Cutting Edge: A Novel Mechanism for Rescue of B Cells from CD95/Fas-Mediated Apoptosis

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J Immunol 1999; 163:2378-2381; http://www.jimmunol.org/content/163/5/2378

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CD95-induced apoptosis contributes to the maintenance of homeostasis in both B and T lymphocyte-mediated immunity. B cells increase CD95 expression in response to activation signals and become susceptible to CD95-induced apoptosis. Protection from CD95-mediated death signals can be induced in mature B cells by signals delivered through the B cell Ag receptor. In this paper we demonstrate for the first time that rescue from apoptosis can occur independently of de novo protein synthesis. This rescue from apoptosis prevents activation of caspase 8, the apical caspase in the CD95 death pathway, and CD95-FADD (Fas-associated death domain containing protein) association does not occur normally. Thus B cell activation signals can biochemically modify proximal elements of the CD95 death pathway and regulate the sensitivity of cells to apoptosis induction at an early stage in programmed cell death. The Journal of Immunology, 1999, 163: 2378–2381.

Resting B cells constitutively express very low levels of CD95 and are not susceptible to CD95-mediated apoptosis induction. Ligation of CD40, by interaction with an activated T cell expressing CD154 (CD40L), causes up-regulation of CD95 expression on B cells and renders them susceptible to CD95-mediated apoptosis (1, 2). Anergic B cells are effectively deleted by CD95 engagement, whereas B cells that have not been tolerized can be protected from CD95-mediated apoptosis by B cell receptor (BCR) engagement (3–6). Thus, CD95-mediated apoptosis can contribute to the deletion of autoreactive B cells and/or B cells activated by “bystander” interactions with T cells in an Ag nonspecific fashion.

Regulation of apoptosis is a tightly controlled process and has principally been attributed to the transcriptional regulation of various genes. The most notable are Fas-associated death domain-like IL-1-converting enzyme (FLICE)-inhibitory protein (FLIP) (7), Toso (8), and the Bcl family members (9). Overexpression of genes encoding Bcl-2 or Bcl-xL protects B cells from CD95-mediated apoptosis (10, 11). In addition, a previously reported BCR-mediated rescue requires 12–18 h to fully protect and requires de novo protein synthesis (3, 12). Additionally, in other cell types the TNF receptor-associated factors (TRAF) 1 and 2 and inhibitor of apoptosis (IAP) (13) are able to inhibit apoptosis induction by CD95 and TNF. Thus, the susceptibility of cells to apoptosis induction can be regulated by the expression of pro- or antiapoptotic proteins.

The first documented step in CD95-mediated apoptosis is the recruitment of the adapter molecule Fas-associated death domain containing protein (FADD) (14, 15) to CD95, which allows the association of the zymogen form of caspase 8 with the CD95-FADD complex (16). Following the formation of the death-inducing signaling complex (DISC), caspase 8 is cleaved to form an active protease (16). Caspase 8 is the apical protease in the CD95 and TNFR-induced death cascade and is required (17) and sufficient for apoptosis induction (18, 19). Caspase 8 then cleaves downstream caspases, leading ultimately to the demise of the cell (20).

The murine B cell line A20 is susceptible to CD95-mediated apoptosis and can be rescued by signaling through its surface BCR (4). This BCR-mediated rescue has been attributed to the up-regulation of Bcl family members and the lack of caspase 1 activity (4). In contrast, we report here that BCR-mediated rescue occurs independently of de novo protein synthesis and blocks activation of caspase 8 and caspase 3, the initiating and effector proteases respectively. Surprisingly, rescue blocked association of CD95 and FADD. These results show that early biochemical events following BCR ligation interact with proximal signaling components of the CD95 death cascade.

Materials and Methods

Cells
A20, a mature IgG2a+ mouse B cell line derived from a lymphoma of a BALB/c mouse (21), was provided by Dr. David McKean (Mayo Medical Center, Rochester, MN). Cells were grown in RPMI 1640 supplemented with 10% FCS, 10 μM 2-ME, and antibiotics (BCM).

