Epigenetic Determinants of Resistance to Etoposide Regulation of Bcl-xL and Bax by Tumor Microenvironmental Factors

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Background: Epigenetic factors (i.e., alterations of gene activity not involving mutations), as well as genetic changes in surviving cancer cells, may play an important role in drug resistance following cancer chemotherapy—a common cause of tumor relapse. Bcl-2 family proteins are central to the regulation of apoptotic cell death and modulate drug sensitivity. We investigated how survival signals in the cellular microenvironment affect the expression, protein conformation, and protein–protein interactions of the Bcl-2 family proteins Bax and Bcl-xL and how changes in response to microenvironmental signals alter the response of cancer cells to the drug etoposide. Methods: JLP119 human B-lymphoma cells were treated with etoposide (40 μM) and then cultured in the presence of an activating anti-CD40 antibody, vascular cellular adhesion molecule-1 (VCAM-1)—to activate VLA-4 (α4β1) integrin, and interleukin 4. Cell fate was monitored after etoposide treatment with or without these microenvironmental signals. Bcl-xL gene transcription and protein levels of Bcl-xL and Bax were measured by northern and western blotting, respectively. Nuclear translocation of transcription factor NF-κB was monitored by immunofluorescence and inhibited by (E)-capsaicin. Bax conformation and Bax–Bcl-xL interactions were monitored by immunofluorescence and immunoprecipitation, respectively. Results: Microenvironmental survival signals produced statistically significant reductions in etoposide-induced apoptotic cell death, from 84.6% (95% confidence interval [CI] = 76.7%–92.4%) to 21.3% (95% CI = 19.5%–23.0%); P < 0.001. Activation of surface protein CD40 increased Bcl-xL protein levels via an (E)-capsaicin-inhibitable activation of NF-κB; i.e., (E)-capsaicin restored etoposide sensitivity. Interleukin 4 had no effect on Bcl-xL protein levels but accelerated the increase in Bcl-xL protein associated with CD40 activation. VCAM-1- and interleukin 4-mediated signals diminished conformational changes in Bax protein and prevented the etoposide-induced disruption of constitutive Bax–Bcl-xL binding. Conclusions: Microenvironmental factors reduce the sensitivity of a B-cell lymphoma to etoposide in vitro by modulating the expression and functions of Bax and Bcl-xL. This interaction may provide a paradigm for epigenetically induced drug resistance in other tumors. [J Natl Cancer Inst 2000;92:18–23]

Residual disease following cancer chemotherapy is a significant clinical problem. Undetectable subpopulations of tumor cells that survive drug treatment consequently lead to relapse. It is generally considered that this subpopulation of drug-resistant cells arises from genetic changes that either occur spontaneously, often because of inherent genetic instability in cancer cells, or as a result of the clastogenic nature of some therapies (1,2). However, continued cell survival following cytotoxic therapy need not result only from classically defined drug resistance mechanisms but may also be the consequence of changes in the survival potential of a tumor cell (3). Cell survival potential—in this case, the ability to decrease the probability of apoptotic cell death after drug-induced cell damage—is influenced by signals generated by soluble extracellular factors, interactions with the extracellular matrix, and cell–cell contacts (4). The resulting intracellular signaling pathways then modulate the activities of central regulators of survival, such as Bad and other members of the Bcl-2 family (5,6). Together, these survival-promoting mechanisms can prevent drug-induced cell death, resulting in pleiotropic drug resistance.

We considered that residual disease might be the result of subpopulations of tumor cells being rendered resistant to cytotoxic drugs because they are located within a microenvironment that promotes their survival. This is, essentially, an epigenetic mechanism of drug resistance. It would permit surviving cells to repopulate the host either without further genetic changes or with gene mutations because of inherent genetic instability and/or instability resulting from the DNA-damaging therapy that they received. In recurrent follicular lymphoma, for example, patient relapse is initially associated with tumors that continue to respond to therapy before the occurrence of critical genetic changes and transformation into an aggressive, drug-resistant form of the disease (7,8). As a paradigm of this novel type of drug resistance, the drug sensitivity of B-lymphoma cells was determined in suspension or in a microenvironment in vitro that mimics aspects of secondary lymphoid tissue such as the germinal center. In the germinal center, B-lymphoma cells would be exposed to signals elicited by cell–cell contact with infiltrating T cells and with follicular dendritic cells (FDCs) (9–12). The signaling pathways would include those generated by the activation of the B-cell surface molecule CD40, the ligation of the interleukin 4 (IL-4) receptor, and the activation of the integrin receptor VLA-4. After antiproliferative cytotoxic therapy, some of the normal cells that provide these survival signals may be...
come heterogeneously distributed within the germinal center. However, quiescent cells such as FDCs would be predicted to withstand chemotherapy, remaining in sufficient numbers to protect a subpopulation of lymphoma cells in what we term a "survival niche."

