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Bcl-X_L, but not Bcl-2, can protect human B-lymphoma cell lines from parthenolide-induced apoptosis

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Abstract

In this report, we investigated the effects of the natural product parthenolide on human B-lymphoma cell lines. We show that parthenolide inhibited NF-κB transcription factor c-Rel (REL). In addition, the sensitivity of several human B-lymphoma cell lines to parthenolide-induced apoptosis inversely correlated with their levels of anti-apoptosis protein Bcl-X_L. Furthermore, ectopic expression of Bcl-X_L (but not Bcl-2) in two B-lymphoma cell lines decreased their sensitivity to parthenolide-induced apoptosis. Finally, over-expression of a transforming mutant of REL, which increased expression of endogenous Bcl-X_L, decreased the sensitivity of BJAB B-lymphoma cells to parthenolide-induced apoptosis. These results demonstrate that the NF-κB target gene products Bcl-X_L and Bcl-2 can play different roles in protecting B-lymphoma cells from chemical-induced apoptosis.

Keywords

Parthenolide; NF-kappaB; Bcl-X_L; Bcl-2; B-cell lymphoma; Apoptosis

1. Introduction

The nuclear factor κB (NF-κB) family of eukaryotic transcription factors (p50, p52, RelA/p65, RelB, and c-Rel) shows increased activity in a number of cancer cell types [1]. For example, the human *c-rel* (*REL*) gene is amplified in many types of B-cell lymphoma, including Hodgkin's lymphoma, diffuse large B-cell lymphoma (DLBCL), and follicular lymphoma [2]. Moreover, over-expression of wild-type or certain mutant forms of REL can transform avian and human lymphoid cells in culture [3, 4].

The level of nuclear NF-κB activity can be a prognostic marker for several types of cancer. DLBCL has been classified into two subtypes on the basis of gene expression profiling and

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Conflict of interest

No conflict of interest is declared.

NF- κ B activity [5]. Activated B cell-like (ABC) DLBCL cell lines and patient samples have both high NF- κ B activity and NF- κ B target gene expression, and patients with ABC-DLBCL have a poorer clinical outcome in response to standard chemotherapy. In contrast, germinal center B cell-like (GCB) DLBCL cell lines have lower NF- κ B activity and patients with GCB-DLBCL tend to have a better clinical prognosis. Increased expression of the anti-apoptotic protein Bcl-2 is a marker for ABC-DLBCL [6], which has been taken to indicate that ABC-DLBCL patients have a poorer prognosis, at least in part, due to increased expression of pro-survival genes. Of note, the structurally related anti-apoptotic proteins Bcl-2 and Bcl-X_L are both products of NF- κ B target genes, and in many circumstances, these two proteins play redundant roles in cell survival [7].

The natural product parthenolide is the active ingredient in the medicinal plant feverfew (*Tanacetum parthenium*) [8]. Parthenolide belongs to the family of compounds called sesquiterpene lactones, which have been ascribed a variety of biological activities [9]. For example, parthenolide is commonly used for the treatment of migraines, and feverfew extracts can be purchased over-the-counter. Parthenolide contains an epoxide group and an exo-methylene-lactone ring. These reactive groups can conjugate to nucleophiles such as thiols, and parthenolide can inhibit NF- κ B by interacting with cysteine-38 in a DNA-binding region of NF- κ B p65 [10] or with cysteine-179 in the activation loop of the upstream NF- κ B signaling protein I κ B kinase (IKK) β [11].

In this report, we have further investigated the effects of parthenolide on NF- κ B and on the growth and viability of B-lymphoma cells. We show that parthenolide can inhibit the NF- κ B transcription factor REL, a prominent player in B-cell lymphoma, and that the sensitivity of several B-lymphoma cell lines to parthenolide-induced apoptosis can be influenced by their levels of the REL target gene product Bcl-X_L. In contrast, Bcl-2 does not appear to play a role in protecting B-lymphoma cells from parthenolide-induced apoptosis. These results demonstrate that Bcl-X_L and Bcl-2 have different abilities to protect B-lymphoma cells from certain types of chemical-induced apoptosis, and that levels of Bcl-X_L may be predictive of clinical outcome in response to certain drugs.

2. Material and methods

2.1 Plasmids

Retroviral vector plasmids pMSCV-puro and pMSCV-Bcl-2 (containing murine Bcl-2 cDNA subcloned into pMSCV-puro) have been described previously [12]. pMSCV-Bcl-X_L was created by subcloning an *Eco*RI fragment containing the human Bcl-X_L cDNA into the *Eco*RI site of pMSCV-puro. For transfections, pcDNA-FLAG or pcDNA-based expression vectors for human p50, p65, REL and RELC27S were used [13, 14].

