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Advances in Brief

Bcl-2 Inhibits T-Cell-mediated Cytolysis of a Leukemia Cell Line[1]

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Abstract

The *bcl-2* gene becomes dysregulated in its expression in a wide variety of human cancers and has been shown to block both spontaneous and drug-induced cell death, thus conferring a selective survival advantage on malignant cells. The biochemical mechanism by which *bcl-2* promotes cell survival remains enigmatic but appears to involve a downstream event in an evolutionarily conserved cell death pathway. Here we report that gene transfer-mediated increases in Bcl-2 protein levels in the human leukemia line Jurkat render these cells more resistant to induction of DNA fragmentation and cytolysis by a cloned T-cell. The killing mechanism used by these particular T-cells was consistent with apoptosis, as opposed to necrosis, in that DNA degradation occurred as a prelysis event. The findings raise the possibility that dysregulation of *bcl-2* gene expression could play a role in the avoidance of immune surveillance mechanisms by cancer cells.

Introduction

The *bcl-2* gene was first discovered because of its involvement in the t(14;18) chromosomal translocations that frequently occur in non-Hodgkin's B-cell lymphomas and which move the *bcl-2* gene from its normal location at 18q21 into a *cis*-configuration with strong enhancer elements associated with the immunoglobulin heavy-chain locus at 14q32 (1). High levels of Bcl-2 protein production have also been reported in a wide variety of human solid tumors and leukemias in the absence of translocations or other gross alterations in the structure of the *bcl-2* gene, including adenocarcinomas of the prostate and colon, small cell and non-small cell carcinomas of the lung, nasopharyngeal carcinomas, neuroblastomas, acute myelogenous leukemias, and chronic lymphocytic leukemias (for examples, see Refs. 2–6). The Bcl-2 protein has been shown to contribute to neoplastic cell expansion by blocking the normal physiological turnover of cells that occurs due to programmed cell death (reviewed in Ref. 7). In addition, gene transfer-mediated elevations in Bcl-2 protein levels have been shown to render leukemia and tumor cells relatively more resistant to induction of apoptosis by multiple types of chemotherapeutic drugs (8, 9). Bcl-2, therefore, may play a significant role, not only in the origins of cancer, but also in its treatment.

Yet another aspect of tumor biology that determines the potential for individual neoplastic clones to gain a selective growth advantage *in vivo* concerns the issue of the immune surveillance mechanisms that help to eradicate or keep in check abnormal cells in the body. In this regard, both humoral (antibody-mediated) and cellular responses can play a role in host defenses against cancer. Overproduction of the Bcl-2 protein would probably not be expected to protect malignant cells against antibody-based responses, since it does not prevent complement-mediated cell death (10) and does not prevent phagocytosis (11), as might occur, for example, when tumor cells are coated with antibody. The potential impact that Bcl-2 might have on cell-mediated immune responses, however, is more difficult to predict because of the multiple mechanisms that CTLs, NK cells, LAK cells, and tumor infiltrating lymphocytes have at their disposal for inducing target cell death (reviewed in Ref. 12) and because the predominant mechanism used appears to vary among different populations or clones of T-, NK, LAK, and tumor-infiltrating lymphocyte cells. In this regard, cell-mediated cytolysis can occur through mechanisms consistent with either apoptosis or necrosis, depending on the particular immune cell effectors and the target cells studied (reviewed in Ref. 13). For the most part, CTLs, NK, and related cells kill tumor targets through two separate processes: (*a*) secretion of cytotoxic granules which contain a number of potentially lethal molecules including perforin (a protein that resembles the C9 component of complement and that pokes holes in membranes), ATP (which can stimulate apopotosis in some types of cells probably via purinergic receptors), proteases (that trigger DNA fragmentation and apoptosis through a pathway which may require cdk kinases), and TIA-1 (an RNA-binding protein that induces apoptosis through poorly understood mechanisms); and (*b*) expression of genes encoding cytotoxic cytokines, such as TNFc-α, lymphotoxins, and the Fas ligand (14–18). With regards to the latter, gene transfer-mediated elevations in Bcl-2 protein levels have been shown to provide at least partial protection against apoptotic cell death induced by TNF-α and antibodies to Fas (19, 20), suggesting that Bcl-2 could potentially render tumor cells more resistant to CTL-induced killing in scenarios where local secretion of TNF-α or expression of transmembrane forms of TNF-α or Fas ligand play a predominant role in the cytolytic mechanism. On the other hand, if perforin-dependent mechanisms involving loss of osmotic equilibrium and necrotic cell death predominated, Bcl-2 would not be expected to provide resistance, by analogy to the lack of protection seen previously for complement-mediated lysis (10). In fact, it has been reported by others that Bcl-2 does not protect against cytolysis induced by at least some allospecific CTLs (10, 21). We show here, for the first time, however, that gene transfer-mediated elevations in Bcl-2 protein levels can render a human leukemia cell line relatively more resistant to DNA fragmentation and cytolysis induced by a cloned T-cell that induces cell death through mechanisms consistent with apoptosis.

