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PRINCIPAL INVESTIGATOR: Dr. Michael F. Clarke

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                           Ann Arbor, Michigan 48109

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Dr. Michael F. Clarke

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Breast cancer is the second leading cause of cancer death among American women, with over 170,000 new cases and 50,000 deaths each year. Despite advances in detection and treatment, mortality from these diseases remains high. Traditional modes of treatment including radiation therapy, chemotherapy, and hormonal therapy have been useful but are limited by the emergence of treatment-resistant cancer cells. Clearly new approaches are needed to treat these diseases. This project is designed to develop novel approaches to detect breast cancer cells that contaminate peripheral blood and bone marrow, and to remove such contaminating cells. An RT-PCR assay has been developed to detect breast cancer cells, and a novel gene therapy vector has been developed to kill contaminating cancer cells. Blood and bone marrow samples obtained from patients with breast cancer are being collected. These samples will be analyzed to determine whether the K19 RT-PCR assay can be used to predict outcome. Next, a gene therapy vector, the bcl-xs adenovirus, has been developed. This vector has promise as a therapeutic agent for the treatment of breast cancer. With additional support from the National Cancer Institute, this virus is undergoing toxicology testing in order to obtain FDA approval for human clinical trials for the treatment of breast cancer.

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Michael J. Clarke 2/3/99
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PI - Signature   Date

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INTRODUCTION

Breast cancer is the second leading cause of cancer death among American women, with over 170,000 new cases and 50,000 deaths each year. Despite advances in detection and treatment, mortality from these diseases remains high. Traditional modes of treatment including radiation therapy, chemotherapy, and hormonal therapy have been useful but are limited by the emergence of treatment-resistant cancer cells. Clearly new approaches are needed to treat these diseases.

One of the more promising approaches for the treatment of metastatic breast cancer is high dose chemotherapy. Breast cancer is susceptible to chemotherapy in a dose dependent manner. The major dose limiting toxicity of many effective chemotherapeutic agents is hematopoietic toxicity. To overcome this obstacle, autologous bone marrow or peripheral blood stem cells are harvested from the patient prior to the administration of the high dose chemotherapy, and then reinfused after the chemotherapy has been excreted and/or metabolized. Although the initial clinical trials of such strategies are promising, there are other obstacles that need to be overcome to optimize results. The agents used for the systemic treatment of this disease need to be improved. Additionally, bone marrow transplantation for solid tumors such as breast cancer is complicated by the fact that these cancers frequently metastasize to the bone marrow.

To date, two significant advances have resulted from this proposal. A sensitive assay has been developed to identify breast cancer cells in the bone marrow and the peripheral blood (Appendix, Manuscript #1). Next, and adenovirus vector has been developed that is selectively lethal to breast cancer cells, but not normal cells. The results of preclinical studies with this virus have been successful. The National Cancer Institute (NCI) has decided to hold an IND for this virus, and toxicology testing is underway in order to obtain FDA approval for clinical trials. This virus should enter clinical trials for the treatment of breast cancer sometime in the next two years.

BACKGROUND

The use of autologous bone marrow transplant (BMT) as a part of cancer treatment has increased dramatically in recent years. For example, autologous BMT often is used to protect patients from the hematopoietic toxicity of high dose chemotherapy in the treatment of breast tumors [1,2]. However, the success rate for this treatment regimen is jeopardized by contamination of the autologous marrow with low numbers of tumor cells [3-6]. Thus, the ability to selectively kill, or purge contaminating tumor cells in the marrow *in vitro* prior to autologous transplant could significantly improve the chances of long term survival.

The most common technique of purging tumor cells from bone marrow relies upon monoclonal "anti-tumor" antibodies to immunologically remove tumor cells from a suspension of marrow cells [7-9]. However, the success of this approach depends on the availability of well characterized antibodies capable of detecting each tumor cell phenotype. This method of purging rarely removes all tumor cells, and requires repeated purging cycles which can decrease hematopoietic cell viability [10]. Moreover, the specific antigens are rarely "tumor cell specific", often being found on normal cells, albeit at low antigen density. Other purging methods, such as photo-sensitization agents or chemotherapy, also can cause significant hematopoietic toxicity [1,11,12].

Until recently, investigators thought that cancer treatments based on chemotherapy, or radiation therapy, exerted their tumor-killing specificities based on the

different sensitivities of rapidly dividing (versus quiescent) cells to these agents. Recent evidence suggests that radiation and many chemotherapy agents specifically kill tumor cells, while sparing normal cells, by triggering of the Programmed Cell Death (PCD) pathway [13-15]. The induction of PCD is impeded by genes such as *bcl-2*, and its presence in cancer cells thus reduces the efficiency of conventional cancer therapy. Mechanistically, *bcl-2* does not appear to be mitogenic or transforming, but it cooperates with c-myc, and members of the ras family, to cause transformation [16-18]. Additionally, *bcl-2* acts to inhibit apoptosis induced by p53, *myc*, chemotherapy, and ionizing radiation [19-23].

Members of this gene family have been implicated in the progression of a large number of human solid tumors, including lymphomas, cancers of the breast, lung, and prostate, as well as neuroblastoma [24]. These observations suggest that suppression of *bcl-2* expression using gene therapy methods would be a valuable tool in cancer treatment, by increasing the susceptibility of tumor cells to existing chemotherapeutic and radiation treatments.

Other members of the *bcl-2* gene family have recently been isolated and partially characterized. A *bcl-2* homologue, *bcl-x*, gives rise to two mRNA species through alternative splicing. One of these, $bcl-x_L$, functions in a manner similar to *bcl-2*, and inhibits apoptosis. The other, $bcl-x_S$, functions as a repressor to *bcl-2* and acts to promote apoptosis. We recently demonstrated that adenoviral-mediated overexpression of $bcl-x_S$ inhibited the anti-apoptotic role of *bcl-2* and induced PCD in a variety of primary tumors and tumor cell lines [25]. This PCD was augmented by, but not dependent upon, the tumor suppresser p53. In contrast, normal bone marrow hematopoietic stem cells resisted $bcl-x_S$-adenovirus induced PCD apoptosis. We postulated that the resistance of hematopoietic stem cells to the $bcl-x_S$ adenovirus was due to the lack of expression of adenovirus transgenes in hematopoietic stem cells or alternatively to the inability of $bcl-x_S$ to induce cell death in these cells. We report that murine hematopoietic stem cells resist expression of an adenovirally transduced gene. Moreover, a $bcl-x_S$ adenovirus works synergistically with a Herpes virus TK adenovirus to specifically purge tumor cells from *in vitro* hematopoietic cultures, with the preservation of transplantable stem cells. Thus, these results indicate that adenovirus vectors fail to transduce genes into early hematopoietic stem cells. Therefore, this suggests that adenovirus vectors encoding suicide genes such as $bcl-x_S$ or Herpes virus TK would preferentially kill the contaminating tumor cells derived from epithelial tissues found in bone marrow cell populations, thus serving as an excellent means of marrow purging.

Significant progress in the purging of breast cancer cells has been made. We have further defined the use of adenovirus suicide vectors for killing breast cancer cells that contaminate the bone marrow of patients with breast cancer. Last year, we found that a pure population of mouse hematopoietic stem cells are not transduced by an adenovirus vector. We have extended this observation and now show that human hematopoietic stem cells are also not transduced by such vectors. The National Cancer Institute has now decided to do toxicology testing of the *bcl-xs* adenovirus to obtain FDA approval for use of the virus in human clinical trials. We envision such trials to begin within the next two years.

BODY
Significant progress in completion of the goals of this grant has been made.

MATERIALS AND METHODS

Primary Bone Marrow Cells. Human bone marrow cells were obtained from the posterior iliac crest of normal volunteers following informed consent using a protocol approved by the University of Michigan Human Institutional Review Board. Bone marrow mononuclear cells were separated by density gradient centrifugation on Ficoll-Paque (1.077 g/mL; Pharmacia). Cells were collected from the interface and washed three times in Iscove's modified Dulbecco's medium (IMDM; Gibco). Cells were then counted and divided for appropriate infection conditions. To isolate murine stem cells, bone marrow was harvested from the femur and tibia of c57black/Ka mice congenic for Thy 1.1. Bone marrow cells were stained with anti-Thy 1.1, anti-Sca-1, anti-Kit, and an anti-Lin cocktail consisting of anti-FcgII/FcgIIb, anti-Ly5.2, anti-CD3, anti-CD4, Anti-CD5, anti-CD8, anti-erythrocyte-specific antigen, anti-B220, anti-Gr-1, and anti-Mac-1[26]. Hematopoietic stem cells were isolated by FACS sorting twice as previously described (26). Reanalysis of the cells revealed greater than 95% of the sorted cells were Thy 1. 1^{lo}, Sca^{hi}, Kit^{hi}, Lin^{-}.

Adenoviral vectors. The *bcl-x$_S$* adenoviral vector, pAdRSV-bcl-x$_S$, was constructed by cloning a full length *bcl-x$_S$* cDNA into the pAdRSV vector [25]. This vector contains an RSV promoter and SV40 polyadenylation signal and allows high level expression of inserted sequences. Replication deficient virus was produced in the permissive human kidney 239 cell line containing complementary sub 360 sequences. Vectors were similarly constructed containing cDNA for thymidine kinase (TK)(pAdRSV-TK) or LacZ (pAdRSV-LacZ). Adenovirus infection and β-galactosidase assays were done essentially as previously described [25].

Hematopoietic Progenitor Cell Assays. Infection and viability assays were performed with adenoviral vectors as previously described [25]. For purging experiments, each infection condition utilized 1 x 10^6 hematopoietic cells admixed with 1.5 x 10^4 MCF-7 breast tumor cells that were stably expressing the G418 resistance gene. These cell mixtures were infected for four hours at a range of multiplicity of infection (MOI) from 2,000 to 10,000 viruses/cell in serum-free medium containing 1 ng/mL c-kit ligand and 10 ng/mL IL-3 (R&D, Minneapolis, MN). Following infection, cells were washed of virus and cultured for 48 hours in DMEM media containing 10% fetal calf serum, 10% horse serum, 0.1 U/mL Epo, 2 ng/mL IL-3, 5 ng/mL GM-CSF, and 10 ng/mL c-kit ligand. Cells were harvested and hematopoietic progenitor assays using 1 x 10^4 cells per assay were performed in triplicate as previously described [25]. Adenoviral infection of MCF-7 cells was assayed by determining colony development in the presence of 1 mg/mL geneticin (BRL/Gibco, Grand Island, NY) to kill normal hematopoietic cells. After two weeks, developing colonies were stained, scored, and photographed as previously described [25].

Murine Bone Marrow Transplants. All mice (C57bl/6, approximately 25 g) were purchased from Charles River (Wilmington, MA), and were used one week after arrival. Marrow cells for transplantation were obtained from the femora of male mice. Donor bone marrow cells from 4 male mice were pooled to yield a total of ~200 x 10^6 unfractionated marrow cells, which was then equally divided into the following groups

for treatment with adenoviral vectors containing TK, Bcl-x$_S$, LacZ, or mock (identical treatment of marrow with adenoviral free reagents), as well as a group treated with TK containing vector followed by gancyclovir. Treated cells were transplanted into recipient female mice (four per condition) irradiated with two doses of γ-irradiation (6 and 5 Gray delivered at 0.134 Gray/min) separated by three hours in order to decrease gastrointestinal tract toxicity. Bone marrow cells were transplanted by injections into either the tail vein or the retro-orbital sinus of anethestised mice. Survival of the recipients was monitored for up to six months to determine long-term reconstitution.

