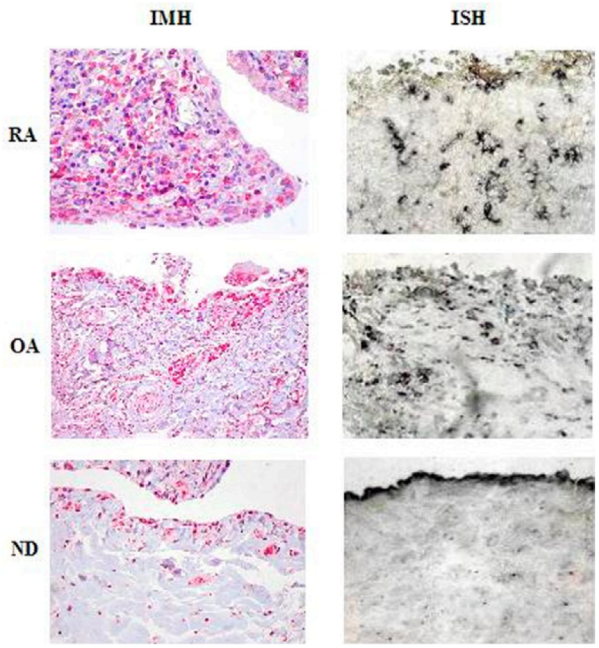
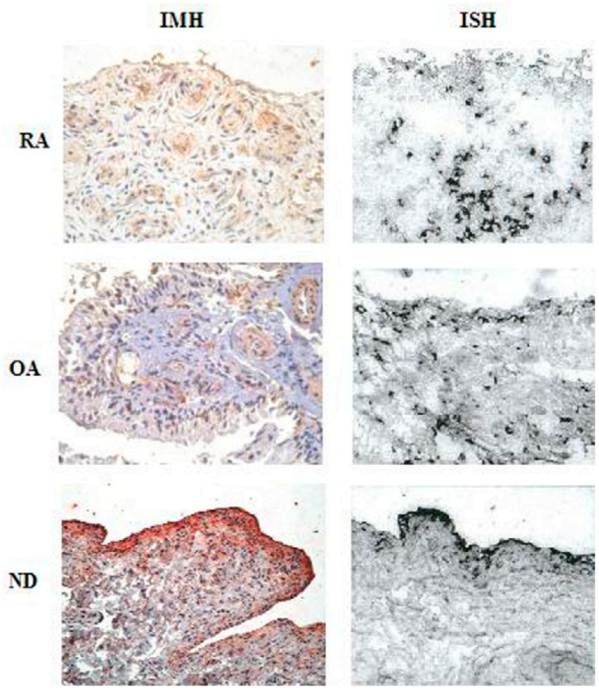
Figure 4



Immunohistochemistry and *in situ* hybridization of synovial tissues for BMP-4. In normal synovial tissue the expression of bone morphogenetic protein (BMP) is localized to the synovial lining layer. In rheumatoid arthritis (RA) and osteoarthritis (OA) tissue samples BMP-4 is expressed less by cells of the superficial synovial layer but more by cells scattered in deeper layers. Original magnifications: immunohistochemistry (IMH): RA, normal donors (ND) ×40, OA ×20; *in situ* hybridization (ISH): RA, OA, ND ×40.

Figure 5



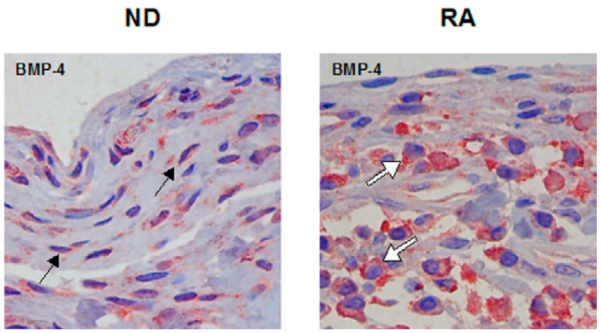
Immunohistochemistry (IMH) and *in situ* hybridization (ISH) of synovial tissues for BMP-5. Histomorphological distribution of BMP-5 is comparable to that of BMP-4 (Figure 4). Original magnifications: immunohistochemistry (IMH): rheumatoid arthritis (RA), osteoarthritis (OA), normal donors (ND) ×20; *in situ* hybridization (ISH): RA, OA, ND ×40. BMP, bone morphogenetic protein.

