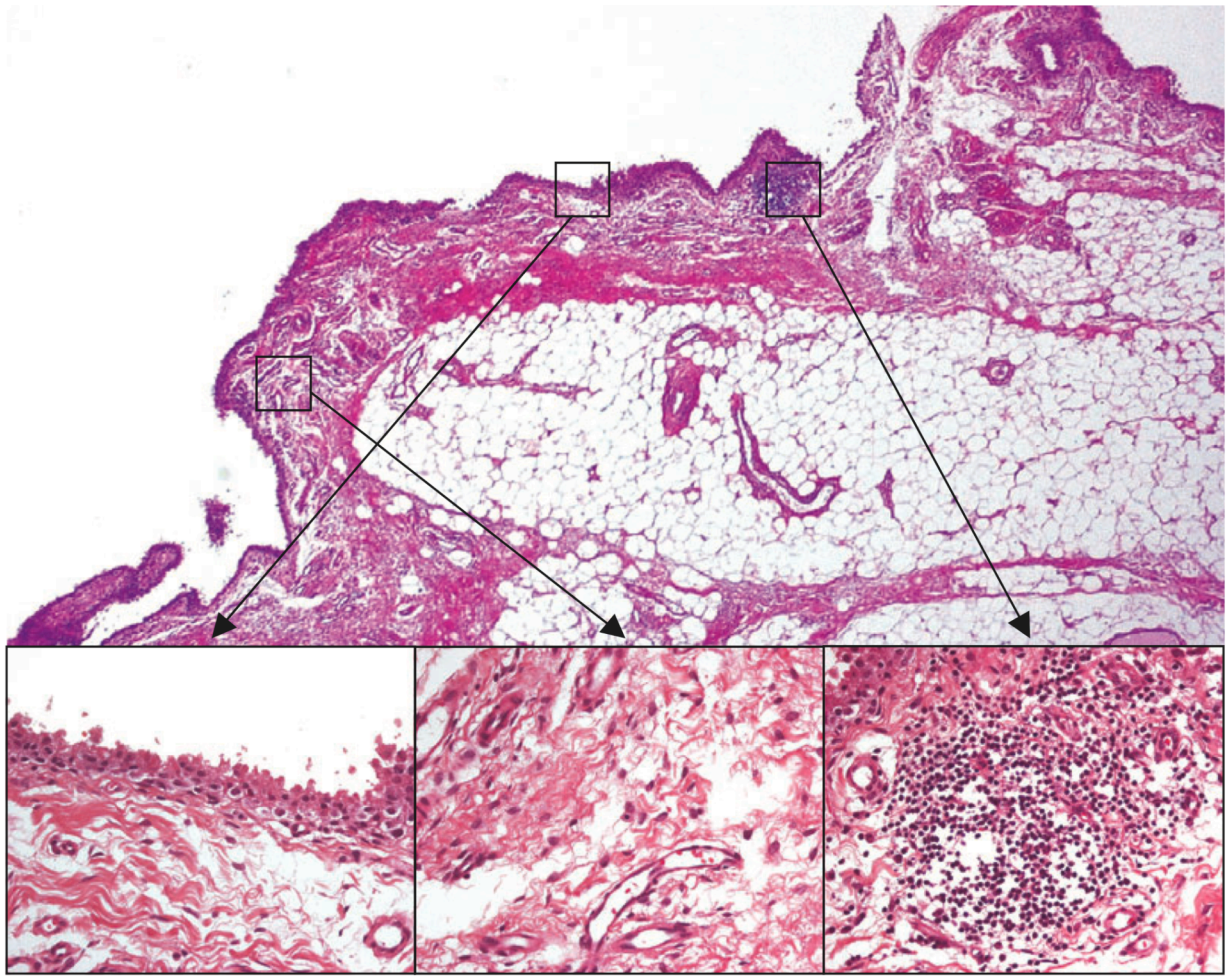

Figure 2. Post-traumatic arthritis. The synovial lining cell layer is slightly enlarged (1 point, left insert), the density of the synovial stroma is slightly increased (1 point, central insert) and there is a small follicle-like lymphocytic inflammatory infiltrate (1 point, right insert). Altogether, a slight synovitis (1 + 1 + 1 = 3 points) can be diagnosed (H&E).

and inflammatory infiltrate), the ranking of alterations being on a scale from none (0), slight (1) and moderate (2) to strong (3) (Table 1, Figure 1). The values of the parameters were summarized and interpreted as follows: 0–1, no synovitis; 2–4, low-grade synovitis; and 5–9, high-grade synovitis (Figure 2). This grading system is based on and resembles an advancement of the grading system first proposed at the 18th European Congress of Pathology.[20]

THE PROBLEM OF INFLAMMATORY HETEROGENEITY IN SYNOVECTOMY SPECIMENS

As inflammatory changes are heterogeneous by nature, analysis was done at the site showing the strongest histopathological alterations, analogous to the established assessment of the differentiation grade of neoplasms. It is advisable to analyse the inflammatory infiltrate under low magnification (× 50–100); on the other hand, the view of the enlarged synovial lining cell layer as well as the cell density of the synovial stroma is better at higher magnification (× 200–400).

Results

Both observers analysed the samples according to the grading system mentioned above. Analysing the synovitis score, the median values for individual clinical diagnoses were as follows: control 1.0, OA 2.0, PtA 2.0, PsA 3.5, ReA 5.0, RA 5.0 (Table 2, Figure 3).

