

Expression of Tn and sialyl Tn antigens in synovial tissues in rheumatoid arthritis

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This study was supported by a Grant-in-Aid for Research (C) (no. 16590992) from The Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and Core Research for Evolutional Science and Technology (CREST) Project from Japan Science and Technology Agency (JST).

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Received on May 27, 2009; accepted in revised form on November 12, 2009.

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Key words: Immunology, rheumatoid arthritis, Tn antigen, sialyl Tn antigen.

Competing interests: none declared.

ABSTRACT

Objective. *The carbohydrate chains represented by mucins (MUCs) are expressed by a variety of normal and malignant secretory epithelial cells and induce a variety of immunoreactions. Tn and sialyl Tn antigens are tumour-associated carbohydrate antigens which are borne on the core proteins of mucins. The purpose of this study is to investigate the existence of tumour-associated carbohydrate antigens in rheumatoid arthritis (RA).*

Methods. *We examined the expression of Tn and sialyl Tn antigens in synovial tissues from RA and osteoarthritis (OA) patients by immunohistochemistry. In addition, mucins from synovial fluid (SF) from RA patients are purified by gel filtration and density gradient ultracentrifugation and the existence of these antigens examined by dot and Western blotting.*

Results. *We found that Tn and sialyl Tn antigens were strongly expressed in synovial cells and infiltrating mononuclear cells on the sublining layer and lymphoid follicles in synovial tissues in RA compared with those in osteoarthritis. Tn and sialyl Tn antigens were detected in purified mucins of SF from RA patients.*

Conclusion. *Tumour-like synovial hyperplasia cells expressed Tn and sialyl Tn antigens. This finding suggests that the mucins exhibiting with abnormal glycosylation may be in part responsible for synovial hyperplasia, leading to the joint destruction in the pathogenesis of RA.*

Introduction

Mucins (MUCs) are glycoproteins with high molecular weights and contained in the mucus produced by epithelial or glandular cells. Their main functions are thought to be lubrication and protection of the epithelial surface in the gastrointestinal tract and articular cartilage surfaces in joints. Mucins are characterised by tandem repeats of identical or highly similar sequences that are rich in serine, threonine and proline residues (1, 2). The specific sequences and numbers of tandem repeats are highly variable and differ from one individual to another. These proteins are highly O-glycosylat-

ed through serine (Ser) and/or threonine (Thr) residues. Mucins have long been implicated in the pathogenesis of cancer, particularly adenocarcinomas. Aberrantly glycosylated mucins produced by cancer cells are found in the sera of cancer patients and used as disease markers. Tn (GalNAc α -Ser/Thr) and sialyl-Tn (Sialyl α 2-6GalNAc α -Ser/Thr) antigens are tumour-associated carbohydrate antigens expressed on mucins in epithelial cancers (3). These are immunogenic in humans and may provide the basis for immunotherapy. Glycopeptides carrying these antigens are of interest for development of cancer vaccines (1, 4).

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by infiltration by lymphocytes and macrophages into the synovium and abnormal synovial hyperplasia. Abnormal synovial hyperplasia along with angiogenesis leads to the formation of a very aggressive tissue called a pannus. Expansion of the pannus induces bone erosion and cartilage thinning, leading to irreversible joint destruction. The rheumatoid pannus can thus be considered a local tumour. Increased proliferation and insufficient apoptosis of synovial cells might contribute to its expansion, and so elimination of proliferating synoviocytes in the rheumatoid synovium seems to be a potentially effective treatment for RA. Alteration of glycoproteins is related with various immunoreactions. The study of sugar chains in autoimmune diseases is intriguing, however, there have been few reports on abnormalities of sugar chains, especially in the pathogenesis of RA. In this study, we found for the first time mucins bearing tumour-associated carbohydrate antigens, Tn and sialyl-Tn, in synovial tissues and synovial fluid (SF) in patients with RA. This finding may provide a new insight into the pathogenesis of RA.

