Activation-induced cell death of aggressive histology lymphomas by CD40 stimulation: induction of bax


CD40 is present on both normal and neoplastic B-lineage cells. CD40 stimulation of normal B cells has been shown to promote normal growth and differentiation, whereas aggressive histology B lymphomas are growth inhibited. The inhibition of neoplastic B-cell growth is believed to occur via activation-induced cell death in which stimuli that typically promote the growth of normal cells prevent the growth of their neoplastic counterparts. We show here that CD40 stimulation using either a soluble recombinant human CD40 ligand (srhCD40L) or anti-CD40 monoclonal antibody resulted in apoptosis of human Burkitt lymphoma cell lines. Additional studies examining the mechanism of CD40-mediated death revealed an increase in bax messenger RNA with a subsequent increase in Bax protein in the mitochondria of the treated cells. In vitro exposure of the cells to bax antisense oligonucleotides resulted in a significant decline in Bax protein levels and partial protection from CD40-mediated death, indicating that induction of Bax was at least one mechanism underlying this inhibitory effect of CD40 stimulation on lymphomas. When immunodeficient mice bearing Burkitt lymphoma were treated with srhCD40L, significant increases in survival were observed indicating a direct antitumor effect as a result of CD40 stimulation in vivo. Overall, these results demonstrate that CD40 ligation of aggressive histology B-lymphoma cells results in inhibition both in vitro and in vivo and thus may be of potential clinical use in their treatment. (Blood. 2002;100:217-223)
inhibiting bax expression partially protected the lymphomas from death by CD40 stimulation. Thus, CD40 stimulation induces bax and this may contribute to the mechanism by which stimulation of CD40 results in AICD in these tumors.

Materials and methods

Tumor cell lines

Daudi and Raji are B-lymphoma cell lines established in cell culture from patients with Burkitt lymphoma (American Type Culture Collection, Rockville, MD). RL is a B-lymphoma cell line established in cell culture from a patient with diffuse large B-cell lymphoma. These lymphomas were kept in culture for no more than 6 weeks before the studies.

Mice

Nonobese diabetic (NOD) severe combined immune deficiency (SCID) mice were bred in our colony (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) and were not used until 6 to 8 weeks of age. NOD/SCID mice were kept under strictly pathogen-free conditions at all times. The mice were housed in microisolator cages and all food, water, and bedding were autoclaved before use. NOD/SCID mice received trimethoprim/sulfamethoxazole (40 mg trimethoprim and 200 mg sulfamethoxazole/520 mL drinking water) in suspension in their drinking water. (Animal care was provided in accordance with the procedures outlined in the “Guide for the Care and Use of Laboratory Animals” [NIH publication no. 86-23, 1985]).

Cell culture and treatments

The Daudi, Raji, and RL cell lines were cultured in RPMI-1640 medium (Biowhittaker, Walkersville, MD), supplemented with 10% fetal bovine serum (PBS; Gibco, Life Technologies, Grand Island, NY), 1% 200 mM L-glutamine (Life Technologies), 1% penicillin-streptomycin and amphotericin B (Fungizone) mix (Biowhittaker). Media was then filtered through a 0.22 μm filter (Nalgene, Rochester, NY). The cells were cultured at 37°C with 5% CO2. Cell lines were subcultured every 2 to 3 days.

Daudi, Raji, and RL cell lines were analyzed by an EPICS flow cytometer ( Coulter Electronic, Hialeah, FL) for CD40 expression. Cells were counted on a Coulter cell counter (Coulter Electronic), and viability was determined microscopically using a hemacytometer and the trypan blue exclusion method. Cells were analyzed by flow cytometry.

In vitro assessment of apoptosis (annexin V–fluorescein isothiocyanate)

Daudi cells were cultured in the presence and absence of 3 μg/mL srhCD40L for 24 hours. Cells (10^5/sample) were washed with PBS and centrifuged at 200g for 5 minutes. Cells stained with annexin were resuspended in 100 μL stain solution (20 μL annexin in 1 mL Hepes buffer; Boehringer Mannheim) before incubation for 15 minutes. Immediately prior to analysis by flow cytometry, 50 μL incubation buffer (10 mM Hepes buffer, 40 mM NaCl, 5 mM CaCl2 in NaOH, pH 7.4) was added to each sample.

