Expression and Localization of the Calcium-Mobilizing Molecules, Calcineurin and NFAT in Germinal Center B Cells.

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Intracellular signaling via the B-cell antigen receptor (BCR) regulates cellular dynamics in B cells, in a similar way to signaling via the T-cell receptor (TCR) in T lymphocytes. Cross-linking of BCR increases Ca\(^{2+}\) influx as a first event, which then activates Ca\(^{2+}\)-dependent signaling molecules. Calcineurin and nuclear factor of activated T cell (NFAT) are Ca\(^{2+}\)-mobilizing elements that are considered to be important in regulating proliferation of T cells. However, little is known about their expression in B cells, especially in the germinal center, where apoptosis and proliferation of B cells actively take place for clonal selection. We investigated the expression and the localization of Ca\(^{2+}\)-mobilizing molecules, including calcineurin and NFAT, in germinal center B cells by immunofluorescence. The results revealed a dramatic increase of intracellular Ca\(^{2+}\), a constitutive expression of calcineurin, and a unique localization of NFATc2. Interestingly, several germinal center B cells expressed nuclear-imported NFATc2, which suggests the activation of NFATc2 and its involvement in the dynamics of B cells in the germinal center. Moreover, double immunofluorescence experiments demonstrated the co-expression of NFAT and cleaved-caspase 3 in apoptotic B cells of the germinal center. Thus, these results indicate that NFAT may participate in the regulation of B-cell dynamics such as apoptosis in the germinal center.

Key words germinal center, B cell, nuclear factor of activated T cell, B-cell antigen receptor

INTRODUCTION

B cell antigen receptor (BCR) is a complex of cellular surface IgM, Ig\(\alpha\) and Ig\(\beta\), that is constitutively expressed on the surface of B lymphocytes\(^1\sim5\). Cross-linking of BCR with anti-IgM or in vivo ligand triggers an intracellular signaling pathway, which regulates cellular dynamics such as differentiation and proliferation\(^1,6\). Molecular analysis revealed that cross-linking of BCR leads to recruitment and activation of several receptor tyrosine kinases, such as Lyn and Syk, via the immunoreceptor tyrosine-based activation motif in the intracellular domains of Ig\(\alpha\) and Ig\(\beta\)\(^1,2,6,7\sim12\). It has also been demonstrated that BCR cross-linking induces a Ca\(^{2+}\) signaling cascade. That cascade is initiated by the activation of phospholipase C-\(\gamma\)2 in response to an increase of intracellular Ca\(^{2+}\) influx by the cooperation between inositol trisphosphate receptor on the endoplasmic reticulum and the calcium-release-activated Ca\(^{2+}\) channels on cellular membranes\(^1,6,9,13\). Moreover, how Ca\(^{2+}\) signaling pathways in B cells are associated with their cellular dynamics has not been fully elucidated. On the basis of previous studies involving T cell receptor-mediated Ca\(^{2+}\) signaling\(^14,15\), calcineurin and nuclear factor of activated T cell (NFAT) are considered to be two of the most important BCR-mediated Ca\(^{2+}\) signaling molecules. It is well known that calcineurin, a Ca\(^{2+}\)/calmodulin-dependent serine/threonine protein phosphatase 2B, induces an IL-2 expression. It activates the NFAT family of molecules by performing their dephosphorylating in T lymphocytes\(^14\sim16\). Calcineurin and its substrate, NFAT, are key determinants that regulate proliferative response of T cells upon Ca\(^{2+}\) mobilization via the T cell receptor (TCR)\(^14,15,17\sim19\).