Materials and methods

Synovial tissues and synovial fluid

The study protocol was approved by the Kyoto Prefectural University of Medicine institutional review board, and informed consent was obtained from each patient. Synovial fluid (n=10)

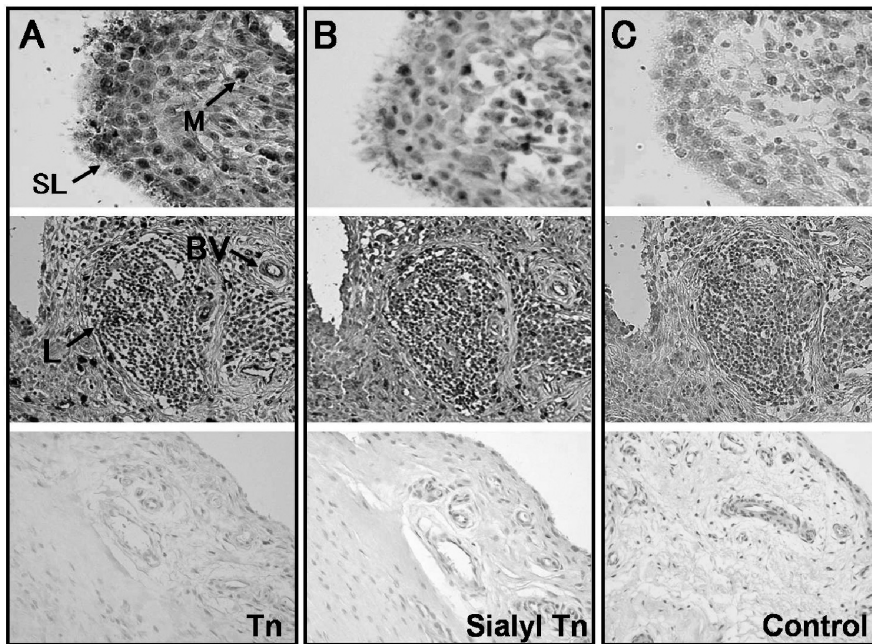


Fig. 1. Immunohistochemical staining of Tn and sialyl Tn antigens in RA and OA synovial tissues. Tn and sialyl Tn positive cells were located in synovial cells and infiltrating inflammatory mononuclear cells in the sublining layer (RA: top, OA: bottom) (Tn: A, sialyl Tn: B, control: C) and RA lymphoid follicles (middle).

Tn and sialyl Tn antigens are strongly expressed in synovial lining cells (immunohistochemical score: mean $\pm$ SE, Tn: 1.57 ± 0.20 , sialyl Tn: 1.40 ± 0.20) and infiltrating mononuclear cells including lymphoid follicles (0.95 ± 0.09 , 0.87 ± 0.13) in RA synovial tissue. In OA tissue, the expression of Tn and sialyl Tn antigens in synovial lining cells (0.25 ± 0.08 , 0.20 ± 0.08) and infiltrating mononuclear cells including lymphoid follicles (0.20 ± 0.11 , 0.20 ± 0.11) were significantly lower than that of RA tissues. ($p<0.05$, Mann-Whitney U-test)

Arrows indicate SL: synovial lining cell; BV: blood vessel; M: mononuclear cell; L: lymphoid follicle.

(top, bottom) magnification $\times 200$, (middle) magnification $\times 100$.

was obtained from patients visiting the rheumatology clinic of our hospital and suffering from RA. Doses of concomitant RA therapies such as non-steroidal anti-inflammatory drugs and oral corticosteroids (less than 10mg/day of prednisolone), disease modifying antirheumatic drugs (DMARDs) including methotrexate (less than 8mg/week) were stabilised. Patients have not been given biologic therapy. Synovial tissues were obtained during joint replacement surgery from 20 patients with RA (5 males / 15 females; mean age $\pm$ SE, 63 ± 2.4 years) (12 from knee, 5 from hand, 2 from elbow) and 10 patients with osteoarthritis (OA) (2 males / 8 females; mean age $\pm$ SE, 67 ± 1.3 years) (8 from knee, 2 from hand) according to the criteria of the American College of Rheumatology.

