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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)

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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.

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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. 1*A*). 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. 1*B*).

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. 2*A*). Dying cells infected with the *bcl-x$_S$* adenovirus morphologically resembled cells undergoing apoptosis. They were shrunken with picnotic nuclei and cytoplasmic blebbing (Fig. 2*A*). 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

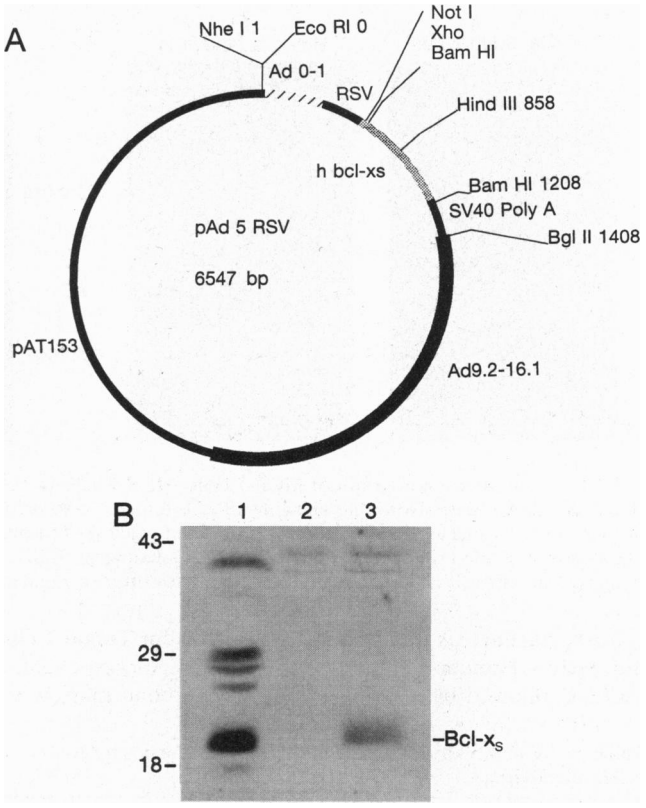

FIG. 1. (*A*) The *bcl-x$_S$* adenovirus. The pRSVAd/*bcl-x$_S$* construct is shown. SV40, simian virus 40; Ad, adenoma virus. (*B*) Immunoblot of lysates from MCF-7 cells. Expression of Bcl-x$_S$ protein was analyzed by SDS/PAGE and immunoblotting with a rabbit polyclonal antiserum (17). Lanes: 1, 200 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. 2*B*). In contrast, cells infected with even the lowest concentration of the *bcl-x$_S$* adenovirus showed a marked cytotoxicity (Fig. 2*B*). 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).

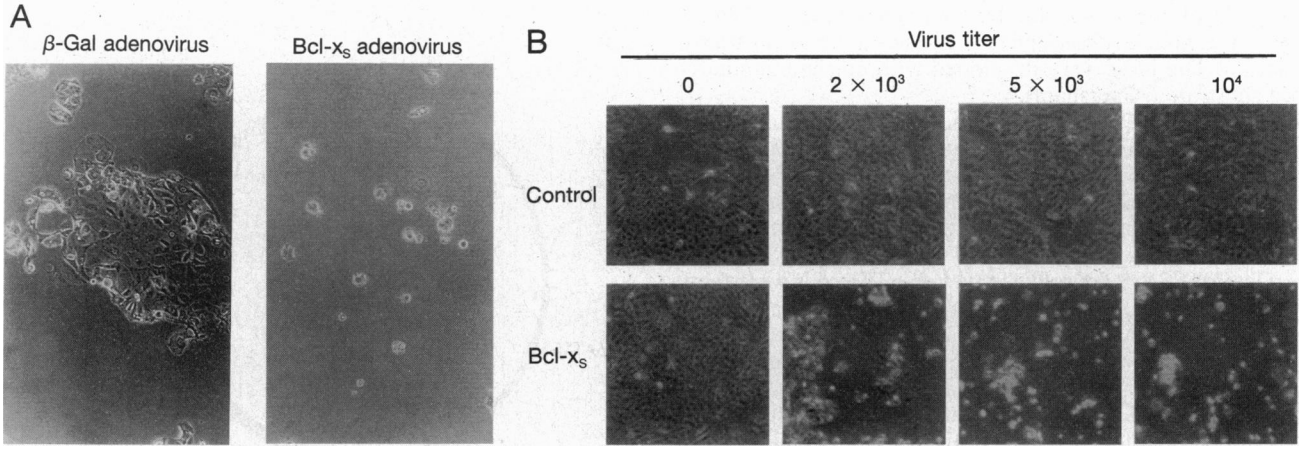

FIG. 2. (*A*) Microphotograph of MCF-7 cells. MCF-7 cells (5×10^5) infected with the indicated virus photographed after 6 days of growth. β-Gal, β-galactosidase. Note that virtually all cells infected with *bcl-x$_S$* adenovirus have died. (×50.) (*B*) Photomicrograph of adenovirus-infected primary breast 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 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, 1×10^6 low-density human bone marrow mononuclear cells were mixed with 1.5×10^4 SHSY-5 human neuroblastoma cells. The lowest virus concentration (2×10^3 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 $2–10 \times 10^3$ 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 1×10^4 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).

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

Cells	Cytotoxicity: β-Galactosidase adenovirus	Cytotoxicity: *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.

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 10^3 of the indicated adenovirus per cell. Approximately 16 hr later, 5×10^6 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 ($P = 0.018$).

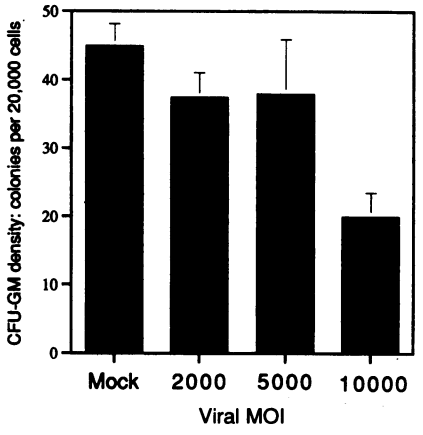

FIG. 3. Mononuclear cells from normal human bone marrow were isolated as described (20). Duplicate samples of hematopoietic cells were infected with the indicated number of *bcl-x$_S$* adenoviruses per cell, and then progenitor assays were done in triplicate. Note that only at the highest virus concentration 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-x$_S$* adenovirus, treated human bone marrow cells were transplanted into Nod/SCID mice. Human bone marrow mononuclear cells were exposed to up to 1×10^5 *bcl-x$_S$* adenovirus vector per cell and cultured *in vitro*. The hematopoietic cells (1×10^7) 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-x$_S$* adenovirus. These data suggest the feasibility of using the *bcl-x$_S$* 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-x$_S$*, 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-x$_S$* adenovirus vector is cell-type specific because normal human bone marrow hematopoietic progenitor cells are resistant to *bcl-x$_S$* 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-x$_S$ 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-x$_S$* adenovirus is uniformly lethal to all solid tumor cells thus far tested. In contrast, the *bcl-x$_S$* 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-x$_S$* 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-x$_S$* 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-x$_S$* 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-x$_S$* 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-x$_S$* adenovirus (G.N., unpublished data). Thus, efficient induction of apoptosis by

Table 3. Human hematopoietic cell engraftment of SCID mice

		Colonies					
			CFU-				
	Human cells, %	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-x$_S$* adenoviruses per cell. The next day, irradiated SCID mice were injected with $\approx 1 \times 10^7$ 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 5×10^3 (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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