Mouse Y-specific Sequence PCR Detection. Engraftment of transplanted male marrow cells was detected by PCR amplification of male-specific Y chromosome sequences in female recipients. Mouse Y specific PCR primers were synthesized with the sequences: Primer 1- 5' CAGTACCAGTCAGCAATATTTGTTG and Primer 2- 5' TTTCTGTATGCATTGTTTTGTGAGT. DNA was extracted from bone marrow of recipient mice using a previously described method [27], and used as a template in the following PCR conditions: 200 mM Tris-HCl pH 8.8, 250 mM KCl, 35 mM MgCl$_2$, 200 nM each dNTPs, 250 nM each primer, 1μg template DNA, 2.5 U *Taq* Polymerase (Gibco/BRL, Grand Island, NY). Cycling parameters were 94°, 1 min; 55°, 1 min; 72°, 2 min; 25 cycles. The expected amplification product size is 316 bp. Reaction products were analyzed on a 1.5% agarose/TBE gel.

Detection of keratin 19 in blood and bone marrow.

Cell Lines, Patients, and Tissue Samples

Human mammary carcinoma cell lines T47D, MCF7 (estrogen receptor-positive), and SK3B3 (estrogen receptor-negative) were used in development of the RT-PCR assay. All three cell lines are positive for K19 by immunohistochemical staining. In addition, two mammary epithelial cell lines established from normal breast tissue obtained from women who underwent reduction mammoplasty were evaluated. To determine the sensitivity of the assay, peripheral-blood mononuclear (PBMN) cells obtained from a normal donor were mixed with decreasing numbers of T47D cells. A total of 1 million cells were present in each sample. Cells were mixed before the RNA preparation thus mimicking the clinical setting for detection of mammary cells in the peripheral blood or bone marrow of patients.

The study population consisted of 34 patients with a histologic diagnosis of breast cancer in different stages, monitored at either the University of Michigan medical Center, Ann Arbor, MI, or at the Medical College of Wisconsin, Milwaukee, WI (Table 1). After approval by the institutional review board, and following informed consent, 2- to 5-mL samples of peripheral-blood or bone marrow aspirate were obtained from patients at various time points during treatment and processed as described later. Samples obtained from the peripheral blood of 10 non-breast cancer patients and bone marrow aspirates from 14 health, BMT donors and 15 patients following allogeneic BMT for chronic myelogenous leukemia (CML) served as negative controls. In addition, four patients with stage IV breast cancer underwent apheresis to obtain peripheral-blood stem cells before autologous BMT. Viably frozen aliquots of these cells were rapidly thawed, washed in phosphate-buffered saline (PBS), and RNA extracted as outlined later. Finally, 3 mL of CSWF was obtained from one patient with breast cancer who had carcinomatous meningitis documented by cytology. And from two additional patients without breast cancer.

Task 1. To test the hypothesis that women with poor prognostic indicators are more likely to present with the presence of blood and/or bone marrow micrometastases is ongoing with sample collections.

-1A. Sample collection and PCR assays. To date, we have collected blood samples from 123 patients including 20 patients with stage I or II cancer, nine with stage III , and 123 with stage IV breast cancer.

-1B. Clinical followup. Followup of the outcome ot the stage I, II and III prognosis patients is being motitored.

Task 2. To use a PCR based assay to detect the mammary cell specific keratin-19 mRNA and evaluate the presence of occult breast cancer cells in patients undergoing BMT.

PURPOSE: Detection of occult carcinoma in patients with breast cancer may aid the establishment of prognosis and development of new therapeutic approaches. To improve on existing methods of detection, we have developed a reverse-transcriptase polymerase chain reaction (RT-PCR) assay for keratin 19 (K19) transcripts to identify mammary carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer. **PATIENTS AND METHODS:** Peripheral-blood or bone marrow samples obtained from 34 patients with stages I to IV breast cancer and 39 control subjects without breast cancer were screened for K19 mRNA by nested primer PCR. **RESULTS:** In reconstitution experiments, K19 RT-PCR reliably detected 10 mammary carcinoma cells in 1 million normal peripheral-blood mononuclear (PBMN) cells. Four of 19 patients with stage IV breast cancer had detectable K19 transcript in peripheral blood. Five of six patients with histologically negative bone marrow biopsies following preablative chemotherapy and before autologous bone marrow transplant (BMT) were positive by this assay. Stem-cell apheresis harvests obtained from one of these patients and three additional patients immediately before BMT were all K19-negative. K19 RT-PCR analysis of CSF from a breast cancer patient with known carcinomatous meningitis was also positive. Thirty-eight of 39 non-breast cancer patients had negative K19 RT-PCR assays. The one exception was a patient with chronic myelogenous leukemia. **CONCLUSION:** RT-PCR of K19 is a sensitive, specific, and rapid method for detection of occult mammary carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer. The presence of residual breast cancer cells in histologically normal bone marrow aspirates but not in stem-cell apheresis harvest is a frequent finding. This assay may be useful in diagnosing metastatic disease, as well as in monitoring the effectiveness of systemic therapy.

Two manuscripts have been published that address this task (see appendix).

-1A. To determine the relative frequency of tumor contamination of marrow versus peripheral blood stem cell harvests. To date, we have collected samples from 59 patients that have undergone BMT. Forty seven samples are from peripheral blood stem cell harvests, and 13 are from bone marrow harvests (one patient had both aphoresis and bone marrow). To date, 20% of the peripheral blood samples have been positive, and 46% of the bone marrow samples have been positive. At the time this grant was written, bone marrow harvest was the source of the hematopoietic cells used for rescue from high dose

chemotherapy. Now, peripheral blood stem cell harvests are used exclusively for rescue from high dose chemotherapy. Since patients routinely get a diagnostic bone marrow prior to high dose chemotherapy, we have amended our protocol in order to analyze peripheral blood, the diagnostic bone marrow specimen, and the aphoresis product for K19.

-1B. Correlation of PCR results with clinical outcome. The positive rate in peripheral blood stem cell harvests is lower than that seen in bone marrow. Since peripheral blood stem cell harvests is used, only about 20% of the patients are K19 positive. Only about 20% of such samples are PCR postive for cancer. Therefore, the sample size is still too small to make any clinical correlations at this time.

-1C. Evaluate the efficacy of BM and stem cell culture purging techniques to elimainate breast cancer cells.

We have published a manuscript describes a novel method for purging contaminating cancer cells from bone marrow hematopoietic stem cells. Many cancers overexpress a member of the *bcl-2* family of inhibitors of apoptosis. To determine the role of these proteins in maintaining cancer cell viability, an adenovirus vector that expresses *bcl-x$_S$*, a functional inhibitor of these proteins, was constructed. Even in the absence of an exogenous apoptotic signal such as x-irradiation, this virus specifically and efficiently kills carcinoma cells arising from multiple organs including breast, colon, stomach, and neuroblasts. In contrast, normal hematopoietic progenitor cells and primitive cells capable of repopulating immune-deficient SCID mice were refractory to killing by the *bcl-x$_S$* adenovirus. This vector may prove useful in killing cancer cells contaminating the bone marrow of patients undergoing autologous bone marrow transplantation. (see manuscript in appendix).

We have previously shown that an adenovirus expressing *bcl-x$_S$* can be used to selectively kill cancer cells that contaminate bone marrow (25). To better understand this observation, we determined whether murine stem cells express a transgene when infected with an adenovirus vector. To do this, Thy 1. 1^{lo}, Sca^{hi}, Kit^{hi}, Lin$^-$ cells, which are the murine long term repopulating hematopoietic stem cell, were isolated from the bone marrow of c57black/KA mice by four-color FACS [26]. Either 1 x 10^3 hematopoietic stem cells or control neuroblastoma cells were exposed to 2 x 10^3-1 x10^4 β-galactosidase adenoviruses/cell. As previously reported [25], neuroblastoma cells infected with even the lowest titer of virus expressed β-galactosidase. In contrast, hematopoietic stem cells infected with even 1 x10^4 viruses/cell did not express β-galactosidase. These data suggest that adenovirus vectors based on the RSV Ad5 system do not transduce hematopoietic stem cells and that such viruses could be used to transduce suicide genes into tumor cells contaminating the bone marrow of patients undergoing high dose chemotherapy and autologous bone marrow transplantation.

In order to assess tumor purging effectiveness of the *bcl-x$_S$* vector, *in vitro* cultures of human hematopoietic cells admixed with MCF-7 cells were treated with adenovirus. Our previous data indicated that an MOI of 2000 viral particles per cell was required to kill neuroblastoma cells. In sharp contrast, an MOI of 10,000 was required to completely kill all MCF-7 breast carcinoma cells. Cells from these mixed hematopoietic/MCF-7 cultures were then cultured in methylcellulose assays in order to assess hematopoietic progenitor cell survival. Numbers of CFU-GM colonies were used as a representative measure of progenitor survival. Some non-specific toxicity was noted

in these cultures, as at a MOI of 2000, there was a slight decrease in CFU-GM numbers, whereas the CFU-GM colony number decrease was greater at a MOI of 10,000. However, this loss was non-specific as control vectors containing LacZ instead of *bcl-x$_S$* caused similar reductions in CFU-GM numbers.

In an attempt to overcome the nonspecific toxicity of the *bcl-x$_S$* adenovirus, a combination of *bcl-x$_S$* and thymidine kinase (TK) containing adenoviral vectors was used in order to reduce the effective concentration of the *bcl-x$_S$* vector. Expression of TK in infected cells leads to a sensitivity to the cytotoxic agent gancyclovir, which is added to the culture media after infection. Many cancer cells derived from cells of epithelial cells overexpress a member of the *bcl-2* family. Since *bcl-2* can inhibit - and *bcl-x$_S$* augment - chemotherapy induced apoptosis, a synergistic effect between TK and *bcl-x$_S$* was expected. Following a treatment with gancyclovir, tumor cell killing in TK/*bcl-x$_S$* treated cultures was found to be as effective as the equivalent MOI of *bcl-x$_S$* alone, but with reduced hematopoietic toxicity. Cultures treated with pAdRSV-*bcl-x$_S$* at a MOI of 10,000 had CFU-GM numbers 26% less than cultures treated with a combination of pAdRSV-TK and pAdRSV-Bcl-x$_S$ at a MOI of 5000 of each vector. A colony replating assay revealed no detectable surviving MCF-7 tumor cells from these cultures (Figure 2B). Importantly, cultures of bone marrow cells mixed with tumor cells were purged equally effectively with either the *bcl-x$_S$* or the *bcl-x$_S$*/TK treatment.

While the above *in vitro* assays indicated the ability of adenoviral vectors to selectively kill tumor cells, we wished to explore the effects of these vectors on the transplantable hematopoietic stem cell. To accomplish this, we used an *in vivo* murine models. The *in vitro* studies showing the inability of adenoviral vectors to directly infect the hematopoietic stem cell were confirmed by *in vivo* transplantation experiments. We determined the effects of adenoviral vectors on murine transplantable stem cells by their ability to reconstitute long-term hematopoiesis following lethal irradiation. Male bone marrow cells were treated *in vitro* at a MOI of 5000 particles per cell, and transplanted into female recipients. Importantly, marrow cells treated with adenoviral vectors containing either TK (with or without subsequent gancyclovir treatment) or *bcl-x$_S$* rescued mice from lethal irradiation, indicating that transplantable stem cells were resistant to adenoviral infection. As expected, control mice receiving mock-infected marrow also survived, while mice receiving no transplant after irradiation had substantially reduced survival. Moreover, both short- (4 weeks) and long-term (six months) hematopoietic reconstitution occurred in these experiments indicating that both the more committed hematopoietic progenitor cells, as well as the hematopoietic stem cell are resistant to adenoviral infection (data not shown). Finally, the contribution of male donor cells to engraftment was confirmed by PCR detection of mouse Y sequences in the female recipients.. These data show that male-specific Y sequences are detected in marrow genomic DNA from all of the female survivors six months after transplant.