In most of the cases, the synovitis score was ± 1 around the respective median value, but almost all

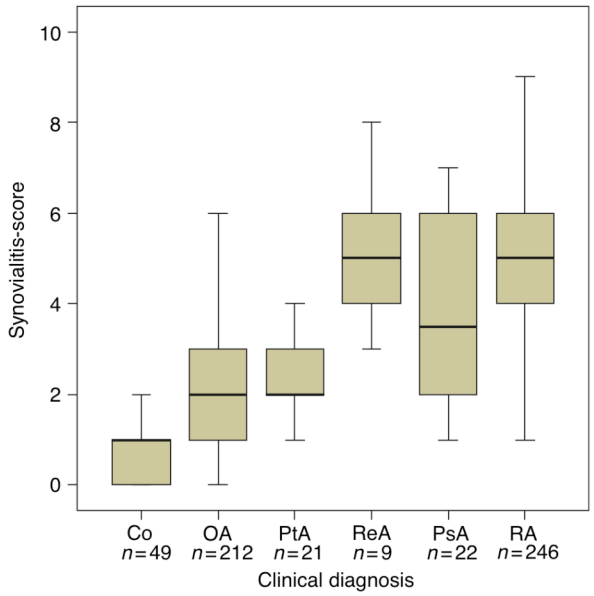

Figure 3. Box plot of the synovitis scores, separated according to clinical diagnoses. The median synovitis scores differentiate most of the diagnoses. However, the range of values is fairly wide so that overlapping is possible. Broad line, median; box, 25th and 75th percentile, respectively; whisker, 5th and 95th percentile, respectively.

diagnoses showed outlying values. So the range of OA was 6 (between 0 and 6), PsA 6 (between 1 and 7) and the range of RA was 9 (between 0 and 9).

Nevertheless, with regard to the synovitis score there were significant differences between controls and all other diagnoses and among many other disease groups. The probability values P (Mann–Whitney test) are given in Table 3. They demonstrate that, only with exception of the comparisons between OA and PtA, PsA and ReA as well as RA and ReA, the synovitis scores were significantly different between the clinical diagnoses. As well as scoring systems for other diseases, this synovitis score was not totally specific for certain joint diseases. For example, there were cases of OA with a rather high synovitis score of 6, whereas some cases of RA had a score of only 2, 1 or even 0 points (Table 2).

Altogether, 277 samples could be regarded as belonging to rheumatic diseases (RA + ReA + PsA). Of these, the synovitis score was between 0 and 4 in n ¼ 106 cases and between 5 and 9 in n ¼ 177 cases. Two hundred and eighty-two samples came from patients with degenerative diseases or controls (Co + OA + PtA). Of these, a synovitis score between 0 and 4 was diagnosed in 271 cases and between 5 and 9 in 11 cases. This gives a sensitivity of 61.7% and a specificity of 96.1% for the histopathological diagnosis of a high-grade synovitis as an indicator of a rheumatic joint disease.

Of 112 patients, there were between two and five samples from different parts of the same joint, giving up to 10 different combinations of correlations. These correlation coefficients were between 0.861 and 0.949. The correlation between the samples was always at least significant ($P < 0.05$), and frequently highly significant ($P < 0.01$).

Grading of the cases by two independent observers showed a marked correlation (Pearson's correlation coefficient r ¼ 0.941, $P < 0.001$).

Discussion

The synovitis score suggested here has proved to be clearly and easily applicable in the evaluation of 559

Table 2. Scoring profile for each particular disease

Synovitis Score Diagnosis	None 0	None 1	Low 2	Low 3	Low 4	High 5	High 6	High 7	High 8	High 9
Co, *n*	19	22	8	0	0	0	0	0	0	0
OA, *n*	12	37	84	39	28	10	2	0	0	0
PtA, *n*	0	3	10	6	2	0	0	0	0	0
ReA, *n*	0	0	0	1	2	2	2	1	1	0
PsA, *n*	0	1	5	5	4	4	2	1	0	0
RA, *n*	2	3	7	19	36	96	43	21	11	8

Co, Control; OA, osteoarthritis; PtA, post-traumatic arthritis; ReA, reactive arthritis; PsA, psoriatic arthritis; RA, rheumatoid arthritis.

Table 3. Probability values for the differences of the synovitis score between each particular disease

	OA	PtA	ReA	PsA	RA
Co	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
OA		$P = 0.587$	$P < 0.001$	$P = 0.001$	$P < 0.001$
PtA			$P < 0.001$	$P = 0.008$	$P < 0.001$
ReA				$P = 0.147$	$P = 0.774$
PsA					$P = 0.049$

Co, Control; OA, osteoarthritis; PtA, post-traumatic arthritis; ReA, reactive arthritis; PsA, psoriatic arthritis; RA, rheumatoid arthritis.

samples. Two independent observers graded the samples with a high correlation ($r = 0.941$). Various joint diseases—degenerative and primarily inflammatory—were assessable by this score. The synovitis scores frequently turned out to be significantly different between degenerative and rheumatic joint diseases, even though the samples showed no characteristic findings of diseases such as rheumatoid granulomas. The result of a high-grade synovitis (synovitis score ≥ 5 points) was an indicator of a rheumatic joint disease. A minor drawback was the fact that some cases of degenerative joint disease displayed synovitis scores of ≥ 5 points and some cases of rheumatic joint disease appeared as low-grade synovitis, leading to a sensitivity of 61.7% and a specificity of 96.1% for this scoring system. Therefore, one might regard the proposed synovitis score as a feasible tool for the standardized histopathological evaluation of synovectomy specimens with diagnostic implications.

In inflammatory diseases the degree of diagnostic reliability and thereby the relevance of histopathological diagnostics depends on the organ system. Diagnostic efficacy ranges widely from definite disease classification, as for example lupus-glomerulonephritis according to the World Health Organization,[21] to only subsidiary diagnoses, as for example in chronic sialadenitis.[22]

There is a considerable number of inflammatory and non-inflammatory diseases within the histological diagnostics of synovitis, given the general acceptance that few diseases show pathognomonic changes in the synovial membrane. The histopathological diagnosis of synovitis is thus subject to inconsistency,[1,3,8] which is why it has long since presented a challenge to pathologists.

