Expression of CD320 in human B cells in addition to follicular dendritic cells

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CD320 has been recently discovered and reported as a follicular dendritic cell (FDC) protein. Although CD320 is known to enhance proliferation of germinal center (GC) B cells, little other information is available. In this study, we investigated its cellular distribution in the GC. Confocal microscopy of human tonsil sections revealed co-localization of CD320 with CD19 and CD38 but not with CD3 indicating that GC B cells expressed CD320 in addition to FDC. In purified GC B cells, CD320 expression was inhibited in the nucleus, membrane and cytoplasm. Reverse transcriptase-polymerase chain reaction confirmed CD320 mRNA expression in B cells. These findings indicate that CD320 is expressed in B cells in addition to FDC, and that its GC activity may be more complicated than previously thought. [BMB reports 2008; 41(12): 863-867]

INTRODUCTION

Germinal centers (GCs) of peripheral lymphoid tissues are pivotal histological sites where massive proliferation and apoptosis of B cells, somatic hypermutation and class switching of immunoglobulins, and the generation of high-affinity plasma cells and memory B cells takes place (1). The major cellular components of the GC are B cells, followed by small numbers of T cells, follicular dendritic cells (FDCs), and tangible body macrophages (2). A myriad of molecules expressed by B cells participate in GC reactions; the molecules include B cell receptor (3), major histocompatibility complex (4), CD40 (5), CD54 (6), CD11a/CD18 (7), CD106 (7), CD95 (8), tumor necrosis factor-alpha (9), and lymphotoxin-alpha (10).

Using a monoclonal antibody (mAb) against FDC-B cell cluster, a molecule designated 8D6 was discovered using expression cloning (11). Subsequently, 8D6 was designated as CD320 (12). It is involved in FDC-dependent growth of a lymphoma of GC origin. In the differentiation of GC B cells, CD320 promotes proliferation of plasma cell precursors, resulting in increased antibody (Ab) secretion (13). Furthermore, CD320 collaborates with CD44 in supporting lymphomagenesis (14). Although 8D6 stains the GC extensively (11), expression of CD320 molecules in GC cells other than FDCs is unknown. The present study investigated whether B cells, the major cellular component of GC, express CD320. The demonstration that GC B cells as well as FDCs strongly express CD320 implies that the cellular and molecular mechanisms of CD320 action are more complicated than has been supposed.

RESULTS

To investigate the cellular distribution of CD320 proteins in peripheral lymphoid tissues, immunohistochemical analyses of human tonsils was performed using 8D6 mAb. CD320 expression was restricted to the follicles and extrafollicular areas were non-reactive (Fig. 1), consistent with the original result (11). DRC-1 and 3C8 were included as positive control mAbs; they reacted with the GC but not with extrafollicular areas. However, the staining patterns obtained by 8D6 and DRC-1 were distinct from that obtained by 3C8. Staining of the GC with 3C8 mAb was less diffuse than the others, implying that fewer numbers of cells reacted with 3C8. Since, unlike 3C8 that recognizes FDCs but not B cells, DRC-1 reacts with both

Fig. 1. Anti-CD320 mAb specifically recognizes the GC. Frozen tonsil sections were stained for immunohistochemical analyses as described in Materials and Methods. Serial sections of a representative GC are shown. Original magnification, 200×.
cell types (15), we examined whether 8D6 recognized B cells in addition to FDCs. Serial tonsil sections were incubated with antibodies specific to FDC, T cell, and B cell subsets in combination with 8D6, and were examined by confocal microscopy (Fig. 2). Co-localization of 3C8 and 8D6 mAbs was obtained, confirming the FDC expression of CD320. CD3-positive T cells were detected inside and outside the GC, and most cells did not co-localize with CD320. In contrast, the majority of CD19 positive cells co-localized with 8D6 indicating that B cells expressed CD320. More widespread co-localization of CD320 with CD19 was observed than that with 3C8, reflecting the fact that B cells are the major cellular component of the GC. Interestingly, CD19-positive cells in the mantle zone surrounding the GC were negative for CD320. This result suggests that naïve B cells and GC B cells differed in the expression levels of this protein. This possibility was assessed by staining the adjacent tonsil sections with antibodies against CD38 and immunoglobulin D (IgD) as a marker antigen of the GC and naïve B cells, respectively. Strong co-localization of CD320 with CD38 but not with IgD was observed, consistent with the expression of CD320 by GC B cells but not by naïve B cells. Co-localization of CD320 was obtained with other markers of GC B cells, (i.e., CD20 and peanut agglutinin; data not shown). To confirm the expression of CD320 protein in B cells, naïve B cells and GC B cells were purified and examined using for confocal microscopy. Unlike the results of immunohistochemical analyses, both cell subsets were positive for CD320 although they exhibited different subcellular distributions (Fig. 3A). CD320 expression in naïve B cells was restricted to the thin rim of the membrane and cytoplasm whereas GC B cells expressed CD320 not only in the membrane and cytoplasm but also in the nucleus. The expression of CD320 in the membrane of both cell subsets was verified by flow cytometry after double staining with anti-CD38 and anti-CD320 Abs. Finally, we examined CD320 mRNA levels in naïve and GC B cells by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Compared to naïve B cells, CD320 mRNA expression was about 6-fold higher.