2.2 Cell culture, transfection, and chemical treatment

Human A293, A293T, RC-K8, SUDHL-4, Daudi, IB4, KMH2 and L428 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biologos, Montgomery, IL, USA) (EF10). BJAB-puro and BJAB-REL Δ TAD1 cells [4] were grown in DMEM supplemented with 20% FBS (EF20). The human B-lymphoma cell lines used in this study have been characterized as follows: RC-K8, ABC-like DLBCL [15], SUDHL-4, reported as both follicular lymphoma [16] and GCB-like DLBCL [17]; BJAB, EBV-negative, GCB-like DLBCL [18,19]; Daudi, EBV-positive, LMP1-negative Burkitt lymphoma [20]; IB4, EBV-transformed lymphoblastoid cell line [21]; L428, KMH2, and HDMYZ, Hodgkin's lymphoma; Namalwa, Raji, and Ramos, Burkitt's lymphoma; and Pfeiffer and SUDHL-6, GCB-like DLBCL.

Twenty-four hours prior to treatment with parthenolide (Fermentek, Jerusalem, Israel), helenalin (ChromaDex, Irvine, CA, USA) or costunolide (AvaChem Scientific, San Antonio, TX, USA), cells were plated into 60-mm or 35-mm plates at approximately 60–70% confluency. Cells were then incubated with the indicated concentrations of compound or the solvent DMSO for the indicated times.

For transfections, A293 were seeded such that they were approximately 60% confluent on the following day when transfections were performed using polyethylenimine (PEI) (Polysciences, Warrington, PA, USA). Transfections were performed by incubating 5 µg of plasmid DNA in 30 µl PEI (1 mg/ml) and 400 µl of DMEM (no serum) at room temperature. The DNA/PEI mixture was then added to 10 ml of EF10 and incubated with a 100-mm plate of 60–70% confluent cells for 24 h. Transfected cells were then passed into four 60-mm tissue culture plates, and cells were allowed to grow for an additional 24 h prior to treatment.

2.3 Generation of stable cell lines

Virus stocks were generated by transfecting A293T cells with pMSCV-puro, pMSCV-Bcl-X_L, or pMSCV-Bcl-2 plus helper plasmid pcL10a1, as described previously [22]. Approximately 48 h later, virus was harvested. Two ml of virus (in the presence of 8 µg/ml polybrene) was used to infect 10⁶ BJAB, SUDHL-4 or Daudi cells using the spin infection method [4]. Two days later, 2.5 µg/ml puromycin (Sigma, St. Louis, MO, USA) was added to the cells and they were maintained under selection for 2–4 weeks.

2.4 Cell proliferation assays

Approximately 10⁵ cells were plated in 16-mm wells in 0.5 ml of EF10. After a 6-h recovery period, cells were treated with the indicated concentrations of parthenolide. Cells in triplicate wells for each treatment were counted 3 days later. For each treatment group, cell numbers were normalized to the number of cells in the control, DMSO-treated plates.

2.5 Electrophoretic mobility shift assays (EMSAs)

EMSAs were carried out as described previously [13]. Briefly, whole-cell extracts were prepared in AT lysis buffer [20 mM HEPES, pH 7.9, 1% (v/v) Triton X-100, 20% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na₄P₂O₇, 1 mM DTT, 1 mM Na₃VO₄, 1 µg/ml PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin]. Cell lysates (30 µg) were incubated with a 26-base pair radiolabeled κB-site probe (κB site: 5'-GGGAAATTCC-3'; 100,000 cpm), 2 µg of poly (dI-dC) in binding buffer [25 mM Tris-HCl, pH 7.4, 100 mM KCl, 6.25 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol] in a 50-µl reaction volume for 30 min at 30°C. Samples were then electrophoresed on a non-denaturing 5% polyacrylamide gel, and protein-DNA complexes were detected by autoradiography.

2.6 Western blotting, and assays for caspase-3 activity and DNA fragmentation

Western blotting was performed essentially as described [13]. Whole-cell extracts were prepared in AT lysis buffer. Samples containing equal amounts of total protein were separated on 7.5 or 10% SDS-polyacrylamide gels, and proteins were transferred to nitrocellulose membranes. Blocking buffer contained 5% non-fat milk in Tris-buffered saline with Tween 20. Primary antisera (shown as dilution; source) against the following proteins were used: poly(ADP-ribose) polymerase (PARP) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); Bcl-X_L (1:1000; Cell Signaling Technology, Danvers, MA, USA); Bcl-2 (1:500; Santa Cruz Biotechnology); Mcl-1 (1:1000; Santa Cruz Biotechnology); Hrk (1:1000; Prosci, Poway, CA, USA); Bim (1:1000; Cell Signaling

Technology); Bik (1:1000; Cell Signaling Technology); Bid (1:1000; Santa Cruz Biotechnology); Bax (1:1000; Cell Signaling Technology); Bak (1:1000; Upstate Biotechnology, Lake Placid, NY, USA); REL C terminus (1:500; gift of Nancy Rice); p65 C terminus (1:4000; gift of Nancy Rice); and tubulin (1:500; Santa Cruz Biotechnology). The appropriate horseradish peroxidase-labeled secondary antiserum was added, and complexes were detected by Supersignal Dura West chemiluminescence (Pierce Chemical, Rockford, IL, USA).

