Up-Regulation of Bcl-xL Expression Protects CD40-Activated Human B Cells from Fas-Mediated Apoptosis

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CD40-CD40L interactions between resting B cells and activated T cells are essential for germinal center formation. It has been shown that CD40L can induce both Fas expression and susceptibility to Fas-mediated killing in B cells, while anti-Ig can partially rescue B cells from Fas-mediated killing. However, the intracellular mechanism for this phenomenon is not known. We examined the expression of Fas and bcl-2 family gene products, such as Bcl-2, Bcl-x, Bax, and Mcl-1, in human tonsilar B cells. The activation of naive B cells by CD40L induced transient expression of Bcl-xL. As the Bcl-xL level decreased in CD40-activated B cells, the cells became susceptible to apoptosis by anti-Fas antibodies. Though anti-Ig did not change the Fas expression, it protected CD40-activated B cells from Fas-mediated killing by up-regulating Bcl-xL expression. The addition of anti-Ig did not significantly change Bcl-2, Bax, and Mcl-1 levels compared to those of B cells activated by CD40L alone. © 1996 Academic Press, Inc.

INTRODUCTION

The maintenance of a balanced immune system is dependent upon the selective survival and apoptotic death of antigen-specific clones during lymphocyte maturation (1, 2). In the humoral immune responses to thymus-dependent antigens, CD40–CD40L interactions between resting B cells and activated T cells are essential for efficient Ig antibody production. Animals with impaired interactions due to CD40 or CD40L mutations fail to produce IgG and IgA antibodies with no germinal center (GC) reaction (3–5).

At the same time, stimulation of naive B cells via CD40 induces Fas expression (6, 7). The apoptosis of activated B cells by Fas–FasL interactions may be an important mechanism for preventing the overexpansion of the antigen-activated clones. This hypothesis is best proven by the lymphoproliferative disorders observed in lpr and gld mice that are deficient in Fas and FasL, respectively (8–10). While the induction of Fas expression renders CD40L-activated B cells susceptible to apoptosis, B cells stimulated by anti-IgM/anti-CD40 are protected from Fas-mediated killing (11, 12). However, the intracellular mechanism for this protection by Ig-receptor engagement is not known.

The survival of cells is regulated at least in part by members of the Bcl-2 protein family (13–15). Some of the members of this protein family are inhibitors of cell death (e.g., Bcl-2, Bcl-xL, and Mcl-1), while others are promoters of apoptosis (e.g., Bax and Bad). While Bcl-2 is known to be critical for maintaining the survival of resting B and T cells (16, 17), Bcl-x appears to play a particularly important role in activated lymphocytes (18–20). Mature resting B cells are typically Bcl-2– Bcl-x+. Recently, it has been shown that Bcl-x is up-regulated transiently in B cells by stimulation with anti-IgM, anti-CD40, and LPS (21). Furthermore, enforced expression of both Bcl-2 and Bcl-x in transgenic mice increased protection against anti-IgD-induced cell death in vivo. However, the physiological function of Bcl-x in human B cell differentiation is unclear.

In this paper, we present evidence that Bcl-xL may play an active role in blocking the Fas-mediated death of CD40-stimulated naive B cells and show that the protection from Fas-mediated death, which is provided by anti-Ig, is not mediated by down-regulation of Fas expression.

MATERIALS AND METHODS

Antibodies

Monoclonal antibody (mAb) anti-Bcl-2 (IgG1) was a generous gift from Dr. D. Y. Mason (Oxford, UK). Monoclonal antibody anti-Bcl-x (IgG2b) was prepared in the laboratory of Dr. Craig Thompson. Rabbit anti-human Bcl-x, Bax, and Mcl-1 polyclonal antisera were prepared and characterized as described elsewhere (19, 22, 23). Other Abs were purchased from different...
companies as follows: anti-Fas mAb (IgM, clone CH-11) from Immunotech (Marseille, France); FITC-conjugated anti-Fas mAb (IgG1, clone DX2) from PharMinigen (San Diego, CA); IgM control mAb from Sigma (St. Louis, MO); FITC-conjugated mouse IgG1 control mAb from DAKO (Glostrup, Denmark); FITC-conjugated anti-CD19, anti-CD20, anti-CD2, anti-CD3, and anti-CD14 from Becton-Dickinson Co. (Mountain View, CA); and rat anti-mouse IgG1 microbeads from Miltenyi Biotec, Inc. (Sunnyvale, CA).

