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Immunohistochemical Analysis of Inflammatory Rheumatoid Synovial Tissues Using Anti-Human Podoplanin Monoclonal Antibody Panel

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Podoplanin (PDPN) is a transmembrane sialoglycoprotein, which is expressed in several normal tissues and malignant tumors. Although PDPN expression in rheumatoid arthritis (RA) has been reported, the role of PDPN in RA and other arthritic conditions has not been fully elucidated. In this study, we examined PDPN expression in inflammatory synovial tissues using an anti-human PDPN (hPDPN) monoclonal antibody (mAb) panel to select the most useful one for evaluation of synovitis. Synovial tissue samples were obtained from 11 RA patients and 9 osteoarthritis (OA) patients undergoing joint surgery. PDPN-positive cells were immunostained by a panel of PDPN mAbs (NZ-1, LpMab-3, LpMab-7, LpMab-10, LpMab-12, LpMab-13, and LpMab-17), followed by cell grading of inflammation and cell counting of PDPN-positivity by a quantitative analyzer. Immunohistochemistry showed that PDPN was markedly expressed in both macrophage-like type A and fibroblast-like type B lining cells of the hyperplastic synovial lining cell layer, and macrophages and fibroblasts in the stroma of RA. Among anti-PDPN mAbs, LpMab-12 showed the highest score. In inflammatory OA synovium, PDPN expression was also detectable. Although LpMab-12 also showed the highest score in OA, the difference was not statistically significant. The inflammatory synovitis score of RA was significantly higher than that of OA. PDPN was expressed in inflammatory lining cells and sublining stroma of RA and OA synovium. In the seven anti-hPDPN antibodies examined, LpMab-12 was the most stainable antibody for PDPN in RA synovitis. Thus, LpMab-12 for PDPN has a possible and promising specific biomarker for evaluating synovitis in RA and inflammatory OA.

Keywords: podoplanin, rheumatoid arthritis, synovitis, inflammation

Introduction

PODOPLANIN (PDPN) is a small mucin-type transmembrane sialoglycoprotein, which is expressed in several normal tissues, including the endothelium of lymphatic vessels, type-I alveolar cells, and glomerular podocytes.[1–4] PDPN is also expressed by malignant tumors such as squamous cell carcinomas, glioblastomas, and osteosarcomas.[5–7] In malignant tumors, PDPN is associated with tumor cell migration and cancer metastasis.[8] Anti-human PDPN (hPDPN) monoclonal antibody (mAb), NZ-1, also inhibited pulmonary metastasis in an experimental metastasis model.[9] Thus, it has been anticipated that anti-hPDPN antibody has the potential application for the treatment of various cancers.

Recently, marked expression of PDPN in fibroblast-like synoviocytes of the active inflammatory synovium of rheumatoid arthritis (RA) was reported. It has been suggested that PDPN can increase the migratory potential of activated fibroblast-like synoviocytes.[10] RA is a chronic inflammatory joint disease, often leading to cartilage and bone damage, resulting in musculoskeletal disability.[11] Many anti-RA drugs, such as conventional disease-modifying antirheumatic drugs (cDMARDs), corticosteroids, and nonsteroidal anti-inflammatory drugs, have been used for their treatment. In contrast, clinical applications of antibody drugs have been spread globally and have contributed to the long-term remission of RA.[12–17] However, resistance to present antibody drugs has been found in 20%–40% of patients with RA.[18,19] Accordingly, the development of novel antibody drugs with

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newly mechanistic properties for the treatment of RA patients, who were resistant to preexisting antibodies, has been in strong demand.

As PDPN is markedly expressed in inflammatory synovium, it has been anticipated as a therapeutic target to control synovitis.[10,18] However, little is known about the potential role of PDPN in inflammation and synovitis in RA patients. Recently, six new anti-hPDPN mAbs were established.[20–25] Comparative studies for the evaluation of inflammatory synovitis using those newly developed anti-hPDPN mAbs have not been performed. Therefore, the aim of this study is to elucidate PDPN expression in inflammatory synovial tissues from surgically treated joint diseases, and to select the most useful PDPN mAb for evaluating synovitis or the degree and property of synovial inflammation.

