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Modulation of Bcl-2 Protein Levels by an Intracellular Anti-Bcl-2 Single-Chain Antibody Increases Drug-induced Cytotoxicity in the Breast Cancer Cell Line MCF-7[1]

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ABSTRACT

Extensive experimental evidence suggests that Bcl-2 promotes cell survival by preventing the onset of apoptosis induced by a variety of stimuli. In addition, Bcl-2 expression has been correlated with resistance and poor response to chemotherapy in a number of cell types. Therefore, this protein represents a logical target for gene therapy strategies designed to achieve selective gene product ablation. In this study, we have developed an approach based upon intracellular expression of single-chain antibodies (sFvs) to achieve modulation of Bcl-2 protein levels in target cells. Using a transient expression system, we show that this intracellular anti-Bcl-2 sFv mediates specific reduction of Bcl-2 levels. This effect significantly enhances drug-mediated cytotoxicity in Bcl-2-overexpressing tumor cells, whereas transfection of the anti-Bcl-2 sFv did not affect the growth rate of the tumor cell lines. This method thus represents a novel and efficient way to selectively abrogate the activity of Bcl-2.

INTRODUCTION

Programmed cell death (apoptosis) is a process that plays an important role in cellular homeostasis. In this regard, an important regulator of apoptosis is the Bcl-2 protein (1–3). The *Bcl-2* gene encodes a M_r 26,000 protein that regulates apoptosis, at least in part, via its interaction with other members of the Bcl-2 family. Previous studies have shown that Bcl-2 is mainly localized as an integral mitochondrial membrane protein, although Bcl-2 is also found to be associated with other membranes, including those of the ER[3] and the nucleus (4). Extensive experimental evidence suggests that Bcl-2 promotes cell survival by preventing the onset of apoptosis induced by a wide variety of stimuli, including essentially all classes of anticancer drugs and X-irradiation (4). A role for Bcl-2 in cancer was initially identified in follicular lymphoma bearing the chromosomal translocation t(14;18) that juxtaposes the *Bcl-2* gene with the immunoglobulin heavy chain locus, thereby up-regulating its expression (5).

Although first described in lymphoma, overexpression of Bcl-2 is also found in a number of nonhematopoietic cancers, including prostate cancer, breast cancer, colon cancer, and glioblastoma (6–11). Recent evidence suggests that, in these cells, Bcl-2 may play an important role in protecting cancer cells from death induced by anticancer drugs. Estrogen-induced increases in Bcl-2 in the context of an estrogen-responsive human breast cancer cell line significantly enhanced their resistance to apoptosis, whereas antisense-mediated reduction in Bcl-2 increased their sensitivity to anticancer drugs (12, 13). In addition, taxol-mediated inactivation of Bcl-2 by phosphorylation in prostate cancer cell lines renders them susceptible to apoptosis (14). Furthermore, it has been shown that Bcl-2 expression in ovarian cancer cells affects the cellular response to apoptosis and modulates their resistance to anticancer drugs (11). In addition to solid tumors, many non-Hodgkin's lymphomas and some acute myeloid leukemias often overexpress Bcl-2 (4, 15). Clinical studies of these hematological malignancies suggest an association between Bcl-2 expression, resistance to apoptosis, poor response to chemotherapy, and shorter patient survival (4, 16). Taken together, these results suggest a central role for Bcl-2 in the promotion of cell survival in solid tumors and hematopoietic malignancies.

Based upon these concepts, molecular therapeutic strategies to modulate Bcl-2 expression have been proposed. In this regard, AS oligonucleotides targeted against Bcl-2 mRNA sequences and plasmid-derived Bcl-2 AS transcripts have been shown to alter the growth and survival of lymphoid cells overexpressing Bcl-2 *in vitro*. In this context, several independent Bcl-2 AS studies have demonstrated a significant increase in apoptosis in treated cells, as well as more effective tumor cell killing after exposure to chemotherapeutic drugs (13, 17–21). *In vivo* models have extended these findings, demonstrating that pretreatment of lymphoma cells bearing the t(14;18) translocation with AS oligonucleotides to Bcl-2 mRNA inhibited the formation of tumors in a severe combined immune deficiency mouse model (22). More recently, preliminary results from a clinical trial using Bcl-2 AS therapy in patients with non-Hodgkin's lymphoma provided the first evidence of down-regulation of the Bcl-2 protein in humans (23). These studies underscore the importance of modulating Bcl-2 protein levels to overcome chemoresistance. Therefore, based on these data and the central role of Bcl-2 in preventing apoptosis induced by anticancer drugs in tumor cells, this protein represents a logical target for gene therapy strategies designed to achieve selective gene product ablation.

To this end, we have developed a novel approach to achieve oncogene ablation based upon expression of intracellular single-chain antibodies. A single-chain antibody (sFv) is the smallest antibody derivative that retains the binding specificity of the parental antibody. This single molecule is constructed by linking the V_H and V_L regions of the antibody using a small flexible peptide linker. Intracellular sFvs have been used as a novel approach for gene therapy to inhibit a variety of HIV proteins, resulting in ablation of replication of the HIV virus in T cells (24–27). In addition, our group and others have used sFvs to abrogate the expression of oncoproteins (28–33). Thus, sFvs can achieve effective functional knock-out of target proteins, potentially reverting dysregulated cellular physiology.

