Detection of gene expression in synovium of patients with osteoarthritis using a random sequencing method

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Background The etiology of osteoarthritis (OA) is multifactorial and current research attributes it to a complex network of biochemical factors. We attempted to identify important molecules in OA joint destruction.

Patients and methods Synovium was collected from 2 women with hip OA. Total RNA was extracted from the combined synovium. Messenger RNAs (mRNAs) were randomly sequenced for identification with the oligo-capping method. mRNA expression of 9 genes that were found to be frequently expressed was compared in synovium from 7 OA patients and 2 control patients with no signs of arthritis.

Results We sequenced 7,339 mRNAs in total and identified 4,247 different kinds, which were ranked in order of frequency. Fibronectin was the protein most frequently expressed (230/7,339), followed by matrix metalloproteinases (MMPs) 1 and 3. The 9 genes selected were those encoding fibronectin 1, MMP1, MMP3, tissue inhibitor of metalloproteinase 3, apolipoprotein L-I (APOL1), syndecan binding protein, insulin-like growth factor binding protein 5, heat shock protein 90, and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5). We investigated expression of these 9 genes in synovium from the 7 individual patients with OA. All 9 genes were expressed in OA and control synovium. Expression of MMP1 mRNA was weak in OA samples, however, while expression of ADAMTS5 and APOL1 mRNAs was weak in the controls and some of the OA samples.

Interpretation ADAMTS5 and APOL1 may have important roles in the mechanism of OA.

It was originally believed that only site-specific mechanical factors have major roles in the pathogenesis of single-site osteoarthritis (OA), but it has been suggested that systemic predisposition may also be important (Felson et al. 1998, Loughlin et al. 2004, Kizawa et al. 2005).

Current research has attributed morphological changes observed in OA, such as cartilage erosion and synovial inflammation, to a complex network of biochemical factors—including for example proteolytic enzymes, cytokines, chemokines, and adhesion molecules (Ishii et al. 2002). Many investigators have studied the expression of specific genes in synovial tissues and compared their expression pattern in rheumatoid arthritis (RA) with that in OA (Sakurai et al. 2001). However, to estimate the relative importance of these mRNAs, it would be better to compare their frequencies in cases with the same disease.

Oligo-capping is a simple method of replacing the cap structure of eukaryotic mRNAs with that of oligo RNAs, which makes it possible to construct full-length enriched cDNA libraries. We sequenced mRNAs in the synovium of OA patients
in a random manner with the oligo-capping method to semiquantitatively identify mRNAs that are present in OA synovium. After we had obtained data on the frequency of expression of mRNAs in OA synovium, we selected 9 frequently expressed genes and compared the expression between OA and control patients by reverse-transcription polymerase chain reaction (RT–PCR).

Patients and methods

A 52-year-old woman and a 74-year-old woman with end-stage OA in the right hip due to acetabular dysplasia were the subjects of this study. No other joint OA except for spondylitis of the lumbar spine was observed in these patients; nor did the laboratory data show any inflammatory signs. At total hip arthroplasty (THA), we collected synovial tissue from the operated joints and immediately froze it at –80°C until analysis. The most inflamed part of the synovium, with villous formation and a reddish appearance, was collected. The synovium from the 2 patients was combined for analysis by the oligo-capping method. For reverse transcriptase PCR (RT-PCR) analysis, we collected synovium from 7 OA patients (mean age 63 (53–75) years; 5 hips and 2 knees; 6 women) and 2 control patients. One control patient was a 26-year-old man who had arthroscopic removal of an injured meniscus but with no signs of arthritis, and the other was a 77-year-old woman who had femoral head replacement for a cervical fracture of the femur. She had no signs of arthritis.

OA was diagnosed by the American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hip (Altman et al. 1991) and knee (Altman et al. 1986). Hip OA patients had acetabular dysplasia and knee OA patients had medial-type primary OA. Written consent was obtained from subjects in accordance with the Declaration of Helsinki. This study was approved by our Institutional Review Board.

We constructed a full-length enriched cDNA library, as described previously (Maruyama and Sugano 1994, Suzuki et al. 1997). Briefly, 1–2 mg of total RNA was isolated from the synovium using TRIzol (Invitrogen Life Technologies, Carlsbad, CA). The poly(A)+ RNA was purified with Oligo-<ref>kos-dT30</ref> (Takara Bio Inc., Otsu, Japan), treated with bacterial alkaline phosphatase (Takara Bio) and tobacco acid pyrophosphatase, and ligated with 5’-oligo RNA using RNA ligase (Takara Bio). Thereafter, the cap structure, which is located at the 5’-end of full-length mRNA, was replaced with that of synthesized oligo RNA. The cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen). Full-length cDNAs were then amplified selectively by PCR using the primers specified for the synthesized oligo RNA and poly(A) tail, after which the PCR products were inserted into the DraIII sites of a mammalian expression vector, pME18S (GenBank accession number AB009864).