In recent years histopathological scores have been established with the purpose of correlating the clinical grades of severity of RA and synovitis[5,12] and objectifying the inflammatory infiltration and the effects that medical therapy has on synovitis.[6,7,9–11] However, they have only confirmed that the majority of histological characteristics show no good correlation with clinical results, although the Larsen grade, the rheumatoid factor titre and the number of joints affected correlate well with the histopathological score of Koizumi.[23]

All of the histopathological scores that have been published to date obviously focus on the grading of the inflammatory activity of RA synovitis in synovial biopsy specimens.[7,9–11] However, histopathological diagnosis requires the type of score that allows for histopathological grading independent of the aetiology of the joint disease. Besides, this score should also be useful in long-term, chronic diseases and equally valid in the evaluation of larger-sized synovectomy samples. The synovitis score presented here considers these requirements and focuses on histological changes that can be easily identified in routine H&E-stained slides.

A major problem in the diagnosis of inflammatory alterations in large tissue samples is the heterogeneity of the inflammatory reaction. So this study recommends selecting for analysis those areas with the greatest alterations, a method that, by analogy, has proven successful in histopathological tumour diagnosis, particularly for the grading of sarcomas.[24]

Due to this score, the assessment of numerous synovectomy tissue samples was reproducible when the analysis of the same tissue sample was repeated by different observers. Thus it is qualitatively comparable to other grading schemes used in tumour pathology, e.g. the Gleason grading system for prostatic carcinoma or the classification of breast carcinoma.[25,26]

Application of the synovitis score has revealed a significant difference between the inflammation grade of rheumatic and degenerative joint diseases, especially between OA and RA. These findings correspond to the results of Koizumi[23] whose, by comparison, elaborate evaluation system included 11 important histological characteristics on a semiquantitative scale with a maximum value of 20. A further significant difference has been recealed between post-traumatic synovitis and synovitis of rheumatoid type. All post-traumatic

synovitis cases showed slight inflammatory lesions, partly with a regular lining cell layer, which have been described in the literature.[27]

The result that is most important for histopathologists is that a synovitis score of ≥ 5 (high-grade synovitis) indicates a rheumatic joint disease with a sensitivity of 61.7% and a specificity of 96.1%. So in serologically and clinically non-specific inflammatory joint diseases, histopathological assessment of the synovial membrane may provide further diagnostic information.

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3 Verwendung der Microarray Technologie und Entwicklung von Strategien zur Interpretation der Ergebnisse

Microarrays, die möglichst alle Gene des humanen Genoms abbilden, liefern zunächst eine schwer zu interpretierende Datenfülle. Statistisch ergibt sich dabei folgendes Problem: Eine gesuchte Veränderung wird immer von Variabilitäten überlagert. Diese können sowohl auf die individuellen Besonderheiten eines Spenders als auch auf technische Unterschiede bei der Probenbearbeitung und Durchführung der Messung zurückgeführt werden. Aufgrund der großen Anzahl von >20.000 verschiedenen Parametern für jede untersuchte Probe [25] können rein zufällig Konstellationen auftreten, die den Suchkriterien entsprechen. Erst durch eine große Anzahl von Versuchen könnte dieses Problem hinreichend kontrolliert werden. Da Microarray Experimente aber relativ teuer sind, werden oft weniger als zehn Proben pro Gruppe untersucht. Deshalb ist es erforderlich, dass die statistisch ermittelten Kandidatengene über alternative Methoden bestätigt werden.

Ein weiterer sehr wichtiger Aspekt ist die Berücksichtigung der zellulären Zusammensetzung. Aufgrund der enormen Expressionsunterschiede zwischen verschiedenen Zelltypen sind Änderungen der zellulären Zusammensetzung einer Probe der wesentliche Grund für Unterschiede in der Expression. Dies trifft vor allem für klinische Proben wie Blut oder Entzündungsgewebe zu. Eine Aufreinigung ist dann hilfreich, führt aber meist zu so genannten *in vitro* Artefakten, da die Manipulation der Probe während der Aufreinigung zu Änderungen der Genexpression führt.

Um möglichst sichere Aussagen liefern zu können und um den Umfang der Validierungsexperimente gering zu halten, sind verschiedene Strategien für Versuchsplanung und Auswertung hilfreich:

- eine schrittweise Untersuchung nach funktionell zusammengehörenden Gengruppen
- eine Reduktion der Komplexität der Probe durch Beschränkung auf eine bestimmte Zellpopulation
- eine gezielte Exposition eines Zelltyps mit einem Stimulans oder einem Inhibitor bei sonst konstanten Versuchsbedingungen

Die erste genannte Strategie schränkt am wenigsten ein bei der Probenauswahl, erfordert aber die größte Nacharbeit bei der Validierung. Die zuletzt genannte Vorgehensweise macht die meisten experimentellen Einschränkungen bei der Datenerhebung, liefert aber dafür die verlässlichsten Kandidatengene.

Die zunehmende Automatisierung und die starke Vereinheitlichung der technischen Durchführung eines Microarray-Experiments bringt weitere Möglichkeiten der Auswertung.

Unter Verwendung von definierten, bereits aufgebauten Genexpressionsdatensätzen, so genannten Signaturen, können kompliziertere Strategien entwickelt werden. Dies ermöglicht auch die Analyse von typischen klinischen Proben wie Blut oder Gewebe, die aus verschiedensten Zellen zusammengesetzt sind und in denen unterschiedlichste gegenseitige Stimulationen vorliegen können. Solche weiterentwickelten Analysestrategien sind ein wesentlicher Schritt hin zu einer systembiologischen Betrachtung und funktionellen Interpretation. Mit den nachfolgend aufgezeigten Arbeiten soll diese Entwicklung beispielhaft aufgezeigt werden.