Materials and Methods

Cells and Cell Culture. The cytolytic properties, immunophenotype, and maintenance in culture of the particular clone of IL-2-dependent CTLL-2 cytolytic T-cells and various transfectants thereof have been described in detail previously (22). CTLL-N-LCK and CTLL-A-LCK cells represent transfectants that received a G418-resistant plasmid in combination with expression plasmids producing either normal p56-Lck or an "activated" version of Lck that contains a tyrosine→phenylalanine substitution at position 505.

Jurkat-NEO and Jurkat-BCL-2 cells are polyclonal populations of G418-resistant Jurkat T-cell leukemia cells that were subjected to electroporation

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(960 μF; 625 V/cm) with 25 μg of *Nde*I-linearized pZIP-NEO or pZIP-BCL-2 plasmid DNA (8), followed by selection in medium containing 0.9 mg/ml active G418. Relative levels of human Bcl-2 protein were assessed by immunoblot assay using specific antisera as described previously (5).

Cytolytic Assays. ^{51}Cr release assays were performed as described previously (22). Briefly, 2–6 × 10^6 target cells were labeled with 150 μCi ^{51}Cr in ~0.4 ml of complete medium (RPMI with 10% fetal bovine serum, 1 mM L-glutamine, 100 units/ml penicillin, and 50 μg/ml streptomycin) for 1.5 h at 37°C, then washed three× with HBSS (GIBCO-BRL, Inc.) and resuspended in 1 ml of complete medium for 0.5 h at room temperature to allow for spontaneous release prior to washing once with complete medium and resuspending at 10^5 cells per ml. T-cells were stripped of IL-2 by incubation for 0.5–1 min in 1 ml of 10 mM sodium citrate (pH 4.0) 140 mM NaCl, followed immediately by dilution into 50 ml of HBSS. After 2 additional washes in HBSS, T-cells were resuspended in complete medium at 10^6 cells per ml with various concentrations (0–100 unit/ml) of purified recombinant IL-2 (a gift of Chiron, Inc.). Various numbers of T-cells (10^4, 5 × 10^4, 10^5, 2 × 10^5, and 4 × 10^5) in 0.1 ml were added to round-bottomed wells of 96-well microtiter plates and incubated at 37° in 5% CO^2 for 4 h to allow for dissipation of IL-2-generated signals in IL-2-deprived T-cells. ^{51}Cr-labeled target cells (10^4 in 0.1 ml) were then added, and after 4 h, ^{51}Cr release into culture supernatants was measured for triplicate samples. The percentage of specific lysis was calculated relative to the total amount of ^{51}Cr-released by treatment with 1% Nonidet P-40 after substraction of spontaneous release from tumor targets incubated without T-cells as described (22).

DNA Fragmentation Assays. Quantitative assays for DNA fragmentation were performed essentially as described by Duke *et al.* (23). Briefly, 2 × 10^6 target cells in 3 ml of complete medium were cultured overnight with 30 μCi of ^{125}I-dUR (ICN Radiochemicals, Inc.), washed twice with HBSS, and once with RPMI 1640 and then resuspended at 2.5 × 10^5 cells/ml in complete medium. Effector T-cells were then combined with ^{125}I-dUR-labeled targets as described above for various times (0–4 h) at an effector:target ratio of 10. Cells were recovered from microwells, pelleted by centrifugation in Eppendorf microcentrifuge tubes, and lysed in phosphate-buffered saline containing 10 mM EDTA-1% Triton X-100 on ice for 10 min. After centrifugation at 16,000 × *g* for 2 min, the resulting pellets and supernatants were separately counted. All assays were performed in quadruplicate, and the data were expressed as mean ± SD.

