sources. The results also demonstrate that selective expression of therapeutic genes in contaminating cancer cells is an efficient approach for purging of hematopoietic stem and progenitor cells.

Methods

Cell lines. The MCF-7, ZR-75-1, BT-20, and SKBR3 breast carcinoma, the A549 lung carcinoma, DU145 prostate carcinoma, SKOV3 ovarian carcinoma, and T98G human glioblastoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown as monolayers in recommended culture medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Human hematopoietic cells. Human PB mononuclear cells were isolated by Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) density gradient centrifugation ($d = 1.077$, 400 g) from leukocyte-enriched leukopaks of healthy donors. Cells were suspended in RPMI-1640 medium containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Bone marrow was obtained from filters used to prepare harvested marrow from normal donors and the mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. Bone marrow stromal cells were isolated by adherence (24).

$CD34^+$ cells were isolated using the Ceprate LC cell separation system (CellPro Inc., Bothell, WA). In brief, BM cells were incubated with a biotinylated mouse anti-$CD34^+$ mAb, washed and then passed through an avidin column. Nonadsorbed cells were removed by washing, and adsorbed cells were eluted from the column. The enriched cells (80–90% $CD34^+$) were maintained in Iscove's MEM containing 12.5% FBS, 12.5% horse serum, and 1 μM hydrocortisone.

Antibody reaction and FACS® analysis. mAbs used were specifically reactive with the cell surface antigens: CD3 (T3, Coulter Immunology, Miami, FL), CD13 (L138, Becton Dickinson, San Jose, CA), CD19 (B4, Coulter Immunology), CD34 (Becton Dickinson), CD51 (integrin αν, clone 1980; Chemicon Inc., Temecula, CA), integrin ανβ3 (LM609, kindly provided by Dr. David Cheresh, Scripps Research Institute, La Jolla, CA) and integrin ανβ5 (25) (clone B5-IA9, generously provided by Dr. Martin E. Hemler, Dana-Farber Cancer Institute, Boston, MA). Cells were incubated with antibody for 30 min on ice. If the antibody was not directly conjugated with FITC or phycoerythrin (PE), a secondary antibody conjugated with FITC or PE (Sigma Chemical Co., St. Louis, MO) was used for indirect fluorescence labeling. Cells were then washed and evaluated by flow cytometric analysis.

Recombinant adenoviruses (Ad). Ad.CMV-βgal, Ad.CMV-tk (20), and Ad.CMV-Luc (kindly provided by Dr. Robert Gerard, University of Texas, Austin, TX) (26) are replication-deficient recombinant adenoviruses in which the luciferase, β-galactosidase, and *HSV-tk* genes, respectively, are under control of the cytomegalovirus (CMV) immediate-early promoter and enhancer. Ad.DF3-βgal and Ad.DF3-tk are recombinant adenoviruses in which the specified genes are under control of the DF3/MUC1 tumor-selective promoter (20, 27). Adenoviral vectors were produced by homologous recombination in the human embryonic kidney cell line 293 as described (28). Large scale production of recombinant adenovirus was accomplished by growth in 293 cells and purification by double cesium gradient ultracentrifugation as described (28). Titers of purified adenovirus were determined by spectrophotometry and by plaque assays.

Adenovirus infection. Cells suspended at 0.5 to 2.0 × 10^6/ml culture medium were infected with adenoviruses at a multiplicity of infection (MOI) of 1 to 1,000 for 2 h, washed, and then resuspended in fresh media. Cells were evaluated for the expression of the transgene at 24 to 48 h after infection.

Assay for luciferase activity. Luciferase activity was measured with D-luciferin (Analytical Luminescence Laboratory, San Diego, CA) using a luminometer. Activity is presented as relative luminescent units (RLU) in an indicated number of cells.

Assays for β-galactosidase. (*i*) Chemiluminescence assay: quantitation of enzyme activity was determined by a chemiluminescence assay using Galacto-Light system (Tropix, Inc., Bedford, MA) that detects 2 fg to 20 ng of β-galactosidase (29). Activity is presented as RLU in an indicated number of cells. (*ii*) Histochemical staining; cells were fixed with 0.5% glutaraldehyde in PBS containing 1 mM $MgCl_2$ for 10 min, rinsed with PBS, and then incubated with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (1 mg/ml), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in PBS for 4 h. (*iii*) FACS®-GAL assay (30): briefly, 0.5–1.0 × 10^6 cells were suspended in 50 μl of serum-free culture medium at 37°C. An equal volume of 2 mM fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Eugene, OR) was added to each aliquot of cells. The cells and FDG were mixed rapidly and incubated for 1 min at 37°C. Thereafter, cells were washed once with 4 ml ice-cold PBS and maintained in ice-cold PBS until analysis.

Tumor cell clonogenic assay. At 24 h after adenovirus infection, ganciclovir (GCV) was added to cells and incubated for 24 h. Serial dilutions of cells were plated on 30-mm culture dishes. Cells were incubated for 2 wk, and colonies (> 50 cells) were stained with crystal violet and counted. Results are expressed as the surviving cell fraction±SEM for the treated groups compared to controls.

Hematopoietic progenitor cell assays. Erythroid burst-forming units (BFU-E) and granulocyte-monocyte colony-forming units (CFU-GM) were assayed in a methylcellulose culture system (Stem Cell Technologies, Vancouver, Canada) containing recombinant human stem cell factor (50 ng/ml), GM-CSF (10 ng/ml), IL-3 (10 ng/ml), and erythropoietin (EPO) (3 U/ml). The numbers of colonies were counted after 2 wk. For more primitive progenitor cells, the number of long-term culture-initiating cells (LTC-ICs) were determined by culturing serial dilutions of $CD34^+$ cells on irradiated bone marrow stromal cells in 96-well plates for 5 wk. The number of wells that contained colonies was then assessed by growth in methylcellulose culture (Stem Cell Technologies) (24). The frequency of LTC-ICs was calculated by plotting the input cell number against the proportion of negative wells as described (24, 31).