3.1 Untersuchung von komplexem Entzündungsgewebe unter Beschränkung auf Gene einer definierten Funktionsgruppe

Ausgehend von früheren Untersuchungen, in denen wir einen Einfluss von „bone morphogenetic proteins“ (BMP) auf Knorpelregeneration und Entzündungshemmung zeigen konnten [26], wurde in der nachfolgenden Arbeit der Fokus auf Homöostase und regenerative Prozesse bei Synovitiden gelegt. Es wurde Microarray-Untersuchungen von Synovialgeweben dazu verwendet, um die Expression von gezielt ausgewählten Genen aus dem Bereich Knochen- und Knorpel-Wachstumsfaktoren zwischen rheumatoider Arthritis, Osteoarthritis und Kontrollgewebe zu vergleichen. Von 12 verschiedenen BMP die auf dem GeneChip HG-U133A Microarray vertreten sind, konnte bei rheumatoider Arthritis eine Erniedrigung von BMP4 und BMP5 festgestellt werden. Die Validierungsexperimente mit RT-PCR, in situ Hybridisierung und Immunhistochemie bestätigten diesen Befund. Es konnte ferner gezeigt werden, dass bei Arthritiden eine Umverteilung der Expression stattfand. In Kontrollgeweben von Gelenken ohne pathologische Veränderungen wurden beide Morphogene in der Deckzellschicht der Synovialmembran produziert. Dies lässt vermuten, dass diese Faktoren im Normalzustand in den Gelenkraum abgegeben werden und Einfluss auf den Knorpel und regenerative Prozesse haben könnten. Bei rheumatoider Arthritis aber auch bei Osteoarthritis waren dagegen BMP-produzierende Zellen in tiefer gelegenen Zellschichten zu finden. Gemessen an der RNA-Menge im Gewebe war die Expressionsrate bei rheumatoider Arthritis am niedrigsten. Gleichzeitig wurden die Befunde mit der Entzündungsaktivität anhand der Expression von Zytokinen (TNF, IL-1) und Destruktionsenzymen (MMP1, MMP3) sowie mit dem Synovitis-Score verglichen. Es wurde eine negative Korrelation insbesondere mit der TNF-Expression aber auch mit der Dauer der Erkrankung gefunden.

Diese Befunde legen nahe, dass BMP4 und BMP5 eine Rolle in der Gelenkhomöostase im gesunden Gelenk spielen und eine Erniedrigung vor allem bei entzündlichen Gelenkerkrankungen mit dazu beiträgt, dass sich neben der Entzündung und Aktivierung von destruktiven Mechanismen zusätzlich das regenerative Potential verschlechtert.

Research article

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Decrease in expression of bone morphogenetic proteins 4 and 5 in synovial tissue of patients with osteoarthritis and rheumatoid arthritis

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Abstract

Bone morphogenetic proteins (BMPs) have been identified as important morphogens with pleiotropic functions in regulating the development, homeostasis and repair of various tissues. The aim of this study was to characterize the expression of BMPs in synovial tissues under normal and arthritic conditions. Synovial tissue from normal donors (ND) and from patients with osteoarthritis (OA) and rheumatoid arthritis (RA) were analyzed for BMP expression by using microarray hybridization. Differential expression of BMP-4 and BMP-5 was validated by semiquantitative RT-PCR, *in situ* hybridization and immunohistochemistry. Activity of arthritis was determined by routine parameters for systemic inflammation, by histological scoring of synovitis and by semiquantitative RT-PCR of IL-1β, TNF-α, stromelysin and collagenase I in synovial tissue. Expression of BMP-4 and BMP-5 mRNA was found to be significantly decreased in synovial tissue of patients with RA in comparison with ND by microarray analysis ($p < 0.0083$ and $p < 0.0091$). Validation by PCR confirmed these data in RA ($p < 0.002$) and also revealed a significant decrease in BMP-4 and BMP-5 expression in OA compared with ND ($p < 0.015$). Furthermore, histomorphological distribution of both morphogens as determined by *in situ* hybridization and immunohistochemistry showed a dominance in the lining layer of normal tissues, whereas chronically inflamed tissue from patients with RA revealed BMP expression mainly scattered across deeper layers. In OA, these changes were less pronounced with variable distribution of BMPs in the lining and sublining layer. BMP-4 and BMP-5 are expressed in normal synovial tissue and were found decreased in OA and RA. This may suggest a role of distinct BMPs in joint homeostasis that is disturbed in inflammatory and degenerative joint diseases. In comparison with previous reports, these data underline the complex impact of these factors on homeostasis and remodeling in joint physiology and pathology.

Introduction

In patients with rheumatoid arthritis (RA), joint pathology is mediated by typical changes in the synovial tissue. Hyperplasia of the synovial lining layer, infiltration of mononuclear cells into the sublining layer, activation of fibroblast-like synoviocytes and the production of catabolic mediators such as IL-1β, TNF-α and matrix metalloproteinases are involved in the joint destruction of patients with RA [1]. Although secondary, synovitis is also found in osteoarthritis (OA) as a response of cartilage degradation and irritation of the lining cells with cartilage matrix components. Eventually, this also induces thickening of the lining layer and aggravates the damage of articular carti-

BMP = bone morphogenetic protein; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; IL = interleukin; ND = normal donors; OA = osteoarthritis; PCR = polymerase chain reaction; RA = rheumatoid arthritis; RT = reverse transcriptase; SSC = standard saline citrate; TNF = tumor necrosis factor.

lage by the release of inflammatory cytokines and destructive proteases [2].

Increases in knowledge about inflammatory cytokines and cytokine networks in chronic joint diseases has promoted the development of a new generation of biological drugs now available as inhibitors of TNF, IL-1 and others. However, little is known about mechanisms that protect and regenerate joints, although it has been shown that the progress of chronic joint diseases is decisively determined by the balance of anabolic and catabolic activities [3,4].

Bone morphogenetic proteins (BMPs) are anabolic candidates with pleiotropic functions in the development, homeostasis and repair of various tissues. Current approaches focus mainly on their ability to regenerate bone and cartilage by the induction of differentiation, apoptosis and proliferation of undifferentiated cells as well as by the stimulation of extracellular matrix formation [5,6]. These stimulatory properties led to the clinical use of recombinant BMP-7 in the treatment of bone nonunions [7]. In contrast, BMP signaling has been shown to be involved in the onset and progression of ankylosing enthesitis in spondyloarthropathies and in the induction of osteophytes in OA [8,9]. Antagonism of BMP signaling was therefore suggested as an attractive therapeutic principle [8,10].