Materials and Methods

Patients and specimens

Eleven RA patients who underwent surgery (total knee arthroplasty: 4, total hip arthroplasty: 3, wrist synovectomy: 2, total shoulder arthroplasty: 1, and metacarpophalangeal joint arthroplasty: 1) for upper and lower limb joint disease were enrolled in this study. Mean age at surgery was 56.7 years (range: 32–79 years). There were four males and seven females. Mean history of RA at surgery was 10.3 years (range: 5–19 years). Eight patients used cDMARDs. Five patients used methotrexate (mean: 9.6 mg/week, range: 6–16 mg/week). Eight patients used steroids (mean: 5.4 mg/day, range: 4–9 mg/day). Eight patients used biologics (Etanercept: 6, Abatacept: 1, Tocilizumab: 1). Accordingly, most of the RA patients were drug-resistant. Mean C-reactive protein at surgery was 1.3 mg/dL (range: 0.1–4.3 mg/dL). All the RA patients fulfilled the 1987 American College of Rheumatology[26] or the 2010 ACR/European League Against Rheumatism (EULAR) Criteria for RA.[27] Synovial tissue samples were obtained at joint surgeries. Nine osteoarthritis (OA) synovial samples were collected for comparison. Mean age of the patients at surgery (total knee arthroplasty: 3, total hip arthroplasty: 6) was 70.1 years (57–82). There were three males and six females (Table 1). This study was approved by the Yamagata University Faculty of Medicine Ethics Committee (approval number: 369, December 13, 2016) and written informed consent was obtained from all the patients.

Table 1. Characteristics of Rheumatoid Arthritis Patients and Osteoarthritis Patients

	RA (n = 11)	*OA (n = 9)*
Age, mean year (range)	56.7 (32–79)	70.1 (57–82)
Gender M/F	4/7	3/6
Disease duration, mean years (range)	10.3 (5–19)	—
cDMARDs, %	72.70	—
Steroids, %	72.70	—
Biologics, %	72.70	—
CRP at surgery (mg/dL), mean (range)	1.3 (0.1–4.3)	

cDMARDs, conventional disease-modifying antirheumatic drugs; CRP, C-reactive protein; OA, osteoarthritis; RA, rheumatoid arthritis.

Assessment of synovitis

The degree of synovitis was evaluated under a light microscope (DMD108; Leica Microsystems, Wetzlar, Germany) by hematoxylin and eosin staining and by Krenn histopathological grading system (0–9 points).[28]

Immunohistochemistry

Synovial samples were fixed in 4% paraformaldehyde. Four micrometers-thick serial sections were deparaffinized in xylene and rehydrated. After blocking of endogenous peroxidase activity with 0.3% H_2O_2 in phosphate-buffered saline for 10 minutes, they were autoclaved at +121°C in citrate buffer (pH 6.0) for 20 minutes. The sections were incubated in 5% bovine serum albumin for 20 minutes to block nonspecific binding and then incubated overnight at +4°C with anti-human CD68 antibody, which can recognize heavily glycosylated 110-kDa membrane protein, and was used as a macrophage marker (5 μg/mL, Agilent Technologies Inc., Santa Clara, CA), and an anti-human CD248 antibody, which can recognize highly sialylated cell surface type I transmembrane glycoprotein, was used as a stromal fibroblast marker[29] (0.5 μg/mL; abcam, Cambridge, MA), anti-hPDPN mAbs (NZ-1: 5 μg/mL, LpMab-3: 5 μg/mL, LpMab-7: 5 μg/mL, LpMab-10: 5 μg/mL, LpMab-12: 1 μg/mL, LpMab-13: 5 μg/mL, LpMab-17: 1 μg/mL). After treatment with biotinylated anti-rat IgG antibody (Vector Laboratories, CA) or anti-mouse IgG antibody (Nichirei Biosciences, Tokyo, Japan) for 1 hour, the immunolabeling was visualized by the biotin–streptavidin–horseradish peroxidase complex (Nichirei Biosciences) and 3,3′-diaminobenzidine tetrahydrochloride (DOJINDO, Kumamoto, Japan). The sections were counterstained with hematoxylin and analyzed under a light microscope (DMD108).[20]