Task 3. Develop additional markers for molecular detection of occult breast carcinoma.

-3A. Evaluate the specificity and sensitivity of PCR based detection of other mammary specific RNA sequences. No new markers have been detected. The K19 marker has been both sensitive and specific. This made this a low priority task, and efforts were concentrated on the K19 effort and the adenovirus vector.

-3B. Develop non-radioactive detection schema. A nested primer approach was used to detect K19 cDNA (see appendix, manuscript #1). PCR experiments using K19 primers

labeled with either 6FAM or HEX. The PCR products were analyzed with an automatic sequencer. Various amounts of MCF-7 breast cancer cell RNA was mixed with normal bone marrow RNA (from 1x 10^6 cells). These mixing experiments showed that after 2 rounds of PCR we were able to detect K19 mRNA in 2/3 samples containing 10 MCF-7 cells, and 2/4 samples containing 1 MCF-7 cell. K19 was not detected in normal peripheral blood. This task is completed, a non-radioactive detection schema has been developed. Either nested PCR with gel electrophoresis, or with fluorescent detection using an automated sequencer, can be used to detect the K19 mRNA.

-3C. Detection of novel breast cancer peptides. Breast cancer cDNA has been made from RNA isolated from SUM 159 human breast cancer cells. To date, no novel cell surface molecules have been identified in.this library.

-3D. As in 3C, no new breast cancer specific peptides have been identified.

Since this grant was awarded, the gene therapy vector (bcl-xs adenovirus) has shown potential utility for the treatment of cancer. Therefore, an amended statement of work was approved by the DOD in order to develop animal models to test the potential clinical utility of this vector.

AMENDED STATEMENT OF WORK (EXPERIMENTAL PLAN)

The initial experiments demonstrate that the bcl-xs adenovirus selectively kills cancer cells, while sparing many non-cancerous cells. One normal cell type that is killed by this virus are replicating endothelial cells. Such cells are critical for formation of the vascular system of cancer cells. We will therefore use animal tumor models and deliver adenovirus by 4 routes of administration in order to determine therapeutic and toxic potentials of this virus. Rats will be inoculated with cancer cells and tumors be allowed to form. The rats will be injected with either a β–galactosidase virus or the bcl-xs virus using the following routes of admistration 1) intravenous virus injected in rats with subcutaneous tumors, 2) intra-arterial injection of virus into rats with subcutaneous tumors, 3) intra-tumoral injection of virus into rats with subcutaneous tumors, and 4) intraperitoneal injection of virus into rats with intraperitoneal tumors. One group of animals will be sacraficed 7 days after injection, and necropsies will be done. The tumors will be analyzed for the pattern of gene transfer (X-gal staining of tumor and major organs in mice given the β–galactosidase vector) and organ toxicity (both groups). The effects of the virus on tumor vasculature will be monitored closesly. A second group of rats will be followed over time. The effects of the virus on tumor progression will be done. Approval for the animal experiments was only recently granted by the DOD. Therefore, a no-cost extension was requested to complete this aim.

As part of this task, we developed a novel approach for the treatment of breast cancer with adenovirus vectors *in vivo*. Replication-deficient viral vectors are currently being used in gene transfer strategies to treat cancer cells. Unfortunately, viruses are limited in their ability to diffuse through tissue. This makes it virtually impossible to infect the majority of tumor cells in vivo and results in inadequate gene transfer. This problem can be addressed by allowing limited viral replication. Limited viral replication facilitates greater penetration of virions into tissue and can improve gene transfer. We have developed a strategy of limited viral replication using AdRSVlaclys, a chemically modified E1-deleted adenovirus, to codeliver an exogenous plasmid encoding the

adenovirus E1 region. This system allows one round of viral replication. We examined the effect of this limited adenovirus replication *in vitro* and *in vivo*. In culture, codelivery of virus and pE1 resulted in a large increase in infected cells when compared with control cells exposed to virus and pUC19. In experiments on nude mice bearing HeLa ascites tumors, intraperitoneal injection of AdRSVlaclys/pE1 resulted in a significantly higher percentage of infected HeLa cells as compared with the PBS controls (p<0.05) or the AdRSV-laclys/pUC19 controls (p<0.01).. These data demonstrate that the transcomplementation of replication-deficient adenovirus with exogenous E1 DNA leads to limited replication, and this controlled replication enhances gene transfer efficiency of adenovirus *in vivo* (see manuscript in appendix).

CONCLUSIONS

Two major advances have resulted from this project. First, a sensitive PCR-based assay for keratin 19 has been developed and been shown to detect breast cancer cells in the bone marrow and in circulating blood. Keratin 19 appears to be a novel and effective marker for RT-PCR detection of breast cancer cells in peripheral blood and the bone marrow. The collection of patient samples now exceeds one hundred RNA preparations. This includes patients with stage I through IV breast cancer, and includes more than fifty patients that have undergone BMT. Next, the bcl-xs adenovirus has been shown to selectively kill breast cancer cells while sparing normal cells. We envision human breast cancer clinical trials to begin in the near future.

As an adjunct to surgery, radiation, or chemotherapy, autologous bone marrow transplants (BMTs) are increasingly used as a method to increase survival of patients with aggressive non-hematopoietic tumors. However, retroviral tagging and PCR studies indicate that autologous marrow is often the source of cancer relapse in these patients. Several methods have been devised to purge marrow of tumor cells prior to transplantation, but each has distinctive shortcomings. Immunologic methods depend on a unique tumor cell surface epitope and a high avidity antibody for efficient negative selection. Chemical techniques can have significant hematopoietic toxicity. We have previously shown that adenovirally mediated transient expression of $bcl-x_S$, a functional repressor of $bcl-2$, would induce PCD in contaminating tumor cells found in bone marrow cell preparations. It is reported here that a pure population of hematopoietic stem cells does not express a transgene when exposed to a recombinant adenovirus. We further postulated that hematopoietic stem cells would retain the ability to repopulate hematopoiesis following treatment with adenoviral vectors.

We show that a combination of $bcl-x_S$ and TK adenovirus are the most effective and least toxic method of killing MCF-7 cells in a mixed tumor/hematopoietic *ex vivo* culture. Of all tumor cell types we have tested to date, MCF-7 cells have proven to be among the most resistant to $bcl-x_S$ adenovirus treatment [25]. By combining a TK adenovirus/ganciclovir treatment with $bcl-x_S$ adenovirus infection of mixed tumor/marrow cultures, MCF-7 cells are purged from the marrow to below detectable levels. In addition, the combination TK/ $bcl-x_S$ treatment results in an equally efficient purging of hematopoietic progenitors compared to an equivalent treatment using only $bcl-x_S$, while resulting in a progenitor cell toxicity that is equal or slightly less. This combined treatment minimizes the non-specific hematopoietic toxicity of these adenoviral vectors, while preserving MCF-7 purging efficiency, although TK treatment alone may be a useful treatment. This data thus supports the hypothesis that $bcl-x_S$ overexpression mediated by adenoviral vectors may be used to effectively purge solid tumor cells from human bone marrow. Although the mechanism behind this observation is unclear, to date MCF-7 cells have proven to be the only cell type for which a combined $bcl-x_S$/TK infection is required to completely purge human marrow *in vivo*. Other cancer cell line, such as the SHEP 1 neuroblastoma line, are efficiently purged by $bcl-x_S$ viral MOIs that do not have any significant effect on hematopoietic cell activity (25).

In this work, hematopoietic stem and progenitor cells are shown to exhibit resistance to greater MOIs than that required to infect 100% of neuroblastoma cells with an adenovirus marker gene. Previous work has shown that mouse hematopoietic stem cells can be isolated on the basis of the phenotype Thy-1.1^{lo} Lin^{-} Sca-1^{+} [30]. As few as 30 of these cells can rescue 100% of lethally irradiated mice, producing long-term, multilineage reconstitution (26). By purifying homogeneous populations of murine stem cells, we were able to directly target them *in vitro* with adenovirus at MOIs greater than those necessary to kill tumor cells. After such treatment, murine stem cells retained their

viability but did not express the adenoviral LacZ gene, whereas tumor cells expressed this marker. Confirming and extending this data, our *in vivo* studies show that murine marrow infected with bcl-x$_S$, TK, or LacZ adenovirus retains the capacity for long term, apparently multilineage, engraftment upon transplant into lethally irradiated syngeneic mice.

The ability to selectively kill tumor cells, while sparing all of the hematopoietic cells in bone marrow prior to autologous transplantation represents a novel method in purging/transplantation as a treatment of many human neoplasms. Previous methods involving immunologic, mechanical, or chemical based tumor purging have had limited success, require extensive marrow processing, or are useful for treatment of only one specific cell type. We conclude that bone marrow purging using an adenoviral-based method (that can be used alone or in conjunction with other purging strategies) represents a simple, quick, and efficient method for purging a wide variety of non-hematopoietic tumor cells while retaining hematopoietic stem cell activity.

The bcl-xs adenovirus will begin toxicology testing for the FDA this year. We anticipate clinical breast cancer clinical trials to begin with this virus within one to two years.

Finally, a human breast cancer cDNA has been made and will be used to attempt to identify novel peptides expressed by breast cancer cells. Any such peptides identified will be used to develop new diagnostic markers for breast cancer.

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Sensitive Detection of Occult Breast Cancer by the Reverse-Transcriptase Polymerase Chain Reaction

By Yvonne H. Datta, Paul T. Adams, William R. Drobyski, Stephen P. Ethier, Valeri H. Terry, and Mark S. Roth

Purpose: Detection of occult carcinoma in patients with breast cancer may aid the establishment of prognosis and development of new therapeutic approaches. To improve on existing methods of detection, we have developed a reverse-transcriptase polymerase chain reaction (RT-PCR) assay for keratin 19 (K19) transcripts to identify mammary carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer.

Patients and Methods: Peripheral-blood or bone marrow samples obtained from 34 patients with stages I to IV breast cancer and 39 control subjects without breast cancer were screened for K19 mRNA by nested primer PCR.

Results: In reconstitution experiments, K19 RT-PCR reliably detected 10 mammary carcinoma cells in 1 million normal peripheral-blood mononuclear (PBMN) cells. Four of 19 patients with stage IV breast cancer had detectable K19 transcript in peripheral blood. Five of six patients with histologically negative bone marrow biopsies following preablative chemotherapy and before au-

tologous bone marrow transplant (BMT) were positive by this assay. Stem-cell apheresis harvests obtained from one of these patients and three additional patients immediately before BMT were all K19-negative. K19 RT-PCR analysis of CSF from a breast cancer patient with known carcinomatous meningitis was also positive. Thirty-eight of 39 non-breast cancer patients had negative K19 RT-PCR assays. The one exception was a patient with chronic myelogenous leukemia.

Conclusion: RT-PCR of K19 is a sensitive, specific, and rapid method for detection of occult mammary carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer. The presence of residual breast cancer cells in histologically normal bone marrow aspirates but not in stem-cell apheresis harvests is a frequent finding. This assay may be useful in diagnosing metastatic disease, as well as in monitoring the effectiveness of systemic therapy.