Cell Cultures

Tonsillar B cells were prepared as described previously (24). B cells were further separated according to their density using a discontinuous gradient of Percoll (Pharmacia, Uppsala, Sweden) consisting of three layers of 80, 60, and 50% Percoll solutions in a 15-ml conical tube. B cells (1 x 10⁹) were laid at the bottom of the gradient and centrifuged for 10 min at 2000g at 20°C. B cells recovered at 60–80% interface were referred to as high-density or naive B cells which were mostly IgD⁺ (65 ± 5%), less than 2% CD38high, and less than 2% Fas⁺ as analyzed by FACSscan. Fas⁺ naive B cells were cultured in RPMI 1640 containing 10% FCS (Irvine, Santa Ana, CA) in the presence of HK cells for various days. HK cells have been shown to enhance DNA synthesis of both GC B cells and naive B cells in the presence of mitogens and share the function of FDC in GC B cell differentiation (25). We found that HK cells did not affect Fas expression in the presence of CD40L or anti-Ig, but did enhance cell viability by 1.5 to 2 times at 72 hr (data not shown). In this culture system, we could recover a large number of B cells with GC markers which would undergo apoptosis in the conventional culture system.

Flow Cytometry

For cell surface antigen staining, cells were stained with a panel of antibodies unconjugated or directly conjugated with FITC. Unconjugated mAb was detected by FITC-labeled goat anti-mouse Ig. In brief, cells were incubated with the appropriate concentration of antibody for 15 min at 4°C. After being washed with PBS containing 0.2% BSA and 0.1% sodium azide, cells were fixed and analyzed by FACS.

For intracellular Bcl-2 and Bcl-x staining, cells were fixed by 1% paraformaldehyde overnight and permeabilized by incubation with 0.3 mg/ml saponin in PBS for 15 min at 4°C followed by incubation with anti-Bcl-2 or anti-Bcl-x antibody and then with FITC-labeled goat anti-mouse Ig.

Fas-Mediated Apoptosis

Naive B cells were cultured in 24-well plates (1 x 10⁶ cells/well) at 5 x 10⁵ cells/ml over irradiated HK cells (2 x 10⁴ cells/well) in the presence of CD40L (1 µg/ml, Immunex, Seattle, WA) or anti-Ig (2.5 µg/ml, Irvine). At various time points, anti-Fas mAb CH-11 or control IgM was added to the cultures at a final concentration of 100 ng/ml. After an additional 8 hr of incubation, cells were harvested, washed once, and then fixed by 1% paraformaldehyde for FACS analysis. Using forward and side scatter, the viable population and the apoptotic population were determined after debris and large HK cells were excluded as described by Swat and colleagues (26). This method was comparable to the TUNEL technique (data not shown).

The percentage of viability is calculated as:

\[
\text{% of viability} = \frac{\% \text{ of viable cells}}{\% \text{ of viable cells} + \% \text{ of apoptotic cells}} \times 100\%.
\]

Western Blotting

Western blotting was performed as described previously (19). Twenty micrograms of total protein from each cell lysate was separated on a 15% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. The membranes were blocked with 5% dry milk in TBS containing 0.05% Tween 20 at 4°C overnight and then incubated with TBS containing an appropriate concentration of antibody for 45 min. After being washed, the blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit Ig (1:2000, Amersham, Buckinghamshire, UK) for 30 min and the proteins were visualized by using the ECL kit (Amersham).

