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Original article

Immunohistological localization of Notch receptors and their ligands Delta and Jagged in synovial tissues of rheumatoid arthritis

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Abstract

Background. The interaction of Notch receptors with their transmembrane ligands Delta and Jagged plays an important role not only in the organization of a variety of tissues but also in several genetic disorders and cancer development. The functional involvement of the Notch signaling in rheumatoid arthritis (RA) has been reported previously, but the expression profile of Notch-related molecules, as well as their relation with clinicopathological parameters, remains unclear.

Methods. In this study, we analyzed the immunohistochemical staining pattern of four Notch receptors (Notch1–4) and their ligands (Delta1 and Jagged1) in 14 synovial tissues obtained from 14 RA patients.

Results. Notch2 and Notch4 were expressed in limited areas in a few samples or in small blood vessels, respectively. Notch1, Notch3, Delta1, and Jagged1 were overexpressed in the synovial lining and sublining cells on synovial hyperplastic lesions in all samples. Notch1 expression was also observed in T and B lymphocytes of lymphoid follicles independently. Notch1 and Notch3 expression overlapped with that of Jagged1, as determined by confocal microscopy. Activation of Notch1 signaling in the RA synovium was identified using a specific antibody to the cleaved form of Notch1. The expression of these molecules did not show any correlation with clinicopathological parameters.

Conclusions. Our results suggest that Notch signaling is activated in RA synovium but does not necessarily reflect the pathological condition of RA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial proliferation and lymphocyte infiltration. The inflamed synovium often forms a panus and invades bone and cartilage by expressing numerous inflammatory cytokines and proteolytic enzymes, a process that ultimately leads to progressive joint destruction. The proliferating synoviocytes consist of macrophage-like type A cells and fibroblast-like type B cells; the latter cells are known to play a central role in synovial lining hyperplasia and cytokine expression.[1] Lymphocytes, including T and B cells, and macrophages accumulate at various densities in the intimal layer; they are also involved in synovial proliferation as they recruit endothelial cells for new vessel formation. Tissue-infiltrated lymphocytes are occasionally arranged in follicles that can potentiate an aberrant immune reaction.[2]

The Notch signaling pathway is highly conserved beyond species and plays a critical role in a variety of cellular functions, including cell proliferation, differentiation, and apoptosis.[3] To date, four Notch receptors (Notch1–4) and five of their ligands (Delta1, 3, 4; Jagged1, 2) have been identified in mammals. All of them are single-span transmembrane polypeptides that act through cell-to-cell contact.[4] The Notch–ligand interaction induces nuclear translocation of the intracellular domain of Notch as a result of proteolytic cleavage at the juxtamembrane portion. In the nuclei, the intracellular domain interacts with CSL [CBF1/RBP-Jk, Su(H), Lag-1] DNA-binding proteins and transactivates target genes, such as hairy enhancer of split-1/5 and Herp-1/2, transcriptional repressors.

Several studies have reported the functional involvement of Notch pathway in the pathophysiology of RA synovitis. For example, the expression of Notch1, Notch4, and Jagged2 in the RA synovium was reported by Ando and colleagues.[5] Other studies also showed the involvement of tumor necrosis factor-α (TNFα) in the up-regulation of Notch1, which leads to synovial proliferation.[6] However, the expression profile of Notch-related molecules as well as the relation between clinicopathological findings remains poorly understood.

