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Germinal Center B Cell Apoptosis Requires Both Caspase and Cathepsin Activity

Marco van Eijk and Cornelis de Groot1

Follicular dendritic cells (FDCs) select B cells during germinal center (GC) reactions. The B cells that are able to bind to the FDCs receive a signal that leads to the termination of endonuclease activity in the nuclei of those B cells. This signal must be in addition to the signals transferred through the cross-linkage of the B cell receptors and signals resulting from the interactions of the adhesion molecules lymphocyte function-associated Ag-1 and very late Ag-4 with ICAM-1 and VCAM-1, respectively. In this report, we present evidence that the FDCs silence all apoptotic processes in GC B lymphocytes and additionally switch off pre-present endonuclease activity. We also show that GC B cell apoptosis requires cathepsin activity downstream of caspase-3. This cathepsin activity is directly connected to endonuclease activity and therefore may be an interesting target for the antiapoptotic factors produced by FDCs. The Journal of Immunology, 1999, 163: 2478–2482.

Germinal centers (GCs)2 are specialized microenvironments in lymphoid follicles of secondary lymphoid organs. Here, B lymphocytes undergo affinity maturation of their B cell receptors (BCR) and Ig isotype-switch, resulting in the formation of memory B cells (1–3). During a GC reaction, B cells are selected and their Ag specificity is checked at different levels (4). Antiapoptotic signals provided by follicular dendritic cells (FDCs) are crucial in this selection process. Native Ags are presented to GC B cells in the immune complexes present on FDCs, allowing B lymphocytes with high affinity BCRs to bind. As a result of this highly competitive binding, the programmed cell death of the GC B cells is cancelled in the binding cells only (5, 6). FDCs protect the attached B lymphocytes in such a way that endonuclease, which is pre-present in the nuclei of GC B cells, is switched off within a few hours (7).

The precise mechanism of this action is largely unknown. BCR cross-linkage with Ag in the immune complexes on FDCs is an important prerequisite; however, interactions between the adhesion molecules ICAM-1 (CD54) and VCAM-1 (CD106) with lymphocyte function-associated Ag-1 (LFA-1) and very late Ag-4 (VLA-4) (CD49d), respectively, also play a role in the intimate contact between B lymphocytes and FDCs (8–10). Thus far, the apoptosis of GC B cells could be postponed by cross-linkage of LFA-1, VLA-4, CD21, CD40, BCR, or CD40 and BCR; however, none of these signals could switch off endonuclease activity in GC B cells (5, 7, 9–13).

To gain insight into the specific rescue mechanism of FDCs, it is necessary to know what routes are used to trigger apoptosis in GC B cells. Cysteine proteases fulfill crucial roles in apoptosis. For instance, the family of IL-1-converting enzyme-like proteases (now called caspases) (14–16) forms an important cascade that links triggering signals such as Fas ligation to the final activation of DNA fragmentation (17–19). This cascade is highly redundant; however, in general, the activation of various members of the caspase family may lead to the activation of caspase-3, resulting in cleavage of various substrates that are crucial in the execution phase of apoptosis. In addition, it was shown recently that members of the papain family of cysteine proteases may be involved in apoptotic processes as well. For example, calpains are involved in the upstream regulation of thymocyte apoptosis (20, 21) and recently, cathepsin W, also called lymphopain, was found in CD8+ T lymphocytes and NK cells, suggesting a role in the apoptosis pathway that is used for target cell killing (22, 23).

In the present paper, we have addressed the role of FDCs on GC B cell apoptosis, and especially the enzymes involved in the regulation of endonuclease activity. Our experiments indicate that both caspase and cathepsin activity are required in the apoptotic cascade of GC B cells. Furthermore, we show that the cathepsin activity acts downstream of caspases and is probably the last proteolytic step involved in the activation of DNA fragmentation. FDCs, therefore, may act on endonuclease activity directly because that is the only apoptotic parameter present in freshly isolated GC B cells (F-B) that is switched off.