RESULTS

Induction of the Fas Expression by CD40L but Not by Anti-Ig

Since it was not known which signals change the phenotypes of naive B cells into GC B cells, we investigated the role of CD40L and anti-Ig in changing the phenotypes of naive B cells into GC B cells. Naive B cells are Bcl-2⁺ Fas⁻ (6). When Fas⁻ resting B cells were cultured in the presence of CD40L, Fas expression was induced at 24 hr and continued to increase up to 3 days (Fig. 1A). However, anti-Ig did not induce a detectable level of Fas (Fig. 1B). The failure of the Fas expression by anti-Ig was not caused by the less efficient activation by anti-Ig because there was a substantial amount of [³H]thymidine uptake in the cultures stimulated by anti-Ig (data not shown). In three separate experiments, when both CD40L and anti-Ig were added to the cultures, Fas expression did not increase to a higher level than that of CD40L alone [i.e., mean fluorescence intensity (MFI) 40 ± 9.1 vs 38 ± 9.2]. This result essentially confirms recent data indicat-
Cross-Linking of Fas Molecules Induces Apoptosis in the Late Stage of CD40L Activation, and Anti-Ig Protects the Cell from Fas-Mediated Killing

To investigate the functional significance of CD40L-induced Fas expression and the role of anti-Ig in this culture system, naïve B cells were treated with anti-Fas mAb, CH-11 (27). In the presence of CD40L, Fas− B cells were resistant to anti-Fas-mediated killing up to 48 hr and became susceptible afterward (Fig. 2). This result is in agreement with the previous observations made by others (6, 28) that anti-Fas induced cell death in the late stage of CD40 activation. The killing appears to be mediated by Fas–anti-Fas interactions because Fas− B cells activated by anti-Ig alone were not killed by anti-Fas (data not shown).

Although anti-Ig did not affect Fas expression, it inhibited Fas-mediated killing of CD40L-activated B cells (Table 1). Approximately 67% of CD40L or CD40L/anti-Ig-activated B cells remain viable at 72 hr in the presence of the control antibody. While an additional 30.4 ± 8.5% of CD40L-activated B cells were killed by cross-linking with anti-Fas, only 8.6 ± 4.5% were killed when anti-Ig was added. The partial prevention of anti-

### Table 1

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<th>Experiment</th>
<th>CD40L</th>
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<tr>
<td></td>
<td>IgM</td>
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</tr>
<tr>
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</tr>
<tr>
<td>2</td>
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*High-density B cells were cultured in the presence of CD40L or CD40L/anti-Ig and HK cells for 72 hr. CH-11 or control mouse IgM antibody was added to the cultures for 8 hr. The cells were then harvested, washed, and fixed by 1% paraformaldehyde. The viable and apoptotic populations were determined as described under Materials and Methods. The percentage of viable cells is shown.*
ive B cells were negative for Bcl-x. Six hours after CD40L activation, Bcl-x expression increased, reaching its peak at 48 hr, and then decreased. However, addition of anti-Ig enhanced Bcl-x expression more than CD40L alone (Fig. 3C). At 72 hr, Bcl-x remained elevated in the presence of CD40L/anti-Ig, while Bcl-x expression of CD40L-activated B cells decreased significantly; the specific MFI was 68 vs 35 (Fig. 3A). Anti-Ig alone induced only a minimal amount of Bcl-x protein accumulation during the culture period. This result was reproducible in three separate experiments.

Although Bcl-2 is highly expressed in naive B cells, it was down-regulated after 72 hr of activation by CD40L (MFI from $50 \pm 10$ to $10 \pm 2$, mean of two experiments). Adding anti-Ig did not change the levels of Bcl-2 (MFI $11 \pm 2$), suggesting that anti-Ig-mediated resistance to Fas-induced killing was not attributable to the level of expression of Bcl-2 (Fig. 4). Other apoptosis regulator gene products, Bax and Mcl-1, were constitutively expressed in naive B cells. There was no significant change in their expression whether B cells were activated by CD40L or CD40L/anti-Ig (Fig. 3B).