Results

Establishment of a Model Cytolytic T-Cell/Tumor Target System. CTLL-2 is an IL-2-dependent cytolytic T-cell line that was originally established in culture from mice immunized with allogeneic tumor cells and which displays major histocompatibility complex-nonrestricted cytolytic activity against allogenic and syngeneic tumor cells (24). This murine T-cell line thus has cytolytic properties similar to LAK cells (25) and also can lyse some xenogeneic tumor targets, including the T-cell leukemia line Jurkat (22). Relative to early passage cultures, currently available CTLL-2 cells have lost much of their cytolytic activity, a phenomenon that commonly occurs among LAK cells when maintained for long periods of time in culture (26). During our previous studies of CTLL-2 cells, we discovered that these T-cells contain relatively low levels of the protein tyrosine kinase p56-Lck compared to freshly isolated T-cells. Furthermore, we demonstrated that IL-2 induces rapid and concentration-dependent increases in the specific activity of Lck kinase in these T-cells, and we showed that gene transfer-mediated elevations in Lck kinase activity markedly increase their cytolytic effector function without influencing their dependence on IL-2 for proliferation and survival (22). For this reason, CTLL-2 cells, in which p56-Lck was restored by gene transfer, were used for most studies.

Fig. 1 shows the cytolytic response of parental CTLL-2 as well as CTLL-N-LCK and CTLL-A-LCK cells to Jurkat leukemia cells. For

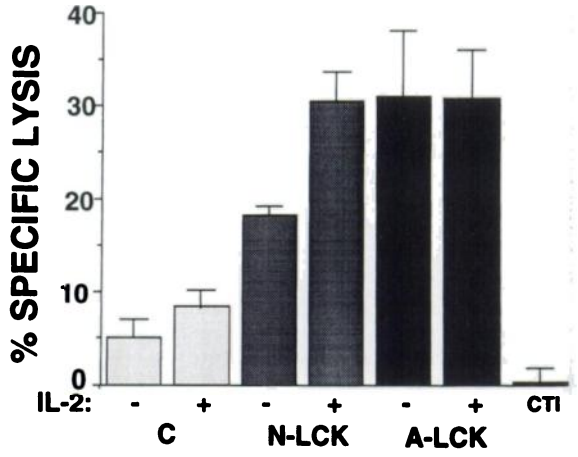

Fig. 1. Characterization of cytolytic effector activity of parental and *lck*-transfected CTLL-2 cells. *C*, parental CTLL-2 cells (for control); *N-LCK*, normal *lck* (Y505)-transfected CTLL-2 cells, and *A-LCK*, activated lck (F505)-transfected CTLL-2 cells were incubated with ^{51}Cr-labeled Jurkat target cells at an effector:target ratio of 10 for 4 h in the presence (+) or absence (−) of 100 units/ml of recombinant IL-2. Cold-target inhibition assays were performed by adding a 10-fold excess of unlabeled Jurkat cells to the wells. In other experiments (data not shown), the addition of a 10-fold excess of the non-target lines WEHI-164 or P815 caused only 28 and 32%, respectively, inhibition of CTLL-2-mediated lysis.

these experiments target cells were loaded with ^{51}Cr and mixed with effector cells at an effector:target ratio of 10. Specific ^{51}Cr-release was then measured in standard short-term (4 h) cytolytic assays. As shown, parental CTLL-2, and genetically modified CTLL-N-LCK and CTLL-A-LCK cells all induced lysis of Jurkat leukemia cells, but the percentage of tumor cell lysis was significantly higher for the *lck*-transfected CTLL-2 cells. CTLL-A-LCK cells, which contain a constitutively activated form of Lck kinase, also displayed less dependence on IL-2 for lysis of Jurkat cells. The lysis of Jurkat cells by these cytolytic T-cells was largely dependent on cell-cell contact as shown by cold-target inhibition experiments (Fig. 1) but was independent of protein synthesis in either the T-cells or the tumor targets (Ref. 22 and data not shown).