RNA preparation

Daudi cells were treated with 10 μg/mL srhCD40L or anti-CD40 (SGN-14 clone, mouse IgG1) and incubated for 24, 48, and 72 hours. Before RNA extraction, cells were isolated and pelleted to remove culture media. Trizol (Gibco, Life Technologies) was added to each sample tube according to manufacturer’s instructions. Following a 5-minute incubation, 20% chloroform (CMS, Houston, TX) was added to the tube and shaken vigorously for 15 seconds. After a 3-minute incubation, samples were centrifuged at 12 000g for 15 minutes. Fifty percent isopropyl alcohol (Sigma, St Louis, MO) by volume to the amount of Trizol used was added to the tube and shaken vigorously for 15 seconds. After a 3-minute incubation, the aqueous phase only was removed from the sample tubes and transferred to tubes containing isopropanol. The tubes were mixed gently and allowed to rest for 10 minutes. The samples were then centrifuged at 12 000g for 15 minutes. The supernatant was discarded and the RNA pellet was transferred to a 1.5-mL Eppendorf tube and washed twice with 70% ice-cold ethanol. RNA pellets were dried by vacuum centrifugation, resuspended in DEPC water (Quality Biologicals, Gaithersburg, MD), and placed in a 50°C water bath for 5 minutes.

Ribonuclease protection assay

To analyze mRNAs in the hcl-2 family of genes, Pharmingen 45004K or 45014K (transcription/ribonuclease protection assay [RPA] kits) were used unless otherwise noted (Pharmingen). Reagents were brought to room temperature before use. Probe synthesis and electrophoresis was carried out according to manufacturer’s instructions. Following electrophoresis, the gel was absorbed to blotting paper (VWR Products, Media, PA) and dried in a vacuum gel dryer at 80°C for 1 hour. The dried gel was placed on a film cassette with x-ray film and developed at ~70°C for 1 to 3 days, depending on application. Following development, the gel was placed in a phosphorimager with an intensifying screen for 24 hours before analysis on a scanner.
(Storm 860, Molecular Dynamics, Sunnyvale, CA) to obtain densitometric analysis. Each experiment was performed 3 times.

**Protein isolation and Western blot**

Raji and RL cells were treated with 10 μg/mL CD40L or anti-CD40 mAb SGN-14 for 6, 24, 48, and 72 hours and analyzed for Bax using a previously described protocol. Lysis of Raji and RL cells following treatment was performed in isotonic buffer (200 mM mannitol/70 mM sucrose/1 mM EDTA/10 mM Hepes, pH 6.9) by Dounce homogenization. Unbroken cells, heavy membranes, and nuclei were pelleted and discarded. The mitochondrial and cytosolic fractions were separated by centrifugation at 18 000g for 10 minutes. The cytosolic fraction (supernatant) was transferred to a clean Eppendorf tube and stored.

For detection of Bax or Erk by Western blot, cell equivalent samples (10 μL aliquots) of the fractions were separated by SDS-polyacrylamide gel electrophoresis on 12% Tris-glycine gels (Novex, San Diego, CA), and transferred to 0.2-μm polyvinylidene difluoride membranes (Novex). Blots were probed with a rabbit polyclonal antiserum specific for the amino terminal of Bax (N20; Santa Cruz Biotechnology, Santa Cruz, CA), or with a rabbit polyclonal antibody specific for Erk (Santa Cruz Biotechnology), followed by the appropriate secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology). The blots were then visualized by enhanced chemiluminescence (Pierce, Rockford, IL) as indicated in the manufacturer’s protocol. Each experiment was performed twice.

