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Expression of Mucin 3 and Mucin 5AC in Arthritic Synovial Tissue

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Objective. **Rheumatoid arthritis (RA) is a chronic inflammatory disease that is characterized by hypertrophy of the synovial tissue, leukocyte infiltration, angiogenesis, and ultimately joint destruction. Mucins (MUCs) are a family of heavily glycosylated proteins that protect epithelial membranes and are used as ligands for cell adhesion. MUC gene expression has been found to be altered in many cancers and inflammatory states. This study was undertaken to examine its expression in synovial tissue (ST) and role in arthritis.**

Methods. **We performed immunohistochemistry, Western blotting, and reverse transcriptase–polymerase chain reaction to determine expression patterns of MUC1, MUC2, MUC3, and MUC5AC in RA, osteoarthritic (OA), and normal human ST.**

Results. **MUC3 was expressed in synovial lining cells, macrophages, and fibroblasts. Significantly more RA (n = 12) and OA (n = 13) synovial lining cells expressed MUC3 than did normal synovial lining cells (n = 7) (22% and 24% versus 0.4%, respectively; $P < 0.05$). Additionally, macrophages in RA and OA ST expressed significantly more MUC3 than did macrophages in normal ST (50% and 51% versus 10%, respectively; $P < 0.05$). MUC5AC was expressed at low levels in synovial lining cells, macrophages, and endothelial cells in RA and OA ST, and was barely expressed in normal ST. MUC1 and MUC2 proteins were not detected in ST. Messenger RNA (mRNA) for MUC3 and MUC5AC was detected in ST, and mRNA for MUC3 was detected in cultured ST fibroblasts.**

Conclusion. **These data demonstrate up-regulated MUC expression by ST cells and suggest a novel role of MUC3 and MUC5AC in the pathogenesis of arthritis.**

Synovial inflammation, including infiltration of monocyte-derived macrophages and lymphocytes into synovial tissue (ST), is characteristic of the chronic arthritic diseases rheumatoid arthritis (RA) and, to a lesser extent, osteoarthritis (OA). RA is also characterized by fibroblast proliferation and synovial lining hyperplasia (1). Additionally, these RA synovial fibroblasts display a transformed phenotype and can invade and destroy adjacent cartilage.

Mucins (MUCs) are a heterogeneous family of heavily *O*-glycosylated glycoproteins that are present in the mucus coating of epithelial surfaces in normal and neoplastic tissue. There are 18 members of the MUC family, which are classified as either transmembrane, gel forming, or soluble, based upon known functions and protein structural similarities (2). MUC gene expression is regulated at the transcriptional, translational, and posttranslational levels. MUCs undergo posttranslational modifications including *O*-glycosylation, multimerization, and proteolytic cleavage (2). MUCs were originally understood to be produced only by stratified epithelia, but they are now known to also be expressed by corneal endothelium, vascular endothelium, immune

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cells such as T and B lymphocytes, and dendritic cells (3–5).

MUC proteins have been implicated in the pathogenesis of several disease states. Neoplastic transformation is associated with dysregulated MUC expression and may be due to alterations of the oligosaccharide chains (6). It has been suggested that MUC5AC and genetic variants of MUC3 may be involved in the pathogenesis of inflammatory bowel diseases (7). Abnormalities in MUC3 expression by intestinal epithelium have been shown in patients with Crohn's disease (8). MUC5AC expression coincides with macrophage accumulation in a model of lung inflammation, indicating that macrophages may induce MUC expression by other cells (9). Additionally, cytokines have been shown to up-regulate MUC3 and MUC5AC expression (10).

The transmembrane MUC3 has been shown to be expressed in small intestine, colon, gall bladder, lung, breast, and salivary gland tissue. MUC3 exists in a membrane-bound form containing a transmembrane region and cytoplasmic tail, and in soluble forms arising from either alternate splicing of the MUC3 gene or proteolytic cleavage of the MUC3 protein (11). Additionally, MUC3 is coded for by 2 genes resulting in 2 different proteins, MUC3A and MUC3B. MUC5AC is expressed in the lung, stomach, pancreas, gallbladder, and endocervix. This is the first report to describe the expression of MUC family members MUC1, MUC2, MUC3, and MUC5AC in normal and diseased ST.