Bcl-2 Partially Protects Jurkat Target Cells from T-Cell-mediated Cytolysis. To explore the effects of Bcl-2 on cell death induced by CTLL-2 cytolytic T-cells, we stably transfected Jurkat leukemia cells with either a Bcl-2-encoding or control (NEO) expression plasmid, thus establishing the lines Jurkat-BCL-2 and Jurkat-NEO. Immunoblot analysis of lysates prepared from these cells revealed markedly elevated levels of Bcl-2 protein in Jurkat-BCL-2 cells relative to Jurkat-NEO cells (Fig. 2). This level of Bcl-2 protein was comparable to that found in lymphoma cell lines that contain a t(14;18) translocation involving *bcl-2* (data not shown).

Next, the cytolytic activity of parental CTLL-2, CTLL-N-LCK, and CTLL-A-LCK cells against Jurkat-BCL-2 and Jurkat-NEO cells was compared in ^{51}Cr release assays. Regardless of their Lck status, CTLL-2 cells were able to kill Jurkat-BCL-2 cells only about one-half as efficiently as Jurkat-NEO cells (Fig. 3). Thus, the cytolytic mechanism by which CTLL-2 cells kill Jurkat leukemia cells is at least partially repressible by overproducing Bcl-2 protein in these neoplastic target cells.

Bcl-2 Partially Abrogates CTL-mediated DNA Fragmentation. One of the hallmarks of apoptotic cell death is the activation of endonucleases that digest the genomic DNA. This genomic digestion occurs early in the process of apoptotic cell death, before cell lysis, and has been described in the setting of CTL-induced death of target cells (23). In contrast, DNA degradation typically occurs as a postlysis event when necrosis is the mechanism involved (27). To determine whether killing mediated by CTLL-2 cells involved induction of DNA

[5] The abbreviations used are: CTL, cytolytic T-cells; NK, natural killer; LAK, lymphokine-activated killer; TNF, tumor necrosis factor; HBSS, Hanks' buffered salt solution; IL-2, interleukin-2; ^{125}I-dUR; ^{125}I-deoxyuridine.

degradation, and if such DNA degradation occurred as a pre- or postlysis event, the DNA of tumor target cells was metabolically labeled with ^{125}I-dUR. Release of the ^{125}I-dUR label into the soluble fraction was then measured at various times after mixing target cells with effector T-cells. In parallel, ^{51}Cr released from target cells was monitored as an indicator of cell lysis. As shown in Fig. 4, DNA degradation reached maximal levels within 2 h of combining ^{125}I-dUR-labeled target cells with CTLL-A-LCK cells. In contrast, ^{51}Cr release did not reach maximal levels until at least 4 h. Consistent with the data presented in Fig. 3 above, Jurkat-BCL2 cells were more resistant to both cell lysis and induction of DNA degradation than Jurkat-NEO cells, demonstrating the protective effect of the Bcl-2 protein. Gel-electrophoretic analysis of DNA recovered from ^{125}I-dUR-labeled target cells after exposure to cytolytic T-cells revealed a "ladder" of bands representing integer multiples of the internucleosomal DNA distance (~180 base pair), thus providing further evidence that death was consistent with an apoptotic mechanism (data not shown). Incubation of ^{125}I-dUR-labeled P815 cells (which are not lysed by CTLL-2 cells; Ref. 22), with CTLL-N-LCK and CTLL-A-LCK cells failed to result in the release of ^{125}I-label into the soluble fraction or production of a DNA ladder (data not shown), thus confirming the specificity of the results.