**Antisense experiments**

Daudi and Raji cells were initially cultured with Bax antisense (TGCTC-CCCGGACCCGTOOFT) (Gibco) or mixed sense (CCGCTGCGCCAGTC-) Daudi and Raji cells were initially cultured with Bax antisense (TGCTC- Daudi (A,B) and Raji (C) cells were incubated either alone or with log dilutions of CD40L or CD40 antibody (SGN-14 clone, mouse IgG1) for 72 hours. Measurement of proliferation was performed using a microculture tetrazolium (MTT) assay as described in "Materials and methods." Data are presented as the mean with SD. Viability of the cells (D) was assessed using the trypan blue exclusion method. Treatment of Daudi and Raji cells with either CD40L or CD40 antibody (at 10 μg/mL concentration) resulted in a significant (P < .001) decrease in proliferation, as well as a decrease in viability in these cells versus untreated cells as indicated by the asterisk.

**Results**

**Effects of CD40L and anti-CD40 on human B-cell lymphoma growth**

Incubation of the tumor cells with either srhCD40L or antibody compared with an isotype-matched antibody control depending on the cell line used (Figure 1A-C). Viability was tested at 72 hours using the trypan blue exclusion method. A 31.1% to 49.5% decrease in viability was seen in the groups treated with srhCD40L or anti-CD40 mAb (Figure 1D). These results are in agreement with earlier studies showing inhibitory effects of anti-CD40 antibodies on Burkitt lymphoma cells. These results demonstrate that srhCD40L and an agonist anti-CD40 mAb are capable of inhibiting human B-lymphoma proliferation in vitro.

**CD40 stimulation causes apoptosis in human B-lymphoma lines**

We then investigated whether this inhibitory effect of CD40 stimulation was due to apoptosis. This was assessed by incubating Daudi lymphoma cells with srhCD40L or anti-CD40 and quantifying DNA strand breaks using TUNEL assay or annexin V staining. The cells were cultured with 10 μg/mL anti-CD40 for 24, 48, and 72 hours before staining and flow cytometric analysis. A 5-fold increase in the number of apoptotic Daudi cells occurred after 24 hours’ incubation as determined by TUNEL assay (from 1.4% to 58%; Figure 2A). Using annexin staining, the fraction of apoptotic cells increased from 11% to 53% after incubation of Daudi cells with 3 μg/mL srhCD40L (Figure 2C). Similar results were seen with the anti-CD40 mAb (data not shown). These results indicate that CD40 stimulation induces apoptosis in human B lymphomas in vitro.

**CD40 stimulation causes an increase in bax mRNA**

We then determined whether treatment of Daudi cells with srhCD40L or anti-CD40 SGN-14 caused an increase in apoptosis-related genes. The cells were cultured with 10 μg/mL srhCD40L and analyzed at 12, 24, and 48 hours to obtain cellular viability.

**Optimal inhibition of 40% to 60% was seen with 10 μg/mL srhCD40L or antibody compared with an isotype-matched antibody control depending on the cell line used (Figure 1A-C). Viability was tested at 72 hours using the trypan blue exclusion method. A 31.1% to 49.5% decrease in viability was seen in the groups treated with srhCD40L or anti-CD40 mAb (Figure 1D). These results are in agreement with earlier studies showing inhibitory effects of anti-CD40 antibodies on Burkitt lymphoma cells. These results demonstrate that srhCD40L and an agonist anti-CD40 mAb are capable of inhibiting human B-lymphoma proliferation in vitro.**
or anti-CD40 SGN-14 for 24, 48, and 72 hours before RNA extraction and analysis by RPA. We used this concentration of antibody and ligand because we have shown that this resulted in optimal growth inhibition of the lymphomas (Figure 1). We observed that there was an increase in bax mRNA as well as increases in mRNA of other proapoptotic genes such as bak, bik, and bcl-xL after CD40 stimulation by either antibody or ligand (Figure 3). Interesting, both bfl-1 and mcl-1, which have been associated with inhibition of apoptosis were also detected. L32 is a control gene, similar to GAPDH. There was an absence of the antiapoptotic gene bcl-2 in the untreated as well as treated groups at each time point, in agreement with previous reports on the bcl-2 status of these cell lines. There was a significant (P < .01) increase in bax mRNA levels in both the srhCD40L and anti-CD40 (SGN-14)–treated groups at 24, 48, and 72 hours compared to an untreated control shown by densitometric analysis of the gels (Figure 4). Thus, CD40 stimulation, by either antibody or soluble ligand, increases bax mRNA levels in Burkitt lymphoma cell lines.