Fig. 2. CD320 is expressed by GC B cells and FDCs. Frozen tonsil sections were stained with designated Abs and were examined using confocal scanning fluorescence microscopy. The pictures show a germinal center in adjacent sections. Original magnification of panels, 200×.
in GC B cells (Fig. 4). The specificity of the result was verified by sequencing the amplified PCR products.

**DISCUSSION**

Our current findings extend previous results by revealing the cellular distribution of CD320 among mononuclear cells. Presently, we demonstrate that CD320 co-localizes with CD19 and CD38 in situ in immunohistochemical analyses. Purified naïve and GC B cells are positive for CD320 using confocal microscopy, with expression of CD320 on B cells confirmed by flow cytometry. Naïve and GC B cells express CD320 mRNA, while T cells and monocytes do not express CD320 proteins. Based on these observations, we conclude that human B cells express CD320.

Confocal microscopy examination tonsil sections revealed co-localization of CD320 with CD38 but not with IgD, implying that GC B cells are positive but naïve B cells are negative for CD320. However, CD320 was clearly expressed in naïve B cells as well as GC B cells when isolated B cells were analyzed by confocal microscopy, fluorescence-activated cell sorting (FACS), and RT-PCR. Considering the similar levels of CD320 expression on the surface of naïve and GC B cells as determined by FACS, the immunohistochemistry finding that naïve B cells are negative for CD320 may reflect the absence of this molecule inside the nucleus of naïve B cells. In contrast, most CD320 molecules were present in the nucleus of GC B cells. This difference may have resulted in the preferential binding of 8D6 mAb to the GC but not to the mantle area where most IgD⁺ B cells are located. In support of the localization of CD320 in the nucleus, CD320 nucleotide has a putative nuclear localization signal (NLS) sequence. The classical NLS consists of a monopartite or bipartite signal. The former is defined by a single cluster of four to six positively charged amino acids.

In GC B cells (Fig. 4), the specificity of the result was verified by sequencing the amplified PCR products.
charged amino acids, while the latter consists of two stretches of positively charged residues separated by a spacer region of any 8-10 residues (16). According to this rule, the presumed sequence of CD320 contains one NLS (11). The localization of CD320 in the nucleus suggests that this molecule may function as a novel transcription factor.

CD320 molecule expressed by FDC may augment GC B cell growth through interactions with an as-yet unidentified receptor of CD320 (11). Since GC B cells as well as FDCs express CD320, the molecular and cellular modes of CD320 action appear to be more complicated than has been thought. CD320 molecules may be involved in the cellular interactions between FDCs and B cells in addition to interactions between FDC and B cells. CD320 of B cells may stimulate FDC to produce B cell growth factors. Further studies will be necessary to deduce the specific CD320 receptor and whether it is a secreted cytokine, whether B cell growth enhancement is the major function of CD320, the function of CD320 in GC B cell nuclei, and the nature of regulation during differentiation of naive B cells to GC B cells. Ongoing biochemical characterization of the CD320 proteins should facilitate the development of Abs that can be utilized in immunoblotting and immunoprecipitation studies.