In this study, we report the construction and the functional activity of an anti-Bcl-2 sFv targeted to the ER. Using a transient expression system, we show that this sFv mediates specific reduction of the Bcl-2

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[3] The abbreviations used are: ER, endoplasmic reticulum; AS, antisense; V_H, variable heavy; V_L, variable light; CMV, cytomegalovirus; LMP, latent membrane protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; HRP, horseradish peroxidase; CDDP, *cis*-diamminedichloroplatinum; MTS, 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H* tetrazolium; AdpL, adenovirus-polylysine-DNA.

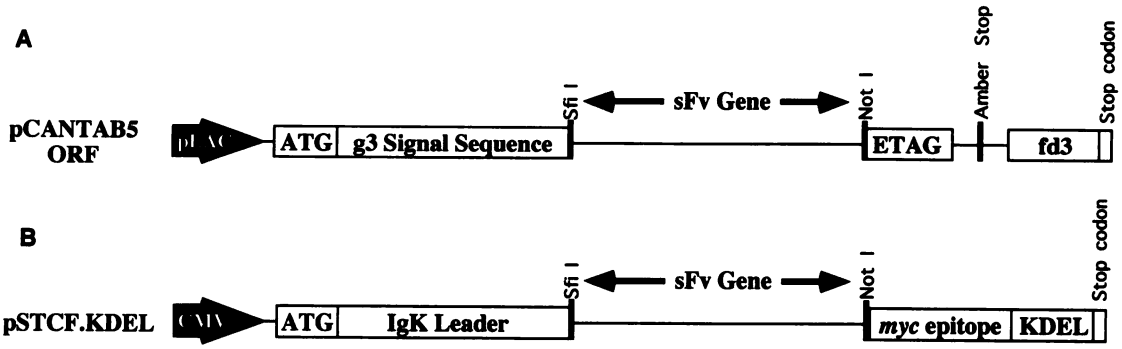

Fig. 1. *A*, pCANTAB5 vector showing control regions. The sFv cDNA (750 bp) is introduced between the *Sfi*I and *Not*I sites. The g3p leader sequence directs transport of the protein to the inner membrane/periplasm of *E. coli*. The sFv is expressed as a fusion protein with the E-tag peptide to allow easy detection. *B*, pSTCF.KDEL eukaryotic vector expressing *sFv* genes. Expression of the sFv protein is driven by the CMV promoter. The sFv cDNA is introduced between *Sfi*I and *Not*I. The IgK leader sequence directs the sFv protein to the ER, and the KDEL signal at the COOH terminus leads to retention in this cellular compartment. The sFv open reading frame is also fused with a c-myc epitope to allow easy detection by Western blot.

protein levels and enhances drug-induced cytotoxicity in Bcl-2-expressing tumor cells.

MATERIALS AND METHODS

Derivation of sFv Constructs. The murine hybridoma cell line 4D7, which expresses a monoclonal antibody against the human Bcl-2 protein, has been described previously (34). This hybridoma was used to generate cDNA from purified mRNA. sFv constructs were generated with the recombinant phage antibody system (Pharmacia Biotech, Piscataway, NJ) according to the instructions provided by the manufacturer. Briefly, the V_H and V_L chains were amplified from the cDNA by PCR using mouse variable region primers (Pharmacia Biotech, Piscataway, NJ). The V_H and the V_L DNA fragments were linked together by overlap extension PCR using a $(Gly_4Ser)_3$ linker to generate a 750-bp sFv construct with flanking *Sfi*I and *Not*I restriction sites. The sFv DNA fragments were cloned into *Sfi*I/*Not*I sites of the prokaryotic expression vector pCANTAB5 (Pharmacia Biotech, Piscataway, NJ). Screening of recombinant clones expressing a sFv against Bcl-2 was accomplished by colony lift assay, as described previously (35).

Plasmids. The phagemid pCANTAB5/sFv contains the anti-Bcl-2 sFv DNA under the control of the inducible *lac* promoter. This vector also encodes a peptide tag (Etag) located at the 3′ end of the sFv to allow easy immunological detection of sFv protein expression. The Bcl-2 expression plasmid pRc-CMV/hBcl-2 contains wild-type human Bcl-2 cDNA under the control of the CMV promoter. The pGEX-hBcl-2 encodes the human Bcl-2 and was used to purify the Bcl-2 protein. The ER-targeting vector pSTCF.KDEL has been described in detail elsewhere[4]. The anti-Bcl-2 sFv fragments generated by *Sfi*I/*Not*I digest of pCANTAB5/sFv were ligated into the *Sfi*I/*Not*I sites in pSTCF.KDEL just upstream from and in-frame with the c-myc/KDEL sequence. The ER-targeted anti-LMP1 sFv (cloned into pSTCF.KDEL) was used as a control in various experiments. LMP1 of EBV is not expressed in any of the cell lines used in this study and therefore provided a good plasmid control to evaluate nonspecific sFv effects.

Purification of Bcl-2 and BAG-1 Proteins. The Bcl-2 protein was produced from the pGEX-hBcl-2 vector in *Escherichia coli* cells. A single colony was cultured at 37°C in Luria-Bertani medium with 100 μg/ml ampicillin. Induction was carried out at an A_{600} of 0.8 with 1 mM isopropyl β-D-thiogalactoside at 37°C for 6–8 h before recovering cells by centrifugation. Cells were resuspended in 5 ml of lysis buffer (0.1% Triton X/1 mM phenylmethysulfonyl fluoride in PBS) and incubated on ice for 20 min before brief sonication to reduce the viscosity. Pellets were collected by centrifugation at 15,000 rpm for 20 min. Clear supernatants were incubated with rotation at 4°C for 2 h with 300 μl of glutathione Sepharose 4B beads (Pharmacia Biotech), which had been washed with 50 mM PBS/0.1% Triton X. The beads were collected by brief centrifugation and washed once with PBS/Triton X buffer. The immobilized Bcl-2 protein was then eluted with 10 mM glutathione and 50 mM Tris-HCl (pH 8.0) and dialyzed against PBS overnight at 4°C. The BAG-1 protein was purified using the same protocol. Purified proteins were stored at −20°C until needed and subsequently characterized by SDS-PAGE (10% gels), followed by Coomassie staining.