Antibodies
Goat or sheep anti-mouse IgM or IgG and F(ab’)2 were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and Sigma.
(Saint Louis, MO). Anti-CD95 (Jo2) was purchased from PharMingen (San Diego, CA). Anti-TNP, clone UC8–169, Syrian hamster IgG (an isotype control for anti-CD95) was obtained from the American Type Culture Collection (Manassas, VA). Goat anti-caspase 8 and rabbit anti-caspase 3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ab to mouse FADD was kindly provided by Dr. Astar Winoto (University of California, Berkeley) (22).

Assay for apoptosis

Cells were stimulated with anti-CD95 mAb (100 ng/ml) to induce apoptosis or a combination of anti-CD95 and additional Abs (10 μg/ml). At various times the cells were harvested, fixed in 70% ethanol, and stored at −20°C until further analysis. The samples were washed with PBS and resuspended in 0.5 ml phosphate-citrate DNA extraction buffer for 5 min. Following removal from the DNA extraction buffer, the cells were resuspended in 200 μg/ml RNase A and 40 μg/ml propidium iodide. DNA content of the cells was determined using a Becton Dickinson (San Jose, CA) FACScan benchtop flow cytometer. Cells containing less than 2 N DNA were counted as apoptotic. Cycloheximide (CHX) was purchased from Sigma and used at a final concentration of 10 μM. Cells were pretreated with CHX for 30 min before the addition of stimuli.

Caspase activity assay

Caspase 8 activity in cells was determined using a kit from Clontech (Palo Alto, CA) following the protocol supplied by the manufacturer. Cells were stimulated with anti-CD95 (1 μg/ml) or anti-CD95 plus anti-BCR or isotype control Abs at 10 μg/ml. The cells were lysed in the provided buffer supplemented with 50 μg/ml PMSF, 50 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin. Cellular debris was removed by centrifugation. The lysates were then incubated with substrate for caspase 8 (IETD-AFC) or caspase 3 (DEVD-AFC) (Biomol Research Laboratories, Plymouth Meeting, PA) for 1 h at 37°C. The samples were read on a spectrofluorometer, set at 400 nm excitation and 505 nm emission wavelengths.

Caspase activity was also assessed by Western blotting for caspase 3 or caspase 8. Briefly, cells were stimulated for 2 h with anti-CD95 at 1 μg/ml with or without anti-BCR or isotype control Abs at 10 μg/ml. Cells were lysed in 1% Nonidet P-40 lysis buffer containing protease inhibitors. Samples containing lysates from equal cell numbers were loaded onto SDS-polyacrylamide gels. Western blotting was conducted as described in Hsing et al. (23). Anti-caspase 3 or anti-caspase 8 were diluted 1:200 for blotting. Secondary Abs, anti-rabbit-HRP or anti-goat-HRP, were diluted 1:4000. Immunoprecipitation of CD95 was conducted essentially as described by Zhang and Winoto (22) except that in some samples anti-BCR Abs were included, as in the apoptosis assay. Blots were probed for FADD (66 ng/ml) and Fas (1 μg/ml).

Results and Discussion

B cells can be rescued from CD95-induced apoptosis by signals delivered through the BCR (4), but the precise mechanism of this rescue is poorly understood. Fig. 1 shows that A20 cells, when stimulated with agonistic anti-CD95 Abs, underwent apoptosis rapidly and nearly completely. However, simultaneous addition of anti-CD95 and anti-IgG F(ab′)2, abrogated the induction of apoptosis. Similar results were obtained using either sheep or goat anti-BCR Abs. Binding of anti-CD95 to the cells was not changed by the addition of anti-IgG F(ab′)2, as assessed by flow cytometry (data not shown); thus the block in apoptosis induction is not a result of the anti-IgG F(ab′)2 interfering with binding of anti-CD95. The addition of anti-IgM F(ab′)2, an isotype not expressed on A20, failed to modify CD95-induced apoptosis. To exclude the possible involvement of Fc receptors the experiments were performed with anti-IgG F(ab′)2 fragments (Fig. 1).