MATERIALS AND METHODS

Antibodies and reagents

mAb 8D6 developed as previously described (17) was provided by Dr. Y. S. Choi (Ochsner Clinic Foundation, New Orleans, LA). mAbs 8D6 and 3CB were purified and conjugated with biotin using a biotinylation kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. Other Abs and reagents used in this work were DRC-1 (R4/23); biotin-conjugated mouse IgG1; fluorescein isothiocynate (FITC)- or phosphatidylethanolamine (PE)-conjugated streptavidin (Dako, Glostrup, Denmark); mouse IgM and IgG1; horseradish peroxidase-conjugated goat anti-mouse IgG1 and anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA); PE-conjugated anti-CD3, anti-CD14, anti-CD19, and anti-CD38 (BD Biosciences, San Jose, CA); and propidium iodide (Sigma-Aldrich, St. Louis, MO).

Isolation of lymphocytes and monocytes

Tonsillar B cells were prepared as described previously (18). In brief, tonsillar mononuclear cells were depleted of T cells by rosetting with sheep red blood cells. B cells were further separated using a discontinuous gradient of Percoll. B cells recovered at the 60%-80% Percoll interface were referred to as high density B cells and those recovered at the 35%-50% interface represented low density B cells. Naïve B cells were further purified from the high density B cells by magnetic cell separation (Miltenyi Biotec, Auburn, CA) using anti-IgD mAb HJ9. The purity of the final positive fraction was 98% by quantifying the expression of IgD as analyzed by FACS Calibur (Becton Dickinson, Sunnyvale, CA). GC B cells were obtained from the low density B cells by depleting CD44+ cells. The purity of GC B cells was more than 98% as ascertained by estimating IgD- CD38+ cells. Peripheral blood mononuclear cells were obtained using a discontinuous density gradient of Ficoll. Monocytes were isolated by plastic adherence. The non-adherent T and B cells and adherent monocytes were subjected to FACS analysis.

Immunohistochemistry and confocal scanning fluorescence microscopy

Immunohistochemical analyses and confocal microscopy of human tonsil sections were carried out as described previously (19, 20). Briefly, cryostat sections of human tonsils were fixed in cold acetone for 10 min. The sections were rehydrated in phosphate buffered saline and blocked for 10 min with Protein block (Dako). The slides were incubated with control Abs or mAbs 3CB, 8D6, DRC-1 for 1 h at room temperature, and then stained using an UltraTek HRP kit (Scytek, Logan, UT) according to the manufacturer’s instructions. Peroxidase activity was detected by adding AEC substrate solution (Dako). The slides were counterstained with hematoxylin. For confocal microscopy, the slides were stained with biotin-labeled 8D6 and FITC- or PE-conjugated Abs, followed by FITC- or PE-labeled streptavidin. The coverslips were mounted onto slides using fluorescent mounting media (Dako). Purified naïve and GC B cells were cytospun, and the slides were incubated with 8D6 and propidium iodide for nuclear staining. The relative positional distribution of the fluorochromes was visualized and scanned using a Fluoview FV300 confocal laser microscope (Olympus, Tokyo, Japan).

Flow cytometry

Unfractionated tonsillar B cells or peripheral blood mononuclear cells were double-stained with unlabeled 8D6 mAb, followed by FITC-labeled goat anti-mouse IgG Ab. Unoccupied Fab sites of goat Abs were blocked with mouse IgG1 Abs. The stained cells were further incubated with PE-conjugated anti-CD3, CD14, CD19, or CD38 Abs. Flow cytometric analyses were performed using a FACS Calibur instrument (21).

Semi-quantitative RT-PCR

RT-PCR was carried out as described previously (19). The CD320 primers were AGTGTCACTCTCTCGAG (sense) and GAGGCTCTGTTCTCGAC (antisense), and GAPDH primers were CTTCTCCAAATCAAGTGGGG (sense) and CGCACCAG TTTCGAGGG (antisense). To evaluate mRNA expression semi-quantitatively, PCR was carried out using 5-fold dilutions of target cDNA. Thirty cycles of amplifications were performed with the annealing temperature of 55°C. The GAPDH control was amplified with 25 cycles. The reaction products were subjected to 1% agarose gel electrophoresis and visualized with a UVBio Imaging gel documentation system (UVP, Upland, CA Systems).
Statistical analysis
Statistical analysis and graphic presentation were carried out with GraphPad Prism 4.0 software (GraphPad, LaJolla, CA). Results are presented as means ± standard error of the means (SEM) of triplicate assays. The statistical significance of differences was determined by Student’s unpaired t-test; P < 0.05 was considered significant.

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