Discussion

Inflammation and destruction are leading pathomechanisms in chronic joint diseases. In recent years, however, aspects of regeneration and homeostasis have become more and more important. Members of the TGF-β family, especially BMPs, are pivotal factors in skeletal tissue development and may contribute to the repair of various other tissues. We investigated the expression of BMPs in the synovial tissue compartment under normal and pathologic conditions by using microarray technology. All BMPs from BMP-2 to BMP-11, BMP-14 and BMP-15 revealed low to very low signal levels. Of these experiments, BMP-4 and BMP-5 were significantly decreased in RA in comparison with ND. This difference was confirmed by semiquantitative PCR. In addition, PCR analysis revealed a reduced expression of BMP-4 and BMP-5 in OA tissue in comparison with normal tissue. This variance of BMP expression levels in OA tissue in comparison with normal or RA synovial tissue may be explained by technical differences in sensitivity and resolution between PCR and microarray hybridization. However, the groups analyzed by PCR and microarray were independent. BMP expression in OA may therefore be more variable than that in RA. Immunostaining in normal donors revealed the expression of both BMPs predominantly in the synovial lining layer, whereas in patients with RA the expression was more frequently found in the sublining layer. A decrease in BMP-4 and BMP-5 in RA and OA could be correlated with markers of systemic and in part with markers of local inflammation as well as with disease duration. A relation of BMP suppression to therapy with steroids and disease-modifying anti-rheumatic drugs administered only in RA was excluded because BMP expression in synovium of OA patients was affected similarly, although to a lesser extent.

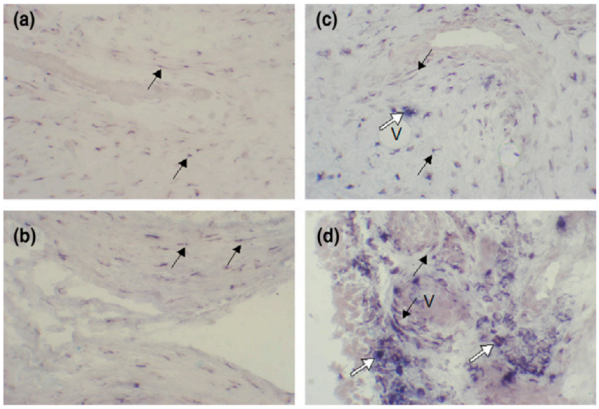
Expression of BMPs in synovial tissues was investigated recently by Lories and colleagues [18]. They compared synovium from RA and spondyloarthropathies with synovium from traumatic joint diseases and found BMP-2 and BMP-6 to be expressed most consistently with a calculated relative expression in the range 0.002 to 0.2% compared with β-actin. This confirms our own observations of a low expression level in the synovial tissue compartment. Similarly to their results, we could not detect differential expression of BMP-2 and BMP-6 mRNA in RA compared with normal tissue. *In vitro*, however, Lories and colleagues found an increase in BMP-2 and BMP-

Figure 6



Expression of BMP-4 in fibroblastoid (black arrow) and macrophagocytic (white arrow) cells by immunohistochemistry. Original magnifications: normal donors (ND), rheumatoid arthritis (RA) ×100. BMP, bone morphogenetic protein.

Figure 7



Fibroblasts (black arrows) expressing bone morphogenetic protein (BMP)-4 **(a)** and BMP-5 **(b)** in areas with fibrosis in osteoarthritis synovial tissue (original magnification ×20). Macrophagocytic (white arrows) and fibroblastoid (black arrows) appearance of cells adjacent to vessels (V) expressing BMP-4 **(c)** and BMP-5 **(d)** in rheumatoid arthritis synovial tissues (original magnification ×40).

6 expression on stimulation of cultivated synovial fibroblasts with TNF-α and IL-1β. These data seem in part controversial to our observation that in synovial tissue the expression of the BMPs investigated (BMP-4 and BMP-5) was decreased. In addition, BMPs were negatively correlated with local or systemic parameters of inflammation as well as the duration of the disease. This discrepancy might depend on differences in the biological function and regulation of individual members of the BMP family. In fact, Lories and colleagues [18] also reported that BMP-4, in contrast to BMP-2 and BMP-6, was not increased by stimulation with IL-1β or TNF-α. Furthermore, local differences between stimulatory and inhibitory mechanisms for BMP production could explain our observed differences in the histomorphological distribution of BMP-expressing cells in RA compared with controls. A similar distribution and predominant expression of different BMPs in fibroblastoid and macrophagocytic cells was also shown by Lories and colleagues [18] and van Lent and colleagues [26].