Immunofluorescence colocalization studies

The sections were incubated with the primary antibodies, monoclonal mouse anti-human CD68 antibody (5 μg/mL; Agilent Technologies, Inc.), monoclonal rabbit anti-human CD248 antibody (0.5 μg/mL; abcam), and with a rat anti-hPDPN mAb (NZ-1), followed by incubation with secondary antibodies, Alexa Fluor® 488-conjugated goat anti-mouse IgG antibodies (Thermo Fisher Scientific, Inc., Waltham, MA), Alexa Fluor® 568-conjugated goat anti-rat IgG antibodies (Thermo Fisher Scientific, Inc.), Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibodies (Thermo Fisher Scientific, Inc.), or Alexa Fluor® 546-conjugated donkey anti-rabbit IgG antibodies (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Nuclei were counterstained with DAPI (Thermo Fisher Scientific, Inc.). The images were observed under a fluorescence digital microscope (BZ-X 710 digital microscope; Keyence, Osaka, Japan).

Quantitative histomorphometric analyses

PDPN immunopositive cells that typically inflamed synovial lining layers in both RA and OA were scanned using a BZ-X 710 digital microscope. The area of immunopositive cells per reference area was calculated digitally using BZ H3C Hybrid Cell Count Software (Keyence, Osaka, Japan).[30]

Statistical analysis

Mann–Whitney *U* test and Kruskal–Wallis test were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which was a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it was a modified version of R commander designed to add statistical functions frequently used in biostatistics. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

In actively inflamed synovium of RA, CD68-positive ($CD68^+$) macrophage-like type A lining cells and macrophage infiltrates of the sublining stroma (Fig. 1A) and CD248-positive ($CD248^+$) fibroblast-like type B lining cells and stromal fibroblasts were observed (Fig. 1B). In synovium of OA with inflammatory change, $CD68^+$ macrophage-like type A lining cells and $CD248^+$ fibroblast-like type B lining cells were also observed, but immunoreactivity of macrophage and fibroblast in stroma were modest (data not shown). Inflammatory synovitis scores of RA were significantly higher than for OA (Fig. 1C, RA: 7.4 ± 1.0, OA: 4.3 ± 1.7, $p < 0.01$).

Immunohistochemistry showed that PDPN was markedly expressed in the hyperplastic synovial lining layer and stromal macrophages and fibroblasts of RA (Fig. 1D–J). In OA samples with inflammatory reaction, immunoreactivity of PDPN was modestly observed in the superficial lining layer and slightly in the stromal macrophages and fibroblasts