MATERIALS AND METHODS

Tissue specimens. RA ST and OA ST specimens were obtained from patients undergoing total joint replacement, and normal ST specimens were obtained at autopsy, shortly after death. A total of 70 tissue specimens were obtained, 30 from patients with RA (mean age 59.2 years), 25 from patients with OA (mean age 64.5 years), and 15 from autopsy subjects (mean age 61.5 years). Seventy-nine percent of the subjects were women. ST specimens were either frozen for cryostat sectioning and RNA purification or used fresh for isolation of synovial fibroblasts. While the majority of the arthritic tissue specimens were obtained from knee surgeries (79%), tissue specimens from hip, hand, and ankle surgeries were also included. All specimens were obtained with Institutional Review Board approval.

Immunohistochemistry. Four-micrometer sections of ST were immunostained using Elite ABC Kits (Vector, Burlingame, CA). Acetone-fixed slides were blocked with diluted serum and incubated with monoclonal antibodies (mAb) to either anti-human MUC1, MUC2, MUC3, or MUC5AC (LabVision, Fremont, CA), or an isotype-specific IgG control (Beckman Coulter, Fullerton, CA). Slides were counterstained with hematoxylin. ST components, including lining cells and sublining cells (macrophages, lymphocytes, fibroblasts, smooth muscle cells, and endothelial cells), were graded for immunostaining above background by a frequency of staining scale, scored 0–100%, where 0% indicates no staining and 100% indicates that all cells were immunoreactive (12). Five 400× fields were examined per section by a single pathologist (GKH) who was blinded with regard to ST type. Selected sections were analyzed by 2 additional observers (MVV and AEK). Sublining macrophages and endothelial cells were identified by morphology, which was confirmed by reactivity with anti-CD11c (Becton Dickinson, Mountain View, CA) and anti–von Willebrand factor (Dako, Carpinteria, CA), respectively. Synovial fibroblasts and smooth muscle cells were differentiated by location; spindle cells associated with vascular walls were identified as smooth muscle cells, and spindle cells in the stroma were categorized as sublining fibroblasts.

Synovial fibroblasts. Seven RA and 4 OA ST specimens were minced and digested in a solution of Dispase, collagenase, and DNase (12). Synovial fibroblasts were cultured in RPMI 1640 plus 10% fetal bovine serum plus 0.1% gentamicin (Gibco BRL, Grand Island, NY). The cells were used at passage 3 or later, at which time they were a homogenous population of fibroblasts verified by vimentin staining (data not shown).

Polymerase chain reaction (PCR). Total RNA was prepared from ST or synovial fibroblasts by an acid phenol method (12). Following DNase treatment, RNA (10 μg) was reverse-transcribed using oligo(dT) primers and Superscript II RT (Gibco BRL). PCR was performed in a buffer containing 1.5 m*M* $MgCl_2$, 0.2 μM each dNTP, 0.2 μM each oligonucleotide primer, and 2.5 units of platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The thermal cycling conditions were as follows: 30 cycles of 95°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute. The oligonucleotide primers were as follows: for MUC3, 5′-CCTCATTGCAAACTTCACTC-3′ and 5′-AGCCCACATTTTCTGTACTG-3′; for MUC5AC, 5′-TACAACAACATCATCACGAGTGCG-3′ and 5′-CTAGGGTGCTAGGAGCTGTCACAG-3′; and for GAPDH, 5′-GAACGGGAAGCTCACTGGCATGGC-3′ and 5′-TGAGGTCCACCACCCTGTTGCTG-3′. Bands were identified on agarose gels using ethidium bromide.

Western blotting. Synovial fibroblasts were lysed in lysis buffer containing nonionic detergent and protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN). Twenty micrograms of protein was subjected to 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose membranes (12). Membranes were blocked with 5% nonfat milk followed by incubation with either anti-MUC3 mAb (1:200; LabVision), anti-MUC3 polyclonal antibody (pAb) (1:500; Biomeda, Foster City, CA), or rabbit antiactin (1:1,000; Sigma-Aldrich, St. Louis, MO) in blocking buffer. Blots were then incubated with a goat anti-mouse or goat anti-rabbit horseradish peroxidase–conjugated antibody (1:10,000; Amersham, Piscataway, NJ). All blots were developed using enhanced chemiluminescence reagents (Amersham). Bands were detected using a Storm 860 PhosphorImager and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation. ST were homogenized in lysis buffer and then centrifuged and filtered through a 0.45-μm filter. Four hundred micrograms of protein extract was prein-