PCR analysis. $CD34^+$ cells, CFU-GM, and BFU-E picked from methylcellulose culture were digested at 56°C for 1–2 h with proteinase K (2 mg/ml) in cell lysis buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin (Sigma Chemical Co.), 0.45% NP-40, and 0.45% Tween 20, as described (32). Samples were then heated at 95°C for 5 min. DNA was amplified using the GeneAmp PCR reagent kit (Perkin Elmer/Cetus Corp., Norwalk, CT). The β-actin gene was used as an internal control and amplified using the primers 5′TCACCCACACTGTGCCCAT3′ and 5′GCATTTGCGGTGGACGATG3′. The adenovirus *E1A* gene was amplified using primers 5′ATTACCGAAGAAATGGCCGC3′ and 5′CCCATTTAACACGCCATG3′. The adenovirus *E2B* gene was amplified using primers 5′TCGTTTCTCAGCAGCTGTTG3′ and 5′CATCTGAACTCAAAGCGTGG3′ as described (33).

Statistical analysis. Results are presented as means±SEM. Data comparisons were made by ANOVA. Pairwise comparisons were made using Fisher's PLSD (34) with STATVIEW 4.0 software (Abacus Concepts, Inc., Berkeley, CA).

Results

The efficiency of adenovirus-mediated reporter gene expression was first evaluated in hematopoietic cell preparations using Ad.CMV-Luc and Ad.CMV-βgal. Luciferase and β-galactosidase activities were low but detectable in unfractionated PB and BM mononuclear cells at MOIs of 10 and 100 (Fig. 1 *A*). By contrast, there was little if any detectable reporter gene expression in these cells when using Ad.DF3-βgal (Fig. 1 *A*). Similar studies in MCF-7 breast cancer cells demonstrated a marked increase in efficiency (five orders of magnitude) of Ad.CMV-Luc and Ad.CMV-βgal-mediated reporter gene expression (Fig. 1 *B*). Moreover, as previously demon-

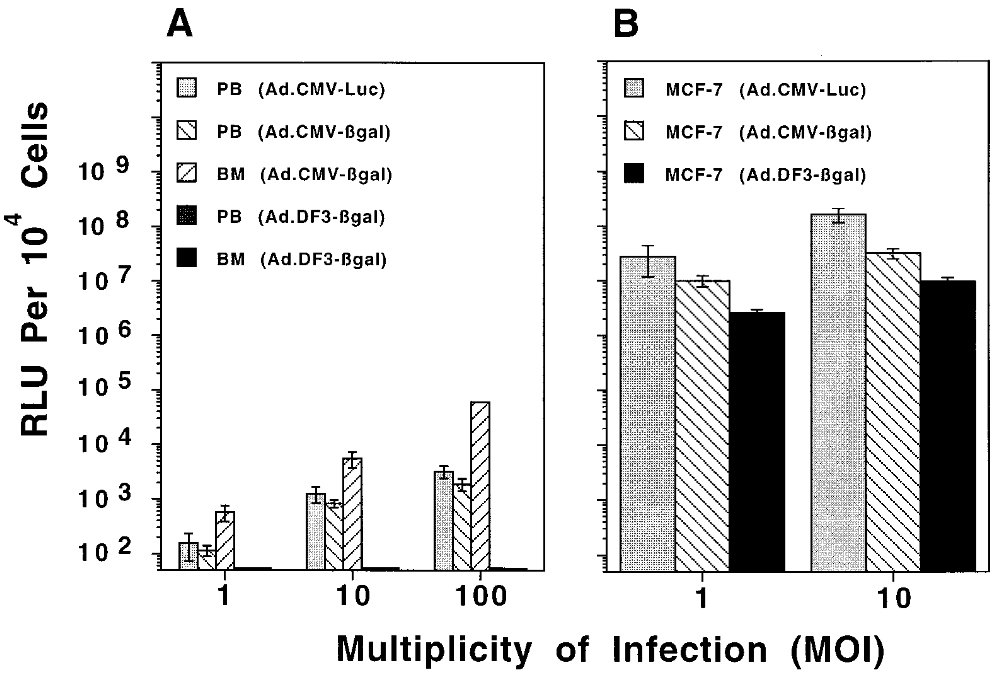
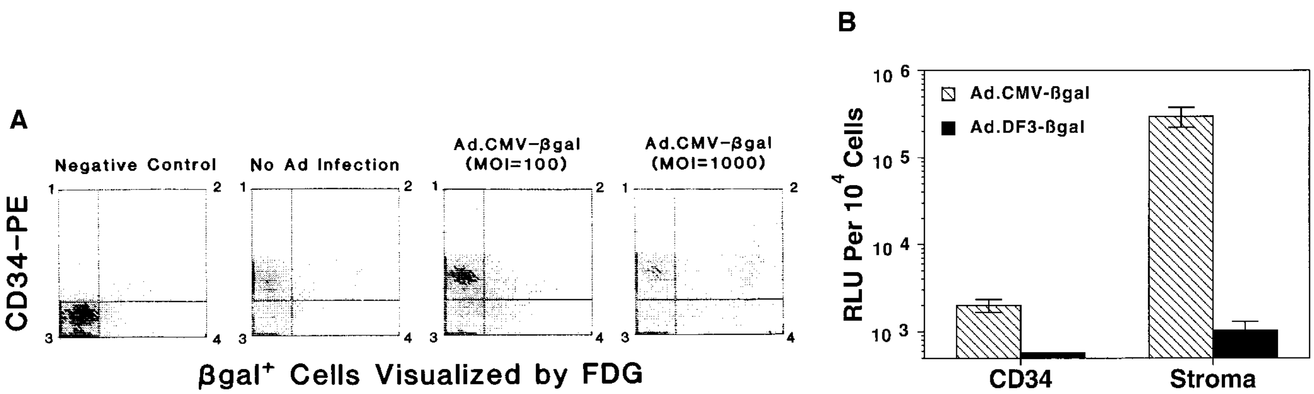


Figure 1. Analysis of adenovirus-mediated reporter gene expression in PB, BM, and breast cancer cells. (*A*) PB and BM mononuclear cells. (*B*) MCF-7 breast cancer cells. Cells were infected with Ad.CMV-Luc, Ad.CMV-βgal, or Ad.DF3-βgal at the indicated MOIs for 2 h at 37°C, washed, and cultured for 48 h. Cells were then lysed and assayed for luciferase or β-galactosidase activity. The results are presented as relative luminescent units (*RLU*) in the indicated number of cells (mean±SEM). Results were obtained from four to nine experiments.