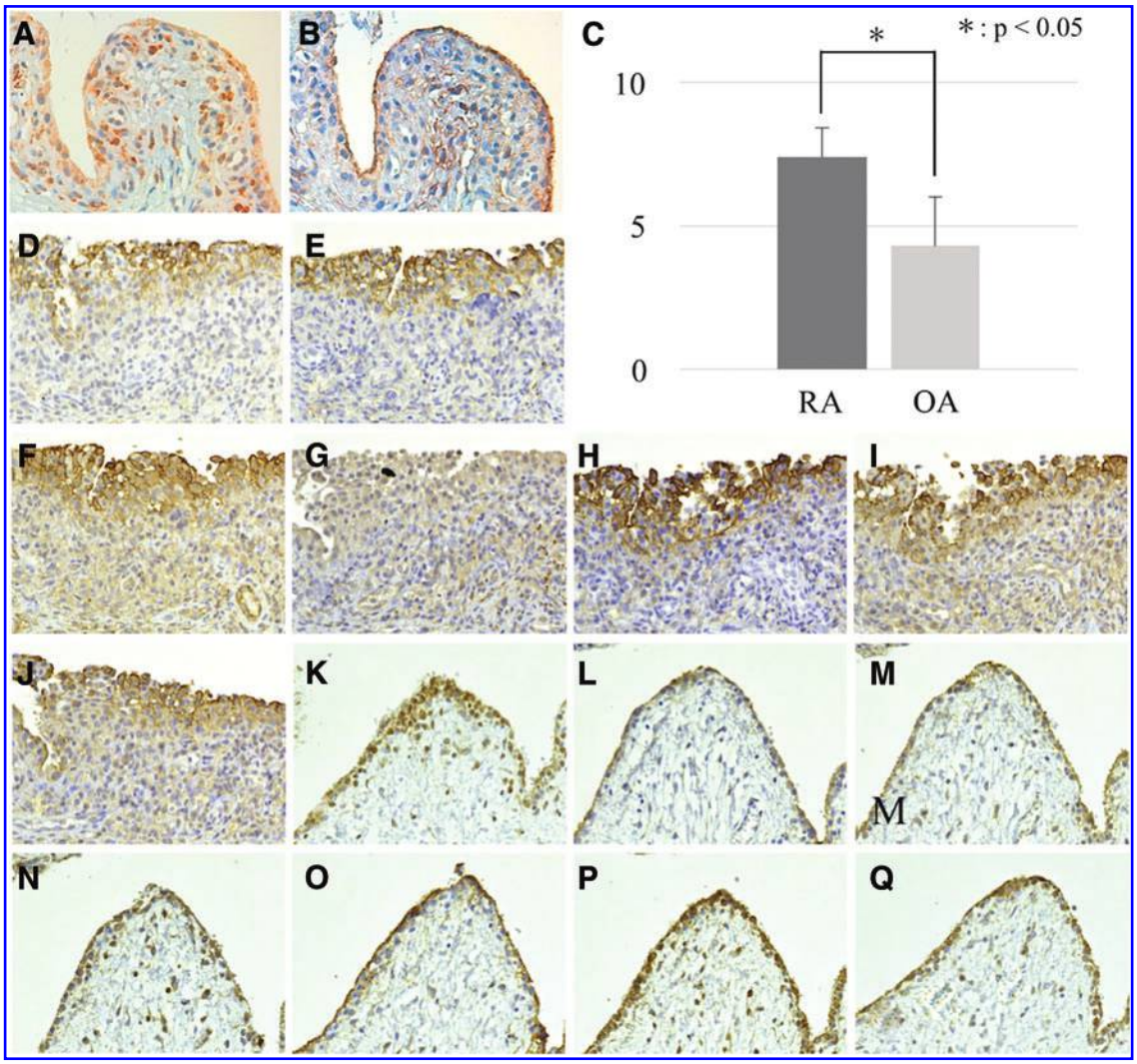

FIG. 1. Immunohistochemistry of RA synovium. **(A)** $CD68^+$ macrophage-like type A lining cells and macrophage infiltrates of the sublining stroma were observed. **(B)** $CD248^+$ fibroblast-like type B lining cells and fibroblasts of the stroma were also observed. **(C)** Inflammatory synovitis score of RA using Krenn histopathological grading system was significantly higher than that of OA (RA: 7.4 ± 1.0, OA: 4.3 ± 1.7, $p < 0.01$). Immunohistochemistry using anti-human PDPN mAbs of RA and OA synovium (RA: **D–J**, OA: **K–Q**). Marked expression of PDPN was detected in the hyperplastic synovial lining layer and stromal fibroblasts and macrophages of RA **(D:** NZ-1, **E:** LpMab-3, **F:** LpMab-7, **G:** LpMab-10, **H:** LpMab-12, **I:** LpMab-13, **J:** LpMab-17). PDPN was modestly observed in the superficial lining layer and slightly in the macrophages and fibroblasts of the sublining stroma of OA **(K:** NZ-1, **L:** LpMab-3, **M:** LpMab-7, **N:** LpMab-10, **O:** LpMab-12, **P:** LpMab-13, **Q:** LpMab-17). mAb, monoclonal antibody; OA, osteoarthritis; PDPN, podoplanin; RA, rheumatoid arthritis.