The double-stranded DNA was sequenced with the dideoxy nucleotide-chain termination method using an ABI7700 Autosequencer (Perkin-Elmer Applied Biosystems, Foster City, CA).

The tag sequences were searched against NCBI RefSeq Human database 14759 (ftp://ncbi.nlm.nih.gov/refseq/H_sapiens/mRNA–Prot/hs.fna.gz) and Unique Human database 95928 (ftp://ncbi.nlm.nih.gov/repository/UniGene/Hs.seq.uniq.gz) with the aid of BLAST (e-value was 1.0 × e-50) (Altschul et al. 1997). To assess whether a given alignment constituted evidence for homology, we calculated the bit score, identity, and E-value using the formulae obtained with BLAST. The bit score represents the number of nucleotides of a given alignment that are identical to the specific mRNA sequence, while the identity represents the percentage of identical nucleotides. Finally, the e-value represents how likely an alignment can be expected from chance alone.

From the frequently expressed genes list (Table 2, see supplementary article data), we selected 8 genes whose products were considered to have something to do with matrix metabolism: fibronectin 1, matrix metalloproteinase (MMP) 1, MMP3, tissue inhibitor of metalloproteinase 3 (TIMP3), apolipoprotein L-I (APOL1), syndecan binding protein (SDCBP), insulin-like growth factor binding protein 5 (IGFBP5), and heat shock protein 90 (HSP90). We also selected genes encoding a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 5, which have been reported to be expressed in OA synovium (Vankemmelbeke et al. 2001). We compared the expression of these 9 genes in synovium of 7 OA patients and the 2
control patients without arthritis using RT-PCR. Frozen tissue pellets were ground down to powder with liquid nitrogen in a mortar on dry ice, and total RNA was extracted from the tissue using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. After treatment with RNase-free deoxyribonuclease I (Takara Bio), 500 ng of total RNA from each sample was reverse transcribed using SuperScript II (Invitrogen). The reaction time was 60 min at 42°C. Thereafter, 1 µL of each reaction product was amplified in 20 µL PCR mixture containing 0.5 U TaKaRa EX Taq (Takara Bio) and 10 pmol of each primer to detect mRNA specific to each molecule. Amplification was performed in a Program Temp Control System (DNA Engine PTC-200; MJ Research Inc., Waltham, MA) for 35 cycles after an initial denaturation step at 94°C for 5 min, with denaturation at 94°C for 30 seconds, annealing for 30 seconds at 58°C, and extension at 72°C for 60 seconds, with a final extension at 72°C for 5 min. PCR products (10 µL) were electrophoresed in 3% agarose gels and detected by ethidium bromide staining. Nucleotide sequences of the primers for each of the 9 genes are shown in Table 1. PCR experiments were performed with primers that could amplify only a cDNA sequence because the primer sequences were located in different exons or contained an exon-exon junction.

Results

Results of sequencing

We sequenced 7,339 mRNAs in total, investigated each of them with a computer system, and identified 4,247 different kinds of genes.

To verify the accuracy of the results, we analyzed bit scores, identities, and E-values (Table 1). The average identity of fibronectin mRNA, which was the most frequently detected mRNA, was 100% and the lowest identity (230th) of fibronectin was 98%.

The mRNAs were ranked in order of frequency and 126 with more than 6 tags are shown in Table 2. There were 49 kinds of mRNA with 5 tags, 75 with 4, 158 with 3, 456 with 2, and 3,385 with 1 tag. The mRNAs that are not shown in Table 1 (fewer than those with 6 tags) included interferon γ receptor 1 (5 tags), stromal cell-derived factor 1 (4 tags), and leukotriene A4 hydrolase (4 tags).

The mRNAs identified as 16 (similar to C. elegans FAT-3 alcohol dehydrogenase), seventieth (Homo sapiens chromosome 6 open reading frame 11), 83 (Homo sapiens hypothetical protein) and one-hundred-and-second (no hit) in order of frequency are novel because they have not yet been identified as human genes.

Results of reverse transcriptase PCR

All of the 9 genes were expressed in OA and control synovium. However, the expression of MMP1 mRNA was weak in OA samples, while expression of ADAMTS5 and APOL1 mRNAs was weak in the control samples and some of the OA samples (Figure).

Discussion

We identified 126 kinds of mRNA in order of frequency...
mRNA expression levels from 9 selected genes in synovial tissue from OA and TA synovium by RT–PCR. All 9 genes were expressed in OA and TA synovium. The expression of mRNAs encoding MMP1, APOL1, and ADAMTS5 was weak in some samples from OA, while expression of APOL1 mRNA was weak in control samples (TA). FN: fibronectin 1; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinase; APOL1: apolipoprotein L-I; SDCBP: syndecan binding protein; IGFBP: insulin-like growth factor binding protein; HSP: heat shock protein; ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs; OA: osteoarthritis; TA: traumatic arthropathy.

reported to be induced during early proteoglycan degradation, and to contribute to the subsequent type II collagen cleavage and to progression in diseased joints such as seen in OA and RA (Yasuda and Poole 2002). Expression of fibronectin splice variants in the synovial membrane in patients with RA and OA has been reported (Kriegsmann et al. 2004). We found that fibronectin mRNA most frequently occurred in OA synovium, which supports these findings.