**Figure 2.** CD40 stimulation causes apoptosis in Burkitt lymphoma. Daudi cells were cultured in medium in the presence or absence of srhCD40L or anti-CD40 antibody (SGN-14 clone, mouse IgG1). TUNEL (A,B) and annexin V–fluorescein isothiocyanate (FITC; C) stains were performed following incubation. Twenty-four hours after culture initiation, Daudi cells were analyzed for nuclear DNA damage by TUNEL and for phospholipid externalization to the cell surface by annexin and flow cytometry. The results are expressed as the number of cells (percent of total) that have either DNA strand breaks or that bind annexin V–FITC and are therefore undergoing apoptosis. Culture with 10 μg/mL srhCD40L (A) caused a 60% increase in DNA strand breaks compared to an untreated control (from 1.4% to 58%). Dark gray shade (A) is the untreated control group.

**Bax protein levels are increased as a result of CD40 stimulation**

Bax has been previously demonstrated to play a critical role in the death of neoplastic cells. To correlate levels of corresponding Bax protein with bax transcription, we analyzed Bax protein levels by Western blot analysis. In these experiments, we also assessed the levels of Bax in a diffuse large-cell lymphoma cell line, RL, after CD40 stimulation. We have previously shown that RL cells are inhibited by CD40 stimulation both in vitro and in vivo. Protein was extracted from Daudi, Raji, and RL cells after culture with 10 μg/mL srhCD40L or anti-CD40 SGN-14 at various time points. Cell lysates were quantified using Western blot for Bax concentration in both cytosolic and mitochondrial fractions of RL and Daudi cells and mitochondrial fractions of Raji. There was an increase in the levels of Bax protein in RL cytosolic fractions as determined by Western blot and densitometric analysis relative to Erk levels, which was used as a loading control (Figure 5A,B). Erk was chosen as our control because, although it is posttranslationally activated by exogenous stimuli, it is expressed constitutively. The actual loading of the gels for Western blots was based on a fixed cell number per lane, whereas there was no adjustment for the amount

**Figure 3.** RPA analysis of apoptotic mRNA after CD40 stimulation. Daudi cells were cultured in the presence or absence (U) of either 10 μg/mL srhCD40L (L) or 10 μg/mL anti-CD40 SGN-14 (A) for 24, 48, and 72 hours before RNA extraction. RNA samples were analyzed by RPA with probes for the bcl-2 family of genes for changes in mRNA levels. Increases in bax mRNA were seen in each treatment group at each time point compared to an untreated control. Increases in other proapoptotic genes such as bcl-xL/S, bik, and bak were also seen at each time point. These data are representative of 3 experiments.

**Figure 4.** Densitometric analysis of RPA gels. Quantitative levels of bax, bak, bik, and bcl-xL mRNA levels in Daudi cells were established through densitometric analysis of the RPA gel. The gel was exposed to a intensifying screen for 24 hours before analysis on a scanner. Levels of these genes are expressed in ratios to the housekeeping gene GAPDH. There was an increase in all of these gene transcripts compared to the untreated controls. These data are representative of 3 experiments.
of protein loaded. Thus, the Erk levels show that there was no major change in the protein content per cell at this time point following CD40 ligation, whereas there was an actual increase in the quantity of Bax present per cell.