That BMPs might provide a beneficial effect on joint repair can be assumed from their role in joint development [27], their induction of chondrogenic differentiation in adult mesenchymal stem cells [28,29] and their effect on cartilage formation in tissue engineering with chondrocytes [5]. Similarly, the decrease in BMP-7 expression and the increase in BMP antagonists found in osteoarthritic cartilage suggests that a loss of BMP signal might reduce the regenerative capacity of cartilage [12,30]. However, the role of BMPs in the homeostasis of joints and the regeneration of cartilage is still unclear. BMP-2 was found to be increased in osteoarthritic cartilage and stimulated in culture with the proinflammatory cytokines IL-1 and TNF [31]. In contrast, other BMPs were unchanged [32]. Furthermore, the expression of BMP-6 and BMP-7 was also decreased in articular cartilage of TNF-transgenic mice, suggesting that loss of BMP expression could be also involved in chronic inflammatory and not only degenerative joint diseases [33]. The overall decrease in BMP-4 and BMP-5 in the synovial membrane therefore presents a new and additional aspect in the imbalance of joint homeostasis in chronic joint diseases.

As well as a possibly beneficial effect of BMPs on arthritic joints, intra-articular TGF-β injection was shown to induce osteophyte formation, a typical morphological change in OA [34]. Moreover, recent studies suggested that other factors such as BMP-2 and BMP-4 might be involved as downstream mediators of the TGF-β effect and that these BMPs might be released by macrophages of the synovial lining layer [26]. However, these data are derived from a mouse model with TGF-β injected into normal joints. Furthermore, the dosage of TGF-β applied was at least 1,000-fold higher than the TGF-β concentration found in normal or even osteoarthritic joint synovia [35]. Nevertheless, these data demonstrate that uncontrolled high levels of morphogens may exert a negative influence. It is intriguing that inhibition of BMP signalling in a papain-induced OA mouse model could prevent osteophyte formation and synovial fibrosis but at the same time increased the loss of proteoglycan from the cartilage matrix, thereby certainly promoting the damage of the joint surface [10].

Thus, regenerative triggers in the treatment of joint diseases will depend on a balanced action of stimulators and inhibitors of BMP signalling with precise modulation of specific BMPs. The histomorphological distribution may be also important. Expression in deeper layers as seen in the samples of our RA and OA patients may influence predominantly cells of the surrounding tissue, thereby contributing to synovial fibrosis. In contrast, expression in the synovial lining layer may be more relevant for stable or increased levels of BMP in the synovial fluid, where these morphogens may potentially influence artic-

ular cartilage. As BMP-4 and BMP-5 were found to be decreased in the synovium and their expression was attributed to the synovial lining layer in normal joints, they could be favorable candidates for therapeutic application. Nevertheless, it will be important to understand precisely the network of morphogen action and regulation in the joint, because injection of BMP-2 induced osteophyte formation in a murine model [9]. Thus, the interaction of BMPs and inhibitors not only in the synovium but also in cartilage has to be elucidated. Although studies in developmental biology have contributed considerably to the understanding of the BMP network [27], the role of these morphogens in adult tissues is still unclear.

Conclusion

BMP-4 and BMP-5 are expressed in normal synovial tissue and were found to be decreased in OA and RA. Furthermore, the histomorphological distribution of both morphogens showed a dominance in the lining layer in the normal tissue, whereas their expression in RA and OA tissue was also scattered across deeper layers. These results suggest that BMP-4 and BMP-5 may be important in joint homeostasis and are therefore potential candidates for joint regeneration.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CPB and TH performed patient recruitment, PCR, immunohistochemistry and data interpretation and drafted the manuscript. UU was involved in *in situ* hybridization and PCR. VK was involved in patient recruitment and performed the 'synovitis score'. AP and CK conducted part of the patient recruitment and data evaluation. FS, GAM and GRB provided substantial input into data evaluation. All authors read and approved the final manuscript.