Immunostaining

To enhance antigen-antibody reactions

on immunohistochemistry using the avidin-biotin peroxidase complex system, tissue sections were immersed in Histofine (Nichirei Co., Tokyo, Japan), and heated at 75°C for 20 min. Ten $\mu\text{g}/\text{ml}$ of anti-Tn and anti-sialyl Tn mAbs (MLS 128 and MLS 132 mAb) (5, 6), and control normal mouse IgG in PBS were used. The specificity of MLS 128 and MLS 132 mAb was verified elsewhere (6-8).

For each of the tissue specimens from RA and OA patients, the extent and intensity of staining with the anti-Tn and anti-sialyl Tn mAbs in synovial lining cells, macrophages, and fibroblasts were blindly graded on a scale of 0 to 3+ by two observers. A 3+ grade implies maximally intense staining, whereas 0 implies no staining. Differences in the scores for the extent and intensity of immunostaining with anti-Tn, or anti-sialyl Tn mAbs between the two patient groups were analysed by means of the

Mann-Whitney U-test. $P<0.05$ was considered significantly different.

Purification of mucins from the RA patients' synovial fluid

RA patients' synovial fluid was pretreated with the hyaluronidase enzyme solution (Seikagaku Co. Tokyo, Japan) according to the modified Tolksdorf's method. We purified mucins from SF by a conventional procedure including gel filtration and CsCl density gradient centrifugation. Mucins were detected by Western and dot blot analysis with anti-Tn and anti-sialyl Tn mAbs (MLS 128 and MLS 132 mAbs, respectively) (5, 6).

Results

Immunohistochemical staining of Tn and sialyl Tn antigens in RA and OA

Immunohistochemistry was performed in all patients with RA (20 of 20) and OA (10 of 10). We found markedly enhanced expression of Tn and sialyl Tn antigens in synovial lining cells and infiltrating mononuclear cells including lymphoid follicles in RA synovial tissue significantly stronger than in OA tissue. (Immunohistochemical score analysis; $p<0.05$). Immunostaining with normal mouse IgG was negative in all patients with RA (Fig. 1).

Isolation of mucins from synovial fluid of RA patients

We tried to fractionate the synovial fluid obtained from RA patients ($n=10$) on Sepharose 6B (Fig. 2A). Tumour-associated antigens, Tn and sialyl Tn antigens, were detected in these fractions on dot blot analysis, as shown in Figure 2B.

Purification of a mucin from RA patients' synovial fluid

The collected Tn and sialyl Tn antigen-positive fractions by gel filtration (Fig. 2 Fraction no. 26-30), which were excluded from the gel, were pooled and treated with 0.6M perchloric acid. The resultant supernatant was further subjected to CsCl density gradient centrifugation followed by detection of Tn and sialyl Tn antigens in each fraction (Fig. 3A). This method for purification is described before (8). Tn and sialyl Tn antigens detected in fraction no.2

(Fig. 3B). The purity of the mucin was examined by SDS-PAGE followed by both protein and PAS staining (Fig. 3C, lanes a, b). In general, mucins are known to be silver stained very poorly. It is natural that no protein could be detected. Western blot analysis confirmed that the mucins were Tn and sialyl Tn antigens (Fig. 3C, lane c).