Caspase-3 activity in cell extracts prepared with CHAPS lysis buffer was measured using a fluorescent Ac-DEVD-AMC peptide substrate (Enzo Life Sciences, Farmingdale, NY, USA) as described previously [14]. To measure DNA fragmentation, low-molecular weight DNA was isolated and then analyzed by 1.5% agarose gel electrophoresis as described [14].

3. Results

3.1 Parthenolide inhibited REL DNA-binding activity

The NF- κ B family transcription factor REL plays a major role in the growth and survival of B-cell lymphoma [2]. Parthenolide has previously been shown to be able to inhibit DNA binding by NF- κ B p65 but not p50 [10]. To determine whether parthenolide can also inhibit REL, A293 cells were transfected with expression vectors for p50, p65 and REL. Cells were then treated with increasing concentrations of parthenolide for 2 h, and extracts were analyzed in an EMSA using an NF- κ B binding site probe. At 20 μ M, parthenolide dramatically inhibited DNA binding by p65 and REL, but not p50 (Fig. 1A). Mutation of cysteine-27 in a DNA recognition loop of REL (mutant C27S) slightly reduced the dose-dependent inhibition of REL DNA binding by parthenolide.

3.2 Parthenolide inhibited REL DNA-binding activity and the growth of RC-K8 and SUDHL-4 cells, but only induced apoptosis in SUDHL-4 cells

The DLBCL cell lines RC-K8 and SUDHL-4 have mainly REL/p50 complexes as their active nuclear NF- κ B DNA-binding activity; however, their levels of nuclear κ B-site DNA-binding activity differ considerably (RC-K8 is high, while SUDHL-4 is low) [15, 17]. To determine whether parthenolide could inhibit REL DNA-binding activity in a physiological setting we assessed the effect of parthenolide on κ B-site binding activity in these two cell lines. Cells were treated with increasing concentrations of parthenolide for 6 h, and extracts were analyzed by an EMSA. Treatment with 25 μ M parthenolide greatly reduced κ B-site DNA-binding activity in both SUDHL-4 and RC-K8 cells (Fig. 1B). These results show that parthenolide can inhibit the DNA-binding activity of REL in B-lymphoma cell lines with naturally active REL DNA-binding activity.

To determine whether parthenolide could reduce cell proliferation in RC-K8 and SUDHL-4 cells, these cells were treated with increasing concentrations of parthenolide for 72 h and were then counted. Parthenolide reduced the proliferation of both RC-K8 and SUDHL-4 cells by approximately 50% at 5 μ M (Fig. 1C).

The ability of parthenolide to induce apoptosis in the RC-K8 and SUDHL-4 cell lines was next measured by three methods: 1) by measuring apoptosis-induced DNA fragmentation ("DNA laddering") by gel electrophoresis; 2) by measuring caspase-3-like activity in whole cell extracts; and 3) by monitoring cleavage of the caspase-3 substrate PARP by Western blotting. Treatment of cells with parthenolide for 6 h increased all three measures of apoptosis---DNA laddering, caspase-3 activity, and PARP cleavage---in a dose-dependent manner in SUDHL-4 cells, but not in RC-K8 cells (Fig. 2A). Based on the consistency of these three assays, apoptosis was generally assessed by a single measure, PARP cleavage, in the rest of these studies. When SUDHL-4 and RC-K8 cells were treated with 25 μ M

parthenolide for increasing amounts of time, PARP cleavage was induced in a time-dependent manner in SUDHL-4 cells, but not in RC-K8 cells (Fig. 2B). Taken together, these results indicate that parthenolide can inhibit REL DNA-binding activity and cell proliferation in both RC-K8 and SUDHL-4 cells, but the sensitivity of these two cell lines to parthenolide-induced apoptosis is different.

Costunolide and helenalin are two sesquiterpene lactones with reported half-maximal anti-NF- κ B activities of 50 μ M and 10 μ M, respectively [23]. As with 25 μ M parthenolide, 50 μ M costunolide induced apoptosis in SUDHL-4 cells, but not in RC-K8 cells (Fig. 2C). In contrast, helenalin did not induce apoptosis in either SUDHL-4 cells or RC-K8 cells at 50 μ M (Fig. 2C), a level that is five-fold above the reported concentration required for helenalin's anti-NF- κ B activity [23]. These results indicate that some, but not all, sesquiterpene lactones behave similar to parthenolide in terms of inducing apoptosis in B-lymphoma cells.