strated (20), β-galactosidase expression was readily detectable in MCF-7 cells transduced with Ad.DF3-βgal (Fig. 1 *B*). These findings indicated that MCF-7 cells are transduced more efficiently than hematopoietic cells by adenoviral vectors and that the tumor-selective DF3/MUC1 promoter can confer even greater selectivity of transgene expression. Two-color FACS®-analysis further indicated that monocytes and macrophages ($CD14^+$ cells) are the major cell types in PB that express β-galactosidase when infected with Ad.CMV-βgal and not with Ad.DF3-βgal, while T ($CD3^+$) and B ($CD19^+$) cells



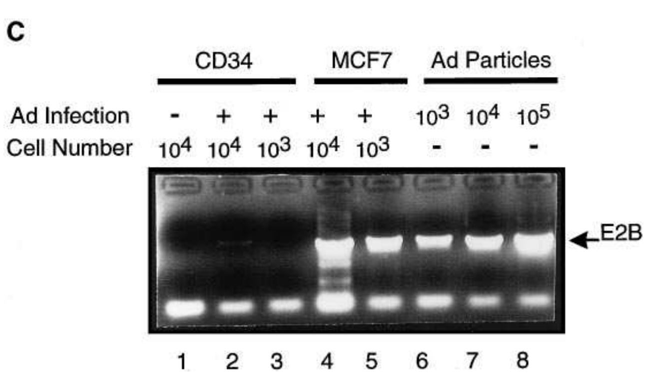


Figure 2. Analysis of adenovirus-mediated gene transfer in $CD34^+$ and bone marrow stromal cells. (*A*) Two-color FACS analysis to detect β-galactosidase activity in enriched $CD34^+$ cells infected with Ad.CMV-βgal or Ad.DF3-βgal. At 24 h after infection, $CD34^+$ cells were stained with PE-conjugated anti-CD34 mAb. Cells expressing β-galactosidase were visualized by the fluorescence substrate, FDG. (*B*) β-galactosidase activity in enriched CD34 cells and bone marrow stromal cells infected with Ad.CMV-βgal or Ad.DF3-βgal. Cells were infected at a multiplicity of infection (*MOI*) of 10 for 2 h at 37°C, washed, and cultured for 48 h. β-galactosidase activity was measured by a luminescence assay. The results are presented as relative luminescent units (*RLU*) in the indicated number of cells (mean±SEM for three samples). (*C*) Determination of adenoviral DNA in adenovirus-infected $CD34^+$ and MCF-7 cells. Cells were infected with adenovirus (MOI = 10) at 37°C for 2 h. Cells were incubated with trypsin/EDTA solution at 37°C for 5 min and washed three times with medium. DNA extracted from 10^4 (lanes *2* and *4*) and 10^3 (lanes *3* and *5*) fluorescence-sorted $CD34^+$ cells or MCF-7 cells infected with adenovirus were used for PCR amplification (25 cycles) of a 0.86 kb sequence in the adenoviral E2B gene. Adenoviral DNA equivalent to 10^3, 10^4, and 10^5 pfu was used as a reference control.

express little if any transgene with either vector (data not shown).

To determine if adenovirus mediates transgene expression in CD34$^+$ hematopoietic stem and progenitor cells, we performed two-color FACS® analysis of enriched CD34$^+$ cells infected with Ad.CMV-βgal. At a MOI of 100, < 4% of CD34$^+$ cells expressed the transgene, while ~ 11% of the CD34$^+$ cells were βgal positive at a MOI of 1,000 (Fig. 2 *A*). These findings indicated that transduction of CD34$^+$ cells is inefficient compared with that for MCF-7 cells. A sensitive chemiluminescent assay showed that there was little β-galactosidase expression when enriched CD34$^+$ cells were transduced with Ad.CMV-βgal (~ 2,000 RLU/10^4 cells at a MOI = 10) and no β-galactosidase expression with Ad.DF3-βgal (Fig. 2 *B*). By contrast to the CD34$^+$ cells, bone marrow stromal cells were transduced efficiently by Ad.CMV-βgal (10^6 RLU/10^4 cells at a MOI = 10) (Fig. 2 *B*). Moreover, the stromal cells exhibited little β-galactosidase expression after transduction with Ad.DF3-βgal (Fig. 2 *B*). To substantiate the relatively low infectability of CD34$^+$ cells by adenovirus, we used PCR to determine the relative copy number of virus per cell after infection. Purified bright CD34$^+$ cells infected with adenovirus were obtained by fluorescence sorting and DNA was extracted for PCR analysis of adenoviral E2B sequences. There was no detectable adenovirus in 10^3 CD34$^+$ cells infected at a MOI of 10, while the E2B signal was readily apparent from 10^3 transduced MCF-7 cells (Fig. 2 *C*). A low level signal was obtained when assaying 10^4 infected CD34$^+$ cells (Fig. 2 *C*). By comparison with an adenovirus standard, we estimate that there are ~ 10 copies of virus per MCF-7 cell and < 0.01 copy per CD34$^+$ cell when cells were infected with adenovirus at a MOI of 10.

Adenovirus infection is a two-step process involving the initial attachment of adenoviral fiber protein to a relatively ubiquitously expressed, but yet unidentified, receptor and then internalization through interaction of the adenoviral penton base with αν integrins, particularly ανβ3 and ανβ5 heterodimers (35, 36). FACS® analysis indicated that CD34$^+$ cells had no detectable αν subunits, ανβ3, or ανβ5 (Table I). By contrast, αν subunits were strongly expressed on breast cancer, lung cancer, prostate cancer, and glioblastoma cells (Table I). The tumor cells expressed ανβ5 at high levels and ανβ3 to a lesser extent (Table I). These results indicated that the low level of adenoviral-mediated transduction in CD34$^+$, as compared to carcinoma, cells is attributable at least in part to the absence of integrins that contribute to adenoviral internalization.