Discussion

We examined the expression of Tn and sialyl Tn antigens in synovial fluid and tissues from patients with RA. We found that Tn and sialyl Tn were markedly expressed in superficial synovial cells, fibroblasts and infiltrating mononuclear cells in the synovial tissue of RA patients compared with OA. Moreover, these mucins from synovial fluid from RA patients were purified by a gel filtration and CsCl density gradient ultracentrifugation. From the excluded fractions, Tn and sialyl Tn antigens were detected by Western blotting. Mucins are divided into two classes, *i.e.* such as secreted and cell-surface associated mucins. These classes include different members that have both unique and shared structural features (1). Cell-surface associated mucins are normally expressed on the luminal surface of most glandular epithelial tissues. However, it is highly upregulated and aberrantly glycosylated on tumour cells such as adenocarcinoma (9, 10), and Tn and sialyl Tn antigens have been used most extensively as tumour markers (11). Furthermore, sialyl Tn antigen-specific T-cell proliferation has been demonstrated and modified forms of sialyl Tn antigen exhibit improved antigenicity and promising immunological properties for use as cancer vaccines (1, 4). On the other hand, Tn and sialyl Tn are expressed in minimal amounts by isolated secretory and ductal cells in the salivary glands, colon, pancreas, and esophageal lining; otherwise, their normal tissue distribution is highly restricted (12). Overexpression of Tn and sialyl Tn antigen was significantly correlated with COX-2 overexpression in endometrial cancer tissues. We also reported that mucins induce production of COX-2 in tumour-infiltrating monocytes/macrophages in the

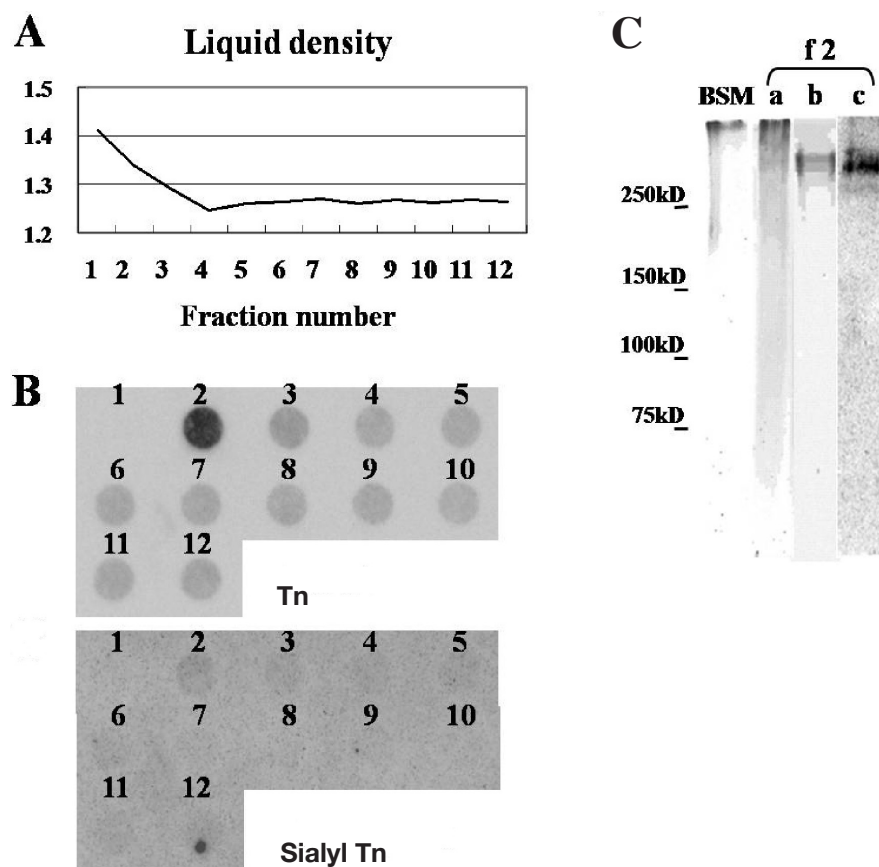
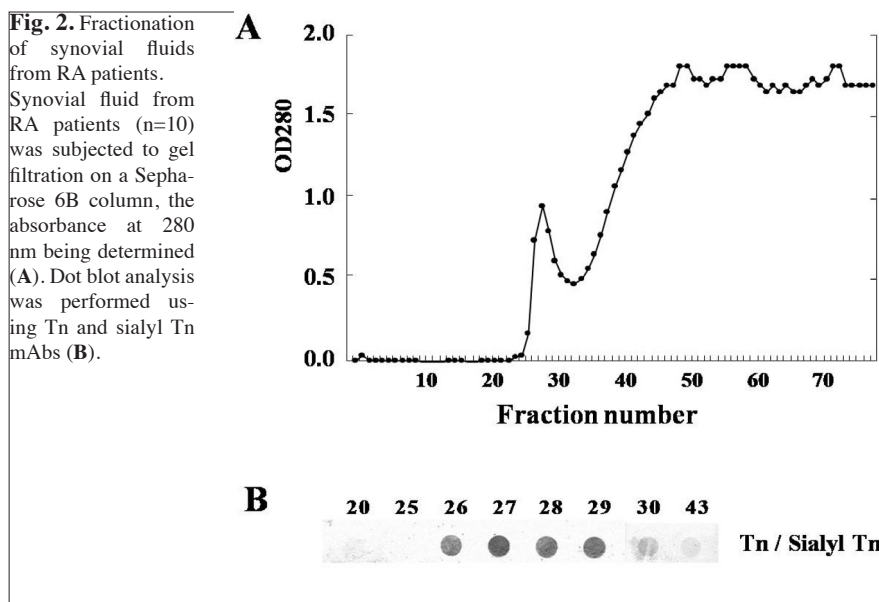