According to the previous reports, the outcome...
of BCR signaling is quite different depending on the differentiation stages of the B cells\textsuperscript{1,6}. In B cells with immature phenotypes such as pro- and pre-B cells, cross-linking of BCR results in dramatic apoptosis, while it triggers a proliferative response in more differentiated B cells, such as the plasma cells\textsuperscript{1,20,21}. Cells representative of the former are, for example, human type I Burkitt lymphoma cells and murine B-cell lymphoma cells, WEHI-231\textsuperscript{1,22}. Moreover, recently, we demonstrated that NFATc2 functioned pro-apoptotically in type I Burkitt lymphoma cells through the BCR signaling cascade\textsuperscript{23}. Interestingly, germinal center B cells share several phenotypes in common with type I Burkitt lymphoma cells, such as, IgM(+) , IgD(−) , CD77(+) , Bel-2(−) and BLR-I(+)\textsuperscript{1,24,25}. Most of these immunophenotypes are unique to immature B cells. On the other hand, physiologically, the germinal center is the only place in peripheral lymphoid tissues where clonal selection takes place in response to stimulation by various antigens\textsuperscript{26}. It is considered that complex signaling pathways are involved in the regulation of apoptosis and rescue of B cells in the germinal center\textsuperscript{26,27}. Therefore, it is expected that Ca\(^{2+}\) signaling via BCR may be involved in cellular dynamics of B cells in the germinal center. However, whether expression of Ca\(^{2+}\) responsive molecules occurs in the germinal center remains still unclear. Here, we demonstrated by immunofluorescence the expression and the localization of these molecules in germinal center B cells, mainly focusing on calcineurin and NFAT.

**MATERIALS AND METHODS**

**Tissues**

Frozen sections of human tonsils were obtained from 5 different patients with chronic tonsillitis. These tissues histologically showed lymphoid hyperplasia with marked secondary follicles. Frozen tissues were sectioned with a cryostat to a thickness of 4.5 μm in thickness and immediately fixed with acetone for 5 min at room temperature.

**Antibodies**

Primary antibodies employed in the immuno-fluorescence study included rabbit anti-human IgM polyclonal antibody (polyAb) (DakoCytomation A/S, Glostrup, Denmark), mouse anti-human CD79α monoclonal Ab (mAb) (DakoCytomation A/S), rabbit anti-phosphorylated elongation factor 2 (phospho-EF2) polyAb (kindly provided by Professor A. Nairn, Yale University), mouse anti-human poly (ADP) ribose polymerase (PARP) mAb (Becton, Dickinson & Co., Franklin Lakes, NJ, USA), rabbit anti-catalytic subunit of calcineurin polyAb (CHEMICON International, Inc., Temecula, CA, USA), mouse anti-human NFATc2 mAb (Becton, Dickinson & Co.), rabbit anti-cleaved caspase 3 polyAb (Cell Signaling Technology, Inc., Beverly, MA, USA), mouse anti-human NFATc1 mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse anti-thioredoxin reductase 3 (TR3) mAb (Becton, Dickinson & Co.). Secondary antibodies were FITC-conjugated goat anti-rabbit IgG(H+L) polyAb (Zymed Laboratories, Inc., South San Francisco, CA, USA) and Cy3-labeled goat anti-mouse IgG(H+L) polyAb (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

**Double immunofluorescence**

Immunofluorescence stainings were performed by a conventional indirect staining method. Frozen sections fixed with acetone were first incubated with fetal calf serum for 10 min at room temperature (RT), and then primary antibodies were applied at the proper concentrations for 1 h at RT after washing with 0.1M Tris-HCl (pH7.5) containing 0.1% Tween 20 (washing buffer). After washing three times with the washing buffer, secondary antibodies were added to the specimens and incubated for 1 h at RT. The specimens were examined with a conventional immunofluorescence microscope or a confocal laser-scanning microscope (Carl Zeiss, Oberko-chen, Germany).

