Lymphoid Chemokine B Cell-Attracting Chemokine-1 (CXCL13) Is Expressed in Germinal Center of Ectopic Lymphoid Follicles Within the Synovium of Chronic Arthritis Patients

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Lymphoid Chemokine B Cell-Attracting Chemokine-1 (CXCL13) Is Expressed in Germinal Center of Ectopic Lymphoid Follicles Within the Synovium of Chronic Arthritis Patients

Kenrin Shi,* Kenji Hayashida,2* Motoharu Kaneko,* Jun Hashimoto,* Tetsuya Tomita,* Peter E. Lipsky,† Hideki Yoshikawa,* and Takahiro Ochi*  

A unique feature in inflammatory tissue of rheumatoid arthritis (RA) is the formation of ectopic lymphoid aggregates with germinal center (GC)-like structures that can be considered to contribute to the pathogenesis of RA, because local production of the autoantibody, rheumatoid factor, is thought to be a causative factor in tissue damage. However, the factors governing the formation of GC in RA are presently unknown. To begin to address this, the expression of B cell attracting chemokine (BCA-1) (CXCL13), a potent chemoattractant of B cells, was examined in the synovium of patients with RA or with osteoarthritis (OA). Expression of BCA-1 mRNA was detected in all RA samples, but in only one of five OA samples. Lymphoid follicles were observed in four of seven RA samples and in two of eight OA samples, and in most of them BCA-1 protein was detected in GC. BCA-1 was not detected in tissues lacking lymphoid follicles. Notably, BCA-1 was detected predominantly in follicular dendritic cells in GC. CD20-positive B cells were aggregated in regions of BCA-1 expression, but not T cells or macrophages. These data suggest that BCA-1 produced by follicular dendritic cells may attract B cells and contribute to the formation of GC-like structures in chronic arthritis. The Journal of Immunology, 2001, 166: 650–655.

B cell attracting chemokine-1 (BCA-1)3 (CXCL13; Ref. 1), a ligand of CXCR-5, is classified as a lymphoid chemokine and is a potent B cell chemoattractant, because CXCR-5 is highly expressed by naive B cells (2, 3). BCA-1 is thought to be expressed predominantly by follicular dendritic cells (FDC) in secondary lymphoid tissues and organs such as lymph nodes and spleen and to play an important role in attracting naive B cells to form germinal centers (GC) in such organs (4, 5).

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease. Within the inflamed synovium, there is an accumulation of lymphoid and myeloid cells, including T cells, B cells, and monocytes. The local activation of these cells is thought essential in perpetuating chronic inflammation and accelerating joint damage (6, 7). T cells and B cells in RA synovium frequently accumulate underneath the synovial surface and organize into lymphocyte aggregates with certain features of GC (8, 9), including the local differentiation of FDC and plasma cells (PC) and the production of large amounts of Ab (10, 11). The development of these structures appears to contribute to the pathogenesis of the disease by leading to the local production of the autoantibody, rheumatoid factor.

The mechanism underlying the formation of lymphocyte aggregates and GC-like structures in RA synovium has not been elucidated. In the present study, we explored the hypothesis that local expression of BCA-1 might play a role. BCA-1 expression in synovia of RA and osteoarthritis (OA) was assessed, and its distribution and relationship to the location of T cells, B cells, macrophages, and FDC were examined. BCA-1 was expressed by FDC in GC of RA and OA synovia and was found in regions of B cell aggregation. Therefore, BCA-1 may play a central role in attracting B cells to form GC in the synovium of patients with chronic arthritis.

Materials and Methods

Tissue samples

Tissue samples were obtained from seven patients with RA (mean age, 54.4 years; range, 29–71 years) and eight patients with knee OA (mean age, 74.1 years; range, 65–86 years) during surgeries. RA was diagnosed according to the American College of Rheumatology (ACR) criteria for the classification of RA (12), and OA was diagnosed according to ACR criteria for the classification of OA of the knee (13). Tissue samples of normal inguinal lymph nodes were obtained from nonarthritic patients during other surgeries.