**METHODS**

**Antibodies and reagents.** Bcl-2 was detected with monoclonal antibody (MAb) 124 (Dako, High Wycombe, U.K.), Bak with MAb TC-100 (Calbiochem, Cambridge, MA), and Bcl-xL with a polyclonal antibody (Transduction Laboratories, Lexington, KY). The antibodies to Bax were used were human specific polyclonal antibody (PharMingen, San Diego, CA) for western blotting and N-terminal epitope-specific anti-Bax Mabs YTH-6A7 for immunoprecipitation and YTH-SB7 for flow cytometry (Trevesgen Inc., Gaithersburg, MD). All primary antibodies were detected on western blots with horseradish peroxidase-conjugated secondary antibodies (Dako) and were visualized with Enhanced Chemiluminescence (Amersham, Little Chalfont, U.K.). Anti-Bax antibody was detected by use of a goat anti-mouse fluoroisothiocyanate (FITC)-conjugated secondary antibody (Dako) in flow cytometric studies. Transcription factor NF-kB was detected by use of MAb G96-337 (PharMingen) and an indocarbocyanine (Cy-3)-conjugated donkey anti-mouse secondary (Jackson Immunoresearch Laboratories, Westgrove, PA). Anti-CD40 antibody M3 was from Genzyme (Cambridge, MA). A goat anti-mouse immunoglobulin G (IgG) used to immobilize anti-CD40 antibody was from Dako. Actin was detected by use of mouse ascites fluid containing anti-actin antibody (Sigma, Poole, U.K.). IgG1 mouse anti-*Aspergillus niger* antibody raised to glucose oxidase (Dako). Etoposide was from Sigma, and (E)-capsaicin was from Calbiochem. All other reagents were from Sigma.

**Cell culture.** The human Burkitt’s lymphoma cell line JLP119 was from K. Bhatia (National Institutes of Health, Bethesda, MD). Cells were maintained in RPMI-1640 medium with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C. VCAM-1/Fc was from M. Humphries (Manchester University, U.K.), and the conditioned medium used in selected experiments to activate CD40 produced by the G28.5 hydridoma was supplied by J. Norton (Paterson Institute, Manchester, U.K.). Recombinant human IL-4 was from Genzyme. All other consumables for cell culture were from Life Technologies, Inc. (GIBCO BRL) (Paisley, Scotland, U.K.). Cells were plated at a density of 2 x 10⁵ cells/mL unless stated otherwise.

**Suppression of etoposide-induced apoptotic cell death by survival signals.** Cells were cultured without fibroblast feeder layers as described previously (12) following a 1-hour exposure to etoposide (40 μM) or drug vehicle (0.2% vol/vol dimethyl sulfoxide) (Fig. 1. A). In some experiments, anti-mouse IgG was omitted and anti-CD40 antibody was, therefore, not immobilized but remained soluble in the culture media. Cells were then resuspended in drug-free media in suspension culture or in the presence of survival signals (Fig. 1. A). The percentage of cells with apoptotic nuclear morphology was determined (13) at 24, 48, and 72 hours after drug addition.

**Effects of etoposide and survival signals on members of the Bcl-2 family.** Western blotting was performed by use of standard methods (14), and 20 μg of protein per sample was loaded onto a 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Equal protein loading was assessed by comparison with actin levels. Immunofluorescence associated with the N-terminal epitope-specific anti-Bax MAb YTH-6A7 for immunoprecipitation (RT–PCR) kit (kit #200420; Stratagene Ltd., Cambridge, U.K.). PCR was then performed with primers designed to amplify the region between base pairs 234–489. The negative control sample did not contain a cDNA template, and the positive control was the pSBS75 plasmid containing the bcl-xL gene (from S. Cosulich, Zeneca Central Toxicology Laboratory, Macclesfield, U.K.).