**Enzyme immunohistochemistry**

Enzyme immunohistochemistry was performed by a conventional indirect staining method. Formalin-fixed and paraffin-embedded sections were deparaffinized in xylene, rehydrated through ethanol gradient and phosphate buffered saline (PBS). After deparaffinization, followed by rinsing in PBS for 5 min, heat-mediated antigen retrieval was performed by boiling the slides in 10 mM citrate buffer, pH6.4, in a microwave oven for 10 min at 600 W. Endogenous peroxidase was blocked by incubation in 3% H\(_2\)O\(_2\) for 20 min. After washing in PBS, the slides were first incubated with goat serum for 10
NFAT expression in germinal center B cells.

mRNA in situ hybridization

mRNA in situ hybridization was performed for the paraffin-embedded specimens from 3 human tonsils according to a method previously described28. To generate the single stranded digoxigenin-labeled riboprobe, a full-length complementary DNA was cloned from a Jurkat cDNA library by the polymerase chain reaction technique, using a pair of primers coding for the regulatory subunit of the human calcineurin. The primers used for cloning the cDNA were: Forward primer, 5'-CGC GGA TCC ATG GGA AAT GAG GCA (with an added Bam HI-site)-3'; reverse primer, 5'-CGC GAA TTC TCA CAC ATC TAC CAC-3'(with an added Eco RI site). The amplified fragment was subcloned into pBlueScript SK+ (Stratagene, La Jolla, CA, USA) to generate the anti-sense RNA probe and the sense RNA probe for a control hybridization.

RESULTS

Expression of calcineurin (calcium/calmodulin-dependent serine/threonine protein phosphatase 2B) in germinal center B cells.

We further investigated the expression of calcineurin (calcium/calmodulin-dependent serine/threonine protein phosphatase 2B) in the germinal center. To function as a protein phosphatase, calcineurin requires the coexpression of both catalytic subunit (CnA) and regulatory subunit (CnB). Calcineurin forms a complex comprised of both subunits in vivo when it becomes fully active16. As shown in figure 1e, germinal center B cells abnormally expressed the catalytic subunit (CnA). On the other hand, mRNA in situ hybridization demonstrated the strong expression of mRNA of the regulatory subunit (CnB) in the germinal center (Fig. 1f). These results indirectly indicate the expression of calcineurin in the germinal center, where calcineurin forms a complex of both subunits.

Expression and localization of NFAT in germinal center B cells.

NFATc2, one of the NFAT family molecules, is known to induce the interleukin-2 (IL-2) to the Th1 subsets of T lymphocytes. NFATc2 is then targeted to the nucleus after specific dephosphorylation by calcineurin14,15,31. Consequently, its activation
Fig. 1. Expression of IgM, CD79α, PARP, phospho-EF2, and calcineurin in lymph follicles of a human tonsil.

a: Enzyme immunohistochemistry for IgM. ×20. b–e: Double immunofluorescence by an indirect method in representative cases. b: IgM (labeled with FITC) and CD79α (labeled with Cy3). ×20. An inset shows a high-magnification view of a germinatal center (×40). c: phospho-EF2 (labeled with FITC) and IgM (labeled with Cy3). ×4. d: IgM (labeled with FITC) and PARP (labeled with Cy3). ×20. e: IgM (labeled with FITC) and calcineurin catalytic subunit (CnA) (labeled with Cy3). ×20. f: mRNA in situ hybridization targeting mRNA of the regulatory subunit (CnB) of calcineurin. ×20. MZ: mantle zone, GC: germinal center.

All 5 tonsil samples employed in the present study showed similar results.
NFAT expression in germinal center B cells.

Fig. 2. Expression of IgM, NFAT, cleaved caspase3, and an TR3 in lymph follicles of the human tonsil demonstrated by double immunofluorescence.

a: IgM (labeled with FITC) and NFATc2 (labeled with Cy3). ×20. b: A high-magnification view of the germinal center shown in Fig. 2a. ×100. Arrowheads indicate nuclear-imported NFATc2 and NFATc2 in transition to the nucleus. The signals were detected by a confocal laser-scanning microscope. c: Cleaved caspase 3 (labeled with FITC) and NFATc2 (labeled with Cy3). ×100. Arrows show double-positive apoptotic cells in the germinal center. The signals were detected by a confocal laser-scanning microscope. d: IgM (labeled with FITC) and NFATc1 (labeled with Cy3). ×40. Inset, ×40. e: IgM (labeled with FITC) and TR3 (labeled with Cy3). ×20.