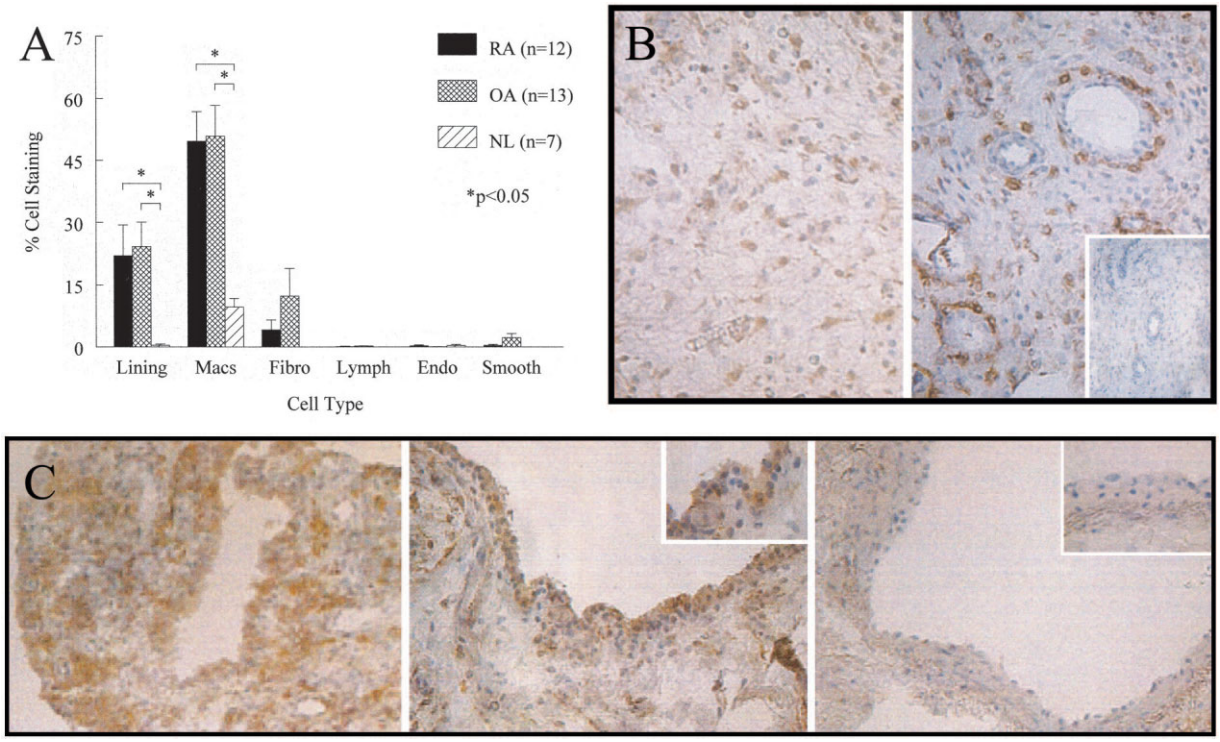

Figure 1. Mucin 3 (MUC3) expression, determined by immunohistochemistry, in synovial tissue (ST) specimens from patients with rheumatoid arthritis (RA) and patients with osteoarthritis (OA), and normal (NL) ST. **A**, Immunohistochemical localization of MUC3 expression in ST. Bars show the mean and SEM. Lining = lining layer; Macs = sublining macrophages; Fibro = sublining fibroblasts; Lymph = lymphocytes; Endo = endothelial cells; Smooth = smooth muscle cells. **B,** MUC3 immunohistochemical staining of RA ST macrophages (left), OA ST macrophages (right), and IgG, the negative control (**inset**) (original magnification × 100). **C,** MUC3 immunohistochemical staining of RA synovial lining cells (left), OA synovial lining cells (center), and normal synovial lining cells (right) (original magnification × 100). **Insets** show higher-magnification views (original magnification × 400).

cubated with protein A–Sepharose (Amersham). Following centrifugation, the supernatant was incubated with anti–MUC3 pAb (Biomeda) and protein A–Sepharose. The MUC3/anti–MUC-3/protein A–Sepharose complex was collected, boiled, separated using SDS-PAGE, and transferred to nitrocellulose membranes. Nitrocellulose membranes were processed as described above for Western blotting, using anti-MUC3 mAb (1:200).