Fig. 3. Purification of mucins from RA synovial fluid, and expression of Tn and sialyl Tn antigens.

The collected Tn and sialyl Tn antigen-positive fractions by gel filtration (Fig. 2 Fraction no. 26-30) were purified by CsCl density gradient centrifugation. An aliquot of the Tn and sialyl Tn antigens-positive fraction (no. 2 in Figure 3 A and B) was subjected to SDS-PAGE followed by silver and PAS staining, and Western blotting using anti-Tn and anti-sialyl Tn antibodies.

A: Density of each purified fraction, **B:** Dot blot analysis using anti-Tn and anti-sialyl Tn antibodies (designated as MLS 128 and MLS 132 mAbs, respectively), **C:** silver staining (lane a), PAS staining (lane b), and Western blotting using anti-Tn and anti-sialyl Tn antibodies (lane c). f2: fraction No.2, BSM: as a positive control.

tumour microenvironment, resulting in PGE₂ production.

In this study, mucins purified from the synovial fluids of patients with RA included tumour-associated carbohydrate antigens such as Tn and sialyl Tn antigens. It is interesting that these mucins are expressed in synoviocytes from RA patients, which can grow like tumour cells. Some potential candidate mechanisms which have been involved in the RA synovial hyperplasia, were reported such as estrogen metabolites (13), receptor for advanced glycation endproducts (RAGE) activation (14). These mucins have possibility that play a part of responsible for synovial hyperplasia, leading to the joint destruction in the pathogenesis of RA.

Several immunologically based clinical trials have targeted mucins that are expressed by adenocarcinomas. These trials included tumour vaccines and monoclonal antibody-based therapies such as involving radioimmunoconjugates and passive immunotherapy. Extensive preclinical and early clinical testing has been conducted with several antibodies that bind to tumour-associated antigens on mucins (15). Such studies that target tumour-associated mucins are in the early phase of development. In RA, antibody therapy against cytokines is very effective for the clinical treatment of RA (16, 17). In this experimental study, it is clear that Tn, sialyl Tn antigens are in synovial fluid and tissues from patients with RA. However, further examination of

the biochemical characteristics and its relevance to RA of this mucin on immunocompetent cells and synoviocytes is needed, and it could be a new target specific for inflamed joints in the treatment of RA. Also, clarification of the mechanism of alteration of mucins or glycosylation related with cellular growth, differentiation and transformation will provide many insights into the pathogenesis of immune-inflammatory diseases.