3.3 The sensitivity of several human B-lymphoma cells to parthenolide-induced apoptosis correlated with cellular levels of Bcl-X_L

We hypothesized that the resistance of RC-K8 cells to parthenolide-induced apoptosis was due to altered expression of anti- and/or pro-apoptosis genes. Two prominent pro-survival NF- κ B/REL target genes encode the anti-apoptotic proteins Bcl-2 and Bcl-X_L. Therefore, we compared the levels of Bcl-X_L and Bcl-2 across a panel of human B-lymphoma cell lines, namely RC-K8, SUDHL-4, BJAB, Daudi and IB4 (Fig. 3A). RC-K8 and BJAB have relatively high levels of Bcl-X_L, whereas Daudi, IB4 and SUDHL-4 have low or barely detectable levels of Bcl-X_L. Of note, SUDHL-4 cells have high levels of Bcl-2 (Fig. 3A), due to a chromosomal translocation [24], but are sensitive to parthenolide-induced apoptosis (Fig. 2A). The levels of the anti-apoptotic protein Mcl-1 were relatively similar across all cell lines. Based on the relative sensitivity of SUDHL-4 vs RC-K8 cells to parthenolide-induced apoptosis, these results suggested that high levels of Bcl-X_L (but not Bcl-2 or Mcl-1) can reduce the sensitivity of B-lymphoma cell lines to parthenolide-induced apoptosis.

To further investigate the correlation between low levels of Bcl-X_L and sensitivity to parthenolide-induced apoptosis, Daudi and IB4 cells (both of which express little or no Bcl-X_L) were treated with increasing concentrations of parthenolide for 6 h. PARP cleavage occurred in a dose-dependent and a time-dependent manner in both Daudi and IB4 cells (Fig. 3B). As with SUDHL-4 cells, IB4 cells express high levels of Bcl-2 but still showed PARP cleavage when treated with parthenolide, further suggesting that the sensitivity of these B-lymphoma cells to parthenolide-induced apoptosis is dependent on levels of Bcl-X_L, but not Bcl-2.

Because Bcl-X_L exerts its anti-apoptotic effect by binding to and sequestering pro-apoptotic proteins, we also measured the levels of several pro-apoptotic Bcl-2 family proteins in these five B-lymphoma cell lines (Fig. 3A). All five cell lines expressed levels of the pro-apoptotic proteins Bid, Hrk, Bax, and Bak, which did not vary by more than three-fold (Fig. 3A, Table S1). However, the levels of Bim and Bik varied to a greater extent among the cell lines (Fig. 3A). Notably, the levels of Bim were very low in the less sensitive RC-K8 cells, but more than 100-fold higher in parthenolide-sensitive SUDHL-4, BJAB cells (see Fig. 6B) and Daudi cells, (Fig. 3A; Table S1), indicating that this pro-apoptotic protein may also play a role in the sensitivity of some B-lymphoma cells to parthenolide.

3.4 Parthenolide inhibited NF- κ B DNA binding and the proliferation of Hodgkin's lymphoma cell lines L428 and KMH2, but only induced apoptosis in KMH2 cells

Because we found that levels of Bcl-X_L, but not Bcl-2, inversely correlated with the sensitivity of RC-K8, SUDHL-4, Daudi and IB4 cell lines to parthenolide-induced apoptosis, we hypothesized that the levels of these two proteins may vary among B-lymphoma cells. Therefore, we used databases from Oncomine to compare the expression patterns of Bcl-2 and Bcl-X_L in DLBCL, follicular lymphoma and chronic lymphocytic leukemia patient samples and cell lines that were previously analyzed by Alizadeh et al. [5]. Interestingly, we found that the levels of Bcl-2 and Bcl-X_L mRNAs show a weak negative correlation value (−0.056), suggesting an inverse correlation between Bcl-2 and Bcl-X_L mRNA expression. To determine whether this correlation was valid at the protein level in B-lymphoma cell lines, additional B-cell lymphoma cell lines were screened for their levels of Bcl-X_L and Bcl-2 protein (Fig. 4A). Overall, ten of thirteen cell lines (Figs. 3A, 4A) expressed either Bcl-2 or Bcl-X_L; only RC-K8 and Pfeiffer expressed both proteins, and Daudi expressed undetectable levels of both proteins. These results suggest that either Bcl-2 or Bcl-X_L is preferentially expressed in B-lymphoma cell lines.