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Table 1. Summary of clinicopathological data

Patient	Age (years)	Sex	Duration (years)	Treatment	Synovial hyperplasia	Lymphocyte infiltration
1	56	F	5	P, D, N	++	+++
2	61	M	10	D, N	+++	+++
3	59	F	15	M, P, D, N	+++	+++
4	50	F	22	P	+	+
5	65	M	10	P, D, N	++	+
6	53	F	25	P, D, N	+++	+++
7	52	F	22	D, N	+++	+++
8	79	F	7	D, N	+++	+++
9	60	F	12	P, N	+++	+++
10	72	F	8	P, N	+++	++
11	38	F	15	P, D, N	++	++
12	65	F	12	P, D	+++	++
13	57	M	9	P, D	+	+
14	58	F	13	P, D, N	+++	+++

P, prednisolone; D, disease-modifying antirheumatic drugs; N, nonsteroidal antiinflammatory drugs; M, methotrexate
The degrees of synovial proliferation and inflammatory reaction were semiquantified by two of the authors (Y.Y., T.M.). +, ++, +++ correspond to mild, moderate, and strong reactions, respectively

In the present study, we performed immunohistochemistry of Notch1–4, Delta1, and Jagged1 in consecutive sections obtained from 14 RA patients. Double staining was also carried out using specific markers to determine the cell phenotype of the positive cells. Using a specific antibody to the cleaved form of Notch1, the results indicated activation of the Notch1 pathway in the RA synovium.

Patients and methods

Tissue samples

A total of 14 RA synovial tissue samples were obtained from 14 patients during joint replacement surgery of the knee joint. The subjects were 11 women and 3 men, with a mean age of 58.9 years (range 38–79 years) and mean disease duration of 13.2 years (range 5–25 years). At the time of surgery, all patients were on antirheumatic drugs (e.g., methotrexate, prednisolone, disease-modifying antirheumatic drugs, nonsteroidal anti-inflammatory drugs) and were categorized as having stage 3 RA. Table 1 summarizes the clinical information for each patient. All patients fulfilled the revised classification criteria for RA proposed by the American College of Rheumatology. The study protocol was approved by the Human Ethics Review Committee of Nagasaki University School of Medicine, and a signed consent form was obtained from each subject.

Tissue samples and immunohistochemistry

To determine the expression profile of each Notch receptor and the expression of Delta and Jagged Notch ligands in the RA synovium, the 14 synovial tissue specimens were subjected to routine histopathological and immunohistochemical analyses. Briefly, deparaffinized sections were preincubated with 3% H_2O_2 in methanol to remove endogenous peroxidase activity and then incubated with one of the specific antibodies listed in Table 2 for 2 h at room temperature. The protein expression was detected by the avidin-biotin-peroxidase complex method with the appropriate secondary antibody and 3-amino-9-ethylcarbazole hydrochloride (AEC) as a substrate. For double staining, the second immunoreactivity was detected by alkaline phosphatase-conjugated secondary antibody and 5-bromo-4-chloro-3-indolylphosphate toluidinium/nitroblue tetrazolium (BCIT/NT). For immunofluorescence staining, Alexa Fluor 488 goat anti-rabbit and 526 donkey anti-sheep antibodies (Molecular Probes, Eugene, OR, USA) were used as the secondary antibody. The stained cells were visualized by confocal laser

Table 2. Antibodies used in the present study

Antibody	Origin	Supplier	Dilution
Notch1	Goat	Santa-Cruz (C-20)	1:200
Notch1	Rabbit	UBI	1:200
Notch2	Rabbit	Santa-Cruz (25-255)	1:200
Notch3	Rabbit	Santa-Cruz (M-134)	1:100
Notch4	Rabbit	Santa-Cruz (H-225)	1:100
CA-Notch1	Rabbit	Cell Signaling	1:100
Delta1	Goat	Santa-Cruz (F-15)	1:100
Jagged1	Goat	Santa-Cruz (C-20)	1:200
L26	Mouse	Dako	1:50
α-SMA	Mouse	Dako	1:100
CD45RO	Mouse	Dako	1:50
Vimentin	Mouse	Dako	1:100

Table 3. Semiquantitative analysis of expression levels of Notch1–4 receptors and their ligands (Delta and Jagged)

