Clonal analysis of B cells in the osteoarthritis synovium

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Abstract

Objectives—Cellular and humoral immunity to collagen and cartilage proteoglycan were shown in patients with osteoarthritis (OA). Inflammatory infiltration containing T and B lymphocytes and macrophages, which are HLA-DR positive, is often seen in the synovial membrane of patients with OA. An analysis of the DNA restriction enzyme patterns of T lymphocytes from the OA synovium showed an oligoclonal pattern of T cell receptor β chain gene rearrangements. No similar studies of B cell clonality have previously been performed. This study aimed at determining the clonal characteristics of the B cells in the OA synovium.

Methods—A reverse transcriptase-polymerase chain reaction of the immunoglobulin transcripts of B cells in the synovial membranes from six patients with OA was performed and the products were analysed by a single strand conformation polymorphism analysis.

Results—Several dominant bands were seen in all samples and some of the dominant bands were common among the two or three separate regions of each synovial sample.

Conclusion—Infiltrated B cells are oligoclonal, and an antigen driven immune response may play a part in the progression of the disease process in OA.

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Osteoarthritis (OA) has been considered to be a degenerative joint disease which results from aging and abnormal stress loading. However, OA synovium often presents features of non-infectious chronic inflammation. In such cases the infiltration of immunocompetent cells, such as CD4 and CD8 positive T cells, B cells, and macrophages was found in the synovial tissue.¹ These findings, together with the presence of cellular and humoral immunity to collagen, cartilage proteoglycan, and chondrocyte surface antigens, suggest that the immune system participates in the pathogenesis of OA.²⁻⁵ Several studies have shown the presence of antigen-specific activation of T and B cells in the synovium of patients with rheumatoid arthritis (RA).⁶⁻⁷ However, it remains unclear as to whether or not the B cell infiltration in the OA synovium is a result of an oligoclonal response to antigen-specific activation or a polyclonal response to antigen non-specific activation.

The immunoglobulin (Ig) heavy (H) and light (L) chains each contain three complementarity determining regions (CDRs) and

four conserved framework areas (FRs). In the H chain, two CDRs (CDR1 and CDR2) are encoded by the VH gene segment. The CDR3 is composed of the 3' end of VH, all of DH, the 5' end of JH, and N nucleotides, which are randomly inserted at both the VH-DH and DH-JH junctions, and is thus the most variable portion of the Ig molecule. By combining the reverse transcriptase-polymerase chain reaction (RT-PCR) of CDR3 of IgH chain transcripts with a single strand conformation polymorphism (SSCP) analysis, we recently developed a new method for analysing B cell clonality.⁸⁻⁹ In an RT-PCR-SSCP analysis, a heterogeneous B cell population, such as peripheral blood mononuclear cells (PBMCs) from healthy subjects, was shown to exhibit a smear pattern because the CDR3 of the IgH chain is diverse. A single B cell clone makes a band. In contrast, a band can be seen in the smear if there is an expanded B cell clonotype in a heterogeneous population.

We examined the clonality of B cells infiltrating the synovial tissues in patients with OA using an RT-PCR-SSCP analysis of the IgH chain CDR3 region.

Patients and methods

PATIENTS

Synovial samples were obtained from the knee joints of patients with OA undergoing replacement surgery. In all patients, no joints other than knee joints were affected and both rheumatoid factor and C reactive protein were negative. All specimens were obtained after receiving the patients' written, informed consent.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA from the cells was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA (3 μ g) was converted into single stranded cDNA using random hexamer oligonucleotides and reverse transcriptase (Superscript, BRL, Gaithersburg, MD). One microlitre of the RT reaction product was amplified using 20 pmol of VH FR3 and μ or γ constant region primers, and 2.5 U of Taq Polymerase (Cetus, Norwalk, CT). A total of 35 cycles of amplification were performed (one minute at 94°C, two minutes at 54°C, three minutes at 72°C). To control for possible contamination, mock PCR reaction mixtures lacking a template or containing products of the first strand cDNA reaction without reverse transcriptase were prepared. None of the controls contained any amplified products visible on ethidium stained agarose gels or product detected by an SSCP analysis.

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Figure 1 (A) Results of a μ RT-PCR-SSCP analysis of B cells from the synovial membranes (SM), bone marrow (BM), and peripheral blood (PB) from patients with osteoarthritis 1–6. The positions of the bands sequence-analysed are shown by arrows. (B) The results of a γ RT-PCR-SSCP analysis.

SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

The amplified DNA was diluted (1:20) in a denaturing solution (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) and was held at 90°C for two minutes. Two microlitres of the diluted sample was electrophoresed in non-denaturing 5% polyacrylamide gels containing 10% glycerol. The gel was run at 35 W constant power for about two

hours. After electrophoresis, the DNA was transferred to Immobilon-S (Millipore Intertech, Bedford, MA) and then was incubated with either a biotinylated internal μ or γ constant region probe. The DNA was visualised by subsequent incubations with streptavidin, biotinylated alkaline phosphatase, and a chemiluminescent substrate system (Phototope detection kit, New England Biolabs, Beverly, MA).

DNA CLONING AND SEQUENCING

Slices containing the specific bands were excised from the polyacrylamide gel. The eluted DNA was then reamplified for 35 cycles with the same FR3 and constant region primers used in an RT-PCR, except that they had a *Bam*HI and *Eco*RI cutting site, respectively, and were purified from 1.1% agarose gel. The recovered DNA fragments were ligated in the *Bam*HI/*Eco*RI site of pBluescript SK- (Stratagene, La Jolla, CA) and used to transform *Escherichia coli* strain XL-1Blue (Stratagene, La Jolla, CA). The clones were picked randomly, and a double strand DNA template was prepared and sequenced using the dyedexy terminator cycle sequencing kit and the Applied Biosystems automatic DNA sequencer (Applied Biosystems, Riossy, France).

PRIMERS AND PROBES

All primers and probes used in this study have been described previously.⁹

Results

RT-PCR-SSCP ANALYSIS OF IgH CHAIN TRANSCRIPTS

Samples from the six patients with OA were subjected to an RT-PCR-SSCP analysis to investigate the B cell clonotypes. In patient 4 synovial membranes were obtained from both knee joints. In the other patients, synovial tissues were all obtained from the right or left knee joint. Except for the synovium samples from patient 5 and from the left knee of patient 4, two or three small pieces were cut out from at least 2 cm separate regions of a single synovial tissue specimen. Each fragment was used for the preparation of total RNA and the subsequent RT-PCR-SSCP analysis. Figures 1A and B show the results of the RT-PCR-SSCP analysis. The results of the analysis of bone marrow (BM) and PBMCs, when available, are also shown. In the μ RT-PCR-SSCP analysis, several dominant clonal bands on the smears were seen in all synovial tissue samples (fig 1A). Some clonal bands were present only in one fragment, whereas others were observed in common among the fragments from different regions and from bilateral synovial samples. BM and PBMCs mainly exhibited smear patterns except PBMCs from patient 5. The PBMCs from patient 5 showed two clonal bands, which were also present in the analysis of synovial membrane. The γ RT-PCR-SSCP analysis of the synovial membranes showed results similar to those from a μ RT-PCR-SSCP analysis (fig 1B). BM and PBMCs showed a few faint and/or dominant clonal bands except for the BM from patient 6. Although two faint bands in BM from patient 1 were present in the synovial samples, no other clonal bands in BM and PBMCs were seen in the synovial membranes.

SEQUENCE ANALYSIS OF COMMON BANDS

To confirm whether the dominant band really represents a clonal population and the common band in different samples contains the same major sequence, and also to examine whether or not a common sequence motif

Table 1 Amino acid sequences of the CDR3 regions

Patient	Sample	VH	N-DH-N	JH
1	SM2	AR	EISSYSSSYD	YDFWF
2	SM1, 2	AG	GGLQFLEWE	DYW
3	SM1, 2	AR	NFRSGDKGYN	YW
4	SM1-3	AR	GTSPGSYLIDF	W
5	SM1	AR	YSEPGTGRRRAH	FDYW
6	SM1, 3	AR	LYGSSPKSAR	YMDVW

exists in CDR3, DNA were eluted from the major bands in the μ RT-PCR-SSCP analysis. One band in SM2 from patient 1, common bands in SM1 and SM2 from patient 2, common bands in SM1 and SM2 from patient 3, common bands in SM1, SM2, and SM3 from patient 4, a band in SM1 from patient 5, and common bands in SM1 and SM3 from patient 6 were sequence-analysed. Figure 1A shows the positions of the bands analysed. These DNA were subcloned, plated, and sequenced. Because such a clonal band is usually located within a smear pattern, several different sequences might be present within each eluted band. Accordingly, a minimum of 12 clones derived from each eluted band were sequenced to determine whether or not there was any dominant sequence. It was thus found that each band showed a major sequence that was present in at least eight of the 12 clones sequenced in every case. In patients 2-4 and 6, the common bands which were sequence-analysed had the same major sequences in each patient. Table 1 shows the protein sequences deduced from the DNA sequences. No common sequence motif was detected.