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SENSITIVE DETECTION of occult carcinoma in bone marrow and peripheral blood of patients with breast cancer may have important therapeutic and prognostic implications.[1-4] Existing methods of detection, including magnetic resonance imaging, bone scintigraphy, flow cytometry, and immunohistochemistry, have demonstrated an improvement over conventional light microscopy, but have limited sensitivity and specificity.[1,5-9] Immunohistochemical staining using one or a panel of monoclonal antibodies directed against cell surface glycoproteins or cytokeratins is perhaps the most widely applied method for detecting occult bone marrow involvement by breast cancer, with an estimated sensitivity of detection ranging from one cancer cell in 10^4 to 10^5 normal bone marrow cells.[8,10] Detection of micrometastases in the bone marrow by this approach in patients with primary breast cancer has been shown to correlate with early disease relapse and decreased survival.[1,2] However, in many studies, the antibodies used in the immunohistochemical reaction cross-react with normal cells, limiting their prognostic value.[1,11]

The detection of occult carcinoma in bone marrow may be of special concern in the setting of autologous bone marrow transplantation (BMT) for breast cancer patients. Sharp et al[4,12] have reported that breast carcinoma cells can be cultured from bone marrow and peripheral-blood stem-cell harvests of patients before receiving ablative chemotherapy. Preliminary results indicate that patients who receive culture-negative bone marrow products are

more likely to remain disease-free when compared with those who receive a culture-positive harvest.[4] Significant efforts have recently been toward purging bone marrow of clonogenic carcinoma cells before reinfusion.[13-15] However, the detection of occult carcinoma by clonogenic assays is laborious (requires several weeks of tissue culture) and the true sensitivity is unknown.[16] To circumvent the problems inherent in immunohistochemical or cell culture techniques, we have evaluated the RNA transcript encoding for the intermediate filament protein keratin 19 (K19) as a marker for mammary carcinoma cells. This class of cytokeratin is found not only in all normal and malignant mammary cells evaluated to date, but also in a number of simple epithelial cells (tongue, epiglottis, esophagus, intestine, and trachea), and in their malignant counterparts.[17,18] K19 mRNA can be targeted as a tissue-specific message for a reverse-transcriptase polymerase chain reaction (RT-PCR). Since K19 is not expressed in

normal hematopoietic tissues, detection of K19 transcript in the peripheral blood or marrow of a patient with known breast cancer should indicate the presence of mammary carcinoma cells.

In this report, we describe the application of K19 RT-PCR for detection of mammary carcinoma cells in the peripheral blood, bone marrow, and CSF of patients with breast cancer. This assay is highly sensitive and specific, and may be useful to establish the diagnosis of metastatic carcinoma, as well as to monitor the effectiveness of various therapeutics.

PATIENTS AND METHODS

Cell Lines, Patients, and Tissue Samples

Human mammary carcinoma cell lines T47D, MCF7 (estrogen receptor-positive), and SKBR3 (estrogen receptor-negative) were used in development of the RT-PCR assay. All three cell lines are positive for K19 by immunohistochemical staining. In addition, two mammary epithelial cell lines established from normal breast tissue obtained from women who underwent reduction mammoplasty were evaluated. To determine the sensitivity of the assay, peripheral-blood mononuclear (PBMN) cells obtained from a normal donor were mixed with decreasing numbers of T47D cells. A total of 1 million cells were present in each sample. Cells were mixed before the RNA preparation, thus mimicking the clinical setting for detection of mammary cells in the peripheral blood or bone marrow of patients.

The study population consisted of 34 patients with a histologic diagnosis of breast cancer in different stages, monitored at either the University of Michigan Medical Center, Ann Arbor, MI, or at the Medical College of Wisconsin, Milwaukee, WI (Table 1). After approval by the institutional review board, and following informed consent, 2- to 5-mL samples of peripheral-blood or bone marrow aspirate were obtained from patients at various time points during treatment and processed as described later. Samples obtained from the peripheral blood of 10 non-breast cancer patients and bone marrow aspirates from 14 healthy BMT donors and 15 patients following allogeneic BMT for chronic myelogenous leukemia (CML) served as negative controls. In addition, four patients with stage IV breast cancer underwent apheresis to obtain peripheral-blood stem cells before autologous BMT. Viably frozen aliquots of these cells were rapidly thawed, washed in phosphate-buffered saline (PBS), and RNA extracted as outlined later. Finally, 3 mL of CSF was obtained from one patient with breast cancer who had carcinomatous meningitis documented by cytology, and from two additional patients without breast cancer.

RNA Preparation

Samples of cultured cells or CSF were spun for 5 minutes at 450 $\times$ g, and the pelleted cells washed with PBS before RNA extraction. Peripheral-blood or bone marrow samples were first centrifuged at 1,030 $\times$ g for 5 minutes and the buffy coats removed into a fresh tube. The contaminating RBCs were lysed by resuspending the buffy-coat cells in a hypotonic RBC lysis buffer (0.15 mol/L ammonium chloride, 0.01 mol/L potassium bicarbonate, and 0.1 mol/L edathamil) followed by gentle agitation for 10 minutes. Cells were counted and viability assessed by trypan blue exclusion. One to 10 million cells were resuspended directly into 500 μL of nucleic acid

extraction buffer (4 mol/L guanidine isothiocyanate, 0.5% Sarkosyl [N-lauroylsarcosine sodium salt (Sigma Chemical Co, St Louis, MO)], 25 mmol/L sodium citrate, pH 7.0) with 0.1 mol/L of 2-mercaptoethanol and frozen at $-70°$C. The samples were thawed, then extracted with phenol/chloroform/isoamyl alcohol, followed by nucleic acid precipitation with 95% ethanol and 3 mol/L of sodium acetate, pH 5.2, as previously described.[19] The RNA was resuspended in 25 to 50 μL of diethylpyrocarbonate-treated water. All patient and control samples were processed at a site separate from amplification and electrophoresis of PCR products to minimize the potential for PCR carryover.

PCR Primers and RT-PCR

Primers were designed from the sequence of the human K19 gene with nomenclature according to Bader et al[20] (Fig 1).

RT-PCR was performed as previously described[19] using approximately 1- to 3-μg aliquots of total cellular RNA. First strand cDNA was generated with 0.05 OD$_{260}$ units of oligo-(dT)$_{20}$ primer, 50 mmol/L Tris, pH 8.3, 50 mmol/L potassium chloride (KCl), 8 mmol/L magnesium chloride, 10 mmol/L dithiothreitol, 0.5 mmol/L deoxynucleotide triphosphate (dNTP), and 20 U of RT (Seikagaku, St Petersburg, FL) in a 50-μL reaction mix, and incubated for 1 hour at 41°C. A 10-μL aliquot of this reaction was subsequently used for first-round PCR using 0.005 OD$_{260}$ units of each primer A and B (Fig 1), added to 40 mmol/L KCl, 0.01% gelatin, 0.1 mmol/L dNTP, and 1 U taq polymerase (AmpliTaq; Perkin Elmer Cetus, Norwalk, CT) in a total volume of 50 μL. Thirty-five cycles were performed consisting of a 50-second denaturation at 94°C and annealing and polymerase extension for 2 minutes 30 seconds at 72°C. All PCR reactions were terminated with a 10-minute extension at 72°C. For the second round of amplification, a 1-μL aliquot of the first-round PCR product was added to 0.005 OD$_{260}$ units of each primer C and D (Fig 1), 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl$_2$, 0.01% gelatin, 2 mmol/L dithiothreitol, 0.2 mmol/L dNTP, and 1 U taq polymerase in a 50-μL final volume. Cycling conditions for the second round consisted of a 50-second denaturation at 94°C with annealing and extension for 2 minutes at 72°C for 35 cycles. More recently, extension times for both first- and second-round PCRs were decreased to 1 minute, 30 seconds. Five- to 8-μL aliquots of the PCR products were electrophoresed on 2% agarose gels and analyzed by direct visualization after ethidium bromide staining. The presence of a 1,069-base pair (bp) first-round product or a 745-bp second-round product indicated the presence of K19 transcripts. All positive K19 RT-PCR assays were repeated from a second aliquot of RNA. If a separate assay gave discordant results, the data point was discarded. The presence of intact RNA and adequate cDNA synthesis was confirmed by a single round of RT-PCR using ABL sequence-specific PCR primers as previously reported.[19] For evaluation of CSF samples, a second round of PCR was performed to detect ABL signal using one of the first round primers and a nested ABL sequence primer previously reported.[19] Mock RNA preparations were routinely processed as negative controls, while a dilution of MCF7, T47D, or SKBR3 cell-line RNA served as a positive control.

RESULTS

Sensitivity and Specificity

Nested primers were designed to generate a PCR product that spanned all five K19 gene introns and produce an mRNA-specific product (Fig 1). To avoid amplification

from the kno were further the gene and 2 shows the serial dilution Using the apr PCR assay cc cells mixed w was able to d

Table 1. Patient Characteristics and K19 RT-PCR Results

Patient No.	Sample Source	Stage	Sites of Metastatic Disease	Therapy*	ABL†	K19
1	Blood	I	None	None	+	−
2	Blood	I	None	Tam	+	−
3	Blood	I	None	None	+	−
4	Blood	II	None	Tam	+	−
5	Blood	II	None	None	+	−
6	Blood	II	None	None	+	−
7	Blood	II	None	None	+	−
8	Blood	III	None	CAMF	+	−
9	Blood	IV	Bone	Tam	+	−
10	Blood	IV	Bone	Aminoglutethamide	+	−
11	Blood	IV	Chest wall	A	+	−
12	Blood	IV	Bone	RT	+	−
13	Blood	IV	Bone	Tam	+	−
14	Blood	IV	Bone, chest wall, liver	CMF	+	−
15	Blood	IV	Lung	Megestrol	+	−
16	Blood	IV	Brain	RT	+	−
17	Blood	IV	Bone, soft tissue	Tam	+	−
18	Blood	IV	Bone, soft tissue	None	+	−
19	Blood	IV	Bone, liver	CMF	+	−
20	Stem-cell apheresis	IV	Lymph node, lung	CAF	+	−
	Blood			BMT‡	+	−
21	Blood	IV	Liver	CAMF, Tam	+	−
22	Marrow harvest	IV	Bone, mediastinum	CAMF, Tam	+	+
23	Blood	IV	Bone	CMF	+	+
24	Blood	IV	Bone, liver, lung	A	+	+
25	Blood	IV	Bone, abdomen	CAMF	+	+
26	Marrow	IV	Bone, lymph node	Tam, CMF	+	+
27	Marrow	IV	Bone	Tam, megestrol, RT	+	−
	Blood			BMT‡	+	−
28	Marrow	IV	Bone, liver	CA	+	+
	Blood				+	+
29	Marrow	IV	Lymph node	CAF, Tam	+	+
	Stem-cell apheresis	IV			+	−
30	Marrow	IV	Skin, bone	CA	+	+
	Blood				+	−
31	Marrow harvest	IV	Bone, liver	CAF, Mito, VP-16, CDDP	+	−
32	Stem-cell apheresis	IV	Liver	CMF	+	−
33	Stem-cell apheresis	IV	Bone	CAMF	+	−
34	Marrow	IV	Chest wall, ovary, bone	CDDP, Velban§	+	+

Abbreviations: Tam, tamoxifen; C, cyclophosphamide; A, doxorubicin; M, methotrexate; F, fluorouracil; RT, radiation therapy; BMT, bone marrow transplantation; Mito, mitoxantrone; CDDP, carboplatin; VP-16, etoposide.

*Refers to active treatment at or before the time of sample collection.

†All patient samples evaluated were RT-PCR–positive for the reference gene ABL (see text).

‡Blood samples were obtained following high-dose ablative chemotherapy and autologous BMT.