Table I. FACS Analysis of Integrin αν Subunit and ανβ3, ανβ5 Heterodimers in CD34$^+$ Cells and Carcinoma Cells

Cell	Type	αν	ανβ3	ανβ5
CD34$^+$	Hematopoietic progenitor cells	−	−	−
MCF-7	Breast cancer	++++	−	+++
BT-20	Breast cancer	++++	−	++++
ZR-75	Breast cancer	++++	−	+++
SKBR3	Breast cancer	++++	−	+++
A549	Lung cancer	++++	+	+++
DU145	Prostate cancer	++++	++	+++
T98G	Brain glioblastoma	++++	++	+++

Cells were stained with mAbs for αν subunit, ανβ3, and ανβ5 heterodimers as described in Methods. Quantitation was determined as described (25) on the basis of fluorescence intensity. ++++, values above 60; +++, between 30 and 60; ++, between 10 and 30; +, values between 5 and 10; −, values under 5.

Table II. Adenovirus-mediated Reporter Gene Expression in Breast Cancer Cells Premixed with PB Mononuclear Cells

MCF-7 per 10^6 PB	RLU per 10^5 cells (mean±SEM)		
	Ad.CMV-Luc	Ad.CMV-βgal	Ad.DF3-βgal
0	$1.1\pm0.2\times10^4$ (1)	$2.1\pm0.7\times10^3$ (1)	$0.5\pm0.3\times10^2$ (1)
2	$2.4\pm1.7\times10^4$ (2.3)	$3.5\pm0.4\times10^3$ (1.6)	$2.0\pm0.8\times10^2$ (4.0)*
10	$1.0\pm0.4\times10^5$ (10)*	$9.9\pm5.7\times10^3$ (5)*	$1.4\pm0.4\times10^3$ (28)*
10^2	$8.7\pm6.1\times10^5$ (83)‡	$4.9\pm3.4\times10^4$ (23)*	$4.4\pm0.8\times10^3$ (90)‡
10^3	$6.4\pm1.9\times10^4$ (615)‡	$1.4\pm0.5\times10^5$ (172)‡	$1.2\pm0.9\times10^5$ (2,359)‡
10^4	$3.2\pm1.7\times10^7$ (5,947)‡	$5.0\pm4.0\times10^6$ (2,354)‡	$0.6\pm0.3\times10^6$ (11,843)‡

MCF-7 cells were premixed with PB cells at the indicated ratios. The cells were incubated with Ad.CMV-Luc, Ad.CMV-βgal, and Ad.DF3-βgal at a MOI of 10 for 2 h at 37°C. At 48 h after infection, cells were harvested, lysed, and assayed for reporter activities using a luminometer. The reporter activities are presented as RLU per 100,000 cells (mean±SEM) obtained from four experiments. A background value of RLU from the uninfected cells was subtracted. The fold increase of reporter activity relative to tumor-free PB cells (MCF-7 = 0) is in parentheses (*$P \leq 0.05$; ‡$P \leq 0.001$).

The finding that adenovirus preferentially transduces carcinoma, as compared to hematopoietic, cells suggested that adenoviral-mediated reporter gene expression could be used to detect contaminating cancer cells in PB and BM. To address this issue, MCF-7 cells were premixed with PB cells at ratios of 1:10^2 to 2:10^6. Reporter activity of Ad.CMV-Luc- and Ad.CMV-βgal–infected cell mixtures reflected the number of contaminating MCF-7 cells (Table II). The level of luciferase activity mediated by Ad.CMV-Luc infection was significantly increased at a ratio of 10 MCF-7 cell/5 × 10^5 PB mononuclear cells. Higher ratios were associated with increases in reporter gene expression (Table II). Similar results were obtained with Ad.CMV-βgal (Table II). Studies performed with Ad.DF3-βgal demonstrated a lower background with uncontaminated PB mononuclear cells and enhanced sensitivity with detection of one MCF-7 cell/5 × 10^5 PB cells (Table II). Similar results were obtained with Ad.DF3-βgal when ZR-75-1 breast cancer cells were mixed with PB cells (data not shown). Other studies were performed on BM cells that had been contaminated (0.1%) with DF3/MUC1-positive breast, lung, prostate, and ovarian cancer cells. The contaminated BM cells demonstrated a marked elevation in reporter activity when using Ad.DF3-βgal (Fig. 3 *A*). Furthermore, contamination of BM with increasing numbers of MCF-7 cells resulted in higher levels of Ad.DF3-βgal–mediated reporter gene expression, while there was no increase in β-galactosidase expression when the BM cells were contaminated with DF3/MUC1 negative T98G glioblastoma cells (Fig. 3 *B*).