Materials and Methods
Isolation of GC B cells from human tonsils

B lymphocytes were isolated from tonsils according to the method of Lindhout et al. (7). Briefly, tonsillar cell suspensions were depleted of T cells using 2-aminoethylisothiouroniumhydrobromide (Sigma, St. Louis, MO)–treated SRBCs (24) followed by density centrifugation on a Lymphoprep (1077 mg/ml; Nycomed, Oslo, Norway) to remove rosetted cells. The final cell population contained >98% CD19+ cells (B cells) and <2% CD3+ cells (T cells). This B cell suspension was centrifuged (15 min, 1200 × g, 4°C) on a Percoll gradient (Pharmacia, Uppsala, Sweden) consisting of three layers (1043, 1067, and 1077 mg/ml). Cells at the 1043/1067 interface were collected and incubated with Abs against surface IgD (MAS 590p, Harlan Sera-Lab, Loughborough, U.K.) and anti-CD39 (AC2, Immunotech, Marseilles, France). Labeled cells were depleted using sheep anti-mouse Ig-coated Dynabeads (Dynam AS, Oslo, Norway). The resulting
purified GC B cell fractions consisted of >98% CD38\(^+\) cells and <2% CD39\(^+\) and surface IgD\(^+\) cells.

**Isolation of FDCs**

FDCs were isolated from tonsils as described by Parmentier et al. (25). Tonsils were cut into pieces and treated with a collagenase (200 U/ml collagenase IV, Boehringer Mannheim, Mannheim, Germany), followed by density sedimentation on a cold discontinuous BSA (Path-o-cyte 4, bovine albumin, Instrumentation Laboratory, Little Chalfont, U.K.) gradient in HBSS (Life Technologies, Paisley, U.K.), consisting of layers of 1.5, 2.5, and 5%. The cells at the 2.5–5% interface were harvested and washed in IMDM. These FDC-enriched fractions (Affigel Blue, Bio-Rad Laboratories, Hercules, CA) were used for all experiments.

**Cell cultures**

All standard media used (IMDM, HBSS) contained gentamicin (90 \( \mu \)g/ml). Cells were cultured for 4 h in 24-well culture plates (Costar, Cambridge, MA) in IMDM supplemented with 10% FCS (HyClone, Logan, UT) in the presence or absence of the general cathepsin inhibitor E64d, the cathepsin MA) in IMDM supplemented with 10% FCS (HyClone, Logan, UT) in the presence or absence of the general cathepsin inhibitor E64d, the cathepsin inhibitor Z-phenyl-phenyl-CHN\(_2\) (ZPP) (Enzyme System Products, Dublin, CA), the cathepsin inhibitor Z-phenyl-phenyl-CHN\(_2\) (ZPP) (Enzyme System Products, Dublin, CA) and the broad range caspase inhibitor z-Val-Ala-DL-Asp (ZVAD)-fluoromethylketone (Alexis Corporation, Läufelfingen, Switzerland). The inhibitors were dissolved in DMSO (Sigma) and applied at concentrations ranging from 50 nM to 150 \( \mu \)M as indicated.

FDC-enriched cell suspensions were depleted of T cells using SRBCs and cultured in IMDM supplemented with 10% FCS. After 14 h, FDC-B cell clusters were separated from single B cells by 1 \( \times \) g sedimentation on IMDM with 30% FCS for 30 min at 0°C. Clusters were isolated from the pellet, and single B cells were harvested from the interface. apoptotic parameters were determined as described below.

**Detection of apoptotic parameters**

The reduction of mitochondrial membrane potential (\( \Delta \psi_{\text{m}} \)) was analyzed according to the method of Zamzami et al. (26) using 3,3\'-dihexyloxacarbocyanine iodide (DiOC\(_6\) (3)) (Molecular Probes, Leiden, The Netherlands) in combination with propidium iodide (PI) (Sigma). DiOC\(_6\) (3) was applied to the cells at a concentration of 40 nM in PBS for 15 min at 37°C. The cells were washed and taken up in PBS. PI (50 ng/ml final concentration) was added, and samples were directly analyzed.

DNA strand breaks were analyzed using the In Situ Cell Death Detection Kit (Boehringer Mannheim) according to the manufacturer’s instructions. Briefly, 1–2 \( \times \) 10\(^5\) B cells were washed in PBS with 1% BSA, taken up in PBS and paraformaldehyde (2% final concentration), and permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate. dUTP-fluorescein was coupled to the DNA strand breaks using terminal transferase.

Phosphatidyl serine (PS) exposure was determined using annexin V-FITC (Bender Medsystems Diagnostics, Vienna, Austria) in combination with PI. Cells were labeled with annexin V-FITC for 30 min on ice, washed, and taken up in medium containing PI.

