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Cutting Edge: Cellular Fas-Associated Death Domain-Like IL-1-Converting Enzyme-Inhibitory Protein Protects Germinal Center B Cells from Apoptosis During Germinal Center Reactions

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During germinal center (GC) reactions, follicular dendritic cells are believed to select memory B lymphocytes by switching off apoptosis in the successfully binding B cells. The cellular signals involved in this process are largely unknown. Here, we show that GC B lymphocytes have a long isoform of the cellular homologue of the viral Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein (cFLIPL), which is capable of inhibiting death receptor-induced caspase activation. In isolated GC B cells, cFLIPL decays rapidly even without Fas ligation, and this results in activation of caspase activity and apoptosis. Contact with follicular dendritic cells prevents cFLIPL degradation and blocks all signs of apoptosis, even in the presence of anti-Fas Abs. cFLIPL expression is sustained by CD40 ligation as well, suggesting that at least at some stage of the GC reaction activated T cells may help select B cells to leave the follicular dendritic cell network without becoming apoptotic. The Journal of Immunology, 2001, 166: 6473–6476.

The mechanism by which FDCs silence apoptosis in adhering B cells is not clear. Freshly isolated single GC B cells have little caspase-3 activity, but this increases dramatically within a few hours at 37°C (7). Activation of caspases can be induced by signals derived from death receptors (DR) that belong to the TNFR1 family (8). GC B lymphocytes express Fas/APO-1/CD95, and this receptor is part of a well-studied DR pathway (9). Ligation of this DR results in the formation of an internal death-inducing signaling complex (DISC). When Fas is activated, the adapter molecule Fas-associated death domain will bind with its C-terminal death domain to Fas. The N-terminal death effector domain of the adapter molecule binds to the death effector domain of caspase-8, resulting in activation of this enzyme, followed by activation of caspase-3 (8). Fas ligation can induce cell death by two different routes. The type I cell death route implies the rapid formation of the DISC with high levels of active caspase-8. Alternatively, a type II cell death route involves low levels of DISC formation and caspase-8 activation. In this situation, amplification of the apoptotic signal involves loss of mitochondrial integrity, leading to cytochrome c release and consequent activation of caspase-9 (10).

Fas-associated death domain-like IL-1-converting enzyme-like inhibitory proteins (FLIPs) can inhibit both death routes. FLIPs were first identified as viral products that interfere with DR-mediated elimination of infected cells (11). Cellular homologues of viral FLIPs (cFLIPs) have been found as well (12). At least two splice variants have been described, a short and a long isoform (cFLIPL), which are both capable of inhibiting DR-induced apoptosis. Both cFLIP variants can block caspase-8 activation, but cFLIPL is more potent (13).

Interestingly, GC B lymphocytes have an endonuclease in their nuclei that is readily activated when the B cell is detached from its microenvironment but is effectively silenced as soon as the contact with FDCs is restored in vitro (5, 6). Recently, we have shown that, in addition to and downstream of their caspase cascade, GC B lymphocytes have a thus far unidentified cathepsin activity that controls DNA fragmentation (7).

Here, we show that cFLIPL is highly expressed in GC B cells and is associated with survival of the cells. Its expression is critically dependent on physical interaction of these B cells with
FDCs. In addition, cFLIP<sub>L</sub> expression is sustained by CD40 ligation as well, thereby giving a clue as to how selected GC B lymphocytes might be able to escape the FDC network without undergoing apoptosis (i.e., by interaction with a proper T cell).

Materials and Methods

Isolation and culture of GC B lymphocytes and FDCs

B lymphocytes and FDCs were isolated from human tonsils as described previously (7). A total of 1 × 10<sup>6</sup>/ml purified GC B cells were incubated in IMDM with 10% FCS (HyClone Laboratories, Logan, UT) for 5 h at 37°C. GC B cells were incubated with or without the cathepsin inhibitor E64d (Scientific Marketing Associates, Barnet, U.K.). Fas ligation was done with the anti-Fas Ab CH11 (Immunotech, Luminy, France). CD40 ligation was done with a CD40 ligand (L)-transfected L cell line (the kind gift of Dr. C. van Kooten, Leiden University Medical Center, Leiden, The Netherlands). FDC-enriched fractions were incubated for 16 h at 37°C, and the resultant FDC-B cell clusters were purified by 1 × g sedimentation on 30% FCS (HyClone) in IMDM. These clusters were monitored under a microscope to ensure that no single cells were present. Clusters were incubated at 37°C for 5 h with or without anti-Fas Abs.