(Fig. 1K–Q). Immunofluorescence showed cellular colocalization of NZ-1 in $CD68^+$ macrophage-like type A lining cells and infiltrating macrophages of the stroma (Fig. 2A–D), and in $CD248^+$ fibroblast-like type B lining cells, stromal fibroblasts were observed (Fig. 2E–H). Moreover, $CD68^+$ and $CD248^+$ cells were observed in the inflamed synovial lining layer of RA (Fig. 2I–L), but only in the superficial lining layer of OA (data not shown). Similarly, colocalization of LpMab-3, LpMab-7, LpMab-10, LpMab-12, LpMab-13, and LpMab-17 in $CD68^+$ macrophage-like type A lining cells (Fig. 2M–R) and those in $CD248^+$ fibroblast-like type B lining cells were observed (Fig. 2S–X).

Evaluation of immunoreactivity using BZ H3C showed that LpMab-12 showed the highest score and was significantly higher than that of NZ-1, LpMab-3, and LpMab-17 in RA (NZ-1: 28.8% ± 5.6%, LpMab-3: 29.5% ± 7.6%, LpMab-7: 31.5% ± 6.7%, LpMab-10: 30.1% ± 9.9%, LpMab-12: 41.3% ± 6.6%, LpMab-13: 37.5% ± 7.9%, LpMab-17: 30.4% ± 7.4%) (Fig. 3A). Similarly, LpMab-12 had the highest score in OA. In contrast to RA, the difference was not statistically