**Inhibition of NF-kB by (E)-capsaicin.** Cells were placed in six-well plates (1 x 10⁵ cells/well) and treated for 1 hour with (E)-capsaicin (200 μM) or vehicle control (0.1% vol/vol ethanol). Cells were then stimulated with 10% G28.5 hydridoma-conditioned medium containing anti-CD40 antibody that was immobilized on goat anti-mouse IgG, VCAM-1/Fc, and IL-4. Samples were taken at 0, 1, 3, 6, and 24 hours for RT–PCR and western blotting for Bcl-xL, and for...
analysis of NF-κB cellular location by fluorescence microscopy. To assess the effect of (E)-capsaicin on CD40-mediated resistance to etoposide, culture conditions were as above but excluded VCAM-1/FC and IL-4.

Analysis of NF-κB subcellular localization by fluorescence microscopy. Control cells and those stimulated by all three survival signals were cytopun (5 × 10^6 cells per slide), fixed, and permeabilized in 1:1 vol/vol methanol/acetone at room temperature, air-dried, and stained by use of MAB G96-337 to NF-κB in 0.1% fetal calf serum/PBS for 1 hour at room temperature. The slides were washed and incubated with Cy3-conjugated donkey anti-mouse immunoglobulin M secondary antibody for 1 hour at room temperature in the dark. The slides were washed, and the nuclei were counterstained with Hoechst 33528 (Molecular Probes Inc., Eugene, OR) at 10 μg/mL for 1 minute. Slides were mounted by use of Vectorshield (Vector Laboratories, Inc., Burlingame, CA). Images were obtained with the use of an Axioskop fluorescent microscope (Zeiss; Welwyn Garden City, Herts, U.K.) and the Openlab software package (Improvision, Coventry, U.K.).

Statistical analysis. All statistical evaluations were two-tailed Student’s t tests at the P = .05 level of significance. Analyses were performed with the use of the Microsoft Excel package (version 5.0; Microsoft Corporation, Seattle, WA).

RESULTS

Effect of Single or Multiple Microenvironment-Derived Signals on Etoposide-Induced Apoptotic Cell Death and Growth Arrest

JLP119 Burkitt’s lymphoma cells were treated with etoposide (40 μM for 1 hour) and then maintained for 72 hours in the absence (suspenion culture) or in the presence of signals generated by an immobilized (or soluble) anti-CD40 antibody, IL-4, and a VCAM-1 fusion protein (VCAM-1/Fc). These culture conditions simulate some of the events that would result from B-cell contact with infiltrating T cells and FDCs. When placed in this in vitro survival niche (Fig. 1, A), cells formed tight clusters and adhered to the culture vessel.

After 72 hours of etoposide treatment, 21.3% (95% confidence interval [CI] = 19.5%–23.0%) of the cells in the survival niche exhibited apoptotic morphology in contrast to 84.6% (95% CI = 76.7%–92.4%) of the cells in suspension culture (Fig. 1, B), a statistically significant difference (P < .001). To ascertain the contribution of each survival signal to drug resistance and to compare the effects of the applied survival ligands—individually or in combination—on members of the Bcl-2 family of proteins (see below), cells were treated with etoposide and then provided with only one of the three survival signals. At 72 hours, when maximal etoposide-induced apoptotic cell death occurred in suspension culture (84.6%), VCAM-1/Fc, IL-4, or immobilized anti-CD40 antibody each were individually able to suppress apoptotic cell death induced by etoposide to approximately the same degree, from 84.6% apoptotic cell death down to 35.7% (95% CI = 29.5%–41.8%), 29.3% (95% CI = 23.2%–35.5%), or 32.2% (95% CI = 29.6%–34.8%), respectively. However, the effect of the three signals combined was statistically greater than that observed with any signal delivered alone; comparisons of the suppression of etoposide-induced apoptotic cell death by VCAM-1/Fc, IL-4, or immobilized anti-CD40 antibody with that produced by all three signals combined gave P values of .01, .08, and .002, respectively. Cell stimulation with a soluble anti-CD40 antibody that did not promote cell attachment to plastic suppressed etoposide-induced apoptotic cell death to the same extent observed as when the antibody was immobilized (Fig. 1, C: 31.4%; 95% CI = 19.1%–43.6%). This finding demonstrates that antibody-mediated cell attachment to plastic per se did not cause cell survival after etoposide treat-

ment. All of the subsequent experiments were conducted with the immobilized anti-CD40 antibody.