Patient no.	Notch1	Notch2	Notch3	Notch4	Delta1	Jagged1
1	+	–	+	±	±	++
2	±	–	±	±	±	±
3	++	±	++	+	++	+
4	++	–	++	+	+	++
5	+	–	++	+	+	++
6	++	–	+	±	+	+
7	+	±	+	+	++	+
8	+	–	++	+	+	++
9	+	–	++	–	++	++
10	+	–	++	+	++	+
11	++	–	+	±	++	+
12	+	–	+	±	+	+
13	++	–	++	–	+	++
14	++	–	+	+	+	++

The expression level of each molecule was scored as follows: –, negative; ±, minimum staining in one area of the tissue; +, patchy staining involved in several areas; ++, moderate diffuse staining

microscopy (LSM5 PASCAL; Carl Zeiss, Oberkochen, Germany).

To detect the constitutive active form of Notch1[7] the sections were digested with 0.1% trypsin for 15 min before incubation with H_2O_2. Preparations incubated without the primary antibody served as the control sections.

Synovial hyperplasia and lymphocyte infiltration were semiquantified by two independent observers as follows: +, mild; ++, moderate; and +++, strong. The immunoreactivity in immunohistology was also classified using the following criteria: –, negative; ±, minimum staining; +, patchy staining; and ++, moderate staining.

Results

Expression of Notch1–4, Delta1, and Jagged1

Immunoreactivities to Notch receptors and their ligands were detected in all examined samples except Notch2 and Notch4 (Table 3). However, the expression levels and distribution patterns were distinct among the samples. The expression of Notch1 was detected in the synovial lining cells that showed papillary proliferation associated with lymphocyte infiltration (Fig. 1). In particular, intense staining was noted in the basal layer synovial cells compared with the superficial layer cells. A large number of cells in the intimal layer also expressed Notch1. In addition, Notch1 expression was observed in almost all lymphocytes present in the lymphoid follicles (Fig. 2A). To identify the cell phenotype of Notch1-positive cells in lymphoid follicles, double immunostaining was carried out in several sections

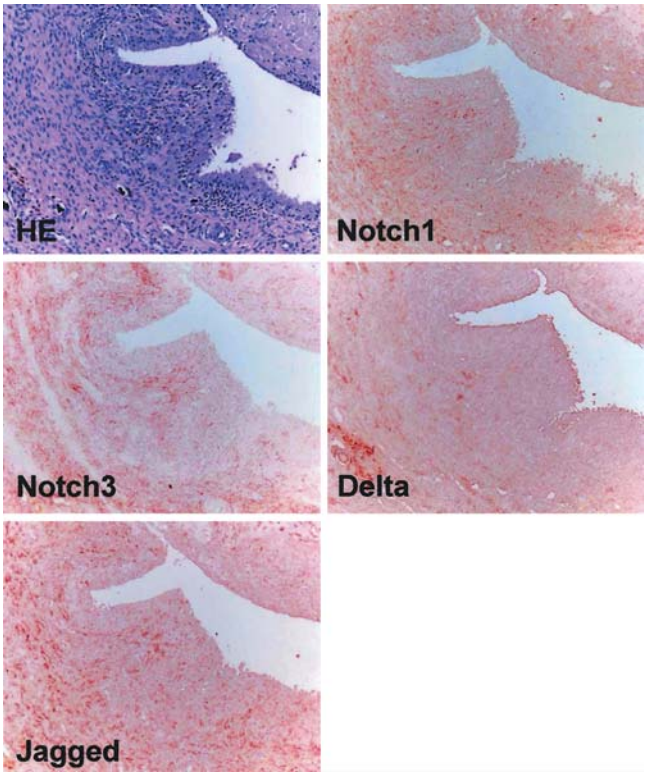

Fig. 1. Expression of Notch1, Notch3, Delta1, and Jagged1 in rheumatoid arthritis (RA) synovium. Five consecutive sections of RA synovia obtained from patient 3 were subjected to hematoxylin and eosin (HE) staining or immunohistochemistry of Notch1, Notch3, Delta1, and Jagged1. Many cellular components, including synovial lining and stromal cells, were stained by each antibody with a similar pattern except for Delta1, which showed a restricted number of immunoreactive stromal cells. ×50