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References

1. Keyszer G, Redlich A, Häupl T, Zacher J, Sparmann M, Engethum U, Gay S, Burmester GR: **Differential expression of cathepsins B and L compared with matrix metalloproteinases and their respective inhibitors in rheumatoid arthritis and osteoarthritis: a parallel investigation by semiquantitative reverse transcriptase-polymerase chain reaction and immunohistochemistry.** *Arthritis Rheum* 1998, **41:**1378-1387.
2. Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M: **Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis – results of a 1 year longitudinal arthroscopic study in 422 patients.** *Osteoarthritis Cartilage* 2005, **13:**361-367.
3. van den Berg WB: **Joint inflammation and cartilage destruction may occur uncoupled.** *Springer Semin Immunopathol* 1998, **20:**149-164.
4. van der Kraan PM, van den Berg WB: **Anabolic and destructive mediators in osteoarthritis.** *Curr Opin Clin Nutr Metab Care* 2000, **3:**205-211.
5. Kaps C, Bramlage C, Smolian H, Haisch A, Ungethum U, Burmester GR, Sittinger M, Gross G, Häupl T: **Bone morphogenetic proteins promote cartilage differentiation and protect engineered artificial cartilage from fibroblast invasion and destruction.** *Arthritis Rheum* 2002, **46:**149-162.
6. Southwood LL, Frisbie DD, Kawcak CE, McIlwraith CW: **Delivery of growth factors using gene therapy to enhance bone healing.** *Vet Surg* 2004, **33:**565-578.
7. Friedlaender GE, Perry CR, Cole JD, Cook SD, Cierny G, Muschler GF, Zych GA, Calhoun JH, LaForte AJ, Yin S: **Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions.** *J Bone Joint Surg Am* 2001, **83-A(Suppl 1):**S151-S158.
8. Lories RJ, Derese I, Luyten FP: **Modulation of bone morphogenetic protein signaling inhibits the onset and progression of ankylosing enthesitis.** *J Clin Invest* 2005, **115:**1571-1579.
9. van Beuningen HM, Glansbeek HL, van der Kraan PM, van den Berg WB: **Differential effects of local application of BMP-2 or TGF-beta 1 on both articular cartilage composition and osteophyte formation.** *Osteoarthritis Cartilage* 1998, **6:**306-317.
10. Scharstuhl A, Vitters EL, van der Kraan PM, van den Berg WB: **Reduction of osteophyte formation and synovial thickening by adenoviral overexpression of transforming growth factor beta/bone morphogenetic protein inhibitors during experimental osteoarthritis.** *Arthritis Rheum* 2003, **48:**3442-3451.
11. Chubinskaya S, Kuettner KE: **Regulation of osteogenic proteins by chondrocytes.** *Int J Biochem Cell Biol* 2003, **35:**1323-1340.
12. Tardif G, Hum D, Pelletier JP, Boileau C, Ranger P, Martel-Pelletier J: **Differential gene expression and regulation of the bone morphogenetic protein antagonists follistatin and gremlin in normal and osteoarthritic human chondrocytes and synovial fibroblasts.** *Arthritis Rheum* 2004, **50:**2521-2530.
13. Ducy P, Karsenty G: **The family of bone morphogenetic proteins.** *Kidney Int* 2000, **57:**2207-2214.
14. Sakou T: **Bone morphogenetic proteins: from basic studies to clinical approaches.** *Bone* 1998, **22:**591-603.
15. Kingsley DM: **The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms.** *Genes Dev* 1994, **8:**133-146.
16. Wozney JM: **The bone morphogenetic protein family and osteogenesis.** *Mol Reprod Dev* 1992, **32:**160-167.
17. Tsumaki N, Nakase T, Miyaji T, Kakiuchi M, Kimura T, Ochi T, Yoshikawa H: **Bone morphogenetic protein signals are required for cartilage formation and differently regulate joint development during skeletogenesis.** *J Bone Miner Res* 2002, **17:**898-906.
18. Lories RJ, Derese I, Ceuppens JL, Luyten FP: **Bone morphogenetic proteins 2 and 6, expressed in arthritic synovium, are regulated by proinflammatory cytokines and differentially modulate fibroblast-like synoviocyte apoptosis.** *Arthritis Rheum* 2003, **48:**2807-2818.
19. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, *et al.*: **The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.** *Arthritis Rheum* 1988, **31:**315-324.
20. Krenn V, Morawietz L, Burmester GR, Häupl T: **Synovitis score: histopathological grading system for chronic rheumatic and non-rheumatic synovitis.** *Z Rheumatol* 2005, **64:**334-342.
21. **Synovitis-Score** [http://www.charite.de/ch/patho/Webpage/pages/diagnostik/biopsie/synovitis/synovitis.htm]
22. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP: **Summaries of Affymetrix GeneChip probe level data.** *Nucleic Acids Res* 2003, **31:**e15.
23. Chomczynski P, Sacchi N: **Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction.** *Anal Biochem* 1987, **162:**156-159.
24. Franz JK, Kolb SA, Hummel KM, Lahrtz F, Neidhart M, Aicher WK, Pap T, Gay RE, Fontana A, Gay S: **Interleukin-16, produced by synovial fibroblasts, mediates chemoattraction for CD4+ T**