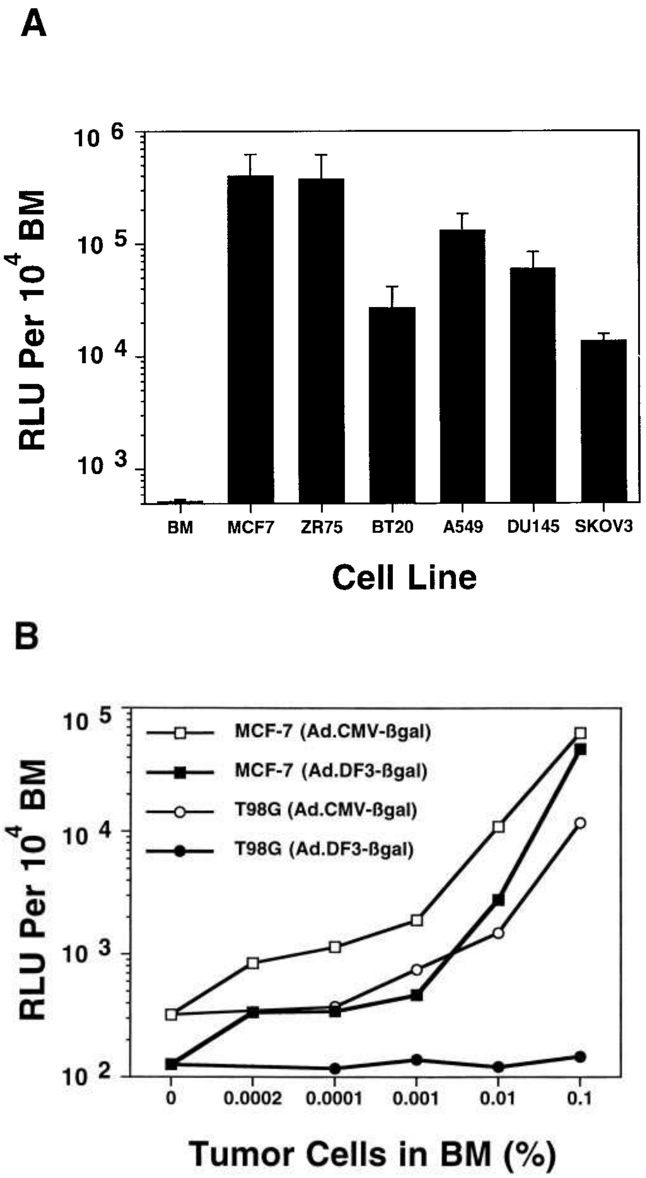


Figure 3. Adenovirus-mediated reporter gene expression in BM contaminated with cancer cells. (*A*) BM mononuclear cells were mixed with 0.1% MCF-7, ZR-75, BT-20 (*breast cancer*), A549 (*lung cancer*), DU145 (*prostate cancer*), and SKOV3 (*ovarian cancer*) cells. The cells were incubated with Ad.DF3-βgal at a MOI of 10 for 2 h at 37°C. After 24 h, cells were lysed and assayed for reporter gene expression by chemiluminescence assay. (*B*) BM mononuclear cells premixed with various ratios of MCF-7 or T98G cells were incubated with either Ad.CMV-βgal or Ad.DF3-βgal at a MOI of 1 for 2 h at 37°C, washed, and cultured for 24 h. β-galactosidase expression was measured by chemiluminescence assay. Similar results were obtained in three separate experiments.

To extend the observation of selective adenoviral-mediated reporter gene expression, we explored other approaches for detection of contaminating carcinoma cells. BM mononuclear cells with and without contaminating MCF-7 cells were infected with Ad.DF3-βgal and then visualized for X-gal staining. Using this approach, the MCF-7 cells could be readily identified by blue staining (Fig. 4, *A* and *B*). The contaminating cells were also readily apparent by fluorescence microscopy after staining with the fluorescence substrate FDG (Fig. 4, *C* and *D*). Cells that expressed β-galactosidase also reacted with mAb DF3 (data not shown), a monoclonal antibody that detects DF3/MUC1 (21). These findings indicated that histochemical, as well as biochemical, approaches can be used for detection of contaminating tumor cells by adenoviral-mediated reporter gene expression.

The selectivity of adenoviral-mediated gene transduction for contaminating tumor cells supported the possibility of using this approach to purge hematopoietic cell populations. Previous studies have documented the strategy of expressing the *HSV-tk* gene for selective killing by GCV (20). To exploit this strategy for purging, adenovirus carrying HSV-tk under control of the CMV or DF3/MUC1 promoters was used to transduce PB cells premixed with tumor cells. As determined by clonogenic survival, infection at an MOI of 10 followed by GCV treatment (10 to 1,000 μM) resulted in the elimination of over 6 logs of contaminating MCF-7 cells. Infection with Ad.DF3-tk at a MOI of 100 and then treatment with 100 μM GCV killed ~ 6 logs of cancer cells (Fig. 5 *A*). In addition, this approach effectively eliminated other contaminating breast, prostate, lung, and glioblastoma tumor cells premixed with BM cells (Fig. 5 *B*).

A potential adverse effect of ex vivo purging is toxicity to hematopoietic progenitor cells. We thus assessed the effects of adenoviral infection and GCV treatment on CFU-GM and BFU-E. Infection with Ad.CMV-tk or Ad.DF3-tk at a MOI of 10 followed by GCV (100 μM) treatment had little effect on CFU-GM and BFU-E as compared with GCV alone (≤ 10% cytotoxicity). Adenovirus infection alone at a MOI of 10 had little if any effect on colony formation (Table III). At a MOI of 100, there was a 17–19% decrease in BFU-E and CFU-GM when Ad.CMV-tk and GCV were used, while there was less of an effect with Ad.DF3-tk and GCV (Table III). Limiting dilution assays were also performed on enriched CD34$^+$ cells to assess the effects of adenovirus and GCV treatment on LTC-ICs. The results demonstrate that infection with Ad.CMV-tk with or without GCV treatment has little if any effect on the regeneration and differentiation of the primitive progenitor cells

Table III. Progenitor Cell Growth of CD34$^+$ Cells Treated with Adenovirus and GCV

Treatment	BFU-E	CFU-GM
Untreated	100	100
+GCV	91±2.8*	90±2.5*
+Ad.DF3-tk (MOI=10)	102±4.1	100±3.2
+Ad.DF3-tk (MOI=10) + GCV	91±3.9	91±2.8*
+Ad.DF3-tk (MOI=100) + GCV	90±3.3*	90±3.0*
+Ad.CMV-tk (MOI=10)	100±3.2	100±3.1
+Ad.CMV-tk (MOI=10) + GCV	90±3.0*	89±3.0*
+Ad.CMV-tk (MOI=100) + GCV	83±3.9‡	81±4.0‡§

Enriched CD34$^+$ cells were treated with the indicated adenovirus for 24 h at 37°C, washed, and cultured for 24 h. GCV (100 μM) was then added for 24 h. The cells were washed and then cultured in methylcellulose for 2 wk. The number of colonies in the treated groups is expressed as the percentage (mean±SEM from four experiments) of that for untreated controls (21.8±7.3 BFU-E and 26±5.9 CFU-GM per 1,000 CD34$^+$ cells). *$P \leq 0.05$, ‡$P \leq 0.001$ vs untreated control; §$P \leq 0.05$ vs GCV alone.