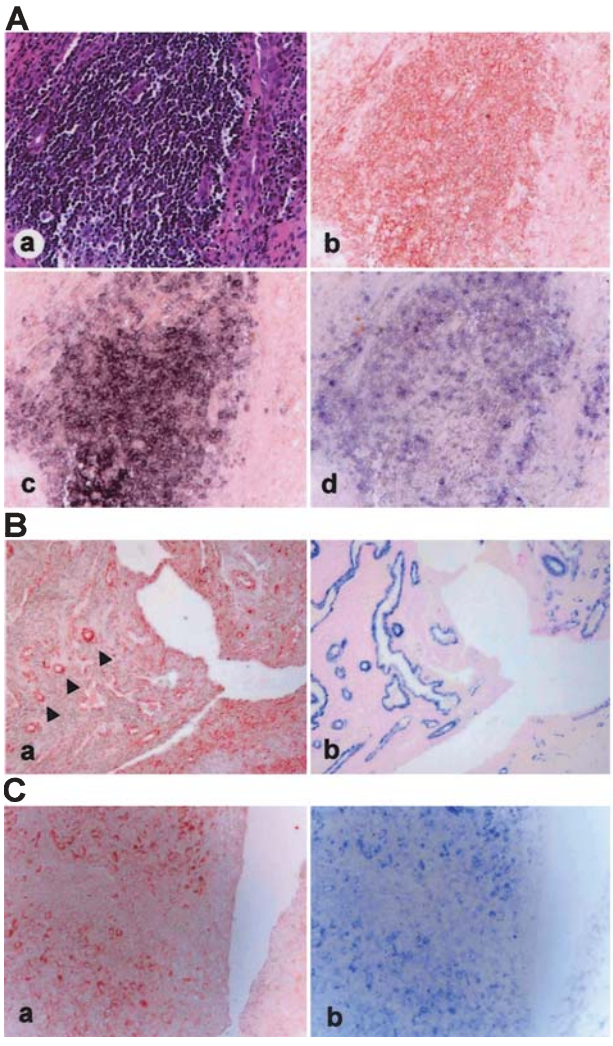

Fig. 2. Expression of Notch1 and Notch3 in various components of the RA synovium. **A** Notch1 expression in lymphoid follicles. Sections from patient 6 were stained with hematoxylin and eosin (**a**) and immunostained with Notch1 antibody and 3-amino-9-ethylcarbazole hydrochloride (AEC) as a substrate (**b**) followed by staining for CD45RO, a marker for T lymphocytes, with alkaline phosphatase-conjugated secondary antibody and 5-bromo-4-chloro-3-indolylphosphate toluidinium/nitroblue tetrazolium (BCIT/NT) substrate (**c**). **d** Immunostaining of B lymphocytes by L26 antibody in an adjacent section. **B** Expression of Notch3 was noted mainly in the smooth muscle cells of small blood vessels. Sections were simultaneously stained for Notch3 (**a**) and smooth muscle α-actin (**b**). After removal of the Notch3 signal by ethanol, the identical section was stained by vimentin. *Arrowheads* indicate preferential expression of Notch3 in small capillaries. **C** Notch1 expression in vimentin-positive cells. A section from patient 4 was double-stained for Notch1 (**a**) and vimentin (**b**). **A** ×100; **B** ×50; **C** ×100