These and other findings with opposing functional implications [5,11,12] demonstrate that the exact role of individual BMPs in degenerative joint diseases is still insufficiently understood.

In this study we focused on the expression of BMP-4 and BMP-5 in the synovial tissue of chronic joint diseases. Both proteins have a fundamental role in embryogenesis and in the induction of cartilage and bone [13,14]. Genetic and expression data suggest that BMP-5 is a key molecule in initiating the formation of particular skeletal elements in mammals [15].

In adult organisms, both BMP-4 and BMP-5, are sufficient to induce the heterotopic formation of bone and cartilage *in vivo* [16]. Moreover, diminished repair after bone fracture in BMP-5-null mutated short-ear mice suggests that BMP-5 might also be required for the growth and repair of skeletal structures after birth [15]. BMP-4 stimulates the synthesis of extracellular matrix in chondrocytes and supports the healing of bone fractures. Overexpression of BMP-4 leads to increased cartilage formation and chondrocyte differentiation without disturbing joint formation [17].

However, little is known about BMPs in synovial tissue. Lories and colleagues [18] demonstrated that BMP-2, BMP-4, BMP-6 and BMP-7 are expressed in the synovial membrane of patients with RA. BMP-2 and BMP-6, but not BMP-4 and BMP-7, are induced in fibroblast-like synoviocytes by stimulation with IL-1β and TNF-α. Moreover, intra-articular injection of BMP-2 induced fibrosis of the synovium [10], suggesting distinct effects of BMPs in synovial inflammation and joint pathology.

Here we have investigated the expression characteristics of BMP-4 and BMP-5, which were identified as differentially expressed BMPs in a comparative microarray study on synovial tissue from normal donors and patients with joint diseases. We confirmed the array data by semiquantitative PCR, *in situ* hybridization and immunohistochemistry. Decreased expression of these morphogens in the inflamed tissues and changes in their histomorphological distribution suggest that distinct members of the BMP family are involved in joint homeostasis. They may be attractive candidates for readjustment of an unbalanced intra-articular milieu dominated by destruction and lack of repair.

Materials and methods

Patients and tissue samples

Synovial tissue samples were obtained from patients with RA ($n = 23$) and OA ($n = 22$) undergoing open synovectomy or total joint replacement and from normal joints *post mortem* ($n = 17$) (tissue bank). Normal samples were derived from macroscopically healthy joints *post mortem*. The cause of death was cerebral bleeding or cerebral infarction. Patient characteristics and age and gender for controls are given in Table 1. No further information about the controls was made available for ethical reasons. Tissue samples for mRNA analysis by microarrays or PCR were snap-frozen in liquid nitrogen in the operating room and stored at -70°C until analyzed. Synovial tissue samples for *in situ* hybridization were embedded in OCT Tissue Tek (Miles, Elkhart, IN, USA) before being frozen. Synovial tissue samples for immunohistochemistry were embedded in paraffin. All patients with RA fulfilled the American College of Rheumatology revised criteria for definite RA [19]. The study was approved by the local ethical committee of the Charité Hospital.

Grading of chronic synovitis

To characterize synovial disease activity and to confirm appropriate sampling before molecular analysis, the synovitis score as published by Krenn and colleagues [20,21] was applied. The histopathological inflammatory scoring system included the following three parameters: hyperplasia/enlargement of synovial lining layer (intima), activation of fibroblastic cells in the sublining stroma, and inflammatory cellular infiltration. All three parameters were graded semiquantitatively (0 = no, 1 = slight, 2 = moderate, 3 = strong) in a manner blinded to diagnosis. The values of all three parameters were added, resulting in a score between 0 and 9; 0 or 1 was interpreted as 'no synovitis', 2 or 3 as 'slight degree of synovitis', 4 to 6 as 'moderate degree of synovitis' and 7 to 9 as 'strong degree of synovitis'.

Table 1

Clinical characteristics of patients

	Microarray			PCR		
	RA ($n = 10$)	OA ($n = 10$)	ND ($n = 10$)	RA ($n = 13$)	OA ($n = 12$)	ND ($n = 7$)
Median age (range), years	60 (39–73)	67 (58–78)	57 (40–76)	69 (29–74)	67 (53–83)	51 (34–61)
Male/female	2/8	1/9	7/3	5/8	2/10	4/3
Median disease duration (range), years	7 (2–37)	10 (1–19)	NA	10 (4–30)	5 (1–38)	NA
Median ESR (range), mm/h	33 (12–78)	20 (10–60)	NA	32 (22–86)	11 (2–29)	NA
Median CRP (range), mg/l	22.1 (6.1–113.3)	6.4 (2–19)	NA	19 (5.9–50.8)	4.8 (0–9.3)	NA
Rheumatoid factor positive, *n* (range, units)	4 (47–400)	1 (29)	-	10 (15–2,450)	-	-
Patients receiving steroids, *n*	8	0	NA	9	0	0
All patients receiving DMARDs, *n*	9	0	NA	9	0	0
Patients receiving MTX	7	0	NA	6	0	0
Patients receiving NSAIDs, *n*	6	5	NA	8	4	0
Synovial tissue from knee	5	10	10	6	5	7
Hip	0	0	0	3	7	0
Hand synovectomy	3	0	0	3	0	0
Elbow synovectomy	2	0	0	1	0	0

CRP, C-reactive protein; DMARDs, disease-modifying anti-rheumatic drugs; ESR, erythrocyte sedimentation rate; NA, not applicable; ND, normal donors; OA, osteoarthritis; RA, rheumatoid arthritis.