Statistical analysis. Data were analyzed using Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

MUC expression in arthritic ST, determined by immunohistochemistry. Monoclonal antibodies against 4 MUC proteins were used to determine their expression in RA, OA, and normal ST specimens, by immunohistochemistry. Immunohistochemical staining of these tissue specimens revealed that MUC3 and MUC5AC were expressed mainly by arthritic ST, while MUC1 and MUC2 were not expressed in ST.

Immunohistochemistry using anti-MUC3 was performed on ST sections from 12 patients with RA and 13 patients with OA, and on 7 normal specimens. RA and OA synovial lining cells, macrophages, and, to a lesser extent, sublining fibroblasts expressed MUC3 protein. In contrast, normal ST expressed little or no MUC3 (Figure 1A). Approximately equal percentages of RA and OA ST macrophages were immunopositive for MUC3 (mean ± SEM 50 ± 7.2% and 51 ± 7.5%, respectively), while only 10% of normal ST macrophages expressed MUC3. Many of the macrophages were located in the perivascular region (Figure 1B). The percentages of RA and OA synovial lining cells immunopositive for MUC3 were also approximately equal (22 ± 7.3% and 24 ± 5.8%, respectively) (Figure 1C). Only 0.4% of normal synovial lining cells expressed MUC3. Smaller percentages of RA and OA synovial fibroblasts were immunopositive for MUC3 (4.1 ± 2.4% and 12.9 ± 6.6%, respectively). Virtually no endothelial cells or lymphocytes (<1%) were immunopositive for MUC3.