Translocation of Bax into the mitochondria has been shown to be associated for the induction of apoptosis.23 There was no Bax protein present in the mitochondria of Raji or Daudi cells at 0 hours or without CD40 stimulation (Figure 6). The Daudi cell line cultured with the anti-CD40 antibody showed a significant level of Bax protein associated with the mitochondria after 6 hours (Figure 6A). Similar results were seen with Raji and RL cells in which the presence of Bax in the mitochondria was detected after 24 hours (Figure 6B,C). Thus, in aggressive histology human B lymphoma cells, CD40 stimulation increased cytosolic Bax levels as well as the amount of Bax protein in the mitochondria and this correlates with the induction of apoptosis observed after treatment.

Bax antisense is protective against CD40-mediated cell death of lymphomas

To determine whether Bax was responsible for the CD40-mediated death in these lymphoma lines, we cultured RL cells with srhCD40L alone, or srhCD40L plus bax antisense. A significant reduction in cytosolic Bax protein was observed by Western blot and densitometric analysis after 48 hours of culture with the antisense (Figure 7A,B). In the next series of experiments, Daudi and RL cells treated with the antisense were also stimulated with either media control or 10 μg/mL anti-CD40 mAb (SGN-1 clone, mouse IgG1) to determine if the reduction of Bax protein by the antisense could protect the lymphomas from CD40-mediated cell death. We have determined that this concentration of antibody or ligand results in a significant (P < .005) increase in viability of the cells was measured by the trypan blue exclusion method. There was a significant (P < .001) increase in viability in both tumor lines treated with anti-CD40 mAb and the bax antisense versus those that received the mixed sense control and anti-CD40 mAb for 48 hours (Figure 8A,B). The viability of control cells not treated with anti-CD40 mAb remained unchanged with either media control, mixed sense, or bax antisense (data not shown). Thus, inhibiting bax by antisense was capable of at least partially protecting aggressive histology B-cell lymphomas from CD40-mediated death suggesting that at least one of the mechanisms underlying the inhibitory effects of CD40 stimulation was through the induction of Bax.

Antitumor effects of CD40 stimulation on NOD/SCID mice bearing B lymphomas

We then investigated whether CD40 stimulation of the tumor alone would be efficacious in treating Burkitt lymphomas in vivo. NOD/SCID mice bearing Raji lymphoma cells were treated with srhCD40L (100 μg/d) every day for 10 IP injections starting on day 1 after tumor injection. Recipient mice received 5 × 10⁶ tumor cells by IV injection. The ligand was used because it has no Fc region and thus cannot mediate antitumor effects by antibody-dependent cell-mediated cytotoxicity (ADCC). Treatment with srhCD40L significantly (P < .005) inhibited tumor growth and promoted survival in these mice (Figure 9). The mice treated with srhCD40L had 60% survival 30 days past the control group. No overt toxicity was observed in the mice receiving repeated administration of the ligand. Similar antitumor results were previously observed using antibodies to CD40 in vivo.2 Thus, CD40 stimulation using a recombinant soluble ligand prolongs survival of Burkitt lymphoma-bearing mice in vivo.

Discussion

We report here that CD40 stimulation of Burkitt and diffuse large-cell lymphoma cell lines induces apoptosis accompanied by an up-regulation in bax mRNA and Bax protein in the mitochondria; in addition, inhibition of Bax production resulted in partial protection from CD40-mediated death. Previous studies have demonstrated that CD40 stimulation can promote the growth and differentiation of normal B lymphocytes while causing a decrease in aggressive histology B lymphocytes.1,7 This is the first report implicating apoptosis via bax as one of the mechanisms by which CD40 stimulation inhibits aggressive histology B-lymphoma growth.