lymphocytes in rheumatoid arthritis. *Eur J Immunol* 1998, **28:**2661-2671.
25. Koziolek MJ, Riess R, Geiger H, Thevenod F, Hauser IA: **Expression of multidrug resistance P-glycoprotein in kidney allografts from cyclosporine A-treated patients.** *Kidney Int* 2001, **60:**156-166.
26. van Lent PL, Blom AB, van der Kraan P, Holthuysen AE, Vitters E, van Rooijen N, Smeets RL, Nabbe KC, van den Berg WB: **Crucial role of synovial lining macrophages in the promotion of transforming growth factor beta-mediated osteophyte formation.** *Arthritis Rheum* 2004, **50:**103-111.
27. Archer CW, Dowthwaite GP, Francis-West P: **Development of synovial joints.** *Birth Defects Res C Embryo Today* 2003, **69:**144-155.
28. Sekiya I, Larson BL, Vuoristo JT, Reger RL, Prockop DJ: **Comparison of effect of BMP-2, -4, and -6 on *in vitro* cartilage formation of human adult stem cells from bone marrow stroma.** *Cell Tissue Res* 2005, **320:**269-276.
29. Schmitt B, Ringe J, Häupl T, Notter M, Manz R, Burmester GR, Sittinger M, Kaps C: **BMP2 initiates chondrogenic lineage development of adult human mesenchymal stem cells in high-density culture.** *Differentiation* 2003, **71:**567-577.
30. Chubinskaya S, Kumar B, Merrihew C, Heretis K, Rueger DC, Kuettner KE: **Age-related changes in cartilage endogenous osteogenic protein-1 (OP-1).** *Biochim Biophys Acta* 2002, **1588:**126-134.
31. Fukui N, Zhu Y, Maloney WJ, Clohisy J, Sandell LJ: **Stimulation of BMP-2 expression by pro-inflammatory cytokines IL-1 and TNF-alpha in normal and osteoarthritic chondrocytes.** *J Bone Joint Surg Am* 2003, **85-A(Suppl 3):**59-66.
32. Bobacz K, Gruber R, Soleiman A, Erlacher L, Smolen JS, Graninger WB: **Expression of bone morphogenetic protein 6 in healthy and osteoarthritic human articular chondrocytes and stimulation of matrix synthesis *in vitro*.** *Arthritis Rheum* 2003, **48:**2501-2508.
33. Bobacz K, Hayer S, Smolen JS, Schett G: **Expression of distinct members of the bone morphogenetic protein family is decreased in cartilage from chronically inflamed joints (abstract).** *Ann Rheum Dis* 2004, **63:**THK0178.
34. Blom AB, van Lent PL, Holthuysen AE, van der Kraan PM, Roth J, van Rooijen N, van den Berg WB: **Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis.** *Osteoarthritis Cartilage* 2004, **12:**627-635.
35. Pagura SM, Thomas SG, Woodhouse LJ, Ezzat S, Marks P: **Circulating and synovial levels of IGF-I, cytokines, physical function and anthropometry differ in women awaiting total knee arthroplasty when compared to men.** *J Orthop Res* 2005, **23:**397-405.

3.2 Transkriptomanalyse von aufgereinigten Synovialfibroblasten und Untersuchung pharmakologischer Einflüsse

Mit der Verfügbarkeit von Genexpressionsdaten zu allen Genen einer Funktionsgruppe stellt sich die Frage, ob und wie diese Gene zusammenwirken bzw. reguliert werden. Untersuchungen zur Entwicklung von Geweben und Organen haben gezeigt, dass die Wirkung der Wachstumsfaktoren abhängig vom Gewebeumfeld und dem Zelltyp ist, in dem sie produziert werden.

In Entzündungsgeweben mit einer Vielzahl verschiedener Zellen ist die Zuordnung der Prozesse äußerst schwierig und damit die Nacharbeit bei Validierungsexperimenten umso größer. Deshalb wurden weitere Experimente zunächst mit aufgereinigten Zelltypen durchgeführt. Damit werden schrittweise die für einen bestimmten Zelltyp charakteristischen Expressionsprofile aufgenommen und die molekularen Veränderungen bei Erkrankung und Behandlung zelltypspezifisch untersucht. Diese Expressionsdaten stehen später für komplexere Analysemethoden als Referenzprofile zur Verfügung.

Um mit den gewebetypischen Veränderungen zu beginnen, wurden in der nachfolgenden Arbeit zunächst Synovialfibroblasten untersucht. Diese waren zum einen von einem pathologisch unauffälligen Gelenk und zum anderen von einem Patient mit rheumatoider Arthritis etabliert und über Transfektion des SV40 T-Antigens immortalisiert worden. Diese Zellen wurden als ein Kompromiss zu den stark schwankenden Eigenschaften nativer Synovialfibroblasten von wechselnden Spendern gewählt. Neben der Untersuchung der Unterschiede zwischen gesund und krank war insbesondere der Einfluss von verschiedenen entzündungshemmenden Medikamenten auf die Genexpression in diesen Zellen von Interesse.

Von den drei verschiedenen Medikamentengruppen, die getestet wurden, zeigte Methotrexat Einfluss auf die Expression von Genen, die mit Wachstum und Apoptose in Verbindung stehen. Dagegen unterdrückte Prednisolon vor allem die Expression von typischen Entzündungsgenen wie IL-1β und IL-8. Diclofenac dagegen als Vertreter der nicht-steroidalen Antirheumatika hatte in diesen Zellen keinen nennenswerten Einfluss auf die Expression von Genen, die charakteristisch für die rheumatoide Arthritis waren.