§Vinblastine; Eli Lilly and Co, Indianapolis, IN.

from the known processed pseudogene of K19, primers were further designed to incorporate differences between the gene and pseudogene at the 3′ ends[20,21] (Fig 1). Figure 2 shows the results of a mixing study performed using serial dilutions of the mammary carcinoma cell line T47D. Using the approach outlined earlier, the nested primer RT-PCR assay could routinely detect 10 mammary carcinoma cells mixed with 1 million normal PBMN cells (Fig 2) and was able to detect a single mammary carcinoma cell mixed with 1 million normal PBMN cells in one of seven occasions (an approximately two- to three-cell detection limit estimated by the Poisson equation; data shown from two samples in Fig 2). The MCF7 and SKBR3 cell lines were also consistently positive for K19 expression by RT-PCR. No amplification product was detected by RT-PCR performed on RNA from all three cell lines in the absence of the RT enzyme, demonstrating that any contaminating DNA derived from the processed pseudogene would not
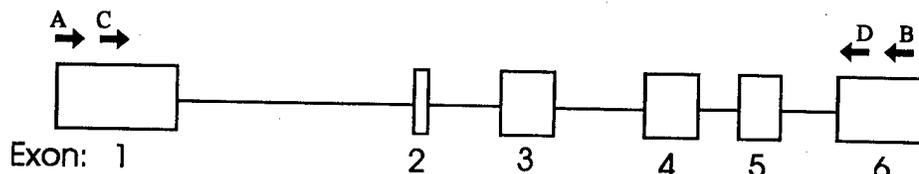
Fig 1. Synthetic oligonucleo-
tide primers for K19 RT-PCR.
Shown is a schematic representa-
tion of the K19 gene with location
of intron (−) and exon (□) se-
quences, as well as the location of
first-round (A,B) and second-
round (C,D) primers. The nucleo-
tides underlined and in bold rep-
resent reported differences from
the pseudogene.

Oligonucleotide	Sequence	Location (5'-3')	Strand
A	AAGCTAACCATGCAGAACCTCAACGACCGC	628-657	+
B	TTATTGGCAGGTCAGGAGAAGAGCC	1697-1673	-
C	TCCCGCGACTACAGCCACTACTACACGACC	757-786	+
D	CGCGACTTGATGTCCATGAGCCGCTGGTAC	1502-1473	-

235 bp —

745 bp —

Fig 3. Detection of oc<
rates of stage IV breast c
bp) is present in all patie
(745 bp) (bottom) is see
Table 1 and text).

amplify using the set of allele-specific primer pairs in Fig 1 (data not shown). RNA prepared from the normal mammary tissue cell lines were K19 RT-PCR−positive, while RNA obtained from normal PBMN cells alone was consistently RT-PCR−negative for K19 expression (data not shown).

RNA prepared from the peripheral blood of 10 control patients (eight females, two males) with hematologic or oncologic conditions other than breast cancer were negative for K19 transcript (data not shown). In addition, 28 of 30 RNA preparations derived from the bone marrow aspirates of 15 patients with CML (12 females, three males) and 14 healthy BMT donors (nine females, five males), were also negative for K19 transcript (data not shown). All of these control patient samples were positive for ABL RT-PCR signal, indicating the presence of intact RNA and successful first-strand cDNA preparation. Bone marrow aspirates collected on two separate occasions from one patient, postallogeneic BMT for CML, were positive for K19 transcripts by RT-PCR. Both of these aspirates were also positive for the BCR/ABL transcript,

demonstrating the presence of minimal residual leukemia (data not shown).

Detection of Circulating Breast Cancer Cells

RNA samples derived from peripheral blood of 27 of 28 patients with breast cancer exhibited successful amplification of ABL sequence and were further evaluated by K19 RT-PCR (Table 1). Nineteen of these patients had stage IV disease, and all but three of these (patients no. 12, 16, and 18) were receiving systemic therapy at the time of sample collection. No stage I, II, or III patients had K19 transcripts detected in their peripheral blood, while K19 transcripts were identified by RT-PCR amplification in four stage IV patients (no. 23, 24, 25, and 28; Table 1). RT-PCR analysis performed on all four stem-cell apheresis samples were K19-negative. All four of these patients underwent autologous BMT with reinfusion of their stem-cell harvests. Three patients (no. 29, 32, and 33) are alive and well at 1, 2, and 2 months, respectively, post-BMT. Patient no. 20 (Table 1) developed carcinomatous meningitis 3 months post-BMT (see following).

Detection of Occult Mammary Carcinoma Cells in Bone Marrow

Bone marrow aspirates and biopsies were obtained from eight patients with stage IV breast cancer (patients no. 22, 26, 27 to 31, and 34; Table 1 and Fig 3). All of the marrow biopsies were negative for mammary carcinoma cells by routine histology; however, six patients (no. 22, 26, 28 to 30, and 34) had bone marrow aspirates positive for K19 transcript in duplicate RT-PCR testing. Bone marrow aspirate samples from six patients were obtained following preablative chemotherapy before autologous BMT (patients no. 22, 28 to 31, and 34; Table
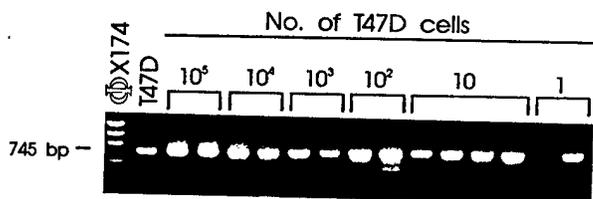

Fig 2. Sensitivity of the K19 RT-PCR assay. Molecular-weight marker is a HaeIII digest of φX174. The number of T47D breast carcinoma cells mixed with 1 million normal PBMN cells is shown. Analyses at 10^5, 10^4, 10^3, and 10^2 T47D cells were performed in duplicate and at 10 cells in quadruplicate.
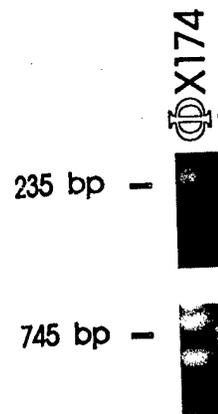
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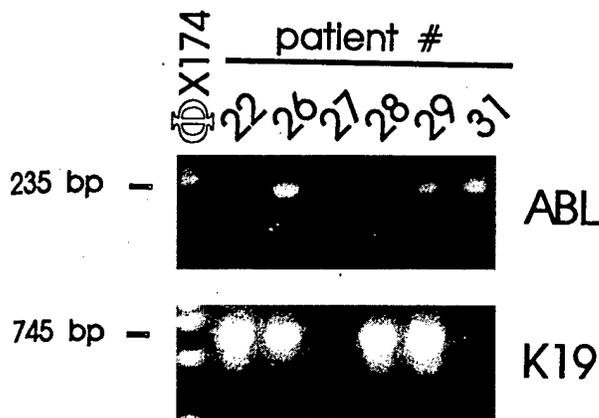(lane 2), headache
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Fig 3. Detection of occult breast cancer cells in bone marrow aspirates of stage IV breast cancer patients. A detectable ABL signal (235 bp) is present in all patient samples (top), while a positive K19 signal (745 bp) (bottom) is seen for patients no. 22, 26, 28, and 29 (see Table 1 and text).

1 and Fig 3). Three of these patients have undergone autologous BMT, receiving marrow (no. 22 and 31) or harvested peripheral-blood stem cells (no. 29). Patient no. 28 had both peripheral blood and bone marrow positive for K19 transcript by RT-PCR following preablative chemotherapy. Progression of liver metastases precluded her from transplant. Patient no. 22 received bone marrow that was RT-PCR–positive for K19. She is now 4 months post autologous BMT, but as yet has no evidence of recurrence. Patient no. 29 received peripheral-blood stem cells that were K19 PCR-negative. She is 1 month postautologous BMT and without evidence of recurrence. Patient no. 31 received harvested bone marrow that was K19 RT-PCR–negative. She remains in clinical remission at 3 months post-BMT. Patients no. 30 and 34, both K19 marrow-positive, are currently awaiting peripheral-blood stem-cell transplants.

K19 RT-PCR Detection of Mammary Carcinoma Cells in CSF

Figure 4 shows the results of K19 RT-PCR performed on RNA derived from the CSF of breast cancer patient no. 20 (Table 1) obtained 3 months post-BMT. A cytospin preparation of the CSF was positive for adenocarcinoma cells by routine microscopy. Lane 1 of Fig 4 represents the first-round ABL signal (235 bp) detected in RNA prepared from the CSF of patient no. 20 (Table 1). Lanes 2 to 4 represent heminested primer ABL signal (185 bp) seen in the CSF samples of a patient with CNS lymphoma (lane 2), headache (lane 3), and a mock RNA prep (lane 4). Lane 5 is the K19 RT-PCR signal seen after one round of PCR for patient no. 20, while lanes 6 to 8 demonstrate

no detectable K19 signal after nested-primer PCR performed on the cDNA samples shown in lanes 2 to 4, respectively. The faint upper (235 bp) band seen in lanes 2 and 3 most likely represents ABL PCR products derived from remaining external (first-round) PCR primers in the heminested PCR amplification mix. WBC counts in the CSF for patients whose amplification products are shown in lanes 1 to 3 and 5 to 7, respectively, were 12/μL, 4/μL and 2/μL.

DISCUSSION

In this report, we have demonstrated that RT-PCR of K19 is a sensitive and specific method for detecting mammary carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer. In addition, the assay was able to identify K19-producing cells in the CSF of a patient with breast cancer and carcinomatous meningitis. This assay is capable of detecting two to three mammary carcinoma cells in 1 million normal PBMN cells, suggesting that it is among the most sensitive assays available for detection of occult breast carcinoma.[8,10] Using immunofluorescence staining with a variety of monoclonal antibodies, previous investigators have demonstrated that the K19 protein is detectable in all invasive primary breast tumors, as well as in all metastatic lesions evaluated to date.[18,22] This widespread expression in breast carcinomas suggests that the K19 RT-PCR assay can be applied to detect occult disease in all women with known breast cancer. In addition to a limited number of normal epithelial tissues, K19 protein expression has also been found in colon carcinoma, small-cell and non–small-cell lung cancer, and prostate cancer.[23-26] These
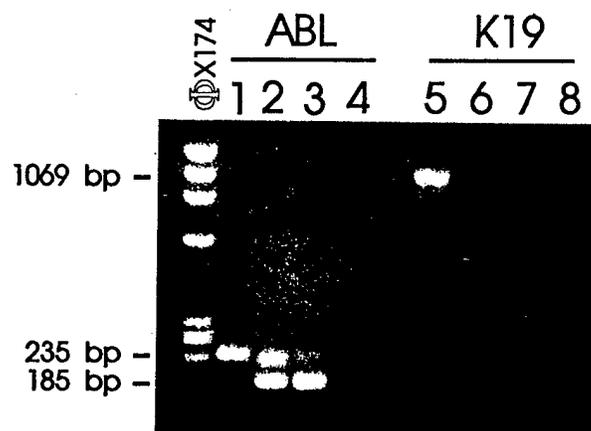

Fig 4. Detection of K19-positive breast cancer cells in CSF. CSF from patient no. 20 (Table 1) was positive for K19 transcripts by RT-PCR in the first round (lane 5). In contrast, two CSF samples from non-breast cancer patients were nested-primer PCR-negative (lanes 6 and 7).

findings potentially broaden the utility of K19 RT-PCR as a detection assay for a number of common malignancies.