Detection of apoptotic parameters

Phosphatidyl serine (PS) exposure was assessed after annexin V and propidium iodide (PI) double staining using the Apo Target annexin V FITC apoptosis kit (Biosource Europe, Fleurus, Belgium). DNA strand breaks were analyzed by dUTP-fluorescein labeling using the in situ cell death detection method (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer. PS exposure and DNA strand breaks were analyzed by FACS. Caspase-8 activity was determined using a caspase-8 fluorometric kit (R&D Systems, Minneapolis, MN) using IETD-7-amino-4-trifluoromethyl coumarin (AFC) as a substrate. AFC release was measured using a Wallac Vitor 1420 multilabel counter (EG & G Wallac, Turku, Finland).

Western blotting of cFLIP was done with 30 μg protein/lane, and expression of cFLIP was assessed using the FLIP-specific mAb Nf6. For caspase-8, 100 μg protein was applied per lane, and caspase-8 was detected using the mAb c1 (both Nf6 and c1 mAbs were kind gifts of P. H. Krämer, German Cancer Research Center, Heidelberg, Germany). Blots were stained with the peroxidase-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark), and Lumi-Light PLUS Western blotting substrate (Roche Diagnostics).

Results

Apoptosis in GC B lymphocytes is a spontaneous process

GC B cells undergo rapid apoptosis upon in vitro culture. This process is autonomous (i.e., it does not require an inducing death receptor signal). Typically, 40–50% of the cells express PS within 5 h (Fig. 1A). This is definitely accelerated after ligation of Fas with the CH11 mAb, indicating that the Fas route is functional in these cells. Ligation of CD40 profoundly inhibits PS expression.

GC B lymphocytes in clusters with FDCs are protected from apoptosis, even after ligation of Fas, indicating that FDCs in some way prevent the Fas-mediated activation of PS expression. PS exposure completely parallels the degradation of the inactive p55 and the simultaneous appearance of the active p20 fragment of caspase-8 (Fig. 1B). Moreover, it is shown in Fig. 1C that caspase-8 enzyme activity strictly correlates with the appearance of the p20 caspase fragment as well as with PS exposure. These data indicate that FDCs potently silence caspase-8 activation, even during Fas ligation.

GC B lymphocytes contain cFLIP<sub>L</sub>, that decays rapidly in single cells

Freshly isolated GC B cells express cFLIP<sub>L</sub>, as shown by Western blotting (Fig. 2). Upon incubation at 37°C, this protein rapidly decays and is virtually gone after 5 h. Remarkably, ligation of CD40 with a CD40L leads to sustained expression of the cFLIP<sub>L</sub> (Fig. 2A). Also, if GC B lymphocytes are cultured with FDCs and the B cells are recovered from these clusters, the expression of cFLIP<sub>L</sub> is maintained (Fig. 2B). These data imply that CD40 ligation (presumably by T cells) and physical contact with FDCs are powerful signals to maintain cFLIP<sub>L</sub> expression in GC B cells and, hence, may prevent DR-induced caspase activation.

cFLIP<sub>L</sub> disappearance is an early process in GC B cell apoptosis

As we recently published, GC B lymphocytes have a cathepsin-like activity that is instrumental in their rapid DNA fragmentation (7). To find out whether this cathepsin may be responsible for the disappearance of cFLIP<sub>L</sub>, the general cathepsin inhibitor E64d was added, and cFLIP<sub>L</sub> expression was followed. As seen in Fig. 3, E64d effectively blocks the formation of DNA strand breaks (Fig. 3B) but leaves PS exposure unhampered (Fig. 3A). Also, addition of E64d to isolated GC B lymphocytes did not prevent the disappearance of cFLIP<sub>L</sub> from these cells. These data indicate that cFLIP<sub>L</sub> decay is independent of cathepsin activity. Similar results were obtained when isolated GC B cells were incubated with the caspase inhibitors z-VAD-fmk or z-DEVD-fmk (data not shown).
FIGURE 2. cFLIP<sub>L</sub> rapidly disappears from isolated GC B cells upon culture, but cFLIP<sub>L</sub> expression is maintained by CD40 ligation and contact with FDCs. Western blotting of cFLIP<sub>L</sub> from GC B cells either directly after isolation (t0) or after 5 h incubation at 37°C (t5). Data are compared with 5 h incubation of single GC B cells with CD40L (A) or with GC B cells recovered from FDC-B cell clusters (B). A total of 30 µg of protein were loaded in each lane. Data are representative examples of at least three different experiments in both A and B.