Gene Expression Profiling of Rheumatoid Arthritis Synovial Cells Treated with Antirheumatic Drugs

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Nonbiological therapeutics are frequently used for the treatment of patients with rheumatoid arthritis (RA). Because the mechanisms of action of these therapeutics are unclear, the authors aimed to elucidate the molecular effects of typical antirheumatic drugs on the expression profile of RA-related genes expressed in activated synovial fibroblasts. For reasons of standardization and comparability, immortalized synovial fibroblasts derived from RA (RASF) and normal donors (NDSF) were treated with methotrexate, prednisolone, or diclofenac and used for gene expression profiling with oligonucleotide microarrays. The cytotoxicity of the antirheumatic drugs was tested in different concentrations by MTS tetrazolium assay. Genes that were differentially expressed in RASF compared to NDSF and reverted by treatment with antirheumatic drugs were verified by semiquantitative polymerase chain reaction and by chemiluminescent enzyme immunoassay. Treatment with methotrexate resulted in the reversion of the RA-related expression profile of genes associated with growth and apoptosis including insulin-like growth factor binding protein 3, retinoic acid induced 3, and caveolin 2 as well as in the re-expression of the cell adhesion molecule integrin α6. Prednisolone reverted the RA-related profile of genes that are known from inflammation and suppressed interleukins 1β and 8. Low or high doses of diclofenac had no effect on the expression profile of genes related to RA in synovial fibroblasts. These data give the first insight into the mechanisms of action of common antirheumatic drugs used for the treatment of arthritides. Synovial fibroblasts reflect the disease-related pathophysiology and are useful tools for screening putative antirheumatic compounds. (*Journal of Biomolecular Screening* 2007:328-340)

Key words: synovial fibroblast, rheumatoid arthritis, drug screening, microarray

INTRODUCTION

RHEUMATOID ARTHRITIS (RA) is a chronic, inflammatory autoimmune disease of unknown etiology that affects primarily the joints, with a variety of extra-articular manifestations. The main attribute of RA is a chronic inflammation in the synovial membrane and in the synovial fluid with infiltrating mononuclear cells from hyperplastic synovial tissue. Hyperplastic synovial tissue results from an increase in macrophage-like and fibroblast-like synoviocytes and from infiltration with T cells, B cells, plasma cells, and dendritic cells. As the disease progresses, the inflamed synovium leads to the formation of aggressive tumor-like pannus tissue that invades and destroys joint cartilage and bone.[1-3]

Current therapeutic approaches focus on the suppression of inflammation, improvement of disease activity, and slowing the disease progression in RA. Therapeutics compose nonbiological compounds such as the disease-modifying antirheumatic drug (DMARD) methotrexate (MTX), the steroidal anti-inflammatory drug (SAID) prednisolone, and the nonsteroidal anti-inflammatory drug (NSAID) diclofenac.[4-6] Newer therapeutic strategies with biological response modifiers antagonize distinct inflammatory cytokines, for example, by administration of interleukin (IL)–1 receptor antagonist or anti–tumor necrosis factor (TNF) agents.[7,8]

In recent years, comprehensive data emerged from gene expression profiling of tissues and cells affected by RA. For instance, expression analysis of synovial tissue and fibroblasts with cDNA microarrays defined a molecular signature of RA that is suggested to be of diagnostic value classifying RA and osteoarthritis.[9] Because global gene expression analysis deepens the understanding of etiopathologic mechanisms underlying RA and other inflammatory autoimmune diseases, expression profiling is expected to lead to better diagnosis, patient classification, and individualized therapy.[10,11] In particular, analyzing synovial tissue in RA is suggested to be of special importance for providing insight into disease progression and for monitoring the effectiveness of antirheumatic therapies.[12] However, comprehensive gene expression data about

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mechanisms of action of currently used antirheumatic therapeutics are limited.

With respect to the scarce information about frequently used antirheumatic drugs, we used SV40 T-antigen immortalized RA (HSE) and normal synovial fibroblasts (K4IM) as a model system to determine the expression profile of RA-related genes affected by antirheumatic treatment. The K4IM cell line expresses typical cell surface molecules, maintains the normal expression kinetics of early growth response 1 upon stimulation with synovial fluid of RA patients or TNF-α, induces HLA-DR after stimulation with interferon-γ, and represents normal, healthy synovial fibroblasts.[13] The synovial fibroblast cell line HSE was derived from a patient with RA (RASF) and expresses typical ILs such as IL1β, IL6, IL8, IL11, IL13, IL16, and IL18 as well as matrix degrading proteases (MMP1, MMP3, cathepsin B, and cathepsin L).[14] Therefore, HSE cells are considered a prototype of activated synovial fibroblasts.