To extend our study of the differential roles of Bcl-X_L and Bcl-2 in protecting B-lymphoma cells from parthenolide-induced apoptosis, we next analyzed two Hodgkin's lymphoma cell lines, L428 and KMH2. Although both of these cell lines have high levels of NF- κ B activity due to deletions in the gene encoding the NF- κ B inhibitor I κ B α [25], we found that they differ in Bcl-X_L and Bcl-2 expression. Namely, L428 expressed high levels of Bcl-X_L but no detectable Bcl-2, whereas KMH2 cells expressed Bcl-2 but had barely detectable levels of Bcl-X_L (Fig. 4A). Therefore, L428 and KMH2 cells were treated with increasing concentrations of parthenolide for 6 h, and apoptosis was then assessed by PARP cleavage. PARP cleavage occurred in a dose-dependent manner in KMH2 but not in L428 cells (Fig. 4B). These results are consistent with the sensitivity of Hodgkin's lymphoma cells to parthenolide also being dependent on levels of Bcl-X_L, but not Bcl-2.

Extracts from L428 and KMH2 cells were also subjected to EMSA analysis (Fig. 4C). NF- κ B DNA binding was inhibited by parthenolide in a dose-dependent manner in both L428 and KMH2 beginning at 25 μ M. To determine whether parthenolide reduced cell proliferation in L428 and KMH2 cells, these cells were treated with increasing concentrations of parthenolide for 72 h (Fig. 4D). In both cell lines, cell proliferation was inhibited by approximately 50% at slightly greater than 5 μ M parthenolide.

3.5 Ectopic expression of Bcl-X_L in SUDHL-4 and Daudi cells conferred resistance to parthenolide-induced apoptosis

To determine whether cells that are sensitive to parthenolide-induced apoptosis and express low levels of Bcl-X_L could be made resistant to parthenolide, we infected SUDHL-4 and Daudi cells (both of which express no detectable Bcl-X_L) with a retroviral vector encoding Bcl-X_L, and created pools of infected cells by selecting for puromycin resistance. Western blotting confirmed over-expression of Bcl-X_L in these retrovirally transduced cells (Fig. 5A, 5B). Control SUDHL-4-puro and Daudi-puro cells and experimental SUDHL-4-Bcl-X_L and Daudi-Bcl-X_L cells were treated with increasing concentrations of parthenolide for 6 h (Fig. 5A, 5B). Parthenolide induced PARP cleavage in SUDHL-4-puro and Daudi-puro cells but PARP cleavage was greatly reduced in SUDHL-4-Bcl-X_L and Daudi-Bcl-X_L cells. Over-expression of Bcl-X_L also blocked DNA fragmentation induced by parthenolide in SUDHL-4 cells (Fig. S1). Taken together, these results show that Bcl-X_L can directly confer resistance to parthenolide-induced apoptosis in these cells. Of note, ectopic expression of Bcl-X_L also protected SUDHL-4 cells from costunolide-induced apoptosis as judged by

inhibition of both PARP cleavage and DNA laddering (Fig. S1). In addition, Bcl-X_L lessened parthenolide-induced inhibition of cell proliferation in SUDHL-4 cells (Fig. 5A).

To confirm that Bcl-2 does not play a role in conferring resistance to parthenolide-induced apoptosis, we infected Daudi cells with a retroviral vector encoding Bcl-2 (Fig. 5C). Daudi-Bcl-2 and Daudi-Bcl-X_L cells were then treated with increasing concentrations of parthenolide for 6 h (Fig. 5C). Parthenolide induced PARP cleavage in Daudi-Bcl-2 but not in Daudi-Bcl-X_L cells. These results provide further evidence that the sensitivity of B-lymphoma cells to parthenolide-induced apoptosis is dependent on the cellular levels of Bcl-X_L but not Bcl-2.

3.6 Over-expression of an activated REL mutant in BJAB cells increased Bcl-X_L expression and blocked parthenolide-induced apoptosis

Bcl-X_L is a REL target gene in some cell types [26]. We have previously shown that over-expression of a REL mutant (REL Δ TAD1) with enhanced transforming activity in chicken spleen cells can increase the oncogenic properties of human BJAB cells and can decrease the sensitivity of BJAB cells to doxorubicin-induced apoptosis [4]. As shown in Fig. 6A, REL Δ TAD1 is expressed at a higher level than endogenous REL in BJAB-REL Δ TAD1 cells and as compared to control BJAB-puro cells. Increased levels of Bcl-X_L were also seen in BJAB-REL Δ TAD1 cells (Fig. 6A). To determine whether increased REL activity can mitigate the sensitivity of BJAB cells to parthenolide-induced apoptosis, we compared the ability of parthenolide to induce PARP cleavage in control BJAB-puro cells versus BJAB-REL Δ TAD1 cells. Control BJAB-puro cells and BJAB-REL Δ TAD1 cells were treated with increasing concentrations of parthenolide for 6 h and PARP cleavage was monitored by Western blotting (Fig. 6B). PARP cleavage was observed in BJAB-puro but not in BJAB-REL Δ TAD1 cells. These results indicate that Bcl-X_L can be up-regulated through a pathway involving activated REL, and suggest that such a pathway can contribute to resistance to parthenolide-induced apoptosis.

