Interleukin-32, CCL2, PF4F1 and GFD10 are the only cytokine/chemokine genes differentially expressed by *in vitro* cultured rheumatoid and osteoarthritis fibroblast-like synoviocytes

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ABSTRACT. Since cytokines and chemokines are important actors in rheumatoid arthritis (RA), the aim of this study was to compare the gene expression profiles in cultured fibroblast-like synoviocytes (FLS) obtained from patients with either RA, or osteoarthritis (OA), focusing our analysis on genes for cytokines and chemokines, and their respective receptors. Gene expression in cultured FLS (third passage) from eight patients with RA (RA-FLS) were compared with gene expression in cultured FLS from nine patients with OA (OA-FLS) using Affymetrix Human Genome U133 Plus 2.0 Array microarray, allowing analysis of over 54,000 transcripts. Among the 171 genes studied (241 probes), limiting the selection of differentially expressed genes to a significant value ($p < 0.05$), and a differential ratio of expression > 1.6, only four genes, namely IL-32, CCL2, PF4F1 and GDF10 were found to be differentially expressed. Out of these four genes, only higher expression of CCL2 has been reported previously in RA. The newly described cytokine IL-32 was the most prominently differentially expressed gene in the present study, with higher expression in RA-FLS than in OA-FLS ($p < 0.0073$). IL-32 might have a previously unidentified pivotal role in RA.

Keywords: rheumatoid arthritis, transcriptomics, microarray, cytokine, chemokine

Rheumatoid arthritis (RA) which affects approximately 0.5 % of the world adult population represents a significant cause of disability. RA is a chronic inflammatory disease characterized by progressive joint destruction involving damage to articular cartilage caused by inflammatory cells, activated synovial fibroblasts and chondrocytes. The broad array of cytokines and factors produced in the affected joints, and the multiple cell interactions, dictate the evolution of arthritis. The levels of pro-inflammatory molecules, particularly the monokines TNF-α and IL-1β, exceed those of anti-inflammatory cytokines such as IL-10 and TGF-β [1, 2]. Biological therapies targeting TNF-α, and now IL-1 and IL-6, have clearly demonstrated the central importance of cytokine production and macrophage-induced inflammation in RA. Nevertheless, these treatments achieve only transient clinical responses, unless given repeatedlyy [3-6]. Moreover, only 40 % of patients reach the American College of Rheumatology (ACR) 50 % response. This highlights the clinical necessity for generating further, novel, pathogenesis-led interventions. To reach this goal, it is crucial to identify and quantify the genes expressed by the cells that are present and which interact in the tissue. A recent review pointed out the interest of the analysis of RA pathophysiology using a genomics approach [7]. Indeed, synovial lesions in RA show complex histopathological manisfestations that render difficult, although informative, the molecular profiling of the synovial tissue [8]. Successful analysis of the pathophysiology of the disease requires the functional understanding of the different cell players in synovium and how they interact. Current evidence indicates that fibroblast-like synoviocytes (FLS), which constitute the synovial lining, are key