Original Article

Apoptosis of Marginal Zone B-Cells in Unimmunized Mice

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Although evidence suggests that peripheral B cell death plays a role in maintenance of B cell homeostasis including elimination of self-reactive B cells, the site of B cell death in unimmunized peripheral lymphoid organs has not yet been studied. Here we demonstrate that cells exhibiting reduced mitochondrial membrane potential and those expressing active caspase 3, both of which are characteristic for apoptosis, are specifically accumulated in the marginal zone (MZ) B cell compartment compared to the other B cell subsets in normal unimmunized mouse spleen. These apoptotic MZ B cells exhibit increased expression of MHC class II and CD22, whose expression is shown to be increased upon B cell activation, but not other activation markers such as CD69 and CD86, suggesting that apoptotic MZ B cells are activated with partial expression of B-cell-activation-associated antigens. These results indicate that MZ is the major site of peripheral B cell apoptosis in unimmunized mice. Apoptosis of MHC class IIh, CD22q MZ B cells may contribute to B cell homeostasis.

Key Words: Apoptosis; Marginal zone B Cell; Caspase 3; CD22

Introduction

Cell death plays a crucial role in shaping B cell repertoire both before and after antigen encounter12. Before antigen encounter, elimination of self-reactive B cells in the bone marrow and in the peripheral lymphoid tissues probably by induction of cell death contributes to self tolerance3, although receptor editing in which self-reactive B cell antigen receptor (BCR) is replaced by non-self-reactive BCR plays a major role in self tolerance in the bone marrow5. When B cells undergo germinal center reaction after antigen encounter, only B cells expressing high affinity BCR are able to survive, whereas low affinity B cells are eliminated by apoptosis, resulting in production of high affinity antibody6. Moreover, antigen-activated B cells appear to undergo apoptosis after elimination of antigen, contributing to termination of immune responses7. Although a large number of apoptotic cells are demonstrated in germinal centers after immunization9. B cell apoptosis in the peripheral lymphoid organs in unimmunized animals has not been extensively studied.

After type 1 transitional (T1) B cells migrate to spleen, they mature to type 2 transitional (T2) B cells, which then undergo maturation to either follicular B cells or marginal zone (MZ) B cells10. MZ B cells respond to blood-borne particulate antigens and rapidly produce antibodies in a T cell-independent manner11, thereby contributing to host defense against bacterial infection. MZ B cell differentiation is augmented in autoantibody-transgenic mice in which frequency of self-reactive B cells is increased, and self-reactive B cells are accumulated in MZ B cells in these mice12, 14. Although self-reactive B cells may generate BCR signaling by interaction with self-antigens, MZ B cell differentiation does not appear to simply depend on increased BCR
signaling. Indeed, the percentage of MZ B cells is reduced in mice in which BCR signaling is augmented, whereas mice deficient in BCR signaling prefers MZ B cell fate. In the present study, we demonstrate that a fraction of MZ B cells especially those expressing higher levels of MHC class II and CD22 undergo apoptosis, whereas frequency of apoptotic cells is very low in both T2 and follicular B cells. These results suggest that MHC class II+ CD22+ MZ B cells constitute a population of B cells that undergo apoptosis.

Materials and Methods

Mice. C57BL/6 and BALB/c mice were purchased from Sankyo Labo Service (Tokyo, Japan). All Procedures followed the guidelines of the Tokyo Medical and Dental University for Animal Research and were approved by the Animal Ethics Committee.

Flowcytometry. Mouse spleen cells were stained with the following reagents: DiOC₃(3) (3,3’-dihexyloxacarbocyanine iodide; Molecular Probes, Inc), CaspACE™ FITC-VAD-FMK In Situ Marker (Promega Corporation), PE-labeled anti-mouse CD23 antibody (e-bioscience), PE-labeled anti-mouse CD69 antibody, biotin-labeled antibodies specific for CD22, CD40, CD43, CD86, CD138 and I-A/I-E (BD Pharmingen), biotin-labeled anti-mouse IgD antibody (Southern Biotechnology Associates), PerCP-Cy5.5-labeled anti-mouse IgM antibody (BD Pharmingen). Biotin-labeled antibodies were visualized by Alexa Fluor647 conjugated streptavidin (Molecular Probe, Inc). Cells were analyzed by flow cytometry using an LSR with CELL Quest software (BD Bioscience).

Immunohistochemical analysis. Spleen sections were prepared as described previously, and were stained with Alexa Fluor647-labeled anti-mouse B220 antibody, biotin-labeled rat anti mouse metallophilic macrophage antibody (MOMA-1; BMA Biomedicals) and affinity-purified rabbit anti-active caspase 3 antibody (R&D systems), followed by reaction with PE-labeled streptavidin (e-bioscience) and FITC-labeled goat anti-rabbit IgG antibody (Southern Biotechnology Associates). Sections were analyzed under a fluorescence microscope Leica DMi6000B (Leica).