References

- HOLLINGSWORTH MA, SWANSON BJ: Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer* 2004; 4: 45-60.
- ANDRIANIFAHANANA M, MONIAUX N, BATRA SK: Regulation of mucin expression: Mechanistic aspects and implications for cancer and inflammatory diseases. *Biochim Biophys Acta* 2006; 1765: 189-222.
- KINNEY AY, SAHIN A, VERNON SW *et al.*: The prognostic significance of sialyl-Tn antigen in women treated with breast carcinoma treated with adjuvant chemotherapy. *Cancer* 1997; 80: 2240-9.
- GEORGE SK, SCHWIENTEK T, HOLM B *et al.*: Chemoenzymatic synthesis of sialylated glycopeptides derived from mucins and T-cell stimulating peptides. *J Am Chem Soc* 2001; 123: 11117-25.
- NUMATA Y, NAKADA H, FUKUI S *et al.*: A monoclonal antibody directed to Tn antigen. *Biochem Biophys Res Commun* 1990; 170: 981-5.
- TANAKA N, NAKADA H, INOUE M, YAMASHINA I: Binding characteristics of an anti-Sialpha2-6GalNAc alpha-Ser/Thr (sialyl Tn) monoclonal antibody (MLS 132). *Eur J Biochem* 1999; 263: 27-32.
- NAKADA H, NUMATA Y, INOUE M *et al.*: Elucidation of an essential structure recognized by an anti-GalNAc alpha-Ser(Thr) monoclonal antibody (MLS 128). *J Biol Chem* 1991; 266: 12402-5.
- NAKADA H, INOUE M, NUMATA Y *et al.*: Epitopic structure of Tn glycoprotein A for an anti-Tn antibody (MLS 128). *Proc Natl Acad Sci USA* 1993; 90: 2495-9.
- LEV R: The mucin histochemistry of normal and neoplastic gastric mucosa. *Lab Invest* 1965; 14: 2080-100.
- HUKILL PB, VIDONE RA: Histochemistry of mucus and other polysaccharides in tumors. I. Carcinoma of the bladder. *Lab Invest* 1965; 14: 1624-35.
- MCGUCKIN MA, WALSH MD, HOHN BG, WARD BG, WRIGHT RG: Prognostic significance of MUC1 epithelial mucin expression in breast cancer. *Hum Pathol* 1995; 26: 432-9.
- YONEZAWA S, TACHIKAWA T, SADAHITO S, SATO E: Sialosyl-Tn antigen. Its distribution in normal tissues and expression in adenocarcinomas. *Am J Clin Pathol* 1992; 98: 167-74.
- CAPELLINO S, MONTAGNA P, VILLAGGIO B, SOLDANO S, STRAUB RH, CUTOLO M: Hydroxylated estrogen metabolites influence the proliferation of cultured human monocytes: possible role in synovial tissue hyperplasia. *Clin Exp Rheumatol* 2008; 26: 903-9.
- STEENVOORDEN MM, TOES RE, RONDAY HK, HUIZINGA TW, DE GROOT J: RAGE activation induces invasiveness of RA fibroblast-like synoviocytes *in vitro*. *Clin Exp Rheumatol* 2007; 25: 740-2.
- PECHER G, HARING A, KAISER L, THIEL E: Mucin gene (MUC1) transfected dendritic cells as vaccine: results of a phase I/II clinical trial. *Cancer Immunol Immunother* 2002; 51: 669-73.
- LIPSKY PE, VAN DER HEIJDE DM, ST CLAIR EW *et al.*: Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. *N Engl J Med* 2000; 343: 1594-602.
- LOPEZ-OLIVO MA, KALLEN MA, ORTIZ Z, SKIDMORE B, SUAREZ-ALMAZOR ME: Quality appraisal of clinical practice guidelines and consensus statements on the use of biologic agents in rheumatoid arthritis: a systematic review. *Arthritis Rheum* 2008; 59: 1625-38.