4. Discussion

In this study, we present evidence that parthenolide can inhibit DNA binding by the REL transcription factor and that high cellular levels of the REL target gene product Bcl-X_L protect B-lymphoma cells from parthenolide-induced apoptosis. Cells with low levels of Bcl-X_L undergo apoptosis in response to treatment with parthenolide by a pathway that leads to caspase-3 activation.

Although parthenolide has previously been shown to inhibit DNA binding by p65 [10], this is the first report to show that parthenolide can inhibit REL DNA binding. The mechanism by which parthenolide inhibits DNA binding by p65 and REL does not appear to be identical because mutation of the analogous cysteine residue in a DNA contact region completely protects p65 from inhibition by parthenolide [10], but only slightly affects REL's ability to be inhibited (REL mutant C27S, Fig. 1A). Of note, p50 DNA binding is not affected by parthenolide at a concentration (20 μ M) that reduces REL and p65 DNA-binding activity [10] (Fig. 1A). The DLBCL cell lines RC-K8 and SUDHL-4 have mainly REL/p50 and REL/REL complexes as their nuclear κ B-site DNA-binding activity [15, 17], whereas the Hodgkin's lymphoma cell lines KMH2 and L428 have mainly p50/p65 complexes [27]. Parthenolide treatment slowed the growth of RC-K8, SUDHL-4, KMH2 and L428 at similar concentrations, possibly through inhibition of REL/NF- κ B. Previous studies have shown that inhibition of NF- κ B activity by introduction of the I κ B α super-repressor slows the growth of ABC-DLBCL cells [15] (including RC-K8 cells [17]) but not GCB-DLBCL cells (including SUDHL4 cells [15]). However, there was no evidence that the I κ B α super-repressor was inducing apoptosis in RC-K8 cells in those studies, again indicating that

inhibition of NF- κ B DNA-binding activity is not sufficient to induce apoptosis in B-lymphoma cell lines. The observation that a parthenolide-sensitive cell line SUDHL-4 undergoes apoptosis more readily than RC-K8, even though cell proliferation and NF- κ B DNA binding are blocked in both cell types, suggests that inhibition of NF- κ B activity is not the primary criterion for whether parthenolide can induce apoptosis in a given cell type.

The exact mechanism by which parthenolide induces growth arrest or apoptosis in B-lymphoma cells is not known. Given that parthenolide can inhibit REL DNA-binding activity and cell proliferation in both RC-K8 and SUDHL-4 cells (Figs. 1B, C), it is likely that inhibition of REL/NF- κ B activity contributes to the parthenolide-induced inhibition of B-lymphoma cell proliferation. This hypothesis is consistent with the proliferation defect seen in B cells from *c-rel* knockout mice, which is due to a failure to make a G1-to-S transition in response to mitogens [28]. Similarly, parthenolide has been shown to induce cell-cycle arrest in human lung cancer cells [29].

In contrast, the ability of parthenolide to induce apoptosis in B-lymphoma cell lines did not correlate with its ability to inhibit REL/NF- κ B DNA-binding activity; that is, short-term treatment with parthenolide blocked REL DNA-binding activity in RC-K8 cells but did not induce apoptosis (Figs. 1 and 2). Moreover, helenalin, which has been reported to be a more potent NF- κ B inhibitor than parthenolide, did not induce apoptosis in SUDHL-4 or RC-K8 cells, even at a concentration (50 μ M) well above that required for inhibition of NF- κ B activity [23]. Of note, costunolide, which induced apoptosis in a pattern similar to parthenolide (Fig. 2B), has a structure that is more similar to parthenolide than is that of helenalin. Namely, parthenolide, costunolide, and helenalin all contain a single exo-methylene-lactone ring, but helenalin has an additional cyclic α,β unsaturated ketone. Therefore, one might speculate that the cyclic α,β unsaturated ketone interferes with helenalin's ability to induce apoptosis in SUDHL-4 cells.

Parthenolide has been shown previously to induce apoptosis by a number of mechanisms [8]. Zhang et al. [30] have shown that parthenolide-induced apoptosis is mediated by sustained activation of c-Jun N-terminal kinase (JNK) in human nasopharyngeal carcinoma cell line CNE1. Zunino et al. [31] have shown that parthenolide can induce the generation of reactive oxygen species (ROS) leading to mitochondrial dysfunction. Specifically, parthenolide has been shown to bind to intracellular glutathione resulting in an imbalance in the thiol buffering system of the cell. This would induce a disruption in the redox balance resulting in ROS generation from the mitochondria. The oxidative stress from mitochondrial ROS generation results in release of cytochrome c from the mitochondria leading to the activation of the caspase cascade.