Primary Ab

Goat anti-human BCA-1 (R&D Systems, Minneapolis, MN) was used to detect BCA-1. For detection of FDC, Abs for CD21 (clone 1F8; Dako, Glostrup, Denmark) and for dendritic reticulum cells (DRC) (clone R4/23; Dako) were used. mAbs directed against CD3-positive T cells (clone PS1; Medac, Hamburg, Germany), CD20-positive B cells (clone L26; Dako), and CD68-positive macrophages (clone KP1; Dako) were also used.

Primary Abs against another lymphoid chemokine, secondary lymphoid tissue chemokine (SLC) (CCL21; R&D Systems), were also used for immunohistochemistry in RA synovium as well as in normal inguinal lymph nodes as a control.
**RNA isolation and RT-PCR**

RA and OA tissues obtained at surgery were minced and weighed. One milligram of the tissue was dissolved with 1 ml of Isogen (Nippongene, Tokyo, Japan) and sonicated. RNA was extracted in accordance with the company's directions. Two micrograms of the extracted RNA was treated with DNase I (Life Technologies, Rockville, MD) to eliminate DNA and reverse-transcribed by SuperScript II reverse-transcriptase (Life Technologies) at 42°C for 70 min using oligo (dT) as a primer (Amersham Pharmacia Biotech, Piscataway, NJ). PCR was conducted with Taq DNA polymerase (AmpliTa gold; Perkin-Elmer Applied Biosystems, Foster City, CA) using 1–3 μl of cDNA (1.5 mM of MgCl₂) on a PCR thermal cycler (PC-700; Astec, Fukuoka, Japan). The primer pair for BCA-1 was CAG AAT CCT GTG GAA CGG ACA CC (3′) and CTT CCA GAC ATT CGG AAT CCT CTG GAA CTT GAG G (5′). Annealing temperatures used for the amplifications were 56°C for β-actin and 58°C for BCA-1. PCR products were resolved by electrophoresis on 1.5% agarose gels and identified with ethidium bromide. The amount of cDNA of each sample precisely, and 58°C for BCA-1. PCR products were resolved by electrophoresis on agarose gel and identified with ethidium bromide staining. To adjust the amount of cDNA of each sample precisely, β-actin expression was examined first using 26–30 cycles of RT-PCR to amplify 0.1, 0.2, and 0.3 μl of cDNA. After resolving the PCR products on agarose gels and identifying the relevant bands with ethidium bromide, the optimal amounts of cDNA for analysis were determined. Chemokine expression in this amount of cDNA was examined using 35, 38, and 40 cycles of PCR amplification, and the results in the linear part of the amplification curve are indicated in Fig. 1.

**Immunohistochemistry**

Tissues from RA and OA patients were embedded in Tissue Tek (Sakura, Tokyo, Japan), and stored at −80°C until use. The tissue was cut into 4- to 7-μm sections with a cryostat, dried at room temperature for 1 h, and fixed in 2% paraformaldehyde at 4°C for 8 min. Normal inguinal lymph nodes were treated in the same manner.

For detection of CD3 and CD20, tissue sections were boiled in 10 mM sodium citrate buffer (pH 6.0) in a 90°C water bath for 90 min to unmask the antigens. After blocking endogenous peroxidase and nonspecific Abs, primary Abs were applied onto tissue sections. Tissues were incubated for 2 h at room temperature for detection of CD3, CD20, CD21, DRC, and CD68 and for 24 h at 4°C for detection of BCA-1 and SLC. Isotype-matched Abs were used for control staining.

Thereafter, detection using the streptavidin biotin-peroxidase complex technique (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan) was conducted. Finally, the sections were developed in 3,3′-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) and counterstained with hematoxylin.