Effect of Survival Signals on Protein Levels of Bcl-2 Protein Family Members

When cells were cultured with all three survival signals for 72 hours, there was no change in Bcl-2, Bax, or Bak protein levels by western blotting, but the level of Bcl-xL was increased at 3 hours and remained elevated (Fig. 2). The CD40 signal alone increased the expression of Bcl-xL, but this was not observed until 24 hours (Fig. 2, B). Although IL-4 stimulation alone resulted in drug resistance (see above), it did not increase Bcl-xL levels (data not shown). However, when cells were cultured with anti-CD40 antibody and IL-4, a more rapid increase in Bcl-xL level was detected (at 3 hours) (Fig. 2, B), exemplifying how microenvironmental survival signals combine to modulate cell survival.

Role of NF-κB in CD40-Mediated Increase in bcl-xL and Drug Resistance

CD40 signaling has been previously shown to activate NF-κB (16), and this was confirmed in JLP119 cells (Fig. 3, A, left-side panels). To determine the relationship between nuclear translocation of NFκB, the increase in Bcl-xL protein level, and resistance to etoposide, cells were pretreated for 1 hour with (E)-capsaicin (200 μM) before culture with the survival signals. (E)-Capsaicin prevents NF-κB-DNA binding (17) and blocked the survival signaling-mediated nuclear translocation of NFκB (Fig. 3, A, right-side panels). Furthermore, (E)-capsaicin completely prevented both the survival signaling-mediated increase in bcl-xL mRNA (Fig. 3, B) and Bcl-xL protein levels (Fig. 3, C). Moreover, Fig. 3, D, shows that treatment with (E)-capsaicin resulted in a statistically significant increase in etoposide-
induced apoptotic cell death in the presence of the CD40 signal (P < .001).

**Regulation of Bax Conformation by IL-4 and VCAM-1 Signaling**

Cells stimulated with VCAM-1/Fc and/or IL-4 were afforded protection from etoposide-induced apoptotic cell death in the absence of increased Bcl-xL protein expression (Fig. 1, C). We asked whether the signals generated by IL-4 and VCAM-1 might act to modulate pro-apoptotic members of the Bcl-2 protein family. Neither etoposide nor VCAM-1/Fc and IL-4 affect the protein levels of Bax (Fig. 4, A, inset); however, on the basis of a recent report by Hsu and Youle (15) showing that the N terminus of Bax is conformationally labile and on our own work on Bak (18), we asked whether etoposide might alter Bax conformation and whether this was modulated by survival signals. The availability of an N-terminal epitope of Bax was examined at 24 hours in fixed, intact cells by flow cytometry. This epitope was cryptic in cells cultured in suspension, and it remained so when cells were stimulated with VCAM-1/Fc and IL-4. Exposure of suspension-grown cells to etoposide resulted in the unmasking of the N-terminal Bax epitope to generate immunofluorescence (Fig. 4, A). This etoposide-induced Bax immunofluorescence, representing the exposure of an otherwise cryptic epitope of Bax, was statistically significantly reduced in cells that received survival signals mediated by IL-4 and VCAM-1/Fc interaction (P = .03; Fig. 4, A and B).

**Effect of Etoposide and Survival Signals on the Binding of Bax to Bcl-xL**

The exposure of an otherwise cryptic epitope of Bax detected by flow cytometry suggests either a drug-induced conformational change in Bax and/or a change in the binding of one or more proteins to Bax. Bax–Bcl-xL protein binding was examined by co-immunoprecipitation at 24 hours in untreated suspension cells, in suspension cells treated with etoposide, in cells stimu-
was immunoprecipitated with the anti-Bax antibody YTH-6A7 (15).

A specific anti-Bax antibody was observed between the cell populations marked by the percentage of cells staining positively with the use of the N-terminal epitope of Bax or Bcl-xL (21). Flow cytometric data were generated from aliquots of the cell samples used to generate the accompanying flow cytometry analysis. The drug-induced decrease in Bax–Bcl-xL protein binding observed in cell suspensions was completely prevented by the addition of VCAM-1/Fc and IL-4.