Proteases have important roles in the joint destruction in OA, while MMPs are key matrix-degrading enzymes that have been studied extensively (Kevorkian et al. 2004, Yuan et al. 2004). In our study, MMP1 (2), MMP3 (3), cathepsin B (48), MMP2 (58), and cathepsin S (83) were detected. As for the inhibitors of proteases, TIMP3 came seventh in order of frequency. Stromal cell-derived factor 1 is reportedly an important tumor necrosis factor (TNF)-independent molecule involved in the migration to and retention of inflammatory effector cells in the joint (Blades et al. 2002). It has been reported that synovectomy is effective in patients with OA or RA because stromal cell-derived factor 1, which can regulate the release of MMP9 and MMP13 from articular chondrocytes (resulting in breakdown of cartilage), is removed by the operation (Kanbe et al. 2004). In our study, we detected 4 tags of this mRNA. It has also been reported that leukotrienes regulate pro-inflammatory cytokine and MMP1 synthesis (He et al. 2002), and we identified 4 tags of mRNA of leukotriene A4 hydrolase.

Insulin-like growth factor 1 or transforming growth factor (TGF) β are reportedly associated with improvement in joint architecture during the development of OA (Clemmons et al. 2002), and injections of high doses of TGF β have been found to induce OA changes (van Beuningen et al. 2000). Thus, some growth factors are associated with progression and/or improvement of OA. In our study, IGFBP5 (26) and TGF β (102) were detected, but their significance in OA remains unknown because it is not clear whether they regenerate the damaged cartilage, or promote cartilage destruction.

We detected immunoglobulin (Ig) λ-H chain (6), α2-macroglobulin (15), α2-macroglobulin receptor (26), HSP70 8 (26), HSP90 lβ (3), Fc fragment of IgG, low affinity IIIa (35), interferon γ-inducible...
protein 16 (41), Fc fragment of IgG, low affinity IIa (41), similar to HSP90 1 α (48), Ig µ-H chain (58), CD4 antigen (17), Ig λ light chain (17), HSP70 1B and/or 1A (83), HLA class II (102), IK cytokine, down-regulator of HLA II (102), polymyositis/scleroderma autoantigen 2 (102) and interferon γ receptor 1 (127, 5 tags), which had been reported to relate to immunological reactions. These results indicate that immunological reactions have certain roles in joint destruction by OA, but expression of inflammatory cytokines, such as TNFα, interleukin 1, and interleukin 6, was not detected. The frequency of expression of such specific cytokines may thus be lower than that of molecules in terms of common immunological reactions.

Complement has been repeatedly implicated in the pathogenesis of RA, on the basis of findings showing reduced levels of native complement components and increased levels of complement metabolites in plasma, synovial fluid, and synovial tissue of RA patients. It has been reported that local complement production and activation may have an important role in RA (Neumann et al. 2002). In our study, complement components 1r subcomponent (4), 1s subcomponent (8), 2 (48), 4A and/or 4B (58) and I factor (complement) (83) were detected. These molecules are thought to have important roles in joint destruction—not only by RA, but also by OA.

Of these molecules, we selected 8 genes from the frequently expressed genes, i.e. those encoding fibronectin1, MMP1, MMP3, TIMP3, APOL1, SDCBP, IGFBP5, and HSP90. We also selected ADAMTS5, which has been reported to be expressed in OA synovium (as a control). Then, we compared the expression of these genes in OA and control patients using RT-PCR. It is difficult to say that the control specimens can be regarded as normal controls, but at least we can say that these are different from those in OA. RT-PCR is not a quantitative or semi-quantitative method in general, but one can usually obtain rough quantification of templates. All 9 genes were expressed in OA synovium. They were also expressed in control synovium, but expression of ADAMTS5 and APOL1 mRNAs were weak. We do not know the function of APOL1 in OA; it may have some role in the pathogenesis of OA, like ADAMTS5. These may have some role in synovitis associated with OA. Synovial tissues usually contain synovial membrane and subsynovial tissue. We don’t mind whether the gene was expressed in synovial membrane or subsynovial tissue. The important issue is that some genes show high expression and they may affect the properties of cartilage. We believe that the genes we found to be expressed frequently are likely to have a stronger influence, but this does not necessarily mean that less frequently expressed genes are not important. The effect of physiologically active molecules is not always proportional to the degree of gene expression, because of translational or posttranslational modifications. After a comparative analysis of the mRNA expression of some candidate molecules, it may sometimes be necessary to analyze the expression levels and function of the corresponding proteins.

Contributions of authors
SW: collected synovium and organized the study. YO, AT, HH, YS, and SS: performed oligo-capping and analyzed the expression. TO, AK, and HN: performed RT-PCR for comparison.

Supplementary article data
Table 2 is available on our website www.actaorthop.org, identification number 0701.

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