How does Bcl-X_L block parthenolide-induced apoptosis? In less sensitive cells, high levels of Bcl-X_L may sequester pro-apoptotic Bcl family proteins such as Bad, Bak, Bax, Bid, and Bim [32] that would normally be freed in response to parthenolide. Conversely, parthenolide-sensitive cells, which have low levels of Bcl-X_L, would be more susceptible to pro-apoptotic Bcl protein-initiated apoptosis. Consistent with this model, ectopic expression of Bcl-X_L in two parthenolide-sensitive cell lines, SUDHL-4 and Daudi, made them less sensitive to parthenolide-induced apoptosis and inhibition of cell growth (Fig. 3). Furthermore, over-expression of REL Δ TAD1, which up-regulates Bcl-X_L, decreased the sensitivity of BJAB cells to parthenolide-induced apoptosis.

It should be pointed out that extended parthenolide treatment (48 h) of RC-K8 cells can induce some PARP cleavage (Fig. S2). Interestingly, cleavage of PARP under these conditions coincides with a reduction in the levels of Bcl-X_L (Fig. S2), further suggesting

that the resistance of RC-K8 cells to apoptosis induced by treatment with parthenolide for 4–12 h (Fig. 2) is due to the high levels of Bcl-X_L in RC-K8 cells.

In contrast to Bcl-X_L, neither over-expression of Bcl-2 nor extremely high endogenous levels of Bcl-2 (as in SUDHL-4 and IB4 cells; Fig. 3A, Table S1) could protect cells from parthenolide-induced apoptosis. Although Bcl-2 and Bcl-X_L have similar anti-apoptotic activities in many circumstances, several reports have shown that they can sometimes have different biological properties. Similar to our results, Luo et al. [33] showed that the sensitivity of the hepatoblastoma HepG2 cell line to apoptosis induced by taxol and doxorubicin depends on the cellular levels of Bcl-X_L but not Bcl-2. Bcl-X_L and Bcl-2 have also been shown to differ in their abilities to protect a murine pre-B cell line and human Ramos B-lymphoma cells from apoptosis induced by a variety of chemotherapeutic agents and Fas ligand, respectively [34, 35]. Moreover, Bcl-X_L and Bcl-2 have different affinities for various pro-apoptotic Bcl proteins, which causes them to interact differentially with such proteins in vitro and in vivo [36–38]. Thus, the higher affinity of Bcl-X_L for certain pro-apoptotic proteins (as compared to Bcl-2) may explain why cellular levels of Bcl-X_L generally predict the sensitivity of several B-lymphoma cell lines to parthenolide-induced apoptosis better than cellular levels of Bcl-2. It is interesting that RC-K8 cells have extremely low levels of the pro-apoptotic protein Bim as compared to SUDHL-4 cells. Thus, the combination of higher levels of Bcl-X_L and lower levels of certain pro-apoptotic proteins may explain the reduced sensitivity of RC-K8 cells to parthenolide-induced apoptosis as compared to SUDHL-4 cells. Similarly, Daudi and IB4 cells, which are sensitive to parthenolide-induced apoptosis, express little or no Bcl-X_L, but have easily detectable levels of several pro-apoptotic proteins.

Further evidence that pro-apoptotic proteins may influence the sensitivity of some B-lymphoma cell lines to parthenolide-induced apoptosis comes from our analysis of BJAB cells. Namely, BJAB and RC-K8 cells express similarly high levels of Bcl-X_L, but BJAB are sensitive to parthenolide-induced apoptosis whereas RC-K8 cells are not. However, BJAB cells express over 300 times more pro-apoptotic Bim than RC-K8 cells (Fig. 3A; Table S1). Indeed, Bim has been shown to predict the sensitivity of some epithelial cancers to drug-induced apoptosis [39]. Nevertheless, even in BJAB cells the induction of higher levels of Bcl-X_L, by over-expression of the oncogenic RELΔTAD1 protein, made these cells less sensitive to parthenolide (Fig. 6B).

Parthenolide and certain parthenolide analogs have shown efficacy towards hematopoietic malignancies in certain preclinical trials [9, 40, 41]. Overall, these results suggest that parthenolide and related chemicals (e.g., costunolide) could be valuable for the treatment of a certain subset of B-cell lymphomas, especially ones expressing low levels of Bcl-X_L.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABC	activated B cell
DLBCL	diffuse large B-cell lymphoma
DMEM	Dulbecco's modified Eagle's medium
EF10	DMEM supplemented with 10% FBS
EF20	DMEM supplemented with 20% FBS
FBS	fetal bovine serum
GCB	germinal center B-cell
IKK	I κ B kinase
PARP	poly (ADP-ribose) polymerase
PEI	polyethylenimine
PMSF	phenylmethanesulfonyl fluoride
ROS	reactive oxygen species

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Fig. 1.