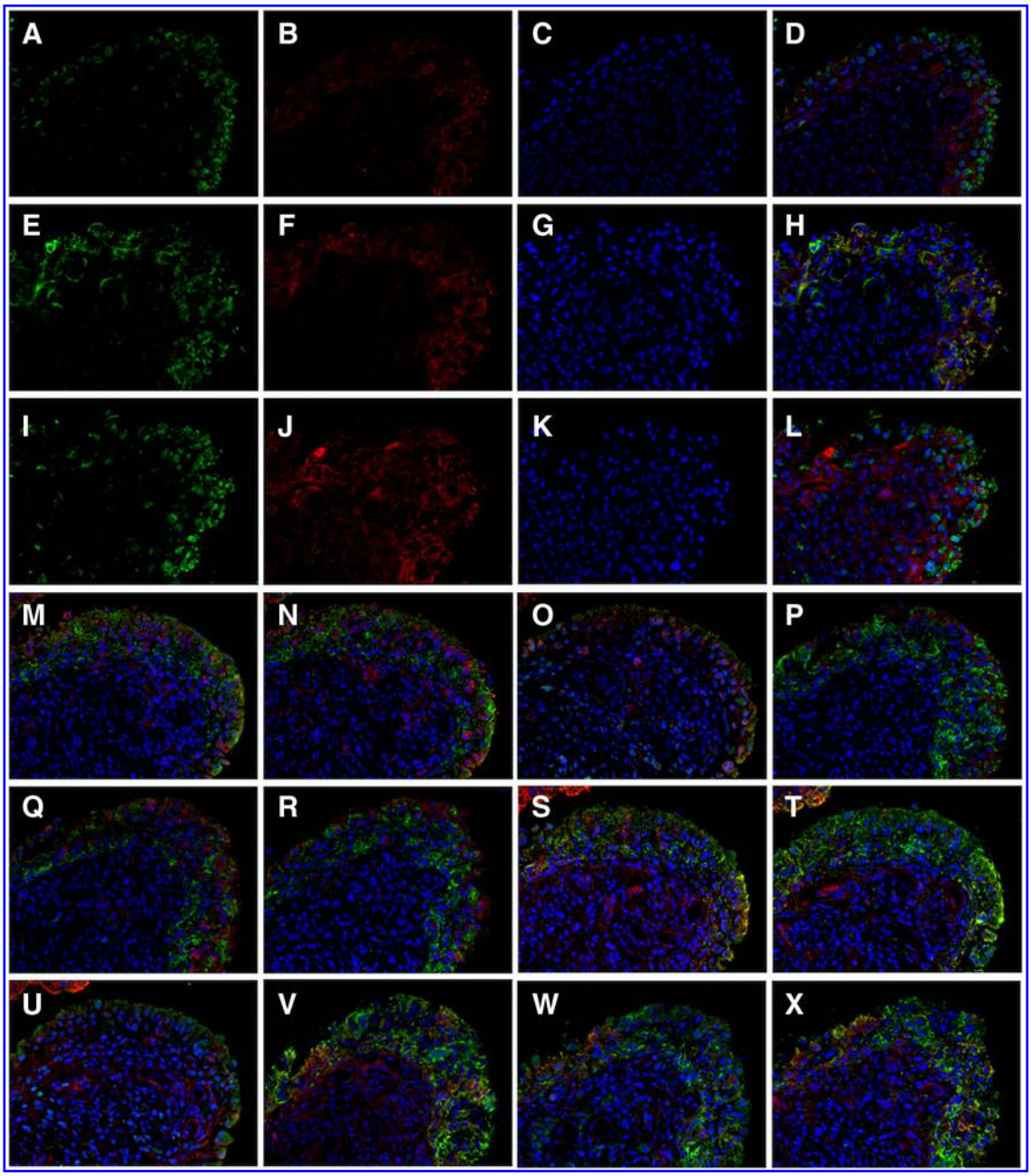

FIG. 2. Immunohistochemical analysis. Double-immunofluorescence staining of CD68 for macrophage **(A, I)**, CD248 for fibroblast **(E, J)**, NZ-1 for PDPN **(B, F)**, and DAPI for nuclei **(C, G, K)** was performed to reveal colocalizations of CD68/NZ-1 **(D)**, CD248/NZ-1 **(H)**, or CD68/CD248 **(L)** with yellow color in overlays. Double-immunofluorescence staining of CD68 and LpMab-3 **(M)**, LpMab-7 **(N)**, LpMab-10 **(O)**, LpMab-12 **(P)**, LpMab-13 **(Q)**, and LpMab-17 **(R)**. Green for PDPN, red for CD68, and blue for nuclei. Double-immunofluorescence staining of CD248 and LpMab-3 **(S)**, LpMab-7 **(T)**, LpMab-10 **(U)**, LpMab-12 **(V)**, LpMab-13 **(W)**, and LpMab-17 **(X)**. Green for PDPN, red for CD248, and blue for nuclei.

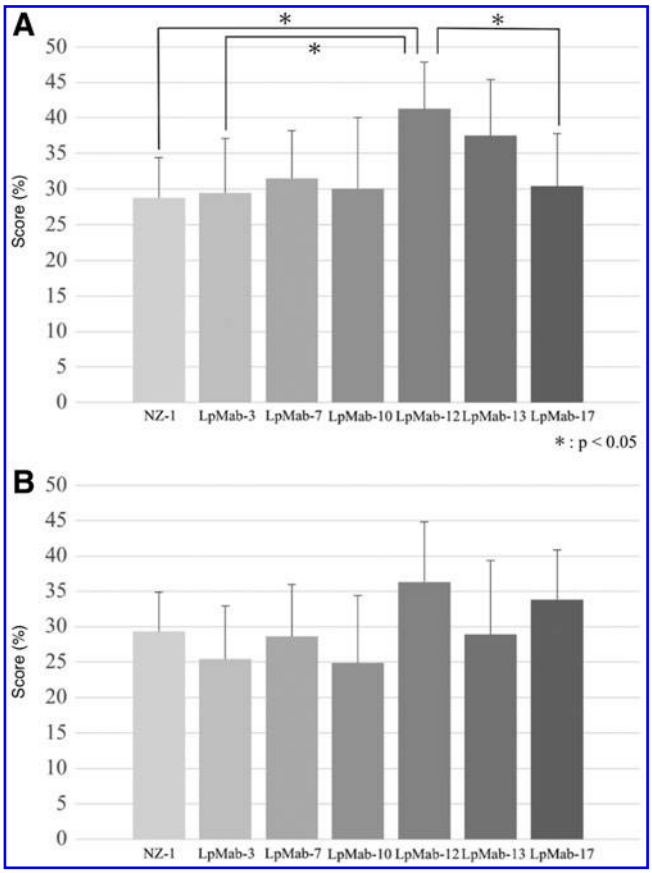

FIG. 3. The result of evaluation of immunoreactivity using BZ H3C Hybrid Cell Count Software. **(A)** LpMab-12 has the highest score, which was significantly higher than that of NZ-1, LpMab-3, and LpMab-17 in RA ($p < 0.05$). **(B)** LpMab-12 has the highest score, but the difference was not statistically significant in OA.