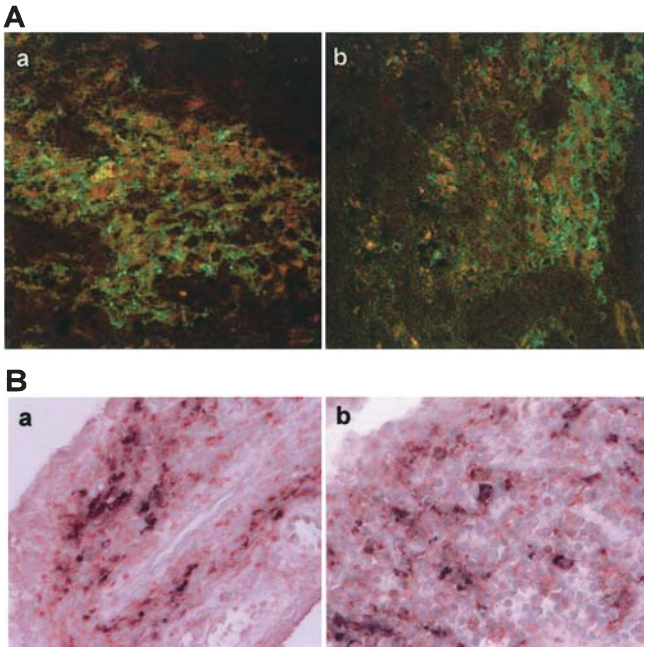

Fig. 3. Immunohistological localization of Notch receptors and Jagged ligand and activation of Notch1. **A** Co-localization of Jagged1 with Notch1 and Notch3. Sections from patient 4 were immunostained with Jagged1 and either Notch1 (**a**) or Notch3 (**b**). Immunoreactivities of Jagged and Notch antibodies were detected by Alexa Fluor 488-labeled (green) or Alexa Fluor 526-labeled (red) secondary antibodies, respectively. Note that not all Jagged-positive synovial cells are stained for Notch. **B** Activation of Notch1 signaling in synovial lining (**a**) and lymphocytes in lymphoid follicle (**b**). Sections from patient 8 were double-stained with Notch1 antibody (red) and a specific antibody raised against a cleaved site of Notch1 (blue). ×200

using antibodies against L26 and CD45RO (specific markers for B and T cells, respectively). The results showed that Notch1 was expressed in both T and B lymphocytes (Fig. 2Ac,Ad).

Similar to Notch1, Notch3 expression was detected in many cellular components, including synovial lining and sublining cells; and Notch3 was also expressed in the perivascular smooth muscle cells of small blood vessels located in the hyperplastic lesions (Fig. 2Ba). Double-staining with smooth muscle α-actin showed that Notch3 was preferentially expressed in small blood vessels rather than well-matured blood vessels (Fig. 2Bb). In contrast, Notch2 could be observed in a highly restricted area of synovial lining cells in two cases (data not shown). In addition, Notch4 expression was restricted to a small number of perivascular smooth muscle cells (data not shown). To identify the phenotype of Notch1- and Notch3-positive cells in the inflamed synovium, we carried out double staining with vimentin, a marker of fibroblasts. The results showed that synovial cells positive for Notch1 were also stained

with vimentin (Fig. 2Ca,Cb). Similar findings were noted for Notch3 (data not shown).

Expression of Delta1 and Jagged1 was detected in all 14 tissue samples, but the expression pattern was different between Delta1 and Jagged1; the number of Delta1-positive cells was small and restricted to stromal cells, whereas Jagged1 was expressed in many cellular components. Because Notch1, Notch3, and Jagged1 showed similar patterns, we performed immunofluorescence staining to determine if the expressions of Notch1 and Notch3 were concomitant with that of Jagged1. Confocal microscopic analysis demonstrated that the expression of Notch1 and Notch3 overlapped that of Jagged1 in most cells, although not all Jagged-positive synovial cells were Notch-positive (Fig. 3Aa,Ab).

Finally, we examined the status of Notch1 receptor in the RA synovium by immunostaining with Notch1 antibody and a specific antibody that recognized the cleaved form of Notch1. Although small in number, Notch1-positive synovial lining and sublining cells (Fig. 3Ba) and lymphocytes in lymphoid follicles (Fig. 3Bb) were noted to be cleaved in the tissue samples.