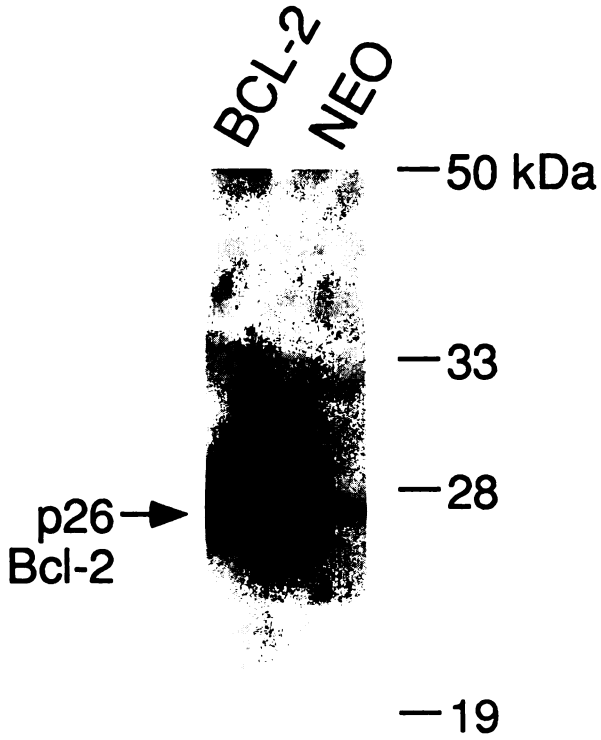

Fig. 2. Immunoblot analysis of Jurkat-BCL-2 and Jurkat-NEO cells. Detergent (1% Triton X-100) extracts were prepared from equal numbers of Jurkat T-cells that had been stably transfected with *bcl-2* expression plasmid (*BCL-2*) or vector only (*NEO*). The relative levels of p26-Bcl-2 protein were compared by immunoblot assay using antisera specific for the human Bcl-2 protein as described (5).

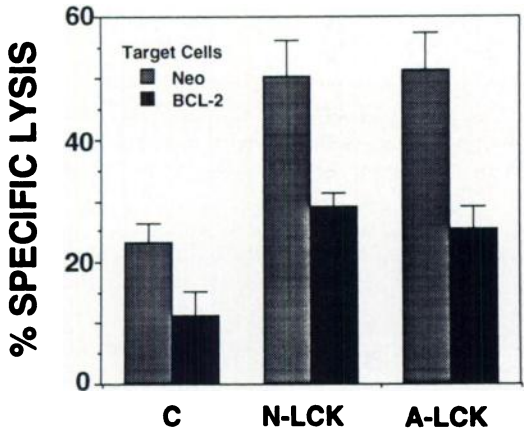

Fig. 3. Bcl-2 protects Jurkat cells from cytolysis induced by CTLL-2 cells. Parental CTLL-2 cells (*C*), *lck* (Y505)-transfected, CTLL-2 cells (*N-LCK*), and *lck* (F505)-transfected CTLL-2 cells (*A-LCK*), were cultured with ^{51}Cr-labeled Jurkat-*BCL-2* (*black bars*) or Jurkat-*NEO* (*shaded bars*) cells at an effector:target ratio of 10 in the presence of 200 units/ml of recombinant IL-2. ^{51}Cr release was measured 4 h later. Data, mean percentage of specific lysis for triplicate determinations; *bars*, SD.

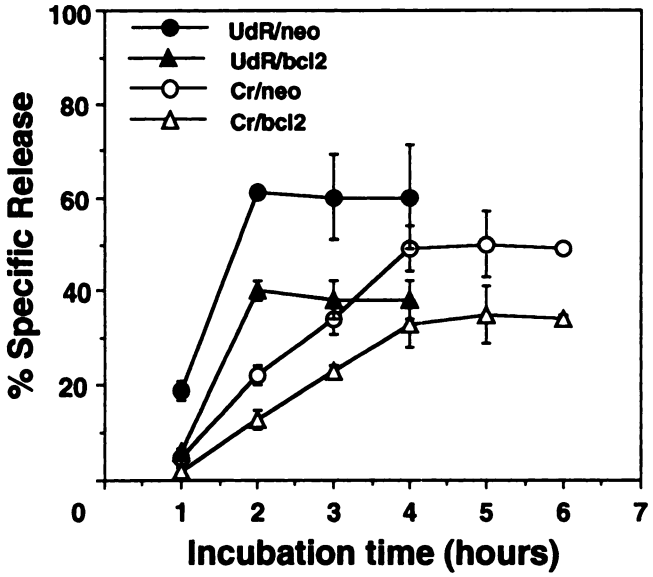

Fig. 4. Bcl-2 protects Jurkat cells from CTLL-2-induced DNA fragmentation. The time course of ^{125}I-dUR and the ^{51}Cr release from labeled target cells were compared. Jurkat-BCL-2 (▲, △) and Jurkat-NEO (●, ○) cells labeled with ^{125}I-dUR and ^{51}Cr were incubated with CTLL-A-LCK cells at an effector:target ratio of 10 in the presence of 100 units/ml of recombinant IL-2. At various times thereafter, release of ^{125}I (●, ▲) and ^{51}Cr (○, △) into the supernatant fraction was measured, and the percentage of specific release was calculated. Data, mean of at least three determinations; *bars*, SD.