significant in OA (NZ-1: 29.3% ± 5.6%, LpMab-3: 25.4% ± 7.5%, LpMab-7: 28.6% ± 7.4%, LpMab-10: 24.9% ± 9.5%, LpMab-12: 36.3% ± 8.5%, LpMab-13: 28.9% ± 10.5%, and LpMab-17: 33.8% ± 7.1%) (Fig. 3B).

Discussion

To the best of our knowledge, this was the first comparative study of PDPN stainability in RA and OA synovium using an anti-hPDPN mAb panel. We demonstrated that LpMab-12 was the most stainable anti-hPDPN mAb for inflamed synovial lining layer of RA patients in immunohistochemistry. Boilard et al. demonstrated that platelets play an important role in the pathophysiology of inflammatory arthritis.[31] Semple et al. proposed that platelets may contribute to the acceleration of the joint destruction in patients with inflammatory arthritis, such as RA.[32] Platelet aggregation stimulating (PLAG) domain is an important site for hPDPN on its function of platelet aggregation.[33] PLAG domain locates on 29–54 amino acid of hPDPN.[34] The minimum epitope of NZ-1, LpMab-3, LpMab-7, LpMab-10, LpMab-12, LpMab-13, and LpMab-17 was Met43-Asp49, Thr76-Glu81, Alg79-Leu83, Glu33-Gly45, Asp49-Pro53, Ala42-Asp49, and Gly77-Asp82 of hPDPN, respectively.[20–25] In particular, LpMab-12 detects the glycosylation on Thr52 of hPDPN.[23] It was reported that sialylated *O*-glycan on Tnr52 was required for the platelet aggregating activity of hPDPN.[34,35] The reason of superiority of LpMab-12 in immunohistochemistry for synovial tissues may be derived from its interaction with platelets which plays an important role in inflammatory synovitis.

In this study, anti-hPDPN mAbs, including NZ-1, LpMab-3, LpMab-7, LpMab-10, LpMab-12, LpMab-13, and LpMab-17, showed immunoreactivity in inflamed the hyperplastic synovial lining layer of RA tissues. In contrast to previous reports, our results showed that PDPN was also observed in OA synovial tissues. Ekwall et al. reported that synovium in OA patients were predominantly PDPN-negative, and immunohistochemistry staining scores of PDPN in RA was significantly higher than OA.[10] In their report, D2-40 (mouse IgG_1) was used as anti-PDPN antibody.[10] The difference for the binding affinity of D2-40, NZ-1, LpMab-3, LpMab-7, LpMab-10, LpMab-12, LpMab-13, and LpMab-17 may lead to the discrepancy of immunohistochemistry. However, in primary cultured fibroblast-like synoviocytes, average PDPN expression was similar in RA and OA (52% ± 24% and 64% ± 6%, respectively).[10] In addition, the reason for the difference of positivity of PDPN between RA and OA among surgically resected fibroblast-like synoviocytes and cultured fibroblast-like synoviocytes was not still elucidated. In contrast to *in-vivo*, cellular activity of fibroblasts may be altered, leading to the increased expression of PDPN in culture conditions. Further investigation is necessary to clarify the discrepancy between *in -vivo* and *in -vitro*.