CD40 cross-linking has been previously demonstrated to have markedly different effects on the growth of B-cell lymphomas, and this has been shown to be dependent on their type and the assay
used to determine effects. CD40 stimulation of multiple myeloma cells, for example, has been reported to either promote or inhibit their growth in vitro.7,28 Additionally, it has been demonstrated that CD40 stimulation by CD40L in indolent lymphomas such as follicular lymphoma and B-CLL in the presence of interleukin 4 will promote clonogenic growth of these neoplasms for a short time in vitro.12,13,15,17 Conversely, stimulation of CD40 on aggressive B-cell lymphomas, such as Burkitt, diffuse large-cell, and EBV-derived lymphomas, results in inhibition of proliferation in vitro and production of antitumor effects in vivo.3,16,18 Antitumor effects associated with CD40 stimulation of lymphomas have been only shown using antibodies to CD40 up until now. Previous studies using anti-CD40 antibodies have shown in vivo efficacy due in part to ADCC,18 thereby making it difficult to discern whether the antitumor effects were due to the direct effects of CD40 stimulation on the tumor or by ADCC. The antitumor effects reported here with srhCD40L confirm that CD40 stimulation has direct antitumor effects that are independent of ADCC in vivo.

Activation-induced cell death is a process by which a signal that would promote growth in a normal cell will cause death in a transformed cell. This process can involve cell cycle arrest, apoptosis, or necrosis.17,19,20 It was observed that CD40 stimulation induced apoptosis in the cell lines tested. Furthermore, CD40 stimulation caused an increase in 4 proapoptotic gene transcripts: bax, bak, bik, and bclx-S. It will be of interest to ascertain the role of the other proapoptotic molecules in CD40-mediated apoptosis. More quantitative analysis by Western blot was focused on Bax, which was a reasonable candidate in light of reports on its critical role in inducing apoptosis in neoplastic cells after exposure to chemotherapeutic agents.31,32 Bax is of particular interest due to its ability to dimerize with itself on the mitochondrial membrane to facilitate ion release through Bax-induced pores as well as cytochrome c release and the induction of caspases.22 Little is known about the direct effects of the other genes. Corresponding with the increase in bax mRNA, there was an increase in Bax protein and a significant increase in Bax in the cytosolic fractions of cells treated with srhCD40L. This effect was also seen in the cells treated with anti-CD40 antibody (SGN-14 clone). Blocking the effects of bax through antisense oligonucleotides was able to protect the cells from death as seen by a rise in viability, suggesting that bax is directly involved in CD40-mediated death in these cells. However, the data showed that only partial protection was achieved with the antisense constructs. It is unclear whether this is related to incomplete blocking of Bax or whether other proapoptotic molecules (ie, bik, bak, bclx-S) also play a role in CD40-mediated inhibition.

There are 2 pathways that can trigger a death signal to the apoptotic machinery in a cell. The first is the activation of death receptors such as CD95/Fas/Apo-1 or TNF receptor 1 by binding their respective ligands.30 This, in turn, causes the recruitment of caspase 8 and the subsequent activation of the other effector caspases.21 The second mechanism is death-receptor independent, in which the apoptotic machinery is triggered directly by a death signal, which then leads to cytochrome c release from the mitochondria to the cytosol.21 In the presence of adenosine triphosphate, this causes the recruitment of caspase 3 and the other effector caspases.31 Cytochrome c release is regulated directly by the members of the bcl-2 family of genes. A number of studies have shown a central role of the p53 tumor suppressor gene in facilitating the death signal as in the second mechanism mentioned above.25,32 It has been demonstrated that p53 induces cytochrome c release from the mitochondria, a process solely dependent on the recruitment of Bax from the cytosol.23 However, diffuse large-cell lymphoma lines have also been shown to be inhibited by CD40...
stimulation. These cells (including the RL cell line) are characterized as having mutations in their p53 gene among other genetic lesions. Interestingly, the RL lymphoma has a t(14;18) translocation and an overexpression of Bcl-2 as result. The results presented here suggest that CD40-mediated inhibition occurs even during overexpression of Bcl-2. These data indicate that the induction of Bax is independent of p53 and apoptosis induced by CD40 stimulation cannot be compensated by Bcl-2. Overall, these results suggest that CD40 agonists (sCD40L or CD40 mAb) may have potential clinical use for the treatment of aggressive histology lymphomas.

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References

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