The general utility of the PCR assay to detect minimal disease in the peripheral blood or bone marrow of patients with hematologic malignancies has recently been reported.[27-29] These assays relied on chromosomal translocations as targets for amplification. These unique markers have an advantage of being found only in the malignant cells. Unfortunately, no consistent chromosomal abnormality has been found in mammary carcinoma cells. Similarly, while point mutations in known oncogenes are frequently associated with breast cancer, no single mutation has been consistently found.[30] Even in those instances in which a mutation may exist, discrimination of a mutated allele from multiple copies of the remaining normal allele in a highly sensitive assay is problematic. Use of tissue-specific RNA transcripts as markers circumvents this problem. Naito et al[31] and Mattano et al[32] targeted neuron tissue-specific transcripts for the detection of residual neuroblastoma cells in patients with neuroblastoma. Detection of the neuronal-specific mRNA transcript PGP 9.5 by RT-PCR had a 100-fold increase in sensitivity compared with routine immunohistochemical studies.[32] However, the mRNA transcripts detected by this assay were present in scant amounts in normal PBMN and bone marrow cells.[32] Smith et al[33] targeted tyrosinase transcription as a tissue-specific marker in melanocytes to detect circulating melanoma cells by RT-PCR. These investigators were able to detect circulating cancer cells in four of seven patients with melanoma following nested-primer PCR, but also reported the detection of some false-positive assays.[33] The possible low-level transcription of tissue-specific genes in nonspecific cells as reported by these investigators may reflect the general process of illegitimate transcription. The existence of such transcripts has been documented for 17 different genes by RT-PCR and has proven useful in the analysis of a number of genetic disorders.[34-36] However, the abundance of these transcripts in inappropriate cells is very low, estimated at one mRNA molecule per 100 to 1,000 cells.[35]

In this report, we carefully assessed for the presence of illegitimate K19 transcripts among individuals without epithelial-derived neoplasms. To exclude also the possibility that normal K19-expressing mammary cells or other K19-expressing epithelial cells existed in the bone marrow or peripheral blood, we studied peripheral-blood or bone marrow samples from 39 healthy controls or patients without breast cancer. Only one patient's bone marrow aspirate was positive for K19 transcript by RT-PCR. This patient had recently undergone allogeneic BMT for CML and had persisting leukemia cells as evidenced by a positive BCR/ABL PCR assay. Although this positive K19 assay could represent illegitimate transcription of K19 by a residual leukemic or other cell, the patient is being evaluated for a second malignancy. The otherwise low frequency of K19-positive PCR products indicates that illegitimate transcription of K19 mRNA or the presence of K19-expressing epithelial cells is, in fact, rare in normal blood or bone marrow. In addition, 22 of 39 control patients had received intensive chemotherapy or BMT, demonstrating that aggressive drug treatment does not induce false-positive assays. The K19 RT-PCR assay offers a significant improvement over immunofluorescent monoclonal antibody stains, which may cross-react with normal cells and relies heavily on the experience of the observer to distinguish stained normal cells from stained tumor cells.[1,11] The RT-PCR assay also requires only a small volume of blood or bone marrow (< 5 mL) and analysis time is rapid, with results available in 24 to 48 hours.

K19 RT-PCR was able to detect circulating breast carcinoma cells in the peripheral blood of four of 19 patients with stage IV breast cancer. Detection of occult malignancy in the peripheral blood of patients with breast cancer without the use of elaborate culture techniques has not previously been reported. Only three of the K19-negative stage IV patients were not receiving systemic therapy near the time of sample collection. Lack of K19 PCR positivity in the blood of stage IV patients may have been due to consistently low numbers of circulating carcinoma cells below the threshold of detection by the K19 RT-PCR assay, or to sequestration of tumor cells at other sites. Peripheral-blood samples from three additional patients with stage IV breast cancer were positive for the K19 transcript in only one of two duplicate RT-PCR assays (data not shown). Although this finding may also be attributed to transcript concentrations near the threshold of detection by our assay, we cannot completely exclude the possibility of PCR carryover. Interestingly, two of the four patients with peripheral-blood–positive K19 assays developed carcinomatous meningitis, while the other two had distant sites of progressive disease around the time of sample collection, which further suggests that a larger tumor burden may correlate with a positive assay.

The K19 RT-PCR assay was particularly powerful in identifying occult mammary carcinoma cells in bone marrow aspirates, with six of eight histologically negative marrows K19-positive by PCR. Five of six BM aspirates harvested from patients immediately before BMT were K19-positive. Interestingly, stem-cell apheresis samples from all four patients analyzed pre-BMT were K19-nega-

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tive Sharp et al,[4,12] using clonogenic tumor assays, found similar results, detecting residual tumor cells in bone marrow harvests at an overall frequency of 42% (13 of 31), while the frequency of detection in peripheral-blood stem-cell harvests was significantly lower at 19% (four of 21). Taken together, these data suggest that apheresis harvests of breast cancer patients are less likely to contain occult tumor cells than bone marrow harvests. The exact role that residual mammary carcinoma cells in marrow or stem-cell harvests play in disease relapse is unknown. In preliminary studies, patients who received tumor-cell culture-negative marrow products were less likely to relapse at distal sites (one of 12 culture-negative patients relapsing) compared with culture-positive patients (eight of 16 distal relapses).[4,12] Occult breast cancer in reinfused marrow or stem cells as detected by the K19 RT-PCR assay may eventually contribute to disease relapse. Alternatively, the sensitive detection of residual cancer cells postchemotherapy may be a marker of inherently more drug-resistant disease. None of the six patients who had a K19 assay performed on marrow or apheresis samples before undergoing BMT in this report (patient no. 20, 22, 29, 30, 32, and 33; Table 1) have relapsed, although follow-up times are short (median, 2.5 months). Since most stage IV patients who undergo BMT ultimately relapse, the detection of occult malignancy by K19 RT-PCR in patients with earlier stages of disease may prove to have a better predictive value for relapse than in pa-tients with advanced (stage IV) disease. Longer follow-up and studies of more patients are needed to address these possibilities and are currently in progress. However, K19 RT-PCR should prove immediately useful in as-sessing the efficacy of purging techniques used on bone marrow before infusion. In addition, application of this approach to identify micrometasteses in lymph nodes, blood, or bone marrow of women with primary breast cancer is currently underway. Detection of K19 tran-scripts at these sites in early-stage patients at the time of diagnosis may eventually identify a cohort of patients at high risk of disease recurrence and aid in selecting candidates for adjuvant therapy.

We conclude that keratin 19 RT-PCR is a specific, highly sensitive, and rapid assay for detection of occult mammary carcinoma cells in patients with breast cancer. The presence of low levels of mammary carcinoma cells in histologically normal bone marrow aspirates, but not in stem-cell apheresis harvests of stage IV patients following aggressive systemic chemotherapy, is a frequent finding. Long-term follow-up is needed to determine whether this assay will be a useful tool in assessing prognosis, guiding therapy, or developing new strategies in bone marrow purging.

ACKNOWLEDGMENT

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A recombinant *bcl-x$_S$* adenovirus selectively induces apoptosis in cancer cells but not in normal bone marrow cells

(*bcl-2*/gene therapy/stem cells)

MICHAEL F. CLARKE*, INGRID J. APEL*, MARY A. BENEDICT†, PETER G. EIPERS*, VENIL SUMANTRAN*, MARIBEL GONZÁLEZ-GARCÍA†, MONICA DOEDENS‡, NINA FUKUNAGA§, BEVERLY DAVIDSON*, JOHN E. DICK‡, ANDY J. MINN¶, LAWRENCE H. BOISE¶, CRAIG B. THOMPSON¶, MAX WICHA*, AND GABRIEL NÚÑEZ†

Departments of *Internal Medicine, †Pathology, and §Radiation Therapy, University of Michigan Medical School, Ann Arbor, MI 48109; ¶Howard Hughes Medical Institute and Department of Internal Medicine, University of Chicago Medical School, Chicago, IL 60637; and ‡Department of Molecular and Medical Genetics, University of Toronto, Hospital for Sick Children, Toronto, ON, Canada M5G 1X8

ABSTRACT Many cancers overexpress a member of the *bcl-2* family of inhibitors of apoptosis. To determine the role of these proteins in maintaining cancer cell viability, an adenovirus vector that expresses *bcl-x$_S$*, a functional inhibitor of these proteins, was constructed. Even in the absence of an exogenous apoptotic signal such as x-irradiation, this virus specifically and efficiently kills carcinoma cells arising from multiple organs including breast, colon, stomach, and neuroblasts. In contrast, normal hematopoietic progenitor cells and primitive cells capable of repopulating severe combined immunodeficient mice were refractory to killing by the *bcl-x$_S$* adenovirus. These results suggest that Bcl-2 family members are required for survival of cancer cells derived from solid tissues. The *bcl-x$_S$* adenovirus vector may prove useful in killing cancer cells contaminating the bone marrow of patients undergoing autologous bone marrow transplantation.

It is becoming increasingly apparent that disruption of the pathways regulating programmed cell death (PCD; apoptosis) is integral to the etiology of a variety of cancers. Expression of certain tumor-suppressor proteins such as p53 can induce some cancer cells to undergo apoptosis (1–4). Oncogenes have also been implicated in PCD. For example, several groups have observed that deregulated expression of c-*myc* can activate the apoptosis pathway (5–7). Furthermore, *bcl-2*, the gene deregulated in most follicular lymphomas, primarily functions to inhibit apoptosis (for reviews, see refs. 8 and 9). *bcl-2* encodes an intracellular membrane-associated protein that has been localized to the mitochondria, endoplasmic reticulum, and perinuclear regions (9). Although expression of *bcl-2* does not stimulate cell proliferation, it can cooperate with c-*myc* (10, 11) to cause transformation. Moreover, expression of high levels of Bcl-2 protein in normal or neoplastic cells delays or inhibits PCD induced by many factors including p53, Myc, chemotherapy, and ionizing radiation (8, 9). A large percentage of epithelial and hematopoietic tumors overexpress Bcl-2 (8, 9). Furthermore, overexpression of Bcl-2 is correlated with poor prognosis and resistance to treatment in patients with neuroblastoma (12), prostatic cancer (13), and some forms of leukemia (14).

Recently, a homolog of *bcl-2*, called *bcl-x*, has been identified and partially characterized (15, 16). As a result of alternative splicing, two *bcl-x* mRNA species, designated *bcl-x$_L$* and *bcl-x$_S$*, were identified in the human. The former, like *bcl-2*, inhibits apoptosis (15, 17). The latter is thought to function as a repressor of Bcl-2, as it enhances apoptotic signals in cells that express Bcl-2 (15). Postulating that inactivation of Bcl-2 or Bcl-x$_L$ might increase the susceptibility of cancer cells to PCD,

an adenovirus vector that expresses Bcl-x$_S$ protein was constructed. Primary carcinoma cells, as well as cell lines derived from solid tumors, rapidly underwent cell death after infection with the *bcl-x$_S$* adenovirus. In primary breast cancer cells and multiple breast cancer cell lines, expression of *bcl-x$_S$* was associated with rapid induction of cell death. In contrast, human hematopoietic progenitor cells exposed to this virus maintained viability and retained their ability to reconstitute the bone marrow of irradiated immune-deficient mice. Blocking Bcl-2 or Bcl-x$_L$ function by the *bcl-x$_S$* adenovirus appears to provide another strategy for inducing apoptosis in tumor cells. These findings have important implications for cancer therapy.

MATERIALS AND METHODS

Construction of the *bcl-x$_S$* Adenovirus. The plasmid pBSbcl-x$_S$ (15) was digested with *Eco*RI, and customized *Bam*HI linkers were ligated onto the ends; the ≈536-bp fragment was purified and ligated into *Bam*HI-digested pAd5RSV plasmid (18). The pAd5RSV *bcl-x$_S$* recombinant virus was isolated by *in vivo* homologous recombination between the linearized pAd5RSV *bcl-x$_S$* plasmid and the replication-deficient sub360 adenovirus that has a partial deletion of the E3 region and deletion of the E1A and E1B genes (18). Three recombinant viruses that expressed Bcl-x$_S$ protein in infected cells were plaque-purified twice. Large preparations of adenovirus were made by infecting 293 cells and purifying crude virus preparations by CsCl centrifugation (19).