**Two-color immunofluorescence staining**

To determine the expression of BCA-1 by FDC, two-color immunofluorescence staining was performed with CD21 and with DRC, respectively. Two primary Abs raised in different species (BCA-1, goat; CD21 and DRC, mouse) were diluted together and applied onto tissue sections for 24 h at 4°C. Isotype-matched Abs were used for negative control.

**Results**

**BCA-1 mRNA is expressed in RA and OA tissues**

Expression of BCA-1 mRNA was assessed by RT-PCR using mRNA directly extracted from RA and OA tissues (Fig. 1). All samples from RA patients expressed BCA-1, whereas only one of five samples from OA patients expressed BCA-1.

**BCA-1 is expressed in lymphoid follicles of RA and OA synovium**

The location of BCA-1 protein expression was determined by immunohistochemistry (Fig. 2). BCA-1 was mostly expressed in lymphoid follicles in RA synovium, but no expression of BCA-1 was recognized in areas of diffuse lymphoid cell infiltration. Notably, BCA-1 was also detected in a few OA synovia with follicular lymphocytic infiltrates, but not so obviously as was observed in RA. No expression of BCA-1 was detected in OA synovia without lymphoid follicle formation.

**FIGURE 1.** BCA-1 mRNA is uniformly expressed in RA synovium. All five RA and one of five OA samples expressed BCA-1 mRNA. Total RNA extracted from freshly isolated synovial tissue of patients with RA or OA was reverse-transcribed and subjected to PCR using specific primers. The optimal amount of cDNA, which was determined for each sample by β-actin expression, was subjected to PCR for detecting BCA-1. PCR products were electrophoresed on agarose gel and identified with ethidium bromide staining.

**FIGURE 2.** BCA-1 is focally expressed in lymphoid follicles of RA synovium. Sections of follicular (A–D) and diffuse (E and F) lymphoid aggregation infiltrates in RA synovium, as well as in OA synovium (G and H), are shown. Focal expression of BCA-1 is observed in lymphoid follicles in RA synovium (B and D), whereas almost no expression is recognized in the area of diffuse lymphocytic aggregation in RA (F) or OA synovium (H). A, E, and G, Hematoxylin and eosin staining. B, D, F, and H, Immunohistochemistry for BCA-1. Higher magnification of the area outlined in B is shown in D, C, Immunohistochemistry with control goat IgG. Original magnification: A–C and E–H, ×200; D, ×600.

FITC-conjugated rabbit anti-goat IgG (Wako, Osaka, Japan) was used as the secondary Ab for BCA-1, and rhodamine-conjugated rabbit anti-mouse Igs (Dako) for CD21 or DRC. Secondary Abs were diluted together and applied onto each tissue section for 30 min at room temperature. Finally, slides were coverslipped with PBS-glycerol with antifading agents (p-phenylenediamine dihydrochloride; Sigma, St. Louis, MO) and observed under a fluorescence microscope (E800; Nikon, Tokyo, Japan) equipped with a standard mercury lamp power supply.
Histological characteristics of the synovium and BCA-1 expression

Histological characteristics of each sample were assessed to determine whether it contained a follicular and/or diffuse lymphocyte infiltrate (Table I). Of seven RA samples, four contained follicular and five diffuse infiltrates. Three of the samples contained both, and one contained no lymphocyte infiltrates. Of eight OA samples, two contained follicular, one diffuse, and five no lymphocytic infiltration.

BCA-1 was detected in each of the synovia with follicles, but not in those without. The expression of BCA-1 was examined in each lymphoid follicle, and BCA-1 positivity was calculated as the ratio of the number of BCA-1-positive follicles to the total number of follicles. The ratio was 92% in RA and 60% in OA.

Table I. Histological characteristics of patients synovial tissue

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)/Sex</th>
<th>Follicular*</th>
<th>Diffuse*</th>
<th>Total No. of Follicles (A)*</th>
<th>No. of BCA-1-Positive Follicles (B)*</th>
<th>No. of Follicles with B Cell Accumulation (C)*</th>
<th>B/A (%)*</th>
<th>B/C (%)*</th>
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<tr>
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<td>3</td>
<td>5</td>
<td>60</td>
<td>60</td>
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* The synovium of each patient was histologically characterized as having a follicular or diffuse infiltrate by hematoxylin and eosin staining.