Binding Analysis by ELISA. The periplasmic extracts were prepared as follows. Bacterial clones containing pCANTAB5/sFvs were induced with 1 mM IPTG for 4 h, centrifuged, and resuspended in ice-cold PBS/1 mM EDTA, followed by incubation on ice for 30 min and centrifugation at 1500 × *g* for 10 min at 4°C. The supernatant, which contains the soluble antibodies, was stored at −20°C until needed. For the ELISA, 96 well plates were coated with 10 μg/ml of purified Bcl-2 protein (200 μl/well) in PBS or BAG-1 protein (10 μg/ml) and incubated overnight at 4°C. The plates were blocked for 1 h with 3% BSA (Boehringer Mannheim Co, Indianapolis, IN) at room temperature and then incubated with various concentrations of sFv protein for 1 h. After washing with PBS, the plates were incubated at room temperature for 1 h with 200 μl of HRP-conjugated anti-Etag antibody (1:8000 dilution; Pharmacia Biotech, Piscataway, NJ). The plates were developed with 2′, 2′-Azino-Bis(3-Ethylbenzthiazoline-6-Sulphonic Acid) Diammonium chromogen reagent (Pharmacia Biotech, Piscataway, NJ) and read on a microplate reader at 410 nm.

Cell Lines. The human breast cancer cell line MCF-7, the human prostate cancer cell line DU145, and the human cervical cancer cell line HeLa were

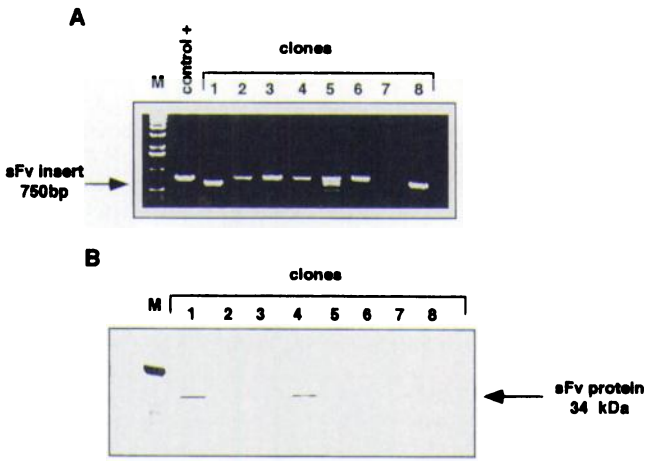

Fig. 2. *A*, screening of positive clones for sFv inserts obtained after the colony lift selection. The plasmid DNA was extracted from positive clones and used in a PCR reaction. The PCR primers were complimentary to the *Sfi*I and *Not*I sites. The resulting sFv DNA products migrate as a 750-bp fragment on an agarose gel (1%). *B*, expression of the anti-Bcl-2 sFv in the *E. coli* strain HB2151. IPTG-induced periplasmic extracts were run on SDS-PAGE gel (12%). After transfer, the membrane was probed with an HRP-labeled anti-E-tag antibody. The anti-Bcl-2 sFv protein has an apparent molecular weight of ~34,000.

[4] J. E. Grim, A. Piché, G. P. Siegal, R. D. Alavarez, and D. T. Curiel. A family of plasmid vectors for the intracellular expression of single chain antibodies, submitted for publication.

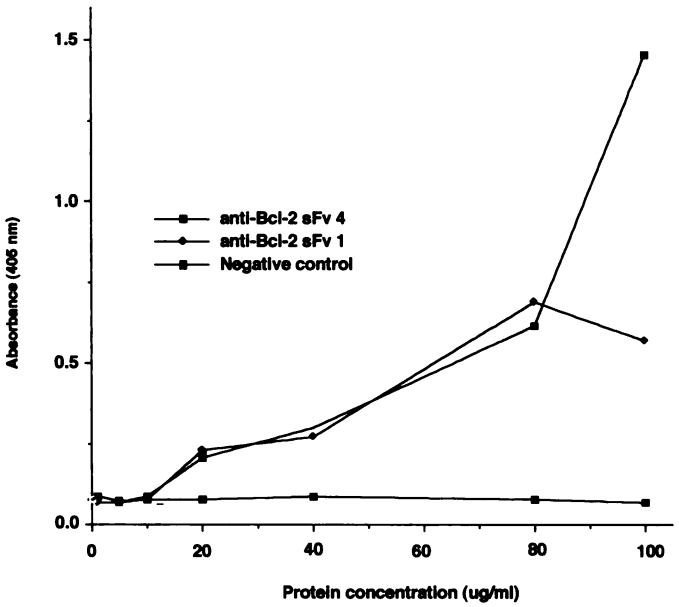

Fig. 3. Binding affinity of the anti-Bcl-2 sFvs to the Bcl-2 protein as measured by ELISA. Various concentrations of periplasmic extracts anti-Bcl-2 sFvs #1 and #4 were added onto a 96-well plate coated with recombinant Bcl-2 protein. A periplasmic extract containing no sFv protein was used as a negative control. After addition of an HRP-conjugated mouse anti-E-tag antibody and the peroxidase substrate, the plate was read at 405 nm. Samples were done in duplicate, and absorbance values are expressed as a mean.

obtained from American Type Culture Collection (Rockville, MD). DU145 and MCF-7 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 300 μg/ml L-glutamine, 100 IU/ml penicillin, and 25 μg/ml streptomycin. HeLa cells were maintained and propagated in DMEM/F12 supplemented with 10% fetal bovine serum, 1% glutamine, and antibiotics. HeLa, MCF-7, and DU145 cells were transfected in six-well plates using the AdpL complex method exactly as described previously (36).