Caspase-3 activity was measured using the ApoAlert CPP32 Fluorescent Assay Kit (Clontech, Palo Alto, CA). Briefly, isolated nuclei were incubated at 37°C for 4 h in TNS buffer (10 mM Tris-HCl, 200 mM sucrose, and 60 mM NaCl (pH 7.5)) followed by the separation of large and small DNA fragments; these fragments were precipitated and run on an agarose gel.

Fluorescence analyses of DiOC\(_6\) (3) signal, DNA strand breaks, and PS exposure using annexin V-FITC staining (Fig. 1a), reduction of \( \Delta \psi_{\text{m}} \) using DiOC\(_6\) (3) staining (Fig. 1b), DNA strand breaks using the in situ cell death detection method (Fig. 1c), caspase-3 activity by monitoring the cleavage product of the caspase-3 specific substrate 7-amino–4-trifluoromethyl coumarin-peptide substrate conjugate (Fig. 2a), cleavage of PARP (Fig. 2b), and endonuclease activity in isolated nuclei of GC B cells (Fig. 2c).

As is shown in Fig. 1, a–c, F-B showed low levels of PS exposure, a high \( \Delta \psi_{\text{m}} \), and very few DNA strand breaks. Caspase-3 is inactive in these cells (Fig. 2a), which is consistent with the finding that the DNA repair enzyme PARP is present in its uncleaved 113-kDa form (Fig. 2b). These data confirm the nonapoptotic phenotype of F-B.
After 14 h however, the single B cell fraction (N-B) showed a largely increased level of PS exposure, reduced Δψm, and increased numbers of DNA strand breaks (Fig. 1, a–c). Moreover, caspase-3 activity could be demonstrated at this timepoint (Fig. 2a). This is in agreement with the finding that PARP was now cleaved (Fig. 2b). The cleavage reaction is observed in the N-B fraction. In c, DNA strand breaks in the N-B fraction is switched off in the C-B fraction. One representative example of at least three experiments is shown with respect to PARP cleavage and endonuclease activity (b and c). The results in a are expressed as mean ± SD (n = 3).

**FIGURE 2.** Caspase-3, PARP, and DNA fragmentation in GC B lymphocytes upon contact with FDCs. a, caspase-3 activity; b, PARP cleavage; c, endonuclease activity in isolated nuclei. Apoptotic parameters were determined in F-B, N-B, and C-B. Caspase-3 activity is expressed as a percentage of the values found in the F-B fraction. In a–c, an increase is observed in the N-B fraction. In c, the latent endonuclease activity in the F-B fraction is switched off in the C-B fraction. One representative example of at least three experiments is shown with respect to PARP cleavage and endonuclease activity (b and c). The results in a are expressed as mean ± SD (n = 3).

Apolipoprotein of GC B cells includes both caspase and cathepsin activity

To investigate whether caspases and other cysteine proteases, such as cathepsins, were involved in the apoptotic machinery of GC B lymphocytes, purified GC B cells were cultured in the presence or absence of various cathepsin inhibitors and the general caspase inhibitor ZVAD (Fig. 3). DNA strand breaks were analyzed after 4 h using the in situ cell death detection assay. Strikingly, DNA strand breaks were blocked not only with the caspase inhibitor ZVAD but also with the general cathepsin inhibitor E64d. However, this was not observed when specific inhibitors for cathepsin B (CA074-Me), cathepsin L (ZPP), or cathepsin S (LHVS) were used. These data imply that both caspase and cathepsin activities are required for the occurrence of DNA strand breaks in GC B cells, and that caspase(s) and cathepsin(s) must act in a sequential manner.

**Cathepsins act downstream of caspase-3 on endonuclease activity in GC B lymphocytes**

To further investigate at which level the cathepsins were involved in the apoptotic cascade, the effects of the cathepsin inhibitor E64d and the caspase inhibitor ZVAD on different apoptotic processes were measured, including PS exposure, reduction of Δψm, caspase-3 activity, cleavage of PARP, and DNA strand breaks. As shown in Fig. 4a, PS exposure was inhibited by ZVAD but not by E64d. Also, ZVAD inhibited mitochondrial damage (Fig. 4b), caspase-3 activity (Fig. 4c), and processing of the caspase-3 substrate PARP (Fig. 4d). E64d did not block any of these processes, indicating that cathepsin activity could not reside upstream of caspase activity. Clear inhibition by E64d was only found when DNA strand breaks were studied (Fig. 4e). These data demonstrate that a thus far unidentified cathepsin activity is involved in one of the very downstream steps of the apoptotic machinery, and that this activity is essential for the execution of DNA fragmentation. Consequently, cathepsin activity must be downstream of caspase-3 activity.

**FIGURE 3.** Inhibition of caspase activity or cathepsin activity blocks the occurrence of DNA strand breaks in GC B cells. Purified GC B lymphocytes were incubated for 4 h at 37°C in IMDM/10% FCS in the presence or absence of the general caspase inhibitor ZVAD (150 μM), the general cathepsin inhibitor E64d (60 μM), the cathepsin B inhibitor CA074-Me (20 μM), the cathepsin L inhibitor ZPP (10 μM), and the cathepsin S inhibitor LHVS (50 nM); DNA strand breaks were analyzed using an in situ cell death detection kit. Both E64d and ZVAD were able to inhibit DNA strand breaks, whereas CA074-Me, ZPP, and LHVS could not. Data are expressed as a percentage of the inhibition of DNA strand break formation. Positive and negative controls were 0% and 100% cells with DNA strand breaks, respectively. Results are expressed as mean ± SD (n = 3).
We have shown previously that the binding of GC B cells to FDCs in vitro results in the silencing of endonuclease activity in the B cell nuclei within 4 h (7). Recently, we have found that as few as 1–2 h may be sufficient for this process (data not shown).

Thus far, the enzymatic activities that are operational during GC B cell apoptosis as well as the mechanisms used by FDCs to rescue the binding B lymphocytes are poorly understood. Many factors contribute to the rescue process, but none of these, either alone or in concert, seem to explain the rapid switch-off of nuclear endonuclease. The data shown for PARP cleavage in d are from one representative example of at least four experiments with different donors. Control indicates GC B cells incubated for 4 h at 37°C without inhibitors. Results shown in a, b, c, and e are expressed as the mean ± SD (n = 4).
of these processes are cancelled upon binding to FDCs. Remarkably, FDCs prevent the up-regulation of multiple, caspase-related events. In addition, they eliminate the latent endonuclease activity that is present in the nuclei of freshly isolated B lymphocytes.

We found that caspase-3 activity is absent in both freshly isolated and FDC-bound GC B cells. Concordantly, PARP is found in its intact form only. These data are in agreement with our previous experiments showing that GC B lymphocytes are resistant to Fas-mediated apoptosis as long as they remain in contact with FDCs (27). The reason for this resistance is still unclear, but it may be speculated that inhibitory proteins such as FLICE-inhibitory protein or Fas apoptosis inhibitory molecule (28, 29) are instrumental in keeping the caspase route silent. Detachment of GC B cells from their natural counterstructures may lead to a reactivation of the caspase route, resulting in the execution of apoptosis. Recently, it was reported that a caspase-activated DNase/DNA fragmentation factor (DFF40) is released from its inhibitor, inhibitor of caspase-activated DNase/DFF45, by cleavage through caspase-3 (17–19).

These findings connect the caspase route directly to the execution of apoptosis (i.e., to DNA fragmentation). Thus, our attempts to demonstrate DFF45 processing in GC B cells by Western blotting have been unsuccessful (data not shown). This may mean either that a different DNase activity is used by these cells or that the activation mechanism of the DNase is different.

Here, we have shown that an additional protease (i.e., a thus far unidentified cathepsin) is part of the apoptotic cascade of GC B lymphocytes. Inhibition of this cathepsin with the inhibitor E64d completely prevents DNA strand breaks but leaves the caspase-dependent PS exposition and PARP cleavage untouched, demonstrating that the cathepsin acts downstream of caspase-3.

It is tempting to speculate that blockade of this very downstream cathepsin activity may well be the target of FDC function. Natural inhibitors of cathepsins belong to the family of cystatins (30, 31), and it has been demonstrated that cathepsins and cystatins are involved in different models of apoptosis. For instance, in a model for bile-salt induced apoptosis in hepatocytes, it was shown that cathepsin B was downstream of caspase-3. Cystatin A could efficiently block apoptosis in this model (32). In addition, increased apoptosis was reported in the brain of the cystatin B knockout mouse (33). Moreover, it was shown that cystatin A could inhibit the virus-induced apoptosis of a carp cell line (34).

Interestingly, cystatin A is present in FDCs (35); it is attractive to consider the possibility that it is transported to B cells during the intimate contact with FDCs. Currently, we are investigating this protein and its relevance in FDC-mediated antiapoptotic signaling in GC B lymphocytes.

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