The susceptibility of cells to apoptosis has been attributed to the transcriptional regulation of genes encoding various pro- or anti-apoptotic proteins. However, the BCR rescue signal was effective with simultaneous addition of the two Abs and occurred quite rapidly (Fig. 1), suggesting early biochemical regulation of the death pathway. To test the possibility that production of antiapoptotic proteins was involved, cells were incubated with 10 μM CHX for 30 min before and during the experiments to block de novo protein synthesis. The CHX concentration used was sufficient to inhibit 97% of protein synthesis during the course of the experiment as measured by [3H]leucine incorporation (data not shown). Fig. 1B demonstrates that the rescue obtained was essentially the same in the presence and absence of de novo protein synthesis. Similar results were obtained with emetine HCl or the RNA synthesis inhibitor actinomycin D (data not shown). This finding eliminates the possibility of up-regulation and synthesis of new antiapoptotic proteins such as Bcl-2 or FLIP as a possible mechanism for BCR-mediated blocking of CD95-induced death in these B cells. This finding also strongly suggests that signals from the BCR act directly on the CD95 apoptosis induction pathway.

The results in Fig. 1 suggested that signaling per se was responsible for the observed rescue. Thus, to test when the cells were irreversibly committed to apoptosis, we stimulated cells with anti-CD95 and added anti-BCR simultaneously or at hourly intervals thereafter. The results shown in Fig. 2 demonstrate that the effectiveness of rescue is dramatically decreased by delaying the addition of the anti-BCR Ab by as little as 1 h, and a delay of 2 h after anti-CD95 virtually abrogated rescue. These data indicate that those cells in a culture that will undergo apoptosis are virtually all those cells in a culture that will undergo apoptosis are virtually all reversibly committed to do so within 2 h of the addition of anti-CD95. CD95-mediated apoptosis is initiated by the activation of caspase 8, which is a member of a class of proteases that are activated during apoptosis. Activation of a caspase occurs when the zymogen form of the protease is cleaved into two or more pieces and two domains of the cleaved protease, of ~10 and 20 kDa, associate and form an active enzyme (16). Caspase 8 is found in association with activated CD95 receptors and is required for

![FIGURE 1. BCR signals rescue A20 from CD95-mediated apoptosis, and rescue is independent of de novo protein synthesis. A, A20 cells were stimulated with anti-CD95 mAb or a combination of anti-CD95 and anti-BCR F(ab′)2 fragments. Cells were harvested at times shown and DNA content was determined. Results are representative of three similar experiments. B, Cells were treated as above except with the addition of 10 μM CHX 30 min before the addition of Abs. The average and SE of three experiments are shown.](http://www.jimmunol.org/)

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CD95-mediated apoptosis (16, 17, 22). The processing sites for caspase 8 suggest that it is autocatalytically cleaved and activated, whereas caspase 3 processing sites suggest it is activated by caspase 8 or a related activity (24). We hypothesize that activation of caspase 8 in cells is an irreversible event in the induction of apoptosis, and predict that it should also be a regulated event. It follows then, that in cells destined to live, very little if any active caspase 8 would be tolerated.

The hypothesis that activation of caspase 8 is an irreversible event in the induction of apoptosis predicts that rescue of cells should require the early addition of the rescuing stimuli. The data presented in Fig. 2 are consistent with this hypothesis. Indeed, activation of caspase 8 occurs very early in CD95-mediated apoptosis, as we have detected increases in caspase 8 and 3 activities as early as 30 min after addition of anti-CD95 (data not shown). Activation of caspase 3 was assessed because it is thought to be directly downstream of caspase 8 and is a major effector caspase (24). To determine the earliest step in the CD95 pathway that is blocked by BCR rescue, we measured caspase activity using two methods: a fluorogenic assay and western blotting for cleavage of caspase 8 or 3. Cells treated with an isotype control Ab or left untreated had low basal levels of caspase 8 or 3 activity (Fig. 3, A and B), but these activities were dramatically increased upon stimulation with agonistic anti-CD95 Ab. However, neither were activated in BCR-rescued cells. Treatment with isotype control Ab had no effect on caspase activity. Similar results were seen in the presence of CHX (data not shown), again demonstrating that BCR signaling rescues cells directly and not as a secondary event.