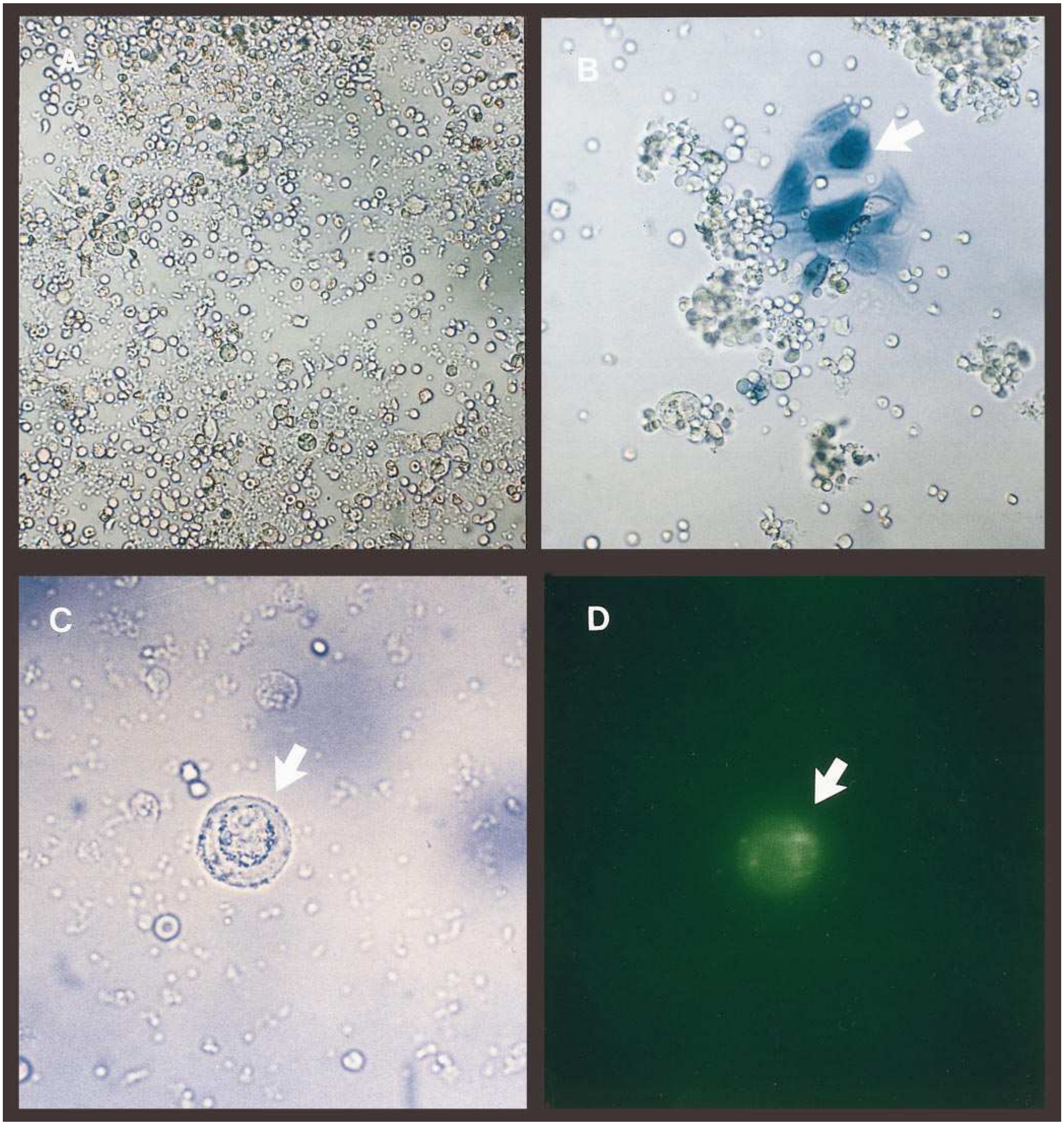


Figure 4. Detection of contaminating breast cancer cells in BM by Ad.DF3-βgal. Cells were incubated with Ad.DF3-βgal (MOI = 10) for 2 h at 37°C, washed, cultured for 24 h, fixed and then stained with X-gal. (*A*) BM mononuclear cells without MCF-7 cells. (*B*) BM mononuclear cells containing 0.1% MCF-7 cells. Magnification is 400. Cells were also incubated with FDG and observed under a fluorescent microscope. (*C*) Bright field. (*D*) Dark field. Magnification is 1000. Arrows indicate breast cancer cells.

(Fig. 6). Additional experiments were performed to determine if adenovirus is detectable in the progeny cells after adenoviral purging of progenitor cells. CFU-GMs and BFU-Es were picked from methycellulose and cultured with 293 cells. No live adenovirus was rescued in three separate experiments. Reverse transcription PCR analysis of CFU-GM and BFU-E colonies failed to detect any transgene expression mediated by recombinant adenovirus (data not shown). Importantly, the finding that PCR analysis did not detect the presence of adenoviral E1a sequences indicated no wild-type adenovirus replication.

Discussion

A major issue for autologous BM or PB transplantation in breast cancer patients is the potential risk of collecting and re-