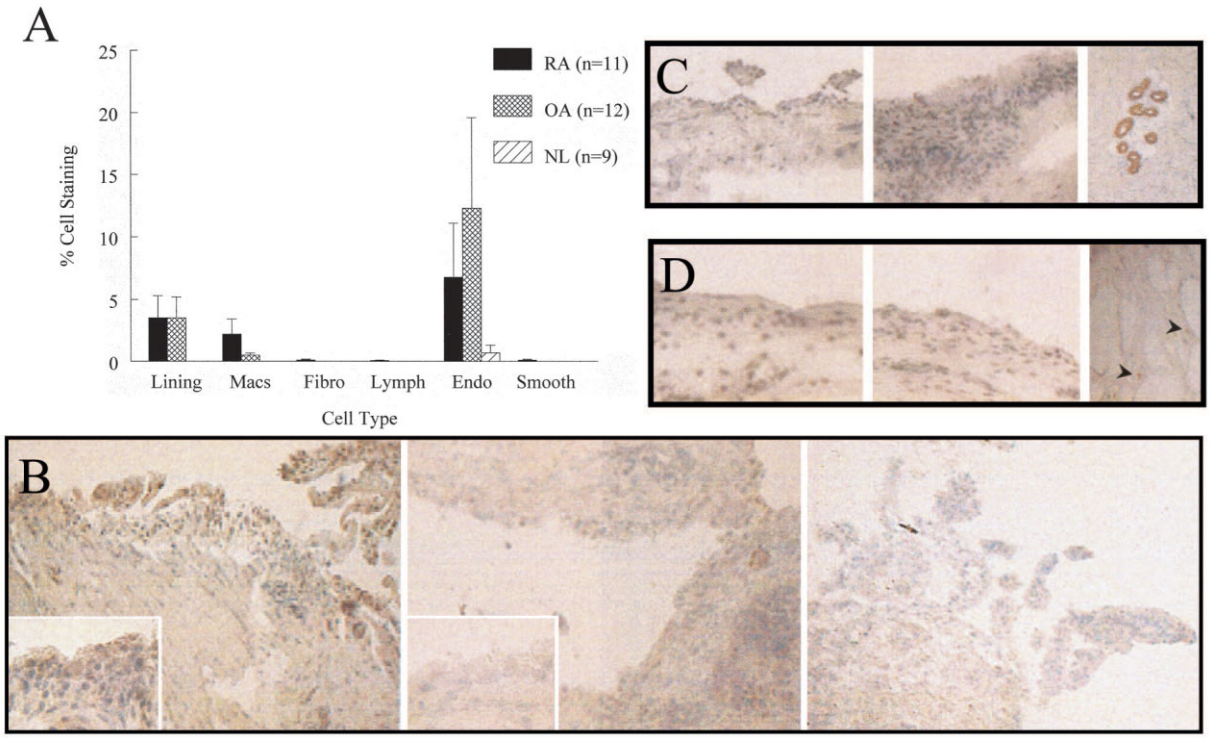

Figure 2. MUC5AC, MUC1, and MUC2 expression, determined by immunohistochemistry, in ST specimens from patients with RA and patients with OA, and normal ST. **A,** Immunohistochemical localization of MUC5AC expression in ST. Bars show the mean and SEM. **B,** MUC5AC immunohistochemical staining of OA synovial lining cells (left), normal synovial lining cells (center), and IgG, the negative control (right) (original magnification × 100). **Insets** show higher-magnification views (original magnification × 400). **C,** MUC1 immunohistochemical staining of RA ST (left), OA ST (center), and breast tissue (right) (original magnification × 100). **D,** MUC2 immunohistochemical staining of RA ST (left), OA ST (center), and breast tissue (right). **Arrowheads** indicate immunopositive cells (original magnification × 100). See Figure 1 for definitions.

Immunohistochemistry using anti–MUC5AC was performed on ST sections from 11 patients with RA and 12 patients with OA, and on 9 normal specimens. MUC5AC was expressed in small percentages of synovial lining cells, macrophages, and endothelial cells in RA and OA ST, but was not appreciably expressed in normal ST, although the difference was not statistically significant (Figures 2A and B). Immunohistochemistry using anti-MUC1 and anti-MUC2 showed no expression of these molecules in arthritic joints (Figures 2C and D).

MUC3 expression in ST, determined by reverse transcriptase–PCR (RT-PCR). To further validate the expression of MUC3 in ST, RT-PCR was performed on ST complementary DNA (cDNA). A specific 233-bp MUC3 band was amplified from ST from patients with RA, ST from patients with OA, and normal ST (Figure 3A). Similarly, the same specific band was amplified from RA synovial fibroblasts from 2 patients, confirming expression of MUC3 (Figure 3A).

MUC3 expression in ST and fibroblasts, determined by Western blotting. To further confirm that arthritic ST expresses MUC3 protein, OA ST protein extract was immunoprecipitated with anti-MUC3 pAb. The immunoprecipitated extract was then probed with monoclonal anti-MUC3, revealing the presence of a 60-kd band (Figure 3B). While this band was smaller than what would be expected for the whole glycoprotein, it was consistent with the findings of previous studies, which have shown that MUC3 exhibits different molecular weights in different cell types (13).

Next, protein extracts from synovial fibroblasts were subjected to Western blotting using either anti-MUC3 pAb (results not shown) or monoclonal anti-MUC3 (Figure 3C). RA and OA, but not normal, synovial fibroblasts expressed a protein that migrated identically to the 60-kd band seen in the immunoprecipitation of OA ST.