Results

A fraction of MZ B cells undergo apoptosis

Apoptotic cells exhibit reduced mitochondrial membrane potential. To assess spontaneous apoptosis of various B cell subsets, we stained untreated spleen B cells from C57BL/6 mice with DiOC₃(3), which accumulates in mitochondria dependently on mitochondrial membrane potential, together with anti-IgM and anti-CD23 antibodies, and measured DiOC₃(3) fluorescence by flow cytometry in CD23+ IgM+ T2 B cells, CD23+ IgM+ follicular B cells and CD23+ IgM+ MZ B cells. Although almost all T2 and follicular B cells exhibited bright DiOC₃(3) fluorescence, DiOC₃(3) fluorescence was reduced in a fraction of MZ B cells (Fig. 1A). Essentially the same result was obtained when we separated B cell subsets using anti-CD21 and anti-CD23 antibodies (data not shown). This result indicated that mitochondrial membrane potential was reduced in a fraction of MZ B cells, and suggested that these cells undergo apoptosis. To confirm this notion, we examined presence of active caspase 3 in various B cell fractions obtained from C57BL/6 spleen by staining the cells with the fluorescence-labeled caspase 3 inhibitor VAD-FMK, which stains cells with active caspase 3 more sensitively than anti-active caspase 3 antibody. In both T2 and follicular B cells, less than 2 % of the cells showed caspase 3 activity (data not shown), whereas percentage of cells with active caspase 3 was significantly increased in MZ B cells compared to that of follicular B cells (Fig. 1B), indicating presence of apoptotic cells in MZ B cells. Essentially the same results were obtained in spleen B cells from BALB/c mice (data not shown).

To further confirm apoptosis in MZ B cells, we stained spleen section of C57BL/6 mice with anti-B220 and anti-active caspase 3 antibodies together with antibody against metallophilic macrophages in MZ. We used anti-active caspase 3 antibody instead of VAD-FMK because VAD-FMK has not been used for immunohistological analysis. Germinal centers were not detectable in the absence of immunization (data not shown). Moreover, only a small number of active caspase 3- cells were present in spleen (Fig. 1C). The low frequency of active caspase 3- cells in spleen section may be due to lower sensitivity to detect active caspase 3- cells by anti-active caspase 3 antibody. Nonetheless, active caspase 3- cells were found almost exclusively in MZ, and were not found in the other parts of spleen including follicles. Taken together, a fraction of MZ B cells undergoes apoptosis in normal mice, and MZ is the major site of B cell apoptosis in unimmunized spleen.
Figure 1. A fraction of marginal zone B cells undergo apoptosis.

(A, B) Flow cytometry analysis. Spleen cells from C57BL/6 mice were stained with DiOC₆(3) (A) and FITC-VAD-FMK (B) together with anti-IgM and anti-CD23 antibodies. Percentages of DiOC₆(3)⁺ (A) and active caspase 3-positive (B) cells in type 2 transitional (T2), follicular (FO) and marginal zone (MZ) B cells are shown. Right panels show mean ± SD of four experiments. Statistical analysis was done by one-way analysis of variance (ANOVA) with Tukey’s posttest (**, p<0.001) and student’s t test (*, p<0.05). (C) Immunohistological analysis. Spleen sections obtained from C57BL/6 mice were stained with anti-mouse B220 (Blue), anti-metallophilic macrophage (MOMA-1) (Red) and anti-active caspase 3 antibodies (Green). FO: Follicle. MZ: Marginal zone. Boxed area in left panel is enlarged in right panel. Objective magnification in left panel: × 40. Bar = 25 µm.
Distinct surface phenotype of apoptotic MZ B cells

To ask whether apoptotic MZ B cells exhibit a distinct surface phenotype, we stained spleen B cells with antibodies against various membrane molecules such as CD22, CD40, CD86 and CD138 together with DiOC<sub>6</sub>(3), anti-IgM and anti-CD23 antibodies. Flow cytometry analysis revealed that DiOC<sub>6</sub>(3)<sup>+</sup> apoptotic MZ B cells express higher levels of MHC class II, CD22 and CD40 than DiOC<sub>6</sub>(3)<sup>-</sup> non-apoptotic MZ B cells, whereas expression levels of other membrane molecules such as CD69, CD86, CD138 and IgD on apoptotic MZ B cells were similar to those on non-apoptotic MZ B cells (Fig. 2). In contrast, expression levels of these membrane molecules including CD22
and CD40 were not different regardless of the DiOC6 fluorescence level in both T2 and follicular B cells. Thus, apoptotic MZ B cells show a distinct surface phenotype from non-apoptotic MZ B cells.