**DISCUSSION**

It appears that three elements are essential for Fas-mediated apoptosis: (1) expression of Fas molecules at sufficient densities, (2) cross-linking of Fas molecules, and (3) a permissive cellular background which allows Fas ligation to generate signals resulting in apoptosis (28). Our observations suggest that Bcl-xL may play a role in inhibiting Fas-mediated killing in B cells. When Bcl-2+ Fas− naive B cells were cultured with CD40L for 24 hr, expression of Fas and Bcl-xL was induced, with peak levels reached at about 48 hr. These B cells were insensitive to Fas-mediated killing. After 48 hr of activation, B cells then became sensitive to anti-Fas-mediated killing and concomitantly levels of Bcl-xL protein, but not Fas, declined. The down-regulation of Bcl-2 that was induced by CD40L might also contribute to Fas-mediated killing. In the presence of anti-Ig, however, the Bcl-xL level remained high and Fas− B cells were resistant to Fas-mediated killing, although these anti-Ig-stimulated B cells expressed comparable levels of Fas and Bcl-2. These results suggest that the transient expression of Bcl-xL suppressed apoptosis. However, we cannot exclude the possibility that anti-Ig stimulation induces either increases in the expression of other genes that block Fas-based cytotoxicity (FAP-1, BAG-1) (29, 30) or decreases in the levels of proteins required for Fas-effective function (FADD/MORTS; YAMA/CPC32p) (31, 32).

In the course of B cell development, signaling through surface Ig causes apoptosis, thereby eliminating self-reactive B cells in the bone marrow, whereas the Fas-mediated death mechanism appears to play a major role in the deletion of activated B cells in the
ROLE OF Bcl-xL IN Fas-MEDIATED APOPTOSIS

FIG. 4. Anti-Ig does not enhance Bcl-2 expression in the presence of CD40L. High-density B cells before culture (A) and after 72 hr culture with CD40L (B) or CD40L/anti-Ig (C) were stained with anti-Bcl-2 mAb as described under Materials and Methods and analyzed by FACS. Control samples were stained with an isotype-matched IgG1 control antibody.

peripheral lymphoid tissues (33). Depending upon the developmental stages of B cells, therefore, apoptotic death appears to be regulated differently. For example, in the immature B cell line WEHI 231, anti-Ig-induced apoptosis was prevented by CD40 stimulation. In WEHI 231 cells CD40L induces an elevation in the levels of Bcl-x protein (34). In contrast, anti-Ig protected from Fas-mediated death of CD40L-activated mature B cells. In both situations, bcl-x gene expression was up-regulated concomitantly with the time when the cells were protected from apoptosis.

Activation of naive B cells by T-dependent antigens in the T-enriched zone in the lymphoid follicles gives rise to B blasts in the GC. Recently it has been shown that CD40L/anti-Ig stimulation is able to induce the expression of some GC B cell markers, such as CD38 and Fas (35). It is also known that some GC B cells express Bcl-x in vivo, as shown by immunohistochemical methods (36). Our data raise the possibility that those Bcl-x+ GC B cells might be generated from CD40L/anti-Ig stimulation.

At the same time, the activation of resting T cells through the T-cell antigen receptor complex (TCR) induces FasL as well as CD40L expression (37, 38). How do T cells expressing both CD40L and FasL affect B cells under physiological conditions? In the early stage of T/B interactions, activated B cells expressing Bcl-x are resistant to killing by Fas–FasL interactions, allowing clonal expansion. At 3–5 days after B cell activation, however, Fas-mediated killing mechanisms become operational, which is coincident with a reduction in Bcl-xL levels. In this situation, stimulation of B cells through Ig receptors, which results in enhanced Bcl-xL expression, appears to rescue B cells from Fas-mediated killing. Taken together, these observations suggest a mechanism for allowing expansion of antigen-binding B cells, while simultaneously selecting against activated B cells that fail to bind antigen. This mechanism could be important in the affinity maturation of antibody responses (39).

REFERENCES

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