Parthenolide inhibits human REL DNA binding. (A) A293 cells were transfected with expression vectors for human p50, p65, REL, and REL mutant C27S. Cells were treated with the indicated concentrations of parthenolide (PN) for 2 h, and cells were then lysed. An EMSA was performed on extracts using a κB -site probe. (B) Equalized cell extracts from SUDHL-4 and RC-K8 cells that had been treated with the indicated concentrations of parthenolide for 6 h were used in an EMSA as in (A). (C) Triplicate wells containing 10^5 cells were treated with the indicated concentrations of parthenolide. Cells were counted three days later. Cell number is relative to DMSO-treated cells (100); error bars indicate standard error.

Fig. 2. Parthenolide induced apoptosis in a dose-dependent and time-dependent manner in some, but not all, B-lymphoma cell lines. (A) SUDHL-4 or RC-K8 cells were treated with parthenolide (PN) for 6 h at the indicated concentrations, and extracts were assessed for DNA laddering (top panel), caspase-3 activity (middle panel), and PARP cleavage (bottom panel). Anti-NF- κ B p65 Western blotting was performed as a loading control. (B) SUDHL-4 or RC-K8 cells were treated with parthenolide (PN) for 6 h with 25 μ M parthenolide for the indicated times. β -tubulin Western blotting was performed as a loading control. (C) SUDHL-4 or RC-K8 cells were treated for 6 h with vehicle (DMSO), 50 μ M costunolide (CT), 25 μ M parthenolide (PN) or 50 μ M helenalin (HL). PARP cleavage was detected by Western blotting.

Fig. 3. Expression of Bcl-X_L is generally correlated with the sensitivity of B-lymphoma cell lines to parthenolide-induced apoptosis. (A) Extracts from several human B-lymphoma cell lines were analyzed by Western blotting for the indicated proteins. Quantification of the bands is shown in Table S1. (B) Daudi and IB4 cells were treated with the indicated concentrations of parthenolide for 6 h or with 25 μM parthenolide for the indicated number of hours. PARP cleavage was detected by Western blotting.

Fig. 4.

Parthenolide inhibits NF- κ B DNA binding and cell proliferation in KMH2 and L428 Hodgkin's lymphoma cells, but induces apoptosis only in KMH2 cells. (A) Extracts from a panel of human B-lymphoma cells were analyzed by Western blotting for the indicated proteins. (B) KMH2 and L428 cells were treated with the indicated concentrations of parthenolide (PN) for 6 h, and PARP cleavage was monitored by Western blotting. (C) A κ B-site EMSA was performed on the same extracts used in (B). (D) Triplicate plates containing 10^5 cells were treated with the indicated concentrations of parthenolide, and cells were counted three days later. Cell number is relative to DMSO-treated control cells (100); error bars indicate standard error.

Fig. 5.

Ectopic expression of Bcl-X_L in SUDHL-4 and Daudi cells decreased their sensitivity to parthenolide-induced apoptosis. SUDHL-4 (A) and Daudi cells (B, C) were infected with a pMSCV-puro control vector or the same vector also encoding Bcl-X_L or Bcl-2. Stable pools of cells were selected with puromycin. In each case, the left panel shows a Western blot to confirm over-expression of the retrovirally transduced protein. (A) SUDHL-4-puro and SUDHL-4-Bcl-X_L cells were treated with the indicated concentrations of parthenolide (PN) for 6 h, and apoptosis was monitored by anti-PARP Western blotting. On the right, cell proliferation in the presence of increasing concentrations of parthenolide was determined as described for Fig. 1C. (B) Comparison of PARP cleavage in Daudi-puro and Daudi-Bcl-X_L, as in (A). (C) Comparison of PARP cleavage in Daudi-Bcl-2 and Daudi-Bcl-X_L cells.

Fig 6.

Over-expression of an activated REL mutant in BJAB cells up-regulated Bcl-X_L and decreased the sensitivity of BJAB cells to parthenolide-induced apoptosis. (A) BJAB cells were infected with a pMSCV-puro control vector or the same vector encoding REL Δ TAD1. Stable pools of cells were selected with puromycin. Western blotting of extracts for REL, Bcl-X_L, and NF- κ B p65. (B) BJAB-puro and BJAB-REL Δ TAD1 cells were treated with the indicated concentrations of parthenolide (PN) for 6 h, and extracts were prepared. PARP cleavage was monitored by Western blotting.