RESULTS

results in the proliferation of T cells as an immune response. In the germinal centers, there were many NFATc2-positive lymphocytes, as shown in figure 2a. Interestingly, among these NFATc2-positive cells, a significant number of the lymphocytes were double-positive for NFATc2 and IgM, which suggests the expression of NFATc2 in germinal center B cells. A high-magnification view of the germinal center revealed that some of the large IgM-positive centroblastic cells contained nuclear-imported NFATc2. NFATc2 was also located between the cytoplasm and the nucleus, indicating that it was in transit between these locations (Fig. 2b, arrowheads). Cleaved caspase 3-positive apoptotic B cells were simultaneously positive for NFATc2 (Fig. 2c, arrowheads). Another subtype of NFAT, NFATc1, was expressed in B cells that localized in the apical region of the germinal center (Fig. 2d inset). As a further step in the analysis, we attempted to examine whether there was expression of any apoptotic effectors known to be regulated by NFATc2. TR3, a human homologue of murine Nur77, functions as a death inducer in response to activation of NFATc2 in the BCR signaling pathway. Immunofluorescence of tonsil samples using anti-Nur77 mAb showed that the expression of TR3 was up-regulated in the germinal center, which corresponded to the expression of both NFATc2 and calcineurin (Fig. 2e).

DISCUSSION

The germinal center is a central place for antigen-driven immune responses, which involve complex apoptotic cascades including BCR signaling. These cascades are activated to regulate clonal selection of B cells. In the present study, we demonstrated the expression and the localization of several Ca2+-related molecules in germinal center B cells, mainly focusing on calcineurin and NFAT. The present results suggest the involvement of Ca2+...
signaling via BCR, which regulates cellular dynamics of B cells in the germinal center. The following elements led to this conclusion. First, enhanced expression of phospho-EF2 in the germinal center indicates that an increase of intracellular Ca\(^{2+}\) influx takes place there. Second, germinal center B cells showed constitutive expression of calcineurin, a specific activator of NFAT, whose activity is known to be dependent upon the increase of intracellular Ca\(^{2+}\) concentration. Moreover, coexpression of the two calcineurin subunits, CnA and CnB, indicates that calcineurin may form a functional complex composed of these two subunits. Third, a T-cell transcription factor, NFAT, was significantly expressed in germinal center B cells, and its activated state was observed in some of these B cells. NFATc2 was expressed primarily in the germinal center including centroblastic B cells, and in some of these cells, nuclear-imported NFATc2 was coincidently expressed in the germinal center. Recently, it has been reported that TR3 is specifically recruited by the activated NFATc2 via BCR cross-linking in Burkitt lymphoma cells, as well as in T cells via TCR-mediated signaling\(^{22-34}\). Therefore, TR3 may be recruited by NFATc2 in germinal center B cell and may be one of the candidates for apoptosis inducers, which function in clonal selection of B cells in the germinal center. Additionally, the distribution of NFATc1-positive B cells was different from that of NFATc2-positive B cells in the germinal center, implying that these subtypes of NFAT molecules function differently in the germinal center. Although the Ca\(^{2+}\) signaling and the mechanisms of clonal selection in the germinal center upon antigen stimulation have not been fully elucidated yet\(^{22-34}\), these findings do suggest the biological importance of the Ca\(^{2+}\)-mobilizing molecules, calcineurin and NFATc2 in the regulation of the cellular dynamics, especially in negative selection of B cell-clones by apoptosis at the germinal center.

ACKNOWLEDGMENTS

We thank Dr. Angus Nairn (Yale University, CT, USA) for kindly providing us with an anti-phospho-EF2 antibody, and Drs. Tadashi Yoshino, Takashi Oka and Kazuhioko Hayashi (Department of Pathology, Okayama University, Okayama) for useful discussions. We also thank Ms. Yuki Onoda and Ms. Mutsumi Okabe for technical assistance.

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