Microarray analysis

Total RNA from synovial tissues was isolated with the Qiagen RNeasy Mini Kit in accordance with the manufacturer's protocols (Qiagen, Hilden, Germany). Total RNA was used for further microarray analysis with the oligonucleotide microarray HG-U133A (Affymetrix, Santa Clara, CA, USA) in accordance with the manufacturer's recommendations. In brief, 5 μg of total RNA was used to synthesize cDNA. Subsequently, *in vitro* transcription (ENZO Biochem, New York, NY, USA) was performed to generate biotin-labeled complementary RNA. Fragmented complementary RNA (15 μg) was hybridized to GeneChips for 16 hours at 45°C. The GeneChips were washed and stained under standardized conditions (fluidic station) and scanned on a Hewlett Packard Genearray Scanner (Affymetrix) controlled by Affymetrix MAS 5.0 software. Raw gene expression data were processed with the Affymetrix GCOS 1.2 software module in accordance with the manufacturer's default settings. Analysis was performed with Affymetrix GCOS 1.2 software to generate CEL files and the robust multiarray analysis (RMA) algorithm for signal calculation [22]. Arrays were adjusted to each other by quantile normalization in RMA.

We followed the hypothesis that BMPs might be involved in the regulation of joint homeostasis. All probe sets ($n = 19$) representing all different genes of the BMP family ($n = 12$) on the HG-U133A array were therefore selected for *t* test analysis. Adjusted *p* values for the 12 genes with Bonferroni-Holm correction ($\alpha = 0.1$) were applied as the threshold of significance.

Semiquantitative kinetic PCR

Tissues were homogenized, treated with phenol–chloroform [23] and total RNA was extracted with RNeasy spin columns (Qiagen). Single-strand cDNA was transcribed by Superscript II RT (Gibco BRL, Karlsruhe, Germany) from 5 μg of RNA in a total volume of 20 μl. The relative expression level of glyceraldehyde-3-phosphate dehydrogenase was used to normalize gene expression in each sample in different concentrations. Semiquantitative PCR was performed as described previously [1]. In brief, oligonucleotides (Gibco BRL) were selected with DNASTAR Primer Select Software (DNASTAR Inc., Madison, WI, USA). Sequences are given with GenBank accession numbers (Gibco BRL) in Table 2. All PCR reactions were performed with AmpliTaq Gold Mix (Perkin Elmer, Weiterstadt, Germany) in a reaction volume of 80 μl, amplifying at 93°C for 1 minute, 62°C for 1 minute, and 72°C for 2 minutes. For quantification of individual genes, 4 μl of each amplification reaction was removed every third cycle covering the linear detection range. Products were separated in a 1% agarose gel containing ethidium bromide and quantified densitometrically (Imager 1D&2D software; Appligene, Oncor, Illkirch, France) within the linear range comparable to the *Ct* value known from real-time PCR. The quality of amplification was controlled by the amplification efficiency as represented by the

Table 2

Oligonucleotides

mRNA	GenBank accession number	Oligonucleotide (5'→3') (up/down)	Product size (bp)	Annealing temperature (°C)
GAPDH	M33197	ATG GGG AAG GTG AAG GTC GGA GTC GAC GCC TGC TTC ACC ACC TTC TTG	797	62
TNF-α	M10988	CTC TGG CCC AGG CAG TCA GA GGC GTT TGG GAA GGT TGG AT	519	62
IL-1β	M15330	CAC CTG TAC GAT CAC TGA ACT GCAC GGC TGG GGA TTG GCC TGC AA	674	60
MMP-1	X05231	CTG CTG CTG TTC TGG GGT GTG GTG GTG GGC CGA TGG GCT GGA CAG	793	62
MMP-3	J03209	TGG AGC TGC AAG GGG TGA GGA CAC CAG GCG GAA CCG AGT CAG GTC TGT	691	62
BMP-4	M22490	ACC CGG GAG AAG CAG CCA AAC TAT AGC GGC ACC CAC ATC CCT CTA CTA	553	62
BMP-5	M60314	GGC ATC CTT GGC AGA AGA GAC CA ACT GCG TCC ATC CCC TGT TTC TG	535	62

BMP, bone morphogenetic protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; MMP-1, collagenase I; MMP-3, stromelysin.

increase in product per cycle. Specificity of the PCR product was confirmed by sequencing. For graphical presentation, data are given as percentages of the glyceraldehyde-3-phosphate dehydrogenase product.

In situ hybridization

In situ hybridization was performed as described previously [24]. BMP-4 and BMP-5 cDNA fragments were derived from the respective PCR products, cloned into pBluescript II (Stratagene, La Jolla, CA, USA) and sequenced. Digoxigenin-labeled riboprobes were transcribed with the PCR-Script Amp-Cloning Kit (Stratagene) and T3 and T7 polymerases (Roche, Mannheim, Germany). For each patient group (RA, $n = 5$; OA, $n = 5$; ND, $n = 4$), frozen sections 6 μm thick were fixed in 3% paraformaldehyde, washed in 2 × standard saline citrate (SSC) for 5 minutes, washed twice in 0.1 M triethanolamine hydrochloride, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride for 30 minutes. After being washed with 1 M triethanolamine hydrochloride, sections were prehybridized for 1 hour with hybridization buffer (50% formamide, 80 μl of 50 × Denhardt's solution, 1.6 ml of 20 × SSC, 200 μl of herring sperm, 100 μl of carrier RNA) without the riboprobe. Hybridization with digoxigenin-labeled riboprobes was performed overnight in hybridization buffer at 50°C. After hybridization, sections were incubated with RNase A (40 μg/ml) for 1 hour at 37°C and subsequently washed for 15 minutes with increasing stringency (1 × SSC, 0.25 × SSC, 0.1 × SSC in 0.1% SDS) at 50°C. The staining procedure was performed with an anti-digoxigenin-alkaline-phosphatase-conjugated Fab by using 5-bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium (all chemicals from Roche). Blocking was performed with 2% horse serum. Sense probes used as negative controls gave no significant signal.