To confirm distinct phenotype of apoptotic MZ B cells, we stained C57BL/6 spleen cells with fluorescence-labeled VAD-FMK together with antibodies to various membrane molecules such as CD22, CD40 and MHC class II. Flow cytometry analysis revealed that MHC class II⁺ CD22⁺ MZ B cells contain higher numbers of cells positive for active caspase 3, although the percentage of cells expressing active caspase 3 was not increased in CD40⁺ cells probably because caspase activity-positive cells are fewer than DiOC6(3)⁺ cells (Fig. 3). Nonetheless, this result indicated that apoptotic cells are accumulated in MHC class II⁺ CD22⁺ MZ B cells, and suggested that these MZ B cells constitute a distinct population of apoptotic B cells.

Discussion

In the present study, we demonstrated that percentages of cells exhibiting reduced mitochondrial membrane potential and those exhibiting caspase 3 activity, both of which are characteristic for apoptosis, are increased in MZ B cells in unimmunized mice. Percentage of cells exhibiting reduced mitochondrial membrane potential was much higher than those carrying active caspase 3, probably due to either difference in detection sensitivity or the fact that reduction in mitochondrial membrane potential precedes to caspase activation. In spleen section, only a few cells were positive for active caspase but they mostly localized in MZ. Lower frequency of active caspase 3⁺ cells in histology than in flow cytometry may be due to the difference in reagents that detect active caspase. Nonetheless, these results indicated that MZ is the major site of B cell apoptosis in unimmunized spleen. Although germinal centers generated after antigen stimulation contain a large number of apoptotic cells⁹, germinal center formation was poor in normal unimmunized spleen. We further demonstrated that apoptotic MZ B cells express higher levels of MHC class II and CD22 than non-apoptotic MZ B cells. Because both DiOC6(3) and VAD-FMK, used for detection of mitochondrial membrane potential and active caspase 3, respectively, generate fluorescence with similar wave length, it was impossible to examine whether the same cell exhibits both reduced mitochondrial membrane potential and increased caspase activity. However, both MZ B cells exhibiting reduced mitochondrial membrane potential and those expressing active caspase 3 showed accumulation of MHC class II⁺ CD22⁺ cells, suggesting extensive overlap between these two cell populations. Taken together, MZ B cells that express higher levels of MHC class II and CD22 constitute the major apoptotic B cell population in unimmunized normal mouse spleen.

Apoptotic MZ B cells express higher levels of MHC class II and CD22, both of which are up-regulated after B cell activation⁸. Thus, apoptotic B cells appear to be activated probably by interaction with antigens. In contrast, expression of the other activation markers CD69 and CD86 was not up-regulated in apoptotic MZ B cells. This finding suggests that apoptotic MZ B cells fail to be fully activated, and that the failure of full activation might cause apoptosis. Because MZ is the site where blood-borne antigens efficiently interact with B cells¹⁰, antigens such as those derived from commensal bacteria may be transported to MZ, and continuously activate B cells. Some of these activated MZ B cells may undergo activation-induced apoptosis thereby limiting the immune response. Alternatively, some of the MZ B cells interact with self-antigens because self-reactive B cells are suggested to be accumulated in MZ B cells¹⁰.¹⁴. Indeed, various autoantibody-transgenic mice including those expressing anti-DNA and anti-Sm antibodies exhibit expansion of MZ B cells and accumulation of self-reactive B cells in MZ¹⁰.¹⁴. Self-reactive B cells preferentially differentiate to MZ B cells probably through continuous BCR ligation by interaction with self-antigens. Apoptosis of MZ B cells might thus contribute to deletion of self-reactive B cells. Further studies are required to elucidate the role of MZ B cell apoptosis in B cell homeostasis.

Although mouse MZ B cells are exclusively located in the splenic MZ, human MZ B cells are also found in many anatomical sites other than the spleen and their transformation leads to lymphomas including MALT (mucosa-associated lymphoid tissue) lymphomas that occur at numerous extranodal sites¹⁹. As is the case for mouse MZ B cells, human MZ B cells may be involved in the first line of defense against infection. This assumption is interesting because Helicobacter pylori infection is suggested to play a role in the development of MALT lymphoma²⁰. MALT lymphomas carry chromosomal translocations such as those generating the API2-MALT1 fusion protein, which
appears to block B cell apoptosis by activating the NF-κB pathway\textsuperscript{21}. Since mouse MZ B cells tend to undergo apoptosis as shown in this study, anti-apoptotic activity of API2-MALT1 may play a role in the development of MALT lymphomas from MZ B cells.

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