Cell Infections with Recombinant Adenoviruses. The number of adenovirus particles in viral stocks was determined by spectrophotometry (18). Adenovirus titers were determined by limiting dilution and plaque formation of 293 cells exposed to the virus dilutions. Absence of replication-competent virus was confirmed by limiting dilution and plaque formation of HeLa cells exposed to the virus dilutions. Each cell line was infected with a stock of the β-galactosidase virus of known titer and then stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) to determine the number of viruses per cell needed to infect 85–98% of each cell line. Unless otherwise indicated, the concentration of the *bcl-x$_S$* adenovirus used to infect cells was identical to the number of β-galactosidase viruses that infected 85–98% of the cells. Cells were exposed to the adenovirus vectors for 4 hr in serum-free medium. The medium was then replaced with tissue culture medium/2% fetal calf serum, and the cells were incubated overnight. The next day the medium was removed and replaced with tissue culture medium/10% serum. Cell viability was measured by trypan blue exclusion.

Abbreviations: PCD, programmed cell death; SCID, severe combined immunodeficiency virus.

Analysis of Bcl-x Protein. Immunoblot analysis of Bcl-x proteins was done as described (17) by using a rabbit anti-Bcl-x antiserum. The blots were developed with epichemiluminescence substrate (Amersham).

Hematopoietic Cell Assays. Bone marrow was harvested from normal human volunteers by using a protocol approved by the University of Michigan Institutional Review Board. Low-density mononuclear cells were isolated using Ficoll/Hypaque centrifugation essentially as described (20). Hematopoietic cells (1×10^6) were mixed with 1.5×10^4 pSV$_2$-neo-transfected SHSY-5 neuroblastoma cells. The cells were then infected with adenovirus in serum-free medium containing kit ligand at 1 mg/ml and interleukin 3 at 10 mg/ml. After 2 days of culture, cells were harvested, and triplicate progenitor assays using 1×10^4 cells were done as described (20). To assay for viability of SHSY-5 cells that had been mixed with hematopoietic cells and then exposed to the *bcl-x$_S$* adenovirus, cells were grown in tissue culture medium containing the antibiotic Geneticin at 1 mg/ml to kill the normal hematopoietic cells. To determine the ability of *bcl-x$_S$* adenovirus-infected cells to engraft nonobese diabetic (Nod)/severe combined immunodeficiency (SCID) mice, 1×10^7 low-density bone marrow mononuclear cells were infected with 0, 2×10^3, 5×10^3, or 10^4 adenoviruses per cell and inoculated into the tail vein of irradiated (400 cGy) Nod/SCID mice (21). After 1 mo, the mice were sacrificed, and the bone marrow was harvested and analyzed for human hematopoietic cells essentially as described (22).

RESULTS

Construction of Adenoviral Vector Expressing Bcl-x$_S$. We and others have recently demonstrated that the overexpression of Bcl-2 oncogene will block p53-induced apoptosis (9). This finding led to the prediction that inhibition of Bcl-2 function might induce apoptosis in tumor cells that express wild-type p53. To test this hypothesis, an adenovirus vector that expresses *bcl-x$_S$*, a functional inhibitor of Bcl-2, was constructed by inserting the *bcl-x$_S$* coding sequences into the pADRSV vector (Fig. 1A). Nine virus plaques were isolated by cotransfecting the pADRSV *bcl-x$_S$* construct with the sub360 adenovirus into 293 human kidney cells (23), and the viruses were amplified. Restriction digests and Southern blots revealed three viruses that contained the *bcl-x$_S$* minigene. Immunoblotting using a rabbit polyclonal antibody raised against the Bcl-x protein revealed that MCF-7 breast cancer cells infected with the *bcl-x$_S$* adenovirus, but not with a control adenovirus that contains a β-galactosidase gene, expressed the ≈21-kDa Bcl-x$_S$ protein (Fig. 1B).

The *bcl-x$_S$* Adenovirus Is Lethal to a Broad Range of Cancer Cells. MCF-7 breast cancer cells (which express high levels of wtp53 and Bcl-2) infected with the *bcl-x$_S$* adenovirus, but not MCF-7 cells infected with the control virus, began dying 2 days after infection. When cells were analyzed 6 days after infection, the MCF-7 cells infected with the β-galactosidase adenovirus had grown and formed colonies. In contrast, MCF-7 cells infected with the *bcl-x$_S$* adenovirus became rounded, subsequently detached from the tissue culture plastic, and died (Fig. 2A). Dying cells infected with the *bcl-x$_S$* adenovirus morphologically resembled cells undergoing apoptosis. They were shrunken with picnotic nuclei and cytoplasmic blebbing (Fig. 2A). Furthermore, DNA degradation, a hallmark of apoptosis, was detected *in situ* in virtually all cancer cells infected with the *bcl-x$_S$* adenovirus but not with the control virus using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay (24) (data not shown). Several other types of cancer cells including cells of breast, colon, and neuroblastoma origin were also killed by the *bcl-x$_S$* adenovirus (Table 1). As reported, the adenovirus containing the β-galactosidase gene alone demonstrated various degrees
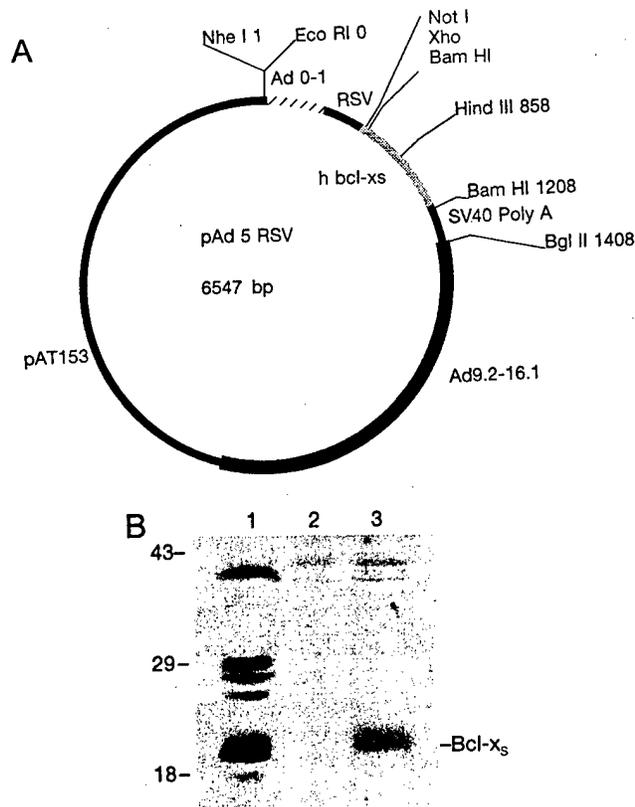
A

B

FIG. 1. (A) The *bcl-x$_S$* adenovirus. The pRSVAd/*bcl-x$_S$* construct is shown. SV40, simian virus 40; Ad, adenoma virus. (B) Immunoblot of lysates from MCF-7 cells. Expression of Bcl-x$_S$ protein was analyzed by SDS/PAGE and immunoblotting with a rabbit polyclonal antiserum (17). Lanes: 1, 200 μg of protein from FL5.12 cells transfected with pSFFV/*bcl-x$_S$* minigene (15); 2, 40 μg of protein from the parental MCF-7 cells infected with β-galactosidase adenovirus; 3, 40 μg of protein from MCF-7 cells infected with *bcl-x$_S$* adenovirus. Molecular size standards are shown at left (in kDa). Note that MCF-7 cells infected with *bcl-x$_S$* adenovirus express the ≈21-kDa Bcl-x$_S$ protein.

of toxicity to some but not all cancer cell lines (25). To determine whether the *bcl-x$_S$* adenovirus can induce cell death in primary cancer cells, breast cancer cells isolated from six patients were exposed to the virus. When infected with β-galactosidase virus at 1×10^3 to 1×10^4 viruses per cell, there was no effect on viability (Fig. 2B). In contrast, cells infected with even the lowest concentration of the *bcl-x$_S$* adenovirus showed a marked cytotoxicity (Fig. 2B). Primary cells isolated from one of the patients would form colonies in tissue culture. Fifty thousand cells from this patient were cultured after infection with zero or 1×10^4 viruses per cell. Although innumerable colonies formed in the control cultures, none formed in the cells infected with the *bcl-x$_S$* adenovirus (data not shown). The *bcl-x$_S$* adenovirus was also lethal to primary breast cancer cells isolated from five other patients (Table 1).

The ability of *bcl-x$_S$* adenovirus-infected cells to grow *in vivo* was tested. Two days after 5×10^4 RKO colon cancer cells were infected with the *bcl-x$_S$* adenovirus, but not the β-galactosidase virus, the cells began dying. By day 6, only a few of the *bcl-x$_S$* adenovirus-infected cells excluded trypan blue (data not shown). Next, 5 million RKO cells were infected with 2×10^3 *bcl-x$_S$* adenoviruses per cell or β-galactosidase viruses per cell. Uninfected cells or colon cancer cells infected with the control virus formed tumors in 7 out of 10 and 2 out of 5 injected nude mice, respectively (Table 2). In contrast, RKO cells infected with the *bcl-x$_S$* adenovirus did not form tumors in any of the 15 mice injected with such cells (Table 2).
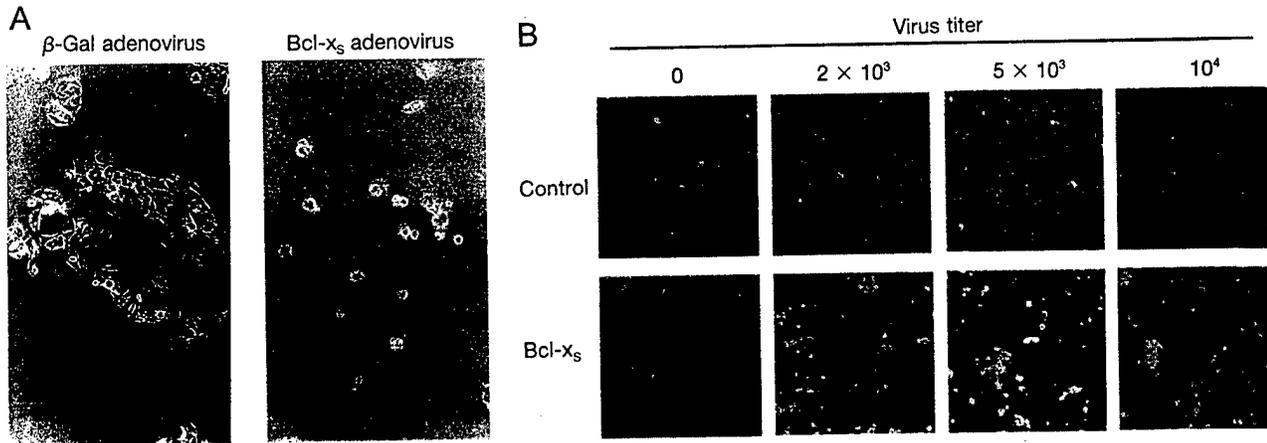
A

β-Gal adenovirus   Bcl-x$_S$ adenovirus

B

FIG. 2. (*A*) Microphotograph of MCF-7 cells. MCF-7 cells (5 × 10⁵) infected with the indicated virus photographed after 6 days of growth. β-Gal, β-galactosidase. Note that virtually all cells infected with *bcl-x$_S$* adenovirus have died. (×50.) (*B*) Photomicrograph of adenovirus-infected primary breast cancer cells. Photomicrographs were taken of primary breast cancer cells infected 2 days previously with the indicated titer of β-galactosidase adenovirus (control) or the *bcl-x$_S$* adenovirus. Cells infected with even the lowest titer of *bcl-x$_S$* adenovirus show evidence of viral toxicity, whereas cells infected with even the highest titer of β-galactosidase virus remained viable. (×270.)