In the present study, to gain insight into the mechanisms of action of frequently used drugs in RA, the molecular effects on RASF and synovial fibroblasts derived from normal donors (NDSF) before and after treatment with typical antirheumatic drugs were determined by gene expression profiling with oligonucleotide microarrays.

MATERIALS AND METHODS

Cell culture

The immortalized synovial fibroblasts HSE and K4IM were derived from primary synovial cells obtained from an RA patient (HSE) and from a normal donor (K4IM).[13,14] Cells were cultured in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% human serum (German Red Cross, Berlin, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin, and 18 µg/ml ascorbic acid at 37 °C, 5% CO_2, and 90% humidity. Seventy-five percent of the culture medium was replaced every other day. Reaching 80% confluence, cells were trypsinized and replated at a density of 10,000 cells/cm^2.

MTS tetrazolium assay

Synovial cells were plated at a density of 3000 cells per well of 96-multiwell plates. Reaching 70% confluence, the culture medium was replaced by 100 µl medium without phenol red containing different concentrations of MTX (0-1 µg/ml, Lantarel; Medac, Wedel, Germany), prednisolone (0-50 µg/ml, Solu-Decortin; Merck, Darmstadt, Germany), and diclofenac (0-150 µg/ml; Ratiopharm SF, Ulm, Germany) in triplicate. After 48 h, 20 µl MTS solution (2 mg/ml in phosphate-buffered saline; Promega, Mannheim, Germany) was added to each well for 3 h according to the manufacturer's instructions. Colorimetric measurement of formazan dye was performed on a spectrophotometer at 492 nm.

Gene expression profiling and data processing

Gene expression profiling was performed in duplicate. Reaching 80% confluency of the cells, the medium was removed, and cells were incubated for 36 h with medium containing 0.2 µg/ml MTX, 1 µg/ml prednisolone, 75 µg/ml diclofenac, or medium as control ($n = 3$ each). Total RNA was isolated using the QIAGEN RNeasy Mini Kit according to the manufacturer's protocols (QIAGEN, Hilden, Germany). Equal quantities of total RNA from each triplicate were combined and processed for HG-U133A microarray hybridization according to the manufacturer's recommendations. In brief, 5 µg of RNA was transcribed to cDNA and used to synthesize biotin-labeled cRNA. Ten micrograms of fragmented cRNA was hybridized to the HG-U133A GeneChips (Affymetrix, Santa Clara, CA) for 16 h at 45 °C. Raw gene expression data were processed 1) with GCOS 1.2 software (Affymetrix) according to the manufacturer's recommendations and 2) by robust multiarray analysis (RMA).[15] Genes were selected when reproducibly regulated >1.3-fold in HSE versus K4IM ($n = 4$ each), HSE treated with antirheumatic drugs versus HSE ($n = 2$ each), or K4IM treated with antirheumatic drugs versus K4IM ($n = 2$ each) as determined by RMA and GCOS 1.2.

To identify general mechanisms of action, those genes were selected that were consistently differentially expressed in HSE after 36 h of drug exposure in all pairwise comparison analyses using GCOS 1.2 ($p \leq 0.002667$ for increased or $p \geq 0.997333$ for decreased) and RMA signals (fold change >1.3 or <–1.3). The list of genes was submitted to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to search objects in pathways. Log-transformed signal ratios of RMA data for all genes in the respective pathways were used to apply a color code for up- or down-regulation of individual genes.

PCR

Total RNA (5 µg) was reverse transcribed after annealing with 500 ng oligo-(dT)$^{12\text{-}18}$ oligonucleotides (Invitrogen, Karlsruhe, Germany) and 5U SuperScript reverse transcriptase (Invitrogen) in 70 µl as described by Gubler and Hoffman.[16] The relative expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize gene expression in each sample in different concentrations. Real-time PCR was performed in triplicate with 1 µl of single-stranded cDNA sample using the SYBR Green PCR Core Kit (Applied Biosystems, Foster City, CA) and the i-Cycler (Bio-Rad Laboratories GmbH, München, Germany). Relative quantitation of differentially expressed genes (**Table 1**) is given as a percentage of the GAPDH product.