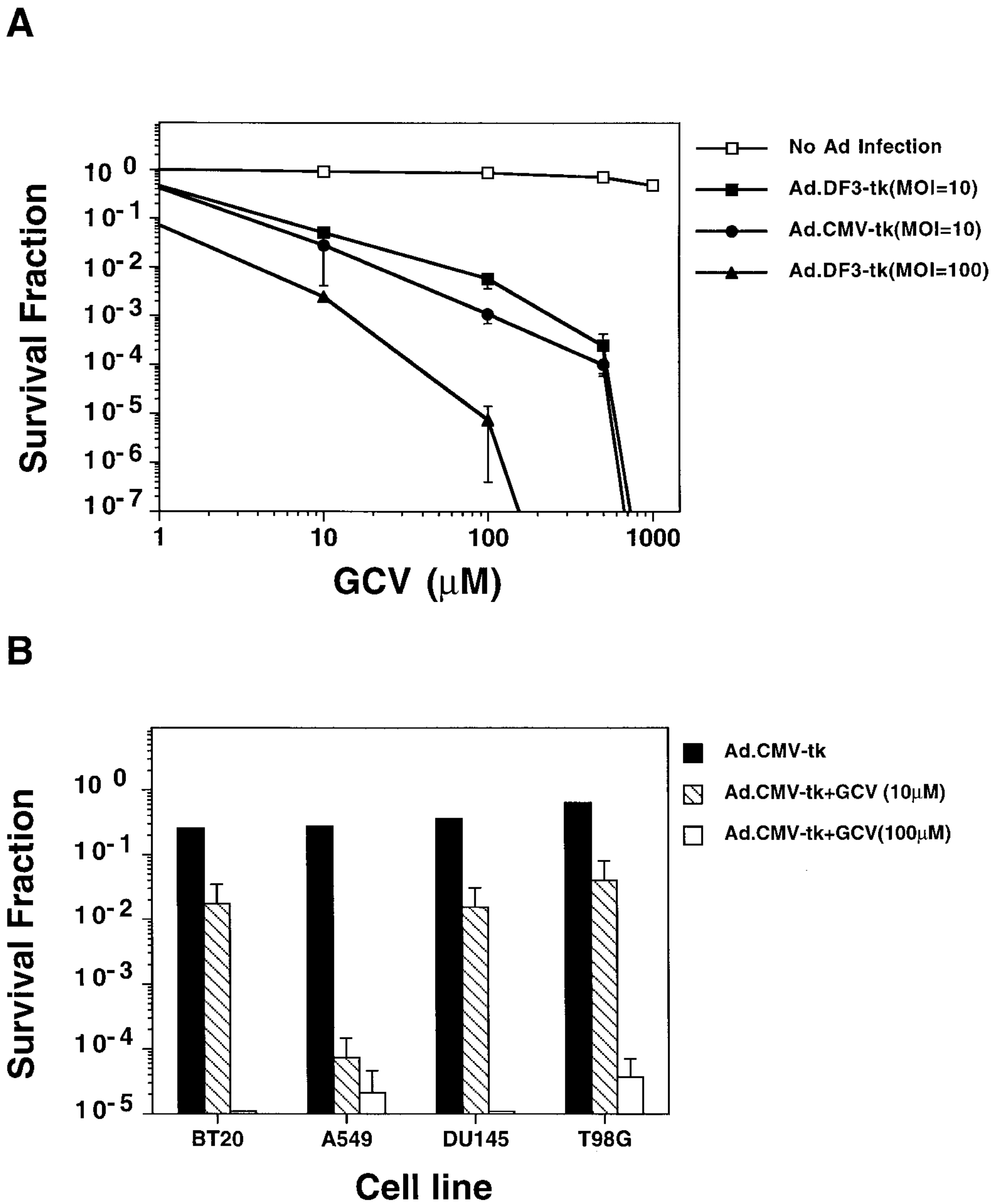


Figure 5. Effects of Ad.CMV-tk and Ad.DF3-tk infection followed by GCV treatment on the survival of cancer cells premixed in PB or BM mononuclear cells. Cancer cells were premixed with 10-fold excess of irradiated PB or BM cells. The cells were then incubated with recombinant adenoviruses at 37°C for 2 h. At 24 h after infection, cells were treated with GCV at the indicated concentrations for 24 h, and were then replated on 30-mm plates in duplicate at serial dilutions ranging from 500 to 10^6 cells per well. 2 wk later, the number of colonies (> 50 cells) was assessed by crystal violet staining. (*A*) Clonogenic assay for MCF-7 breast cancer cells premixed in PB and infected with Ad.CMV-tk or Ad.DF3-tk at the indicated MOIs followed by GCV treatment. The results are expressed as survival fraction, i.e., colony numbers in plates treated with adenovirus and/or GCV as a fraction of that for untreated controls (mean±SEM for two to four experiments). (*B*) Clonogenic assay for carcinoma cells premixed in BM treated with Ad.CMV-tk at a MOI of 10 and GCV.

infusing tumor cells. In this context, a study using histochemical detection has demonstrated BM involvement in 50% of patients with localized breast cancers and both BM (70%) and PB (22.5%) involvement in patients with stage IV disease (5). Gene transfer may provide one strategy for improving the detection and purging of tumor cells in BM or PB preparations. However, the presently available gene delivery systems generally lack target cell specificity. Ligand-DNA complexes, DNA-liposome complexes, and direct transfer of DNA are limited by a low efficiency of gene transduction (37–40). Moreover, the use of retroviral vectors for detection or purging of cancer cells in hematopoietic stem cell preparations could be limited by dependence on replication of the target cell. By contrast, replication-defective adenoviral vectors represent a highly efficient approach for in vitro gene transfer. One potential limitation of this vector system could be transduction of reporter or therapeutic genes into hematopoietic as well as tumor cells. However, the present studies demonstrate that adenovirus is markedly inefficient in the transduction of BM and PB, as compared with carcinoma, cells. Importantly, transduction of purified $CD34^+$ hematopoietic stem and progenitor cells is also inefficient compared with that of cancer cells. Another study has recently reported similar results in BM and $CD34^+$ cell preparations (41). Our results further indicate that the $CD34^+$ cell populations express low to undetectable levels of the $\alpha\nu\beta3$ and $\alpha\nu\beta5$ integrins. Internalization of adenovirus requires interaction of the adenoviral penton base with $\alpha\nu$ integrins (35, 36). Consequently, the absence of detectable $\alpha\nu$ integrin subunits on $CD34^+$ cells and their high level expression on diverse cancer cells provides a mechanistic explanation for the selectivity of transduction.

The finding that adenoviral-mediated gene transduction is inefficient in BM and PB cell preparations compared to carcinoma cells supported the potential for using this approach to detect contaminating tumor cells. Transduction of the luciferase or β-galactosidase genes demonstrated a correlation