Immunohistochemical staining

BMP-4 and BMP-5 was stained in paraffin embedded tissue (RA, $n = 4$; OA, $n = 6$; ND, $n = 4$) with a modified sandwich technique as described previously [25]. Sections 4 μm thick were deparaffinized and endogenous peroxidase activity was quenched for 15 minutes with 0.3% H_2O_2 in methanol at room temperature. Specimens were microwave-heated for 14 minutes and incubated for 30 minutes with pooled, heat-inactivated human serum tested negative for both anti-nuclear antibodies and anti-neutrophil cytoplasmic antibodies. The primary antibodies (polyclonal goat-anti-human BMP-4 and BMP-5 antibodies; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied for 1 hour at room temperature. Slides were incubated for 30 minutes with a horseradish-peroxidase-conjugated secondary rabbit anti-goat antibody at a dilution of 1:50, and afterwards with Dako Envision anti-rabbit antibody. Slides were incubated with the chromogenic substrate 3-amino-9-ethyl-carbazole for 5 minutes at room temperature and counterstained with hematoxylin.

Statistical analysis

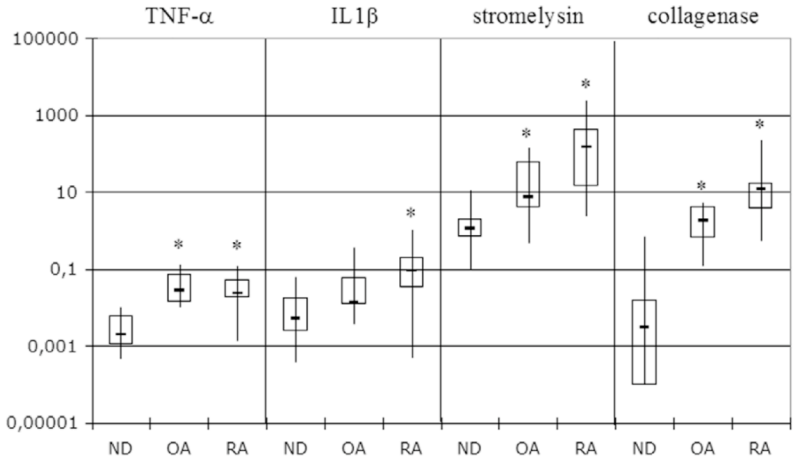
Statistical analysis was performed with GraphPad software (GraphPad Sofware Inc., San Diego, CA, USA). For microarray analysis a *t* test was used with Bonferroni-Holm correction. For comparison between RA, OA and ND (PCR), the Mann–Whitney *U* test was applied. Correlations were calculated by Spearman's rank correlation test.

Results

Validation of systemic and local inflammation

Patients were investigated for systemic as well as local inflammation and disease activity by the analysis of blood and synovial tissue samples. Systemic inflammation was characterized by erythrocyte sedimentation rate (ESR) and C-reactive pro-

Figure 1



Expression of TNF-α, IL-1β, stromelysin and collagenase I in synovial tissues. Results are presented as percentage of GAPDH expression on a logarithmic scale with maximum, minimum, quartiles and median. Where indicated with an asterisk, there were significant differences from normal tissues ($p < 0.05$; Mann–Whitney). Rheumatoid arthritis (RA) versus normal donors (ND): IL-1β, $p = 0.0097$; TNF-α, $p = 0.008$; stromelysin, $p = 0.0009$; collagenase I, $p = 0.0002$. Osteoarthritis (OA) versus ND: IL-1β, $p = 0.1451$; TNF-α, $p = 0.0013$; stromelysin, $p = 0.038$; collagenase I, $p = 0.0012$. RA versus OA: IL-1β, $p = 0.0397$; TNF-α, $p = 0.9591$; stromelysin, $p = 0.0124$; collagenase I, $p = 0.0266$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tein (CRP) (Table 1). Both markers were significantly elevated in RA in comparison with OA (CRP, $p \leq 0.0001$; ESR, $p = 0.0001$). Local inflammation and destructive activity in synovial tissue were quantified by both histological and molecular characteristics. Analysis of the tissues according to the 'synovitis score' described by Krenn and colleagues [20,21] revealed 2.1 (RA), 1.3 (OA) and 0.7 (ND) points for hyperplasia of the synovial lining layer, 1.9, 1.1 and 0.3 points for activation of the sublining stroma, and 2.1, 0.8 and 0.1 points for inflammatory infiltration in RA, OA and ND, respectively. Thus, the synovitis score – assessed in a blinded manner – was increased in all patients with RA (mean score 6.1, 'highly active synovitis') in comparison with those with ND (mean score 1.1, 'no synovitis') and patients with OA (mean score 3.2, 'mild synovitis'). For molecular characterization, expression levels of IL-1β and TNF-α as well as stromelysin and collagenase I were determined by semiquantitative PCR. These parameters were found to be highest in RA with a significantly lower expression in OA (except for TNF-α) and ND. In OA these parameters were also significantly elevated in comparison with ND except for IL-1β (Figure 1).

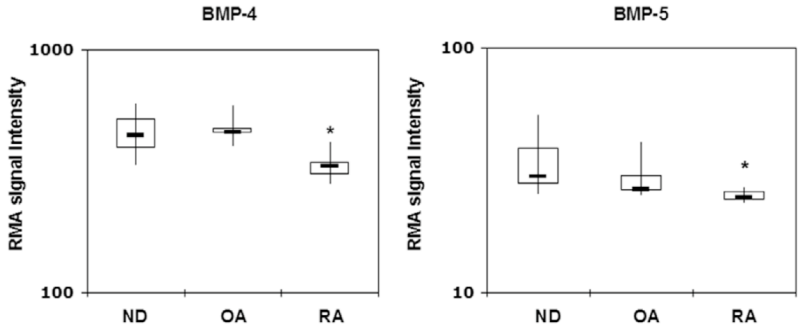
Analysis of BMP-4 and BMP-5 gene expression in synovial tissue

Microarray analysis was performed by investigating 10 samples from each group of donors with RA, OA and normal joints. We exclusively investigated the factors of the BMP family as possible candidates involved in joint homeostasis and cartilage regeneration [5]. BMP-2 to BMP-11, BMP-14 and BMP-15 were represented on the array. In comparison with housekeeping genes, all BMPs revealed low signal levels in all samples investigated. Statistical analysis revealed significantly decreased expression of BMP-4 and BMP-5 in RA in comparison with ND. Moreover, BMP-4 was also lower in synovial tissue of patients with RA than in those with OA. There was no difference of BMP expression between OA and ND (Figure 2).