Altogether, these data indicate that the disappearance of cFLIP<sub>L</sub> is an initial event in the triggering of apoptosis in GC B cells and not a result of apoptosis-associated proteolysis in these cells.

Discussion

Isolated GC B cells readily undergo apoptosis when kept at 37°C. This is accompanied by the rapid activation of caspase-8. Obviously, this activation seems a spontaneous process that does not require Fas ligation. If Fas is ligated with the stimulating CH11 mAb, both caspase-8 activity and PS exposure are dramatically accelerated, indicating that the Fas route is definitely available for the induction of caspase-mediated apoptosis.

A role for Fas in the selection of high affinity B cells has recently been strengthened, because lpr mice (who lack functional Fas) show impaired selection of high affinity B lymphocytes in GC reactions (14). It remains unclear whether Fas must be ligated by FasL or, alternatively, Fas is activated spontaneously. In GCs, an obvious FasL source is lacking, as only few cells express this L. FasL expression has been demonstrated on scattered nonlymphoid cells, but in tonsils, the FasL is mainly expressed on plasma cells (15). Caspase-8 activation is strongly inhibited by cFLIP (13). We have shown here that freshly isolated GC B cells contain the 55-kDa isoform of cFLIP, cFLIP<sub>L</sub>. Naive and memory B cells express much lower amounts (data not shown). None of these B cell fractions expressed the short isoform of cFLIP.

Upon incubation of isolated GC B lymphocytes in vitro, cFLIP<sub>L</sub> is rapidly degraded and is virtually absent after 5 h. Several experiments argue in favor of the idea that this decay of cFLIP<sub>L</sub> is a triggering event for caspase activation rather than a result of general proteolysis during apoptosis. For instance, inhibition of cathepsin activity by the general cathepsin inhibitor E64d did not prevent cFLIP<sub>L</sub> degradation (Fig. 3C). Similar results were found when caspases were blocked by the caspase inhibitors z-VAD-fmk or z-DEVD-fmk (not shown). In addition, Hennino et al. (16) have recently studied the composition of the DISC in GC B lymphocytes at different time points. They demonstrated that cFLIP<sub>L</sub> disappearance from the DISC is virtually complete within 10 min after Fas ligation, indicating that it must be an initial step rather than a result of apoptosis.

Our data suggest an important role of early cFLIP<sub>L</sub> decay and caspase-8 activation typical of a type I route of cell death in GC B lymphocytes. However, as we showed earlier, GC B cell apoptosis also includes reduction of mitochondrial membrane potential (7). Mitochondria are involved in both the type I and II routes of cell death, but their inactivation is not strictly necessary for the type I route (8). Transgenic overexpression of Bcl-2 or Bcl-x<sub>L</sub>, proteins involved in inhibition of mitochondrial cytochrome c release, inhibits GC B cell apoptosis, indicating an important role for the type II cell death route as well (17, 18). In tonsillar B lymphocytes, it has been shown that especially Bcl-x<sub>L</sub> expression is associated with survival, arguing in favor of a mitochondrial involvement in GC B cell apoptosis (19).

By contrast, the recent data of Defrance and coworkers using selective inhibitors of either caspase-8 or caspase-9 strongly suggest that apoptosis of human tonsillar GC B cells in vitro predominantly depends on caspase-8 activity, not on caspase-9 activity (16). This is in line with their earlier data showing that, in CD40L-activated virgin B cells, Fas-induced cell death bypasses the mitochondrial pathway (20).

In our experiments, cFLIP<sub>L</sub> decay is profoundly inhibited when GC B lymphocytes are either in contact with FDCs or with a CD40L-transfected cell line. The CD40-mediated signal requires some time because simultaneous CD40 ligation and anti-Fas treatment does not inhibit apoptosis, whereas CD40 ligation for 4 h followed by anti-Fas treatment results in inhibition of apoptosis (data not shown). The contact with FDCs effectively protects GC B lymphocytes against Fas-mediated cell death. The signaling conditions for this anti-Fas insensitivity are not clear, but cell to cell contact between FDCs and B cells is important (21). The FDC-derived signal acts independently of CD40 signals, Ig receptors, or adhesion molecules (5, 22). Bcl-2 seems not to be involved because this protein is only present at low levels in GC B lymphocytes and is down-regulated on B cells in contact with FDCs (23, 24).

The data presented here may provide a clue as to how selected GC B cells are kept alive in the FDC network. Moreover, because cFLIP<sub>L</sub> expression is maintained by CD40 ligation as well, it may be hypothesized that activated T cells in the GC that can rapidly express large amounts of the L (25) are the permissive factor that help the putative memory B cells to leave the GC for further differentiation.
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