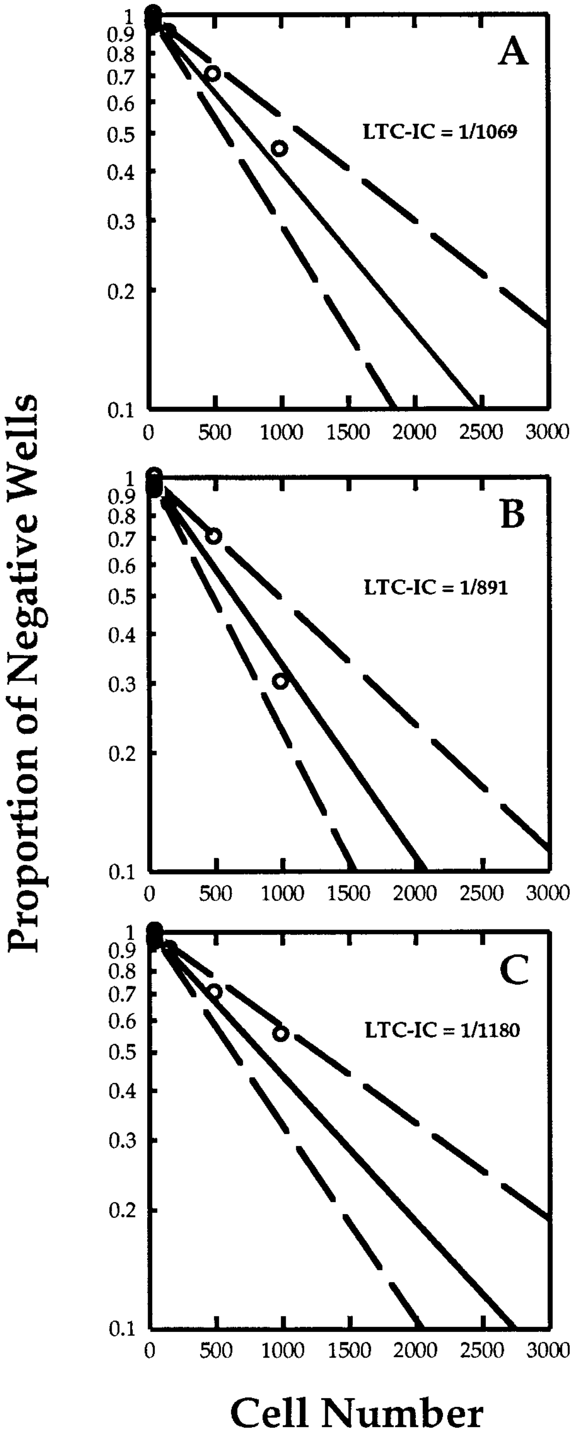


Figure 6. Limiting dilution analysis of LTC-ICs for primitive progenitor cells. Enriched CD34+ cells were treated as described in Table III, and seeded onto irradiated marrow feeders in 96-well plates at concentrations of 55 to 4,000 cells/well and 20 wells per concentration. The number of clonogenic wells was assessed after 5 wk of suspension culture and 2 wk of growth in methylcellulose. The frequency of LTC-ICs was calculated by plotting the input cell number against the proportion of negative wells. The best linear fit and standard errors were determined from the data. (*A*) Untreated control. (*B*) Ad.CMV-tk infection (MOI = 10). (*C*) Ad.CMV-tk (MOI = 10) and GCV (100 μM) treatment. Similar results were obtained in two independent experiments.

between reporter expression and the number of contaminating cells. Expression of the reporter resulted in the detection of one cancer cell in 5×10^5 BM or PB cells. By contrast, an immunohistochemical method using antibodies against cytokeratin detects one tumor cell in 4×10^5 BM cells (5). Moreover, we found that Ad.DF3-βgal transduction can be adapted for histochemical detection and thereby morphological examination by staining with X-gal or FDG. Furthermore, the use of the tumor-selective promoter resulted in lower backgrounds with uncontaminated hematopoietic cell preparations. We previously demonstrated that use of the DF3 promoter in adenoviral vectors provides an efficient and selective approach to target expression of heterologous genes in breast cancer cells (20). There are presently several other tumor-specific or selective promoter sequences that have been used to confer selective expression of heterologous genes in tumor cells (42–45). The present results suggest that use of a tumor-selective promoter in the context of an adenoviral vector can provide a highly sensitive approach for the detection of cancer cells in hematopoietic cell preparations. Studies will now be needed that directly compare the sensitivity of the present approach with other techniques used for detection of contaminating carcinoma cells.

The differential sensitivity of hematopoietic as compared to carcinoma cell transduction by adenoviral vectors further supported the use of this approach to purge contaminating tumor cells. Previous studies have demonstrated that purging BM preparations with 4-hydroperoxycyclophosphamide (4-HC) can lead to 2–3 logs of tumor cell depletion (12, 17). The use of immunomagnetic separation in combination with 4-HC eliminated up to 5 logs of tumor cells (12). However, this approach significantly reduced the recovery of CFU-GM (12). mAbs linked with toxin proteins have also been used for in vitro purging of bone marrow. mAb DF3 linked to ricin resulted in the elimination of 2–3 logs of breast tumor cells (13). However, this approach also resulted in the reduction of CFU-GM formation. The present studies demonstrate that adenoviral mediated gene transduction using Ad.DF3-tk and GCV treatment results in the elimination of 6 logs of contaminating breast cancer cells. Importantly, there was little effect of Ad.DF3-tk transduction and GCV treatment on recovery of CFU-GM and BFU-E. Moreover, the adenovirus-mediated transduction of thymidine kinase to confer GCV sensitivity had little effect on LTC-ICs of enriched CD34+ cells. Since completion of the present studies, another report has demonstrated that adenoviral vectors expressing wild type p53 can be used to purge breast cancer cells mixed with normal bone marrow (41). Other studies have demonstrated that adenoviruses can be used to selectively transduce cancer cells with genes that induce apoptosis (46) and to increase transduction of plasmid vectors coding for toxin genes (41). Thus, combining several adenoviral-mediated strategies could be useful in increasing the efficacy of purging contaminating cancer cells in hematopoietic cell preparations.

Finally, the present results suggest that adenoviral-mediated gene transduction could be useful for the detection and elimination of diverse carcinomas contaminating bone marrow and peripheral blood collections. In addition to studies with breast cancer cells, adenoviral-mediated transduction was highly efficient for cells derived from lung, prostate, and ovarian carcinomas. As the DF3/MUC1 antigen is overexpressed in breast, lung, prostate, and ovarian cancers (21–23), adenoviral vectors containing the DF3/MUC1 promoter could be used in these settings to further increase selectivity of gene transduction. Alternatively, other tumor-selective DNA regulatory elements can be used in a similar context. The present results support the use of replication defective adenoviral vectors with the DF3/MUC1 promoter for purging hematopoietic cell preparations in the clinical setting.

Acknowledgments

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