Immunoblot Analysis. At 48 h posttransfection, cells were lysed using Promega lysis buffer (Promega Corp., Madison, WI). Protein concentration was measured by the Bradford method using the Bio-Rad protein assay, according to the instructions of the manufacturer. Equal amounts of protein (30 μg) were loaded in each lane and separated by SDS-PAGE (12%). A duplicate of the same gel was also stained with Coomassie dye to verify that similar amounts of protein were loaded in each lane. After transfer onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), the membranes were probed with either an anti-Bcl-2 antibody (1:1000; Dako, Carpinteria, CA) or a anti-c-myc tag antibody (1:10000; Invitrogen). A HRP-conjugated rabbit anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) was used at 1:10,000. The immunoblots were developed by chemiluminescence using the Renaissance system according to the instructions of the manufacturer (Dupont, Boston, MA).

Cytotoxicity Assays. Cells were transfected in six-well plates using the AdpL method and replated the next day into 96-well plates (10^4 cells/well). Twenty-four h later, the medium was changed, and fresh medium containing various concentrations of staurosporin (Sigma Chemical Co., St. Louis, MO) or CDDP (Bristol-Myers Squibb, Princeton, NJ) were added. The relative percentage of viable cells was determined 4 days later by MTS reduction assay using the Celltiter 96 kit according to the instructions of the manufacturer (Promega).

RESULTS

Construction of the Anti-Bcl-2 sFv and Expression in *E. coli*. The hybridoma cell line 4D7 produces a monoclonal antibody against the human Bcl-2 protein (34). Specifically, the 4D7 antibody recognizes an epitope localized in the loop region between the BH4 and BH3 domains of the Bcl-2 protein. The cDNA encoding the V_H and the V_L chains of this antibody were linked together as described in "Materials and Methods," and the full-length sFv construct was cloned into the bacterial expression vector pCANTAB5 (Fig. 1*A*). Following screening by colony lift assay, positive clones were analyzed by PCR for the presence of an sFv insert (Fig. 2*A*) and for their ability to generate an M_r ~34,000 sFv protein by Western blot after expression in an *E. coli* system (Fig. 2*B*). All of the clones tested by PCR displayed the expected 750-bp sFv DNA fragment (with the exception of #7) but only two clones, #1 and #4, had detectable expression of anti-Bcl-2 sFv proteins. These two clones were thus further studied for their binding affinity to the Bcl-2 protein.

Anti-Bcl-2 sFvs Binding Activity to the Bcl-2 Protein. To determine the relative binding affinity of the anti-Bcl-2 sFvs #1 and #4, we prepared periplasmic extracts produced from IPTG-induced *E. coli* expressing the respective sFvs. These extracts, which contain soluble sFv proteins, were then used in an ELISA. The untransduced strain and an irrelevant protein (BAG-1) were used as controls. In this analysis, both anti-Bcl-2 sFvs #1 and #4 bound to the Bcl-2 protein (Fig. 3), whereas no binding was observed to an untransduced periplasmic extract or the BAG-1 protein (results not shown). The binding affinity of the anti-Bcl-2 sFv #4 was higher, especially at high concentrations. Thus, we have derived an anti-Bcl-2 sFv that, when expressed in a prokaryotic system, binds to the human Bcl-2 protein.

Intracellular Expression of the Anti-Bcl-2 sFv in Eukaryotic Cells. The intracellular expression of sFvs is a potent way to achieve selective knock-out of cellular proteins (37). Because Bcl-2 is a membrane-associated protein, we have used an eukaryotic expression vector (pSTCF.KDEL) that has the ability to target the anti-Bcl-2 sFv to the ER. Its ability to target the green fluorescent protein to this subcellular compartment has been shown elsewhere[4].

To determine whether the anti-Bcl-2 sFvs could be expressed in eukaryotic cells, we transduced HeLa cells using the AdpL method (>90% transduction efficiency in these cells; data not shown) and