Western blot analysis (Fig. 3, C and D) confirmed data acquired from the fluorogenic assay. Caspase 8 activity can be determined by blotting for the cleaved and active product of ~20 kDa. In resting cells no 20-kDa fragment was visible; however, upon stimulation through CD95 a 20-kDa band appeared. The band was greatly reduced when a BCR signal was delivered, whereas the isotype control, anti-IgM, had no effect. Similar experiments were performed using Ab to caspase 3, except that activation was assessed by the loss of the inactive 32-kDa zymogen. Cells stimulated with anti-CD95 lost the 32-kDa zymogen (Fig. 3D, compare lanes 1 and 2). However, cells that received BCR stimulation maintained their caspase 3 zymogen (lane 3). Isotype control Ab had no effect upon the loss of the 32-kDa band. Thus caspase 3 activation is consistent with the model that these proteins are in a proteolytic cascade initiated by caspase 8. These data show that the activation of the apical protease in CD95-mediated apoptosis, caspase 8, was blocked in BCR-rescued cells, as was the activity of the effector caspase, caspase 3. Our results are thus consistent with the hypothesis that caspase 8 activation is a tightly regulated event in CD95-mediated apoptosis.

The first step in CD95-mediated apoptosis is the recruitment of the adapter molecule FADD to the CD95 receptor (14, 15, 22). As CD95-FADD association precedes caspase 8 activation, the association of CD95 and FADD was assessed by immunoprecipitation experiments to determine whether BCR-induced decreases in caspase 8 activation reflect decreased recruitment of FADD. Fig. 4 shows that FADD associates with CD95 when the cells have received signals through CD95 (lanes 1 and 2). In cells that have received BCR-derived rescue signals, the amount of associated FADD is greatly reduced (lanes 3 and 4). The Fas-FADD association was also blocked at 30 min (data not shown) and 90 min (lanes 5–8). Similar amounts of CD95 were precipitated as assessed by Western blotting. In experiments with F(ab’)2 fragments that provided weaker protection some CD95-FADD association was seen reflecting the amount of apoptotic cells found in the cultures (data not shown). Neither CD95 nor FADD were precipitated with isotype control Ab (data not shown). The lower band in the FADD Western blot is thought to be a degradation product, which is only seen in precipitates of cells that are apoptotic (22). These data show that the earliest event in CD95-mediated apoptosis was blocked in the presence of rescue signals delivered through the BCR and that the second step, caspase 8 activation, was also blocked.
Apoptosis can be characterized as either intrinsic or extrinsic. Apoptosis resulting from growth factor withdrawal or DNA damage is intrinsically induced death and is thought to involve delayed induction of caspase activity through unknown mechanisms. This process is controlled by transcriptional regulation of pro- and antiapoptotic proteins and posttranslational modification of cellular proteins (9, 25, 26). Alternatively, cells may also be susceptible to receptor-mediated apoptosis induction, a cell extrinsic apoptosis. Receptor-mediated apoptosis is characterized by the early activation of caspases and rapid apoptosis of the affected cells. The regulation of receptor-mediated apoptosis has been attributed to the transcriptional control of genes required for apoptosis induction such as Fas (1, 2), Fas ligand (27), caspase 8 (28), or genes whose products interfere with apoptosis induction such as Toso and FLIP (8, 29). Data presented here demonstrate an additional mechanism for the control of CD95-mediated apoptosis. Signals induced via a transmembrane receptor, the BCR, can override the death signal delivered by CD95, and this rescue is a direct effect on the cellular components involved in apoptosis induction. Because rescue does not involve the up-regulation of new antiapoptotic proteins, cell fate can be controlled in a very rapid fashion. In B cells this may aid in retention of useful cells while allowing the elimination of autoreactive cells due to down-regulation of signaling in anergic B cells.

Acknowledgements

We thank Luis Ramirez for technical assistance and Dr. Astar Winoto for providing Ab to FADD.

References