† Total number of follicles (A) and BCA-1-positive follicles (B) were counted, and BCA-1 positivity was given as the ratio B/A (%).

‡ Number of follicles with B cell accumulation (C) was counted after staining for CD20, and BCA-1 positivity was given as the ratio B/C (%).
and colocalized with the expression of CD21 and DRC, respectively (Fig. 5). These findings demonstrated that BCA-1 is expressed by FDC in GC, the B cell-rich area of lymphoid follicles.

**SLC is not detected in synovium of RA**

Immunohistochemistry for another lymphoid chemokine, SLC, showed no notable expression in RA synovium. SLC was not detected even in the area that was rich in CD3-positive T cells in RA synovium, although it was observed to express in T cell-rich areas of normal inguinal lymph nodes that we examined as a positive control (Fig. 6).

**Discussion**

In the present study, we demonstrated that BCA-1 was expressed, most likely by FDC, in GC in the synovium of RA as well as OA and could contribute to lymphoid follicle formation. This paper is the first demonstration of BCA-1 expression in ectopic lymphoid follicles of chronic inflammatory sites.

Lymphoid follicles are usually observed in secondary lymphoid tissues and organs, such as spleen, tonsils, lymph nodes, and mucosa-associated lymphoid tissues, and are thought to be essential structures for the normal immune response in which Ag-specific B and T cells are expanded against Ags. Lymphoid follicle formation is also observed ectopically in chronic inflammatory sites. Although there is no knowledge of the true role of an ectopic lymphoid follicle in chronic inflammation, it could contribute to Ag presentation in situ, to clonal expansion of Ag-specific B and T cells, and to rendering an acute inflammation into a chronic one (10, 11, 14).

RA is a representative chronic inflammatory arthritis, the pathological mechanism of which could be thought of as being an acute inflammation at the onset, followed by progression of the chronic process (6). Although the mechanism underlying the change of the inflammatory course from acute to chronic has not been elucidated, an immune response to Ag proteins derived from joint structures might be one of the causes of prolonged inflammation. From this point of view, the formation of lymphoid follicles, especially those with GC, which is a typical histological feature of RA synovium, seems an important process in the development of chronic arthritis.

The literature illustrates that lymphoid follicles with GC consist of naive B cells in the central zone surrounded by mature B cells, PCs, and CD4-positive T cells in RA synovium, and there, naive B cells are specifically selected, activated, and clonally expanded, exhibiting isotype switching and somatic hypermutation (8–11). PCs in RA synovium have been reported to synthesize high amounts of Igs, including rheumatoid factor, which is thought to participate in the pathogenesis of tissue damage (6, 15–17). Moreover, rheumatoid synoviocytes are reported to support the terminal differentiation of B cells into mature PCs in vitro (18). Based on this knowledge, B cell development in GC might be one of the essential courses in the pathogenesis of RA.

In the formation of GC as well as in the events necessary for B cell development, FDCs are supposed to be involved (19). It is also reported that FDC contribute to the apoptosis of B cells in GC via Fas-Fas ligand (20). As shown in Fig. 3, B cells are localized around BCA-1 expression in the central area of lymphoid follicles in RA synovium. Moreover, FDC characterized by CD21 and DRCs has been observed to express BCA-1 in GC of the follicles (Figs. 4 and 5). These results indicate that FDC might play crucial roles in constructing GC in RA synovium and that BCA-1 produced by FDC might act as a potent chemoattractant of naive B cells into the synovium.