The synovial lining layer is mainly constituted by macrophage-like type A lining cells and fibroblast-like type B lining cells.[36] Kerrigan et al. reported that PDPN was expressed on thioglycolate-elicited inflammatory peritoneal macrophages and lipopolysaccharide-treated RAW264.7 macrophages, but not bone marrow-derived macrophages, alveolar macrophages, and peritoneal macrophages.[37] Croft et al. reported that PDPN was expressed predominately on the lining layer of the synovium, but was seen only on a small number of cells within the sublining layer, whereas CD248 was superiorly expressed in the sublining fibroblasts.[38] Conversely, our results showed that CD248 was clearly positive in synovial lining cells (Fig. 2B). It is possible that the difference between Croft's study and ours with respect to grade of inflammation of synovitis or drugs used at the time of tissue sampling affected the location of CD248-positive cells and their immunoreactivity.

Clinically, 20%–40% of RA patients using biologics are still resistant to the treatment.[18,19] Del Rey et al. reported that there was a strong association between PDPN expression and synovial lymphoid neogenesis in RA synovium, which was reduced after anti-TNF-alpha therapy.[39] Thus, PDPN has been suggested as one of the biomarkers for treatment-resistant inflammatory synovitis, because its expression correlated with local inflammatory grading of the synovitis in groups with biologics or cDMARDs by immunohistochemical analyses using NZ-1.[18] Dakin et al. reported PDPN was increased in diseased compared to healthy tendon tissues and cells, thus concluding that stromal fibroblast activation is a potential therapeutic target in the chronic inflammation.[40] Mechanisms of antibody drugs are as follows; activation of antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), blocking of signal transduction by

means of binding to the receptor, and antibody drug conjugates (ADCs).[41–44] Because RA is an inflammatory disease, not a malignant neoplastic disease, blocking signal transduction or ADCs for abrogation of the activation of inflammatory cascade of synovium may be suitable rather than ADCC or CDC. In our study, LpMab-12 was the most stainable anti-hPDPN antibody in RA synovitis. PDPN, especially LpMab-12, seems not only to be a promising biomarker for inflammatory arthritis both in RA and OA but also may have the potential of being a therapeutic target in RA synovitis by blocking signal transduction or ADCs for suppressing inflammation. Further investigation on PDPN as a therapeutic target molecule for antirheumatic drug is mandatory.

Inflammatory synovium generates proinflammatory mediators, which act as cartilage-degrading factors such as matrix metalloproteinases, tumor necrosis factor, interleukin-1 (IL-1), IL-6, prostaglandin E2, and nitric oxide.[45–48] Inhibition of the cell activation in inflammatory synovial tissues by anti-hPDPN mAbs may have the potential to protect joint destruction leading to the loss of daily function in arthritic patients.

Recently, OA has been reported to be an inflammatory joint disease.[49] In early OA, synovial tissue demonstrated the features of inflammation such as lining layer hyperplasia, $CD68^+$ macrophage infiltration, and vascular proliferation.[50] Synovitis had relationships with not only pain but also physical function in knee OA patients.[51] A recent study of 514 patients without radiographic knee OA revealed that joint effusion and synovitis predicted cartilage loss in the future.[52] In our study, immunoreactivity of PDPN was observed in the lining layer of OA synovium, which was a small number of RA. Thus, PDPN may be also one of therapeutic targets in inflammatory OA synovial tissue as well as in active and/or treatment-resistant RA synovitis.

There are three limitations in this study. First, the study populations were relatively small. LpMab-12 may have a statistically higher score of PDPN positivity in OA synovium from larger samples. Second, synovial samples in this study were collected from joint surgery, which was done for terminally destructive joints. Therefore, it was not possible to compare PDPN expression in early-stage inflamed joint and severe inflamed joint of RA patients. Third, it is likely that steroids may influence PDPN positivity and function in the inflamed synovial lining layer. In fact, 72.7% of patients in this study used oral steroids at the time of tissue sampling. Comparative analyses between steroids naive and steroids user are anticipated.

In conclusion, PDPN was expressed in inflammatory lining cells and sublining stroma of RA and OA synovium. In the seven anti-hPDPN antibodies examined, LpMab-12 was the most stainable antibody for PDPN in RA synovitis. Thus, LpMab-12 has a possible and promising specific biomarker for evaluating synovitis in RA and inflammatory OA. Furthermore, additional analyses of LpMab-12 for the therapeutic target of treatment resistance inflammatory arthritis are required.

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Author Disclosure Statement

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