Chemiluminescent enzyme immunoassay

Supernatants of synovial cells treated with antirheumatic drugs or medium as controls were collected and stored at –20 °C.

ble 1. Oligonucleotide Sequences

Gene Name	*Gene Symbol*	*Accession Number*	*Oligonucleotides (5′→3′) (Up/Down)*	*Product Size (base pairs)*
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_002046	GGC GAT GCT GGC GCT GAG TAC TGG TTC ACA CCC ATG ACG A	149
Insulin-like growth factor binding protein 3	IGFBP3	NM_000598	GCA GCG CCG GCA GTG TGG AGA G TTT GAA TGG AGG GGG TGG AAC TTG	82
Integrin α6	ITGA6	NM_000210	CAG GAC TTG AAA GAA ATG GTG AAT ATA CAG ATA GGG GAG GAA ACA AAA C	143
Hypothetical protein MGC4655	MGC4655	NM_033309	GTG TCC ACG CGC CAG GGT AT GGT CAC GCC ACT CGA TGT CAA A	211
Retinoic acid induced 3	RAI3	NM_003979	CAA ATT CCT GGG GCT GAT ACT ATT AGG AGA CCA TGC CCA CTT AC	103
DNA-damage-inducible transcript 4	DDIT4	NM_019058	TCT TGT TTT TCT GAT CGG AGC AT GCC AGG TGT AAT TTT TCA AGT GTC A	136
Deafness, autosomal dominant 5	DFNA5	NM_004403	GGC AGA GAC CCA AGT ACC AGT TTT T ACC TTC CCC AGT GCA GTC TCC A	160
Caveolin 2	CAV2	NM_198212	CAG GAC TGG TGA ATA TAA ATG ATG AT CCT AGG GCA GTT TTG AAA TGT CTC TT	130
Sialyltransferase 7	SIAT7E	NM_030965	TGA ATG ACG CCC CCA CAC G GGC CCC AGA AGA TGA ACA CG	103
Tissue factor pathway inhibitor 2	TFPI2	NM_006528	CTC AGG AGC CAA CAG GAA ATA AC GCA GCC CCC GTA CAG GAA CT	134
Lymphocyte antigen 96	LY96	NM_015364	GAA GCT CAG AAG CAG TAT TGG GTC AGT CTC TCC CTT CAG AGC TCT GC	288
Transforming growth factor β2	TGFB2	NM_003238	GCT GGA GCA TGC CCG TAT TTA T ACG CAG CAA GGA GAA GCA GAT G	104

IL1β and -8 were measured in duplicate by a quantitative chemiluminescent enzyme immunometric assay using the IMMULITE automated analyzer according to the manufacturer's instructions (DCP-Biermann, Bad Nauheim, Germany). In brief, the samples were diluted in medium and introduced along with alkaline phosphatase-coupled anti-IL antibodies into the test unit, which contained a polystyrene bead coated with monoclonal anti-IL antibodies. During incubation for 1 h at 37 °C, ILs are bound to form an antibody-sandwich complex. Unbound proteins are removed by a centrifugal wash. The chemiluminescent substrate is added, and the test unit is incubated for an additional 10 min. The photon output is proportional to the concentration of ILs.

RESULTS

Morphology of synovial cells and cytotoxicity of antirheumatic drugs

Synovial cells HSE (RASF) and K4IM (NDSF) displayed a typical fibroblast-like appearance (**Fig. 1**). For treatment of the cells with antirheumatic drugs, the in vitro drug concentration was deduced from the dose of the respective drug administered in RA patients. Compared with untreated RASF (**Fig. 1A**) and NDSF (**Fig. 1E**), treatment with 0.2 µg/ml of MTX resulted in a spindle-like morphology of RASF accompanied by a detachment of cells (**Fig. 1B**). NDSF treated with MTX (**Fig. 1F**) took the shape, albeit to a lesser extent than RASF, of spindle-like fibroblasts. Upon administering 1 µg/ml prednisolone, RASF maintained a normal fibroblastic morphology with few cells floating in the medium (**Fig. 1C**). Prednisolone had no effect on the morphology of NDSF (**Fig. 1G**). A high dose of 75 µg/ml diclofenac resulted in an elongation of RASF with few cells detaching and floating in the medium (**Fig. 1D**). Diclofenac had no effect on the morphology of NDSF (**Fig. 1H**).

Cytotoxic effects of antirheumatic drugs on RASF and NDSF were determined by the MTS cytotoxicity assay (**Fig. 2**). Treatment of synovial cells with MTX resulted in a decreased viability of RASF when applying 0.1 to 1.0 µg/ml MTX. NDSF tolerated doses of up to 0.2 µg/ml and showed a reduced viability upon treatment with high doses of MTX (0.5-1.0 µg/ml). Prednisolone had only minor effects on the viability of fibroblasts accompanied by a slight increase of metabolic activity in RASF. A slight decrease in RASF viability was apparent after treatment with 75 µg/ml diclofenac, which was prominent upon treatment with 100 to 150 µg/ml of the drug. Instead, NDSF showed cytotoxic effects as recently as after application of 100 to 150 µg/ml diclofenac. Treatment of cells with a clinically relevant dose of 10 µg/ml diclofenac had neither an effect on the morphology nor the physiological activity of RASF and NDSF (data not shown). Gene expression profiling was performed