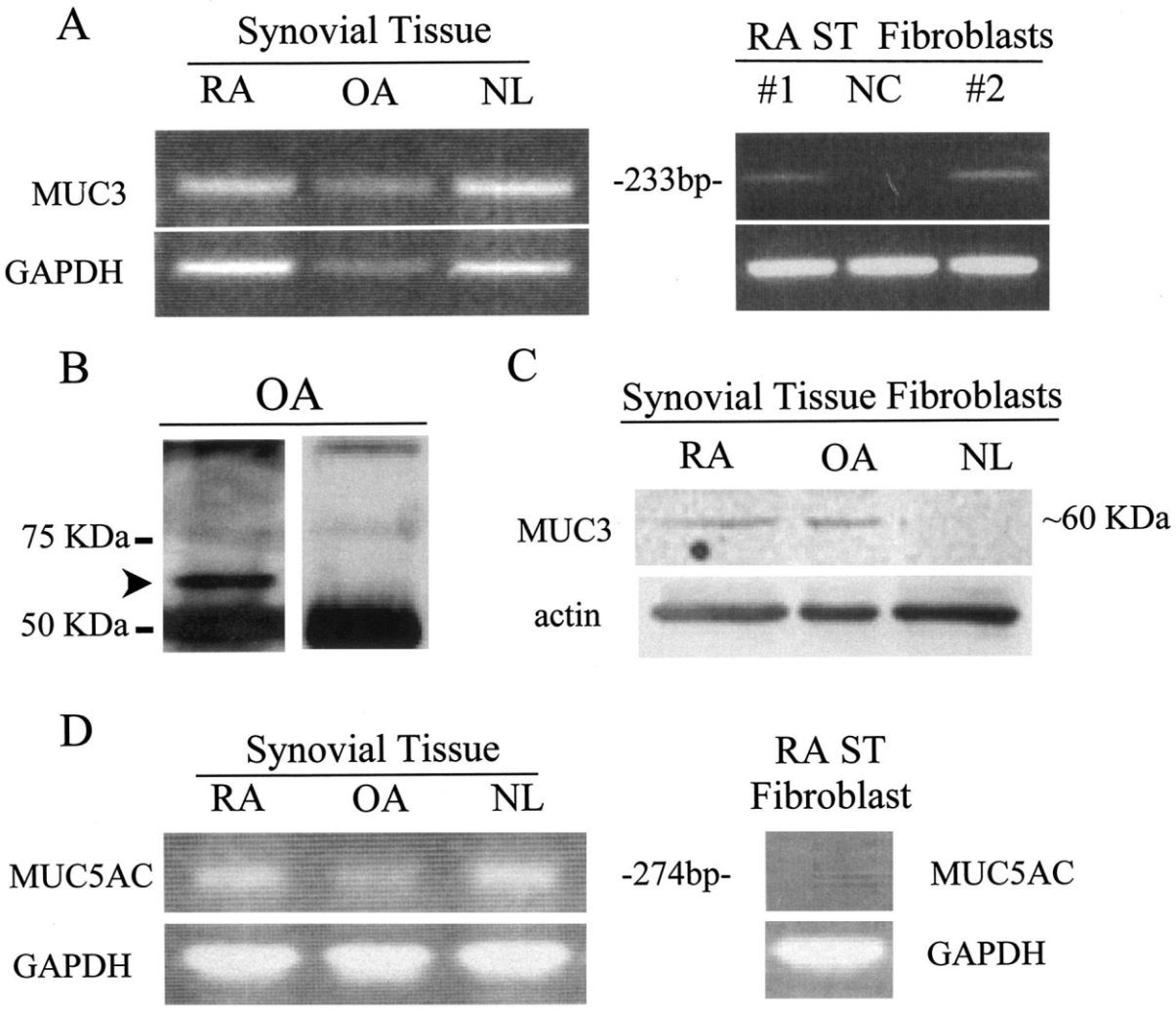

Figure 3. MUC3 and MUC5AC expression, determined by reverse transcriptase–polymerase chain reaction (RT-PCR) and Western blotting, in ST specimens from patients with RA, and patients with OA, and normal ST. **A,** Gel electrophoresis of MUC3 RT-PCR products, using specific MUC3 oligonucleotide primers with ST cDNA (left) and RA synovial fibroblast cDNA (right). Blots are representative of experiments with 3 RA, 3 OA, and 2 normal ST samples. Fibrocartilage was used as the negative control (NC). **B,** Immunoprecipitation of OA ST with a polyclonal anti-MUC3 antibody and detection with a monoclonal anti-MUC3 antibody, showing a prominent 60-kd band (arrowhead) (left), and without primary antibody (right). **C**, Western blot analysis of ST fibroblast MUC3 protein expression using a monoclonal antibody, showing a specific 60-kd band, compared with actin expression determined by probing with an antiactin antibody. Blots are representative of experiments with 4 RA, 4 OA, and 3 normal synovial fibroblast samples. **D,** Gel electrophoresis of MUC5AC RT-PCR products, using specific MUC5AC oligonucleotide primers with ST cDNA (left) and RA synovial fibroblast cDNA (right). Blots are representative of experiments with 3 RA, 2 OA, and 2 normal ST samples and 3 RA ST fibroblasts. See Figure 1 for other definitions.