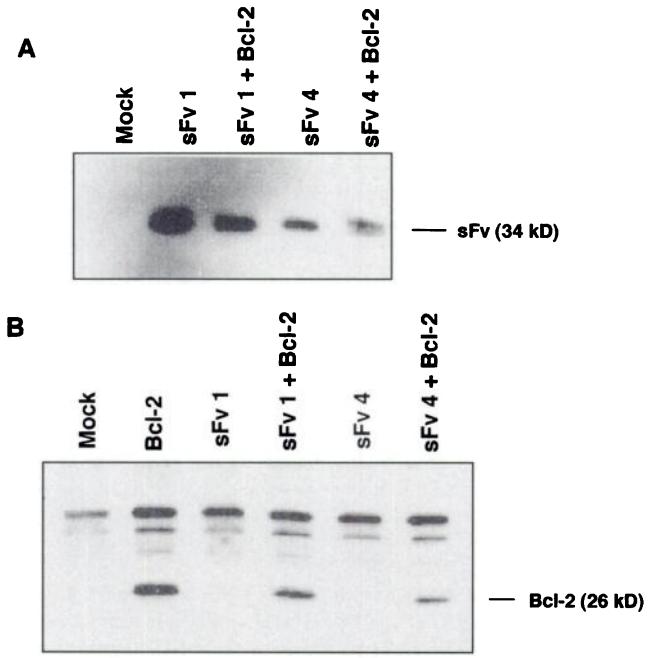

Fig. 4. *A*, expression of the anti-Bcl-2 sFvs #1 and #4 in HeLa cells as determined by Western blot. Cells were transduced with either the anti-Bcl-2 sFvs #1 and #4 or cotransfected with the pRC/CMV/hBcl-2 plasmid (*sFv 1 + Bcl-2*, *sFv 4 + Bcl-2*). In eukaryotic cells, the sFv protein migrates around M_r 34,000. *B*, modulation of Bcl-2 protein levels in HeLa cells as determined by Western blot. *Mock*, cells treated with AdpL only; *sFv 1*, sFv 1 vector only; *sFv1 + Bcl-2*, sFv 1 vector and pRC/CMV/hBcl-2; *sFv 4*, sFv 4 vector only; *sFv 4 + Bcl-2*, sFv 4 vector and pRC/CMV/hBcl-2. Equal amounts (30 μg) of total protein were loaded in each lane.

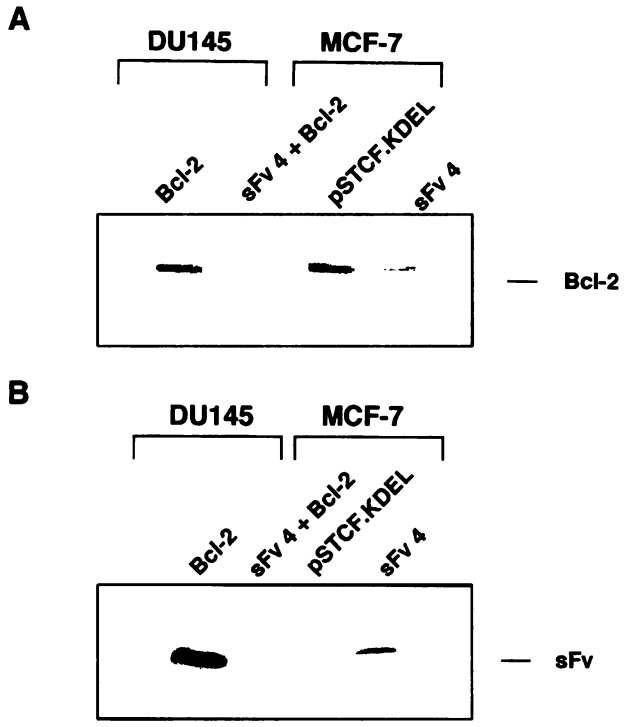

Fig. 5. *A*, Western blot analysis showing a reduction of Bcl-2 protein levels in DU145 and MCF-7 cells. DU145 cells were transfected with either pRC/CMV/hBcl-2 (*Bcl-2*) or pRC/CMV/hBcl-2 plus sFv #4 vector (*sFv 4 + Bcl-2*). MCF-7 cells were treated with AdpL alone (mock) or the sFv #4 vector (*sFv 4*). Equal amounts (25 μg) of total protein were loaded in each lane and separated by SDS-PAGE (12%). The membrane is probed with an anti-Bcl-2 antibody. *B*, expression of the sFv 4 protein in DU145 and MCF-7 cells as determined by Western blot. Equal amounts (25 μg) of protein were separated by SDS-PAGE (12%). The membrane is probed with an anti-c-myc antibody.

analyzed them 48 h later for sFv expression by Western blot. For these experiments, the anti-Bcl-2 sFvs #1 and #4 were introduced into the pSTCF.KDEL vector (Fig. 1*B*). The integrity of these new recombinant plasmids was verified by DNA sequencing. Expression of the sFvs was validated by probing for the incorporated c-myc tag. As shown in Fig. 4*A*, both c-myc epitope-tagged anti-Bcl-2 sFvs were expressed at high levels when cloned into the ER-targeted vector construct. Therefore, as expected, high expression levels of the ER-targeted anti-Bcl-2 sFvs were achieved in mammalian cells using transient transfection.

Reduction of Bcl-2 Protein Levels by an ER-targeted Anti-Bcl-2 sFv in HeLa Cells. To initially assess the functional activity of the anti-Bcl-2 sFv, we chose to use a heterologous system in which we could exogenously introduce the Bcl-2 protein at high levels into cells. The anti-Bcl-2 sFv, or the vector DNA, were cotransfected with pRc/CMV/hBcl-2 into HeLa cells. Forty-eight h after transfection, the cells were lysed, and Bcl-2 expression was determined by immunoblot analysis. A duplicate gel was also stained with Coomassie dye to ensure that the same amount of protein was loaded in each lane. Compared with control, both anti-Bcl-2 sFvs #1 and #4 significantly reduced Bcl-2 protein levels, although clone #4 was more efficient (Fig. 4*B*). This observation is consistent with the fact that the anti-Bcl-2 sFv #4 demonstrated higher binding affinity to Bcl-2 in the ELISA (Fig. 3). In this system, intracellular expression of an anti-Bcl-2 sFv was capable of efficiently reducing heterologously expressed Bcl-2 protein levels.