In this study, BCA-1 expression was recognized in all RA samples, although the formation of lymphoid follicles was observed histologically in four of seven RA patients. The most explanatory reason for this discrepancy could be the sampling issue; the part without lymphoid follicles might be used for histological analysis, whereas another part of such tissues might include lymphoid follicles. A divergence in transcription and translation might also be present.

Recently, several lymphoid chemokines, which are mainly expressed in secondary lymphoid tissues and organs, have been...
stimulated with TNF-α. Lindhout et al. (36) reported that RA fibroblast-like synoviocytes from bone marrow. According to previous reports using knockout model (22). These reports suggest that BCA-1 may contribute to the stimulation in RA synovium. Moreover, RA fibroblast-like synoviocytes are known to produce pro-inflammatory cytokines including IL-1β, TNF-α, and RANTES (31–35). Furthermore, BCA-1 expression was recog-

ized in mucosa-associated lymphoid tissues with Helicobacter pylori infection (21). In addition, BCA-1 was detected in chronic inflammation induced by lymphotoxin-α in a transgenic mouse model (22). These reports suggest that BCA-1 may contribute to the development of chronic inflammation.

The origin of FDC is controversial. Kapasi et al. (23) reported that precursors of FDC exist in bone marrow, move into target organs, and differentiate into FDC, while Humphrey et al. (24) and Yamakawa et al. (25) did not support the idea that FDC originate from bone marrow. According to previous reports using knockout mice, lymphotoxin-α and TNFRI play a crucial role in establishing FDC in vivo (26–30). RA fibroblast-like synoviocytes are known to produce pro-inflammatory chemokines such as IL-8, monocyte chemoattractant protein-1, epithelial neutrophil activating peptide-78, macrophage inflammatory protein-1β, and RANTES (31–35). Furthermore, Lindhout et al. (36) reported that RA fibroblast-like synoviocytes stimulated with TNF-α and IL-1β had intrinsic properties of FDC. However, in this study, BCA-1 was not expressed in fibroblast-like cells in synovium. Moreover, RA fibroblast-like synoviocytes stimulated with pro-inflammatory cytokines including IL-1β, TNF-α, and lymphotoxin-α did not show BCA-1 expression (data not shown). Further investigation is necessary to address the problem of which cell in RA synovium is the precursor of FDC.

Recently, several investigators have reported inflammatory features of OA. Krenn et al. (37) demonstrated highly mutated V β1 genes in B cells, with a characteristic arrangement of B cells and PCs. Nakamura et al. (38) reported the infiltration of T cells in the synovium of OA in the early stage as well as the restricted TCR usage of Vβ-chain among those T cells. These findings suggest that OA can be regarded as one of the chronic inflammatory diseases with a certain immune response resembling autoimmune diseases like RA, although the clinical feature of inflammation is not so severe.

In this study, formation of a few lymphoid follicles with BCA-1 expression was recognized in two of eight OA synovia. The aggregation of CD20-positive B cells was also observed in those lymphoid follicles. The synovium of these patients apparently showed inflammatory changes including villous formation and looked like RA synovium. Although we diagnosed these patients as OA simply by their clinical features and by ACR criteria for the classification of OA, they might have possessed inflammatory properties similar to those in RA in their pathological process. Further follow-up will be necessary to diagnose these patients precisely.

In this study, we examined the expression of lymphoid chemokines other than BCA-1 in RA synovium. Because the expression of SLC was detected by immunohistochemistry in T cell-rich areas of normal lymph nodes but not in RA synovium, even in the area with abundant T cells, SLC might not contribute to T cell accumulation in RA synovium.

Many chemokines have been reported and they are classified into two groups as pro-inflammatory chemokines and lymphoid chemokines. BCA-1 has been classified as a lymphoid chemokine so far, but the results of this study showed its marked expression in chronic inflammatory sites. BCA-1 may play an important role in the induction of immune response in chronic inflammatory diseases, especially in autoimmune diseases such as RA. Further investigation of lymphoid chemokines in inflammatory conditions will be necessary to better understand the immunological pathogenesis in such diseases.

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