There were no relations between the expressions of Notch receptors/ligands and various clinicopathological parameters, including disease duration, drugs associated with immunoreactivity of the examined antibodies, synovial proliferation, and lymphocyte infiltration.

Discussion

In the present study, we characterized the expression profiles of Notch receptors and their ligands Delta1 and Jagged1 in RA synovium. Among the examined Notch receptors and their ligands, Notch1 and Notch3 were expressed in vimentin-positive synovial lining and sublining cells in actively proliferating lesions. In contrast, expression of Notch2 was barely noted, and Notch4 expression was restricted to blood vessels. The results also showed Jagged1 and Delta1 expression in the RA synovium, although the latter was limited to the intimal layer.

Despite using the same antibodies, the expression patterns of Notch2 and Notch4 in our study were slightly different from those in two previous reports that showed relatively high expression of Notch2 and Notch4 in synovial tissues.[5,8] The reason for these discrepancies is so far unclear, but the differences in tissue preparation, such as the frozen procedure and Carnoy-methanol fixation, might lead to enhancement of antigen exposure of Notch2 and Notch4. However, our results demonstrate that the expression of Notch1 and Notch3 was almost concomitant with that of Jagged1, suggesting that the Notch pathway is activated by an autocrine/paracrine mechanism. Furthermore, Notch3 appears to be expressed in smooth muscles of developing vessels as well, indicating that Notch3 is involved in angiogenesis. Notch3 was recently reported to enhance the generation of functional arteries by regulating arterial differentiation and maturation of vascular smooth muscle cells.[9] The same study reported that adult Notch3$^{-/-}$ mice exhibited structurally defective distal arteries together with abnormal arterial myogenic responses. In addition, mutation of Notch3 was reported to cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a hereditary vascular dementia in humans.[10] Taken together, these results suggest that activation of Notch signaling via Jagged binding plays an important role in accelerated angiogenesis and vascularization, which are characteristic processes in the RA synovium.

The functional involvement of Notch signaling in the pathophysiology of RA has been demonstrated previously using cultured synoviocytes. Ando et al. demonstrated that the expression of Notch1, Notch4, and Jagged2 are up-regulated in the RA synovium based on a comparative study of cultured synoviocytes treated with TNFα.[5] Nakazawa et al. also demonstrated that inhibition of endogenous Notch1 expression led to the suppression of TNFα-induced synovial proliferation.[6] In addition, a γ-secretase inhibitor that blocks proteolysis of adjacent membrane portions also suppressed TNFα-induced synovial proliferation. Although the functional difference between Notch3 to Notch1 remains obscure, these results indicate that Notch signaling plays a variety of roles in the pathophysiology of RA.

Among the examined molecules, Notch1 was expressed in infiltrated lymphocytes, although immunoreactivity for Delta1 and Jagged1 could not be detected in the examined samples. Furthermore, using specific antibody to the cleaved form of Notch1, we demonstrated that a substantial number of Notch1-positive lymphocytes were activated. These results suggest that activated Notch1 plays an important role in the formation of lymphocyte follicles. In this regard, Notch1 is involved in the commitment of a bipotential T/B precursor to the T-cell lineage.[11] In addition, T cells in lymphoid follicles are the major source of a variety of inflammatory cytokines, including interleukin-1, TNFα, and interferon-γ,[12] indicating that Notch1 may be also responsible for the inflammatory reaction in RA synovitis.

Despite the small number of tissue samples, our results showed that the expression profiles of these molecules does not necessary reflect the clinicopathological findings; three samples that showed relatively low synovial proliferation and lymphocyte infiltration also reacted strongly with all of the antibodies. The lack of an

obvious difference in the expression of Notch homologues between RA and osteoarthritis[8] suggests that immunohistochemical analysis of the Notch system alone is not sufficient for estimating RA disease activity. Further studies that include tissue samples at other RA stages are required for our understanding of the significance of the Notch system in the RA synovium.

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