MUC5AC expression in ST, determined by RT-PCR. To confirm the expression of MUC5AC in ST, RT-PCR was performed on ST cDNA. A specific MUC-5AC 274-bp band was amplified from ST from patients with RA, normal ST, and to a lesser extent, ST from patients with OA (Figure 3D). RT-PCR was also performed on synovial fibroblasts from 3 patients with RA, but an appropriate-sized band indicating the expression of MUC5AC messenger RNA (mRNA) could not be amplified (Figure 3D).

DISCUSSION

The synovial lining layer consists of fibroblast-like cells and macrophage-like cells. Additionally, syno-

vial fibroblasts and macrophages exist in the sublining ST. Our results indicate that synovial lining cells and sublining macrophages and endothelial cells express MUC proteins. MUC3, a membrane-type MUC, is expressed primarily by synovial lining cells and sublining macrophages. The sublining macrophages that express MUC3 tend to be perivascular, suggesting that either they recently arrived in the synovium from the peripheral blood or they are involved in recruiting other leukocytes into the ST. Additionally, ST from patients with RA or OA expresses significantly more MUC3 protein than does normal ST, suggesting a role of MUC3 expression in both RA and OA.

ST expression of MUC3 protein was confirmed by immunoprecipitation and immunoblotting of a 60-kd band using 2 different anti-MUC3 antibodies. Western blotting experiments with anti-MUC3 antibodies verified that RA and OA synovial fibroblasts, but not normal synovial fibroblasts, also express a 60-kd protein band.

To further validate ST MUC3 expression, MUC3 mRNA expression in ST from patients with RA and patients with OA, and normal ST was investigated by RT-PCR. Surprisingly, the results of these experiments indicated similar MUC3 mRNA expression levels in normal ST and arthritic ST, which differed from the immunohistochemistry and Western blotting data. This apparent difference may be due to the way the immunohistochemical studies were analyzed, with results reported as the percentage of cells that showed staining rather than as the amount of MUC3 protein that was produced. Thus, one possibility is that the 10% of macrophages that are immunopositive in normal ST are responsible for the MUC3 mRNA. Alternatively, MUC3 is known to be regulated at the translational and post-translational levels (11). Taken together, the RT-PCR and Western blotting data provide evidence that ST and synovial fibroblasts express MUC3.

MUC3 has previously been shown to be expressed by epithelial cells from diverse tissue types, but this is the first study to show MUC3 expression in either synovial fibroblasts or synovial macrophages. MUC1, another transmembrane MUC that was originally thought to be expressed solely by epithelial cells, has now been identified in lymphocytes and monocyte-derived dendritic cells, where it may function in leukocyte migration and cell–cell interactions (3,5). We were unable to detect MUC1 in ST, although we did detect it in breast tumor tissue. Thus, MUC3 is the second MUC family member shown to be expressed by a type of leukocyte.

We found that MUC5AC, a gel-forming MUC, was expressed in small percentages of synovial lining cells, sublining macrophages, and synovial endothelial cells in patients with RA and OA. RT-PCR data provide supporting evidence of MUC5AC protein expression by ST. The amplification of MUC5AC mRNA in normal ST suggests that MUC5AC may be regulated at the translational level in ST. We were unable to amplify MUC5AC from synovial fibroblasts, which was consistent with the results of immunohistochemical analysis. Another gel-forming MUC, MUC2, was not detectable in ST by immunohistochemistry.

MUC5AC has previously been shown to be expressed by epithelia and goblet cells in various organs, but this is the first study to demonstrate MUC5AC expression by fibroblasts, macrophages, or endothelial cells (2). MUC5AC has been shown to activate neutrophils on the ocular surface, suggesting a role of MUC-5AC in neutrophil adherence and activation (14).

In conclusion, we have shown that MUC3 is expressed in significantly more synovial lining cells and macrophages in arthritic joints than in normal joints, suggesting that it has a role in arthritis. Additionally, MUC5AC was expressed by a fraction of synovial lining and endothelial cells in arthritic ST. Our findings indicate that MUCs are involved in the pathogenesis of arthritic diseases, including OA and RA.

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AUTHOR CONTRIBUTIONS

Dr. Volin had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study design. Volin, Koch.
Acquisition of data. Volin, Shahrara, Woods, Koch.
Analysis and interpretation of data. Volin, Haines, Woods, Koch.
Manuscript preparation. Volin, Shahrara, Koch.
Statistical analysis. Volin.

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