Discussion

The pathogenesis of OA is still poorly understood, but multiple factors, including genetic, constitutional, and environmental factors are considered to play a part.¹⁰ One of the dominant clinical symptoms in decompensated OA is characterised by a non-infectious chronic inflammatory condition. Cellular and humoral immunity to collagen, cartilage proteoglycan, and chondrocyte surface antigens have been found in patients with OA.²⁻⁵ A histological examination of 38 OA synovial membranes showed that over half the OA synovial membranes contained a lymphoid infiltrate, that 17 showed lymphoid aggregates, and that in seven there were well formed lymphoid follicles. In most cases, HLA-DR+ cells were present in the subintimal region of the synovial membrane.¹ These findings suggest that a local or systemic immune response, or both, may have a role in certain aspects of the pathophysiology of OA.

In all patients analysed, our μ and γ RT-PCR-SSCP analysis data indicate a clonal expansion of B cells in the OA synovial membrane. In addition, the μ RT-PCR-SSCP data, together with the sequence information, indicate the presence of common B cell clones at different separated sites in the synovial membranes in four of six patients. Common B cell clones were also detected in the synovial membranes from both knee joints in one patient. Clonal expansion is unlikely to be driven by polyclonal activators such as superantigens, which stimulate the B cell expressing

members of the specific VH family, thus leading to the polyclonal expansion of these cells without conserving any particular CDR3 sequence. Rather, clonal expansion is a necessary feature of antigen driven immune responses. Specific antigens expressed during *Borrelia burgdorferi* infection induce oligoclonal synovial Ig production.¹¹ The clonal expansion of B cells has also been described in the salivary gland of Sjögren's syndrome.¹²

In the γ RT-PCR-SSCP analysis, BM and PBMCs showed a few faint and/or dominant bands except for the BM from patient 6. The PBMCs from patient 5 showed two bands in the μ RT-PCR-SSCP analysis. Two bands in PBMCs from patient 5 in the μ RT-PCR-SSCP analysis and two faint bands in BM from patient 1 in the γ RT-PCR-SSCP analysis were also present in the analyses of the synovial membranes. At present, we do not know whether the expanded B cell clones in the OA synovial membranes are generated in the synovium or whether they are generated in a secondary lymphoid organ and thereafter migrate to the synovial tissues. In both cases, it is possible that memory B cell clones and plasma cells which have the same antigen specificity as the expanded B cell clones in the OA synovium may circulate in the PB and reside in the BM. When immune responses to joint components mainly occur in a secondary lymphoid organ, clonal B cells may only be detected in BM and/or PB, but not in synovial membranes. The reason why oligoclonal bands in BM were found only in the γ RT-PCR-SSCP analysis is not clear. IgG producing clones may have a stronger tendency to reside in BM than IgM producing ones. The μ and γ RT-PCR-SSCP analyses of PBMCs and BM from healthy subjects showed only a smear pattern.^{8,9} Oligoclonal bands in BM and PB from the patients with OA may be further evidence of the participation of immune responses in the pathogenetic processes of OA.

Many autoantigens are targeted by the immune system in patients with RA.¹³ Most of these autoantigens are also targeted in patients with other autoimmune diseases. Oligoclonal B cell expansion seen in OA synovial membranes suggests that different B cell reactivities are also present in OA joints and some of them, not just one reactive to a single autoantigen, may induce, sustain, and modify the disease course of OA. Locally synthesised Igs may contribute to the chronic inflammatory processes through the activation of complement and resident mononuclear phagocytes. Recent studies disclosed that autoreactive B cells play a part in the activation and diversification of autoreactive T cells, probably as antigen presenting cells.^{14,15} It is tempting to consider that oligoclonally expanded B cells in the synovial membranes activate T cells and thus sustain the chronic inflammation seen in OA joints.

Some clonal B cells are present only at one site in the synovial membrane. To generate such B cell clones, individual infiltrating B cells are considered to be independently stimulated by antigen presenting cells at different locations to proliferate and produce separate clones. The common B cell clones at different and separate sites in the synovial membrane may be generated in a secondary lymphoid organ and thus subsequently migrate to the synovial membrane, where they become activated and induced to proliferate.

Further studies on the immune response and subsequent chronic inflammation in the OA synovial membrane will help us to obtain a better understanding of the pathogenesis of OA and thereby lead to the development of new treatment strategies for OA.

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