This differential expression of BMP-4 and BMP-5 as determined by microarray technique was verified by semiquantitative PCR (Figure 3). A significantly reduced expression of both BMPs was found in OA and RA tissue in comparison with normal synovial tissue ($p < 0.015$). Expression of BMP-4 in RA synovial tissue was also lower than in tissues from patients with OA ($p < 0.02$). For BMP-4, there was no overlap between the ranges of RA and ND expression values: all values of RA tissues were lower than the minimum level found in ND tissues. In OA, expression values of 5 of 12 synovial tissues were within the range of ND expression values. For BMP-5, expression in all patient samples except those from one RA donor were below the range of expression in ND tissues. Thus, PCR analysis confirmed the results for RA versus ND as determined by microarray hybridization.

Correlation analysis of BMP-4 and BMP-5 with each other and with markers of inflammation was performed by combining the data from RA and OA donor groups for the respective parameters. BMP-4 was found to decrease with rising systemic inflammation as represented by ESR ($r = -0.4184$, $p = 0.0298$) and C-reactive protein ($r = -0.5808$, $p = 0.0012$) as well as with disease duration ($r = -0.6343$, $p = 0.0005$). Furthermore, expression of BMP-5 was negatively correlated with an increase in TNF-α expression ($r = -0.4739$, $p = 0.0167$).

Figure 2



Expression of BMP-4 and BMP-5 in synovial tissues detected by microarray technique. Results are presented on a logarithmic scale with maximum, minimum, quartiles and median. Where indicated with an asterisk, there were significant differences from normal tissues ($p < 0.05$; *t* test). Rheumatoid arthritis (RA) versus normal donors (ND): bone morphogenetic protein (BMP)-4, $p = 0.0009$ (adjusted $p \leq 0.0083$); BMP-5, $p = 0.0142$ (probe set ID 205431_s_at; data not shown) and $p = 0.006$ (probe set ID 205430_at) (adjusted $p \leq 0.009$). Osteoarthritis (OA) versus ND: BMP-4, $p = 0.854$; BMP-5, $p = 0.216$ (probe set ID 205431_s_at) and $p = 0.129$ (probe set ID 205430_at) (no significance). RA versus OA: BMP-4, $p = 0.000003$ (adjusted $p \leq 0.0083$); BMP-5, $p = 0.2391$ (probe set ID 205431_s_at) and $p = 0.026$ (probe set ID 205430_at) (no significance).

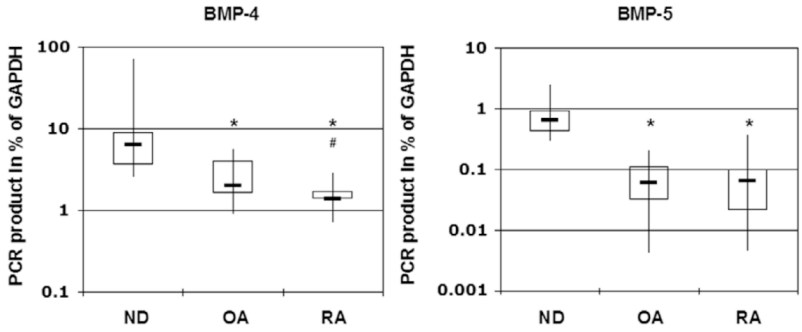
In situ hybridization and immunohistochemistry

Synovial tissue of patients with RA, OA and ND was analyzed to localize the morphological site of BMP-4 and BMP-5 expression by mRNA *in situ* hybridization and immunohistochemistry (Figures 4 and 5). Both techniques present only qualitative morphological results and do not reflect the quantity of transcripts.

In situ hybridization in normal synovial tissue (ND) revealed BMP-4 and BMP-5 expression predominantly on the surface of the synovial membrane. However, in RA and OA tissues BMP-4 and BMP-5 were less dominant in the superficial layer but were also found in cells of the sublining layer. Both morphogens were mostly localized to cells with large nuclei or spindle-like shape (Figure 6). Especially in OA samples with areas of fibrous tissue formation, cells were positively stained for morphogen transcripts (Figure 7a,b). Perivascular cell infiltrates also contained positive cells with large nuclei along with positive cells of spindle-like appearance, thus resembling macrophage and fibroblastoid morphology, respectively (Figure 7c,d).

To confirm the results of *in situ* hybridization, antibody staining for BMP-4 and BMP-5 protein was performed in independent samples. Synovial tissues of all three groups (RA, OA and ND) revealed positive results. The sites of expression of both morphogens were identical to those found by *in situ* hybridization. Both methods therefore documented independently that BMP-4 and BMP-5 expression is related to the synovial lining layer in ND and more to the sublining layer in RA and OA patients (Figures 4 and 5).

Figure 3



Expression of BMP-4 and BMP-5 in synovial tissues detected by semiquantitative PCR. Results are presented as percentage of GAPDH expression on a logarithmic scale with maximum, minimum, quartiles and median. Where indicated, there were significant differences from normal tissues (asterisk) or osteoarthritis (OA) (hash sign) ($p < 0.05$, Mann–Whitney). Rheumatoid arthritis (RA) versus normal donors (ND): bone morphogenetic protein (BMP)-4, $p = 0.0005$; BMP-5, $p = 0.0016$. OA versus ND: BMP-4, $p = 0.0143$; BMP-5, $p = 0.0011$. RA versus OA: BMP-4, $p = 0.0180$; BMP-5, $p = 0.9215$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.