Decreased of Bcl-2 Protein Levels in Breast and Prostate Cancer Cells Expressing Bcl-2. We next evaluated whether the anti-Bcl-2 sFv #4 (chosen based on previous data) could also reduce the levels of Bcl-2 protein in the breast cancer cell line MCF-7. These cells are highly transducible by the AdpL method (data not shown) and express Bcl-2 at readily detectable levels. Previous studies using MCF-7 cells have shown that cell survival after exposure to cytotoxic drugs is modulated by the levels of Bcl-2 (12, 13, 38). Thus, anti-Bcl-2 sFv-mediated decreased of Bcl-2 protein would be expected to augment the sensitivity of MCF-7 cells to apoptotic stimuli. To verify this hypothesis, the anti-Bcl-2 sFv #4 was introduced into MCF-7 cells, and the levels of the Bcl-2 protein were determined. Compared with the control, a marked decreased in Bcl-2 protein levels was noted in cells that received the anti-Bcl-2 sFv (Fig. 5*A*) but not a control plasmid. A similar effect was seen in the prostate cancer cell line DU145. DU145 cells do not express Bcl-2 and thus, for these experiments, Bcl-2 was exogenously introduced. When cotransducted with Bcl-2, the anti-Bcl-2 sFv achieved significant reduction of Bcl-2 protein levels compared with the control plasmid (Fig. 5*A*).

To verify expression of the anti-Bcl-2 sFv protein in these MCF-7 and DU145 cells, the cell lysate were subjected to immunoblot assays using an anti-c-myc tag antibody. As shown in Fig. 5*B*, expression of the anti-Bcl-2 sFv protein was found in both cell lines, although DU145 cells expressed higher levels, despite the fact that equal amounts of total protein were analyzed. The lower levels of anti-Bcl-2 sFv protein detected in MCF-7 cells is consistent with the fact that some Bcl-2 proteins were still detectable in these cells after transfection with the sFv. Taken together, these results demonstrate that intracellular expression of an ER-targeted form of the anti-Bcl-2 sFv is capable of effectively reducing endogenously or exogenously produced Bcl-2 in tumor cells.

The ER-targeted Anti-Bcl-2 sFv Interacts with the Bcl-2 Protein in Mammalian Cells. We performed immunoprecipitation experiments to confirm the direct interaction of the anti-Bcl-2 sFv with Bcl-2 in MCF-7 cells. For these experiments, MCF-7 cells were transfected with either the anti-Bcl-2 sFv, a control sFv, or pSTCF.KDEL vector. The cells were lysed 48 h later, and the anti-Bcl-2 sFv/Bcl-2 protein complexes were immunoprecipitated by antibodies directed against the c-myc tag of the sFv protein. Bcl-2 expression was detected using a murine anti-Bcl-2 antibody. As shown in Fig. 6,

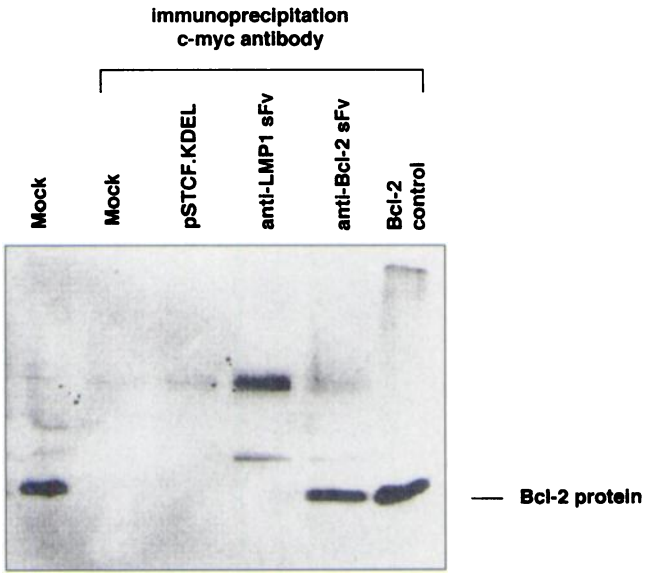

Fig. 6. Immunoprecipitation of Bcl-2 in MCF-7 cells transfected with either pSTCF.KDEL (control), anti-LMP1 sFv (control), or anti-Bcl-2 sFv. A cell lysate from MCF-7 transfected with no DNA (*Mock*) was used as control in the first lane. On the second part of the membrane, cell lysates from MCF-7 cells transfected with no DNA, pSTCF.KDEL, anti-LMP1 sFv, or anti-Bcl-2 sFv were immunoprecipitated with a mouse polyclonal anti-c-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and analyzed by electrophoresis on a 12% SDS-PAGE gel. The position of Bcl-2 is indicated.

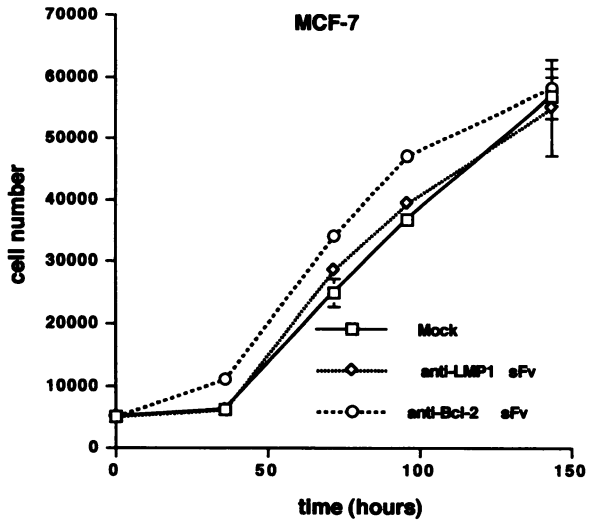

Fig. 7. Expression of the anti-Bcl-2 sFv does not affect the growth rate of MCF-7 cells. These cells were transfected with no plasmid (*Mock*), the anti-LMP1 sFv, or the anti-Bcl-2 sFv and followed over time. The growth rate was determined by MTS assay. Results from four samples are expressed as means; *bars*, SD.

Bcl-2 proteins were only detected in MCF-7 cells transduced with the anti-Bcl-2 sFv, indicating that a direct interaction between Bcl-2 and the anti-Bcl-2 sFv occurs in mammalian cells. These data also suggest that the interaction occurring between the anti-Bcl-2 sFv and Bcl-2 is specific. No Bcl-2 proteins were detected when MCF-7 cells were transduced with the control sFv.

Intracellular Expression of Anti-Bcl-2 sFv Increases Drug-induced Cytotoxicity in MCF-7 Cells. To examine the effects of sFv-induced reduction of Bcl-2 protein levels on the growth of human tumor cells, endogenously Bcl-2-overexpressing MCF-7 cells were transfected with the anti-Bcl-2 sFv or a control sFv, and cellular growth was measured by MTS assay at several times after transfection. The growth kinetics of these cells are presented in Fig. 7. No growth inhibition was observed in cells transduced with the anti-Bcl-2 sFv compared with controls. These results suggest that, in this transient assay system, inhibition of Bcl-2 protein expression does not significantly affect the growth of Bcl-2-overexpressing cells under normal growth conditions. This is consistent with recent data whereby modulation of Bcl-2 levels was reported to have no effect on the proliferation of epithelial tumor cells lines, whereas it did inhibit cell cycle entry by fibroblasts and lymphocytes (39).

Because Bcl-2 expression can modulate the sensitivity of cancer cells to drug-induced apoptosis, we next explored the effect of sFv-mediated reduction of Bcl-2 levels on the relative sensitivity of MCF-7 cells to CDDP- or staurosporin-induced cytotoxicity. Staurosporin is known to inhibit protein kinase activity and can induce apoptosis in essentially all types of normal and neoplastic cells (40). For this analysis, MCF-7 cells were transduced with the anti-Bcl-2 sFv or a control sFv using the AdpL method. One day later, the cells were trypsinized and plated onto 96-well plates, and media containing various concentrations of either CDDP or staurosporin were added the next day. As shown in Fig. 8*B*, MCF-7 cells transduced with the anti-Bcl-2 sFv were more susceptible to cell killing induced by staurosporin. The IC_{50} of the sFv control-transfected cells, estimated to be 160 ng/ml, was reduced approximately 6-fold (25 ng/ml) in cells transduced with the anti-Bcl-2 sFv. A similar effect was observed when MCF-7 cells were transfected with the anti-Bcl-2 sFv and treated with CDDP. In contrast, the percentage of cell killing did not significantly differ from mock-transfected cells when a sFv control was transduced in MCF-7 cells. In this experiment, we observed an approximately 4-fold reduction of the IC_{50} in cells transduced with the anti-Bcl-2 sFv (Fig. 8*A*). The increased drug-induced cytotoxicity mediated by the anti-Bcl-2 sFv was not related to cell growth inhibition because we determined that this sFv did not affect the growth kinetics of MCF-7 cells compared with controls (Fig. 7). Hence, it is clear that the anti-Bcl-2 sFv can reduce Bcl-2 protein levels in MCF-7 cells and that there is a concomitant augmentation of the cytotoxic action of CDDP and staurosporin.

DISCUSSION

Recent studies have shown that the Bcl-2 protein plays a central role in the apoptotic process induced by a wide variety of stimuli (2, 4). In this regard, Bcl-2 can heterodimerize with other members of the