Discussion

The cell death mechanism used by CTLL-2 cells to lyse tumor target cells involved induction of DNA degradation as a prelysis phenomenon, consistent with apoptosis as opposed to necrosis. Microscopic examination also revealed that the target cells underwent cell shrinkage as opposed to swelling, again consistent with an apoptotic mechanism (data not shown). CTLs, NK, and LAK cells, have been reported to induce cell death through both necrotic and apoptotic mechanisms (12, 13, 23, 27). The particular cell death mechanism involved, apoptosis *versus* necrosis, probably varies, depending on the relative amounts of various cytotoxins produce by any particular lymphocyte cell clone. Perforin secretion by CTLs, for example, would be expected to induce death through a necrotic mechanism by creating holes in the plasma membrane of target cells and disrupting their osmotic equilibrium. However, it is also known that some cytolytic cells secrete only small amounts of perforin and that perforin-induced holes in membranes can be transient (28), such that insufficient membrane damage is produced for necrosis. In this case, adequate perforin may be secreted, nevertheless, to allow for transient passage into the target cells of proteases and TIA-1, which are stored in cytotoxic granules with perforin and which are dependent on perforin or perforin-like proteins to gain access to the cytosol of target cells and thus exert their apoptosis-inducing effects. Alternatively, the CTLL-2 cells may use mechanisms for induction of cell death that do

not depend on the injection of molecules across the plasma membrane of the target cell, such as those involving ATP release or interactions of plasma membrane-associated TNF-α, lymphotoxin, and Fas ligand with their specific receptors on target cells. In this regard, we have shown previously that CTLL-2 cells contain mRNA for perforin, granzyme A, TIA-1, TNF-α, and lymphotoxin α, and that they express on their cell surface TNF-α and Fas ligand (22). Thus, these cytolytic cells have a number of potential mechanisms at their disposal for inducing cell death in tumor targets. Furthermore, the data presented here indicate that at least some of these mechanisms can be blocked by overproduction of Bcl-2 in the target cell. Indeed, examination of the effects of some of these and other potential initiators of cell death, including anti-Fas antibodies and ATP, has revealed markedly greater resistance of Jurkat-BCL-2 cells relative to Jurkat-NEO cells.[6]

The finding that Bcl-2 protein can partially abrogate DNA fragmentation and cell death induced by CTLL-2 cells differs from previous reports that have made contrary claims about the ability of Bcl-2 to block cytolysis induced by other CTLs (10, 21). Probably this discrepancy can be attributed to the use of different effector CTLs by other investigators which induce target cell death, predominantly through *bcl-2*-independent pathways. Still another variable in determining whether Bcl-2 is protective, however, is the status of the tumor target cells, whose relative content and state of activation of Bcl-2 partner proteins and dominant inhibitors may vary (reviewed in Ref. 7). As just one example of potential relevance to CTL-mediated lysis, for instance, Bcl-2 has been shown to protect some types of cells from TNF-α-induced cell death but not others (19, 29). Thus, the cellular background on which the Bcl-2 protein is produced may have a significant impact on whether it provides protection from CTL-mediated killing. Given that Bcl-2 protein is found at relatively high levels in a variety of types of human cancer, it is intriguing to speculate that this apoptosis-blocking oncoprotein could play a role in tumor avoidance of immune surveillance mechanisms or that it could thwart efforts to clinically apply adoptive immunotherapy strategies. Further experiments comparing *bcl-2* and control-transfected tumor lines in animal models are required, however, to determine the relative importance of Bcl-2 in tumor avoidance of immune effector mechanism *in vivo*.

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