bcl-x$_S$ Adenovirus Cytotoxicity Is Selective for Tumor Cells but Spares Human Hematopoietic Cells. High-dose chemotherapy followed by infusion of autologous bone marrow to

Table 1. Viability of different cancer cells after exposure to *bcl-x$_S$* adenovirus

	Cytotoxicity	
Cells	β-Galactosidase adenovirus	*bcl-x$_S$* adenovirus
RKO (human colon carcinoma)	−	++++
Primary breast carcinoma cells*	−	++++
Patient 1		
Patient 2	−	++++
Patient 3	+	++++
Patient 4	−	++++
Patient 5	+	++++
Patient 6	−	++++
MDA435		
(human breast carcinoma)	+++	++++
T47D		
(human breast carcinoma)	+++	++++
MCF-7		
(human breast carcinoma)	++	++++
HT29		
(human colon carcinoma)	++	++++
SHSY-5		
(human neuroblastoma)	+	++++
SK-N-SH		
(human neuroblastoma)	+	++++
IMR-32		
(human neuroblastoma)	++++	++++
K-562 (human leukemia)	−	−

The indicated cell lines were infected with *bcl-x$_S$* adenovirus by using a virus titer that resulted in expression of β-galactosidase in >95% of cells infected with the same titer of β-galactosidase virus. In cell lines, each experiment was done in triplicate, and viability was determined 6 days after infection. Degree of cytoxicity was as follows: − (<5%), + (6–25%), ++ (26–50%), +++ (51–90%), and ++++ (91–100%). *Breast cancer cells isolated from either pleural or ascites fluid were collected by the University of Michigan tissue procurement laboratory and stored in liquid nitrogen. These cells were placed in tissue culture medium and exposed to the *bcl-x$_S$* adenovirus. The primary breast cancer cells were infected with identical titers of either β-galactosidase or *bcl-x$_S$* adenovirus that resulted in expression of β-galactosidase in most cells. The primary breast cancer cells infected with β-galactosidase virus remained viable.

rescue the damaged hematopoietic system is felt to cure some children with neuroblastoma (26). Unfortunately, the bone marrow of such patients is often contaminated with neuroblastoma cells that contribute to relapse (27). To mimic the situation in which bone marrow cells collected for bone marrow transplantation are contaminated with cancer cells, 1 × 10⁶ low-density human bone marrow mononuclear cells were mixed with 1.5 × 10⁴ SHSY-5 human neuroblastoma cells. The lowest virus concentration (2 × 10³ viruses per cell) completely inhibited the ability of SHSY-5 neuroblastoma cells to form colonies (data not shown). After exposure of the bone marrow cells to 2–10 × 10³ viruses per cell, which totally inhibited proliferation of the neuroblastoma cells, human hematopoietic progenitor cells remained viable and formed colonies in methylcellulose (Fig. 3). There was a slight decrease in hematopoietic cell colonies after exposure to 1 × 10⁴ viruses per cell. This result was not specific for the *bcl-x$_S$* adenovirus because it was observed with a control adenovirus (data not shown) and is probably secondary to nonspecific viral particle toxicity at very high doses (B.D., unpublished observation).

Hematopoietic Cells Exposed to the bcl-x$_S$ Adenovirus Retained the Ability to Reconstitute Bone Marrow. For the *bcl-x$_S$* adenovirus to be clinically effective in tumor cell purging, human hematopoietic stem cells capable of repopulating the patient must be spared. Recently, transplantation assays for primitive human SCID-repopulating cells (21) have been developed by engrafting human bone marrow or cord blood in irradiated immune-deficient SCID or Nod/SCID mice (22,

Table 2. Tumor formation in nude mice

Adenovirus	Mice injected, no.	Tumors, no.
Mock	10	7
β-Galactosidase	5	2
bcl-x$_S$	15	0

RKO colon cancer cells were infected with 10³ of the indicated adenovirus per cell. Approximately 16 hr later, 5 × 10⁶ cells were collected and injected into the flanks of nude mice. Control cells (either mock-infected cells or β-galactosidase adenovirus-infected cells) were injected into one flank, and *bcl-x$_S$* adenovirus-infected cells were injected into the opposite flank of 15 mice. Mice were examined 4 weeks later for tumors. Statistical analysis using the Wilcoxon signed-rank analysis shows a significant difference in the number of tumors that the control cells vs. the *bcl-x$_S$* adenovirus-infected cells formed (*P* = 0.018).

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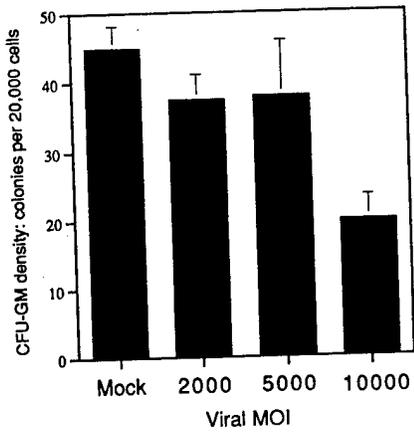SCID-re
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FIG. 3. Mononuclear cells from normal human bone marrow were isolated as described (20). Duplicate samples of hematopoietic cells were infected with the indicated number of *bcl-xs* adenoviruses per cell, and then progenitor assays were done in triplicate. Note that only at the highest virus concentration is there any decline in colony numbers. Data from one experiment are shown. A second experiment using a different donor yielded essentially identical results. MOI, multiplicity of infection.

28). To ensure that SCID-repopulating cells remained functional after exposure to the *bcl-xs* adenovirus, treated human bone marrow cells were transplanted into Nod/SCID mice. Human bone marrow mononuclear cells were exposed to up to 1 × 10⁵ *bcl-xs* adenovirus vector per cell and cultured *in vitro*. The hematopoietic cells (1 × 10⁷) were then infused into the tail vein of irradiated mice according to standard protocols (21, 22). One month after transplantation, DNA analysis with a human-specific α-satellite probe indicated that significant levels of human cells had repopulated the mouse bone marrow (Table 3). In addition, the bone marrow contained multiple lineages of human myeloid and erythroid progenitors, even in mice transplanted with cells exposed to the highest virus titer (Table 3). Quantitatively and qualitatively, these mice were indistinguishable from several hundred mice that we have transplanted with normal human cells, indicating that the SCID-repopulating cells were unaffected by exposure to the *bcl-xs* adenovirus. These data suggest the feasibility of using the *bcl-xs* adenovirus vector to eliminate cancer cells from the bone marrow while sparing normal stem cells.

DISCUSSION

It has been postulated that Bcl-2 may contribute to the malignant phenotype by blocking apoptotic pathways in cancer cells. In this present report, we used an adenovirus vector containing *bcl-xs*, a functional inhibitor of Bcl-2, to induce PCD in human cancer cells derived from a variety of solid tumors. Furthermore, our evidence shows that the cytotoxicity induced by the *bcl-xs* adenovirus vector is cell-type specific because normal human bone marrow hematopoietic progenitor cells are resistant to *bcl-xs* adenovirus-induced apoptosis. Indeed, human hematopoietic cells exposed to these viruses retained the ability to reconstitute the bone marrow of irradiated SCID mice.

Initial descriptions of Bcl-xs suggested that expression of this protein inhibited the ability of Bcl-2 to protect cells from PCD induced by interleukin 3 withdrawal (15). It is notable that the *bcl-xs* adenovirus is uniformly lethal to all solid tumor cells thus far tested. In contrast, the *bcl-xs* adenovirus failed to induce cell death in hematopoietic precursors. The mechanism for this cell-type specificity is presently unknown. Expression of *bcl-x* is obligate for fetal liver hematopoiesis (29). However, it is not known whether expression of Bcl-x$_L$ is necessary for adult hematopoiesis or at what stage of differentiation expression of *bcl-x$_L$* is required for survival. The cell-type selectivity of the *bcl-xs* adenovirus may, at least partly, be due to the fact that these recombinant adenoviruses do not result in prolonged expression of transgenes in hematopoietic stem cells and that expression of Bcl-x$_L$ is not required for such cells to survive. It is also possible that the *bcl-xs* adenovirus does not infect stem cells. Recent evidence shows that adenovirus vectors demonstrate tissue specificity. In lung tissue recombinant adenoviruses do not efficiently transduce columnar epithelial cells *in vivo* (30).

The observation that the *bcl-xs* adenovirus is uniformly toxic to such a diverse number of cancer cells suggests that expression of a *bcl-2* family member may be obligate for cell survival in cancer cells of solid tissue origin. It has been thought that Bcl-2 and Bcl-x$_L$ proteins protect cells from apoptotic signals such as those induced by growth factor withdrawal or DNA damage (8, 9). Our results suggest that such signals might be constitutively present in certain cells. Cells stably transfected with a *bcl-xs* plasmid and grown using selection medium in culture (15) uniformly express a small amount of Bcl-x$_S$ protein compared with cells infected with the *bcl-xs* adenovirus (G.N., unpublished data). Thus, efficient induction of apoptosis by

Table 3. Human hematopoietic cell engraftment of SCID mice

	Human cells, %	Colonies					
			CFU-				
		BFU-E	G	M	GM	GEMM	Total
Mock infection	1–10	3	27	18	0	1	49
Mock infection	0	1	4	0	0	0	5
2K virus infection	10–50	22	43	81	4	1	151
2K virus infection	10–50	38	77	157	4	2	278
5K virus infection	1–10	2	9	13	0	0	24
5K virus infection	N/A	—	—	—	—	—	—
10K virus infection	1–10	2	15	24	1	1	43
10K virus infection	1–10	2	17	16	2	0	37

Low-density mononuclear cells from human bone marrow were collected as described (20) and infected with the indicated number of the *bcl-xs* adenoviruses per cell. The next day, irradiated SCID mice were injected with ≈1 × 10⁷ cells essentially as described (21, 22). After 1 mo, bone marrow cells were harvested. Southern blots were done to determine the percentage of human cells in the bone marrow (22). Low-density mononuclear cells were cultured in duplicate in methylcellulose with human hematopoietic growth factors, and erythroid (BFU-E), granulocyte (CFU-G), macrophage (CFU-M), granulocyte/macrophage (CFU-GM), and mixed granulocyte/erythroid/monocyte (CFU-GEMM) colonies were counted 2 weeks later. One of the mice injected with cells that were exposed to 5 × 10³ (5K) viruses per cell died before analysis. Note that mouse marrow was engrafted with human hematopoietic cells exposed to the highest titer of virus.

the *bcl-x_S* adenovirus may relate to its ability to transduce high levels of Bcl-x_S protein in infected cells. An alternative explanation is that cancer cells infected with the *bcl-x_S* adenovirus express an effector of apoptosis, either an endogenous effector or a virally encoded effector (or both).

Cancer cell contamination of bone marrow used to rescue patients from high-dose chemotherapy is a significant problem in the treatment of neuroblastoma (31) and breast cancer (32). Elegant retrovirus-tagging experiments have shown that reinfusion of malignant cells contributes to the relapse of neuroblastoma (27). In all relapsed patients, biopsies of such tumors showed that virally marked cells were invariably present (27). We have shown that after infection of contaminated bone marrow cells with the *bcl-x_S* adenovirus, the cells can be incubated *in vitro* for a short period to allow the carcinoma cells to die and then be infused into a mouse and reconstitute hematopoiesis. By this method, the *bcl-x_S* adenovirus can be used as a "molecular scalpel," either by itself or in conjunction with other purging techniques (33), to selectively eliminate contaminating tumor cells from bone marrow samples. Together, these observations suggest that strategies such as the *bcl-x_S* adenovirus designed to disrupt the *bcl-2* family pathway may provide alternative therapeutic approaches to cancer treatment.

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