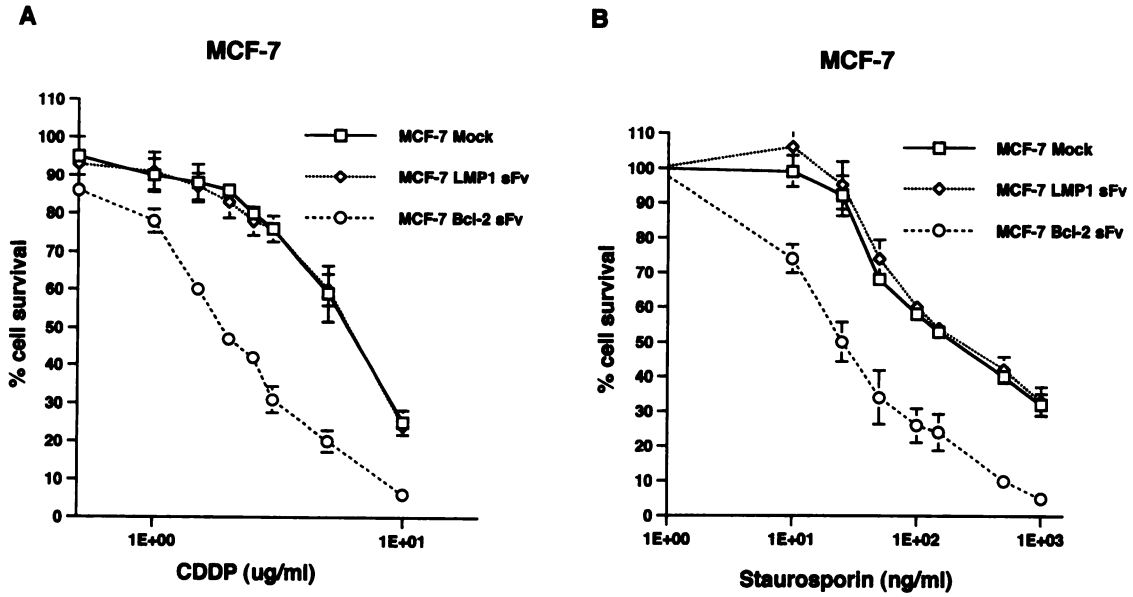

Fig. 8. Expression of the anti-Bcl-2 sFv increases drug-mediated cytotoxicity in MCF-7 cells. In *A*, MCF-7 cells were transduced with no DNA, the anti-LMP1 sFv (control), or the anti-Bcl-2 sFv and treated with various concentrations of CDDP. *B*, MCF-7 cells were transduced with no DNA, the anti-LMP1 sFv (control), or the anti-Bcl-2 sFv and treated with various concentrations of staurosporin. Cell killing was determined by MTS assay at 4 days. Results from four samples are expressed as means; *bars*, SD.

Bcl-2 protein family, such as Bax, and oppose their effects, leading to a prolongation of cell survival (3, 4). Overexpression of Bcl-2 correlates with increased cell protection from apoptosis-inducing anticancer drugs. Consistent with these results, a number of AS studies have demonstrated the potential of Bcl-2 down-modulation for achieving selected chemosensitization (14–23). These studies also underscore the central role of Bcl-2 overexpression in promoting drug resistance in human tumor cells.

Based on these considerations, we designed an approach to achieve functional knock-out of the Bcl-2 protein as a means to chemosensitize tumor cells to anticancer drugs. Using a method based on the intracellular expression of a single-chain antibody directed against the Bcl-2 protein, we have selectively reduced Bcl-2 protein levels in epithelial tumor cell lines. Although the epitope recognized by the anti-Bcl-2 sFv is located in the loop region of Bcl-2, which is cytosolic, we have shown that the sFv was able to directly interact with its target. Consistent with this observation, recent evidence suggests that cytosolic domains of a membrane protein do, in fact, transit in the lumen of the ER before being translocated in the cytosol (41). Thus, this physiology would allow the anti-Bcl-2 sFv to interact with newly synthesized Bcl-2 proteins and entrap them in the ER, thereby preventing their interaction with other proteins in the cytosol. The reason for the decrease in Bcl-2 protein levels is not clear. One possible explanation would be that the complex Bcl-2/Bcl-2 sFv, when sequestered in the ER, is degraded as the result of the mislocalization of the Bcl-2 protein. A similar effect has been observed in our laboratory with other sFvs targeted to the ER.

The anti-Bcl-2 sFv-mediated reduction in Bcl-2 levels enhanced the action of two cytotoxic drugs by 4–6-fold in MCF-7 cells. In addition, the chemosensitizing effect of the anti-Bcl-2 sFv does not appear to be a direct result on cell growth kinetics. Rather, it is probably related to increased apoptotic events in cells treated with the anti-Bcl-2 sFv. The sensitivity of MCF-7 cells to cancer drug may not depend solely on the expression of Bcl-2. Other proteins, such as p53 and Bax, may also affect the cellular response to apoptotic stimuli and therefore modulate drug-induced cytotoxicity. In addition, as shown in Fig. 6*A*, we did not achieve complete depletion of Bcl-2 protein in MCF-7 cells. A complete abrogation of Bcl-2 levels might have resulted in a more potent effect on drug-mediated cell death. Our findings are nevertheless consistent with other studies. For example, Teixeira *et al.* have demonstrated enhanced cytotoxicity to Adriamycin in MCF-7 cells expressing Bcl-2 antisense transcripts (13). Mandal and Kumar also demonstrated that the level of Bcl-2 expression regulates the butyrate-induced apoptosis in breast cancer cells (38). Moreover, the Bcl-2 antagonist protein Bcl-Xs has also been shown to promote chemotherapy-induced apoptosis in MCF-7 cells.

We have shown previously that sFvs can be used to enhance tumor cell chemosensitivity (42). For example, the erbB-2-overexpressing ovarian tumor cell line, SKOV3, was treated with an anti-erbB-2 sFv, CDDP, or a combination of these agents. In this analysis, we showed that intracellular expression of the anti-erbB-2 sFv or CDDP induced cytotoxicity, but a synergistic effect was noted when the two agents were used in combination (42).

The selective ablation of antiapoptotic protein, such as Bcl-2, via the sFv approach to increase the sensitivity of tumor cells to chemotherapeutic agents offers several advantages over the previous methods that have been used to modulate Bcl-2 expression: (*a*) sFvs can be delivered with high efficiency (*i.e.,* via AdpL or recombinant adenoviruses) to a wide variety of epithelial tumor cells, resulting in high levels of sFv protein expression; (*b*) like their parental antibodies, sFvs are selective for their targets. Thus, the likelihood of nonspecific effects is reduced compared with AS oligonucleotides, for example; and (*c*) sFvs have little or no effects on cells that do not overexpress the target protein.

In summary, the anti-Bcl-2 sFv has demonstrated functional and biological activity against Bcl-2 in epithelial tumor cells. This method represents a novel and efficient way to selectively abrogate the activity of the Bcl-2 protein, at least *in vitro*. We are presently exploring the efficacy of this approach in a xenograft model of ovarian cancer.

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Modulation of Bcl-2 Protein Levels by an Intracellular Anti-Bcl-2 Single-Chain Antibody Increases Drug-induced Cytotoxicity in the Breast Cancer Cell Line MCF-7

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