**DISCUSSION**

We have sought to determine, by use of a human B-cell lymphoma as a model, whether survival signals provided to tumor cells within their microenvironment could contribute to etoposide resistance. We investigated the effect of signaling molecules derived from the microenvironment on members of the Bcl-2 protein family, which are key intracellular determinants of cell survival, and on resistance to etoposide. The combination of IL-4 receptor ligation, antibody-mediated activation of CD40, and stimulation of the VLA-4 integrin receptor yielded more surviving lymphoma cells after cytotoxic drug treatment than any of these signals delivered alone. Immobilized anti-CD40 antibody induced the cells to attach to plastic and cluster in vitro, and this change in cell adhesion may contribute to observed CD40-mediated suppression of etoposide-induced apoptotic cell death. Anti-CD40 antibody stimulation of B-lymphoma cells has been shown to promote homotypic adhesion via the binding of ICAM-1 to its receptor LFA-1 (19). However, soluble anti-CD40 antibody similarly suppressed etoposide-induced apoptotic cell death, which suggests that the CD40 signal promoted etoposide resistance in the absence of cell adhesion to plastic.

CD40 signaling elevated the mRNA and protein levels of Bcl-xL, in agreement with previous studies (20,21) (Fig. 2, B). In M12 or Daudi B-lymphoma cells, CD40 signaling activated the transcription factor NF-κB (16). In the JLP119 B lymphoma cells used here, the effect of CD40 on Bcl-xL was mediated via this transcription factor (Fig. 3). These data link a transcription factor that is known to modulate apoptotic cell death [i.e., following tumor necrosis factor-α (TNF-α) stimulation (22)] to the increased level of an established antiapoptotic member of the Bcl-2 family. Recently, TNF-α-mediated activation of NF-κB was also shown to drive the increase in Bcl-2 and Bcl-xL protein expression in primary hippocampal neurons (23). In JLP119 B-lymphoma cells, NF-κB may orchestrate cell survival after drug damage by use of both Bcl-xL-independent and -dependent pathways, all of which would be inhibited by the prevention of NF-κB–DNA binding by (E)-capsaicin. However, because both VCAM-1 and IL-4 alone resulted in resistance to etoposide without increasing Bcl-xL levels, the extra Bcl-xL protein synthesized in response to CD40 signaling probably augments the suppression of etoposide-induced apoptotic cell death in this system. Although IL-4 signaling alone had no effect on Bcl-xL protein levels, it accelerated the increase in this survival protein by the CD40 signal (Fig. 2, B). The mechanism underlying this
facilitative role of IL-4 in increasing the level of Bcl-xL protein is presently unclear and warrants further study.

Of interest, VCAM-1- and IL-4-mediated signals diminished a drug-induced change in the conformation of Bax protein (Fig. 4). This associates temporally with its dissociation from Bcl-xL protein. The two survival signals promoted Bax–Bcl-xL binding and completely blocked their disassociation after drug treatment. A similar damage-induced change in the conformation of Bak protein, which occurs on exposure to a diverse range of cytotoxic agents including etoposide, has been observed in T lymphoma cells and was also associated with a subsequent decrease in binding of Bak to Bcl-xL proteins (18). Staurosporine-induced changes in the conformation of the N terminus of Bax protein in HeLa cells have also been reported recently (24). Taken together, these data suggest that a change in conformation of Bax or Bak protein leads to their release from Bcl-xL protein. Critically, we show that this is abrogated by survival signals. Additional studies are required to determine whether the etoposide-driven N-terminal conformational change in Bax protein is a prerequisite for its release from Bcl-xL protein, and whether this, together with the CD40-mediated increase in Bcl-xL protein levels, is of functional importance in deciding B-lymphoma cell fate.

In summary, the data presented here demonstrate that resistance to etoposide may arise via one or more epigenetic mechanisms. Three survival signals work in concert to suppress etoposide-induced apoptotic cell death in B-lymphoma cells according to the following model: IL-4 and VCAM-1 generate signals that promote Bcl-xL–Bax protein binding, and CD40 signals increase the amount of Bcl-xL available to bind Bax. IL-4 hastens the CD40-mediated effects on Bcl-xL protein levels. The implication from this is that etoposide-induced damage results in an increase in Bax protein in a form that is lethal, an effect prevented by the combination of three microenvironment-derived signals. In vivo, cells that withstand chemotherapy in a survival niche may enter a period of tumor dormancy and are afforded time to repair drug-induced DNA damage, with or without fidelity. The studies presented here dissect out some of the key microenvironmental factors predicted to impact on certain B lymphomas in vivo and may provide a paradigm for other tumor types where tumor relapses after chemotherapy occur. If antitumor strategies are to succeed, the contribution of epigenetic factors such as the integration of multiple microenvironmental signals to promote drug resistance must be understood. This approach would permit identification of nodal points on multiple survival-signal pathways that might represent useful targets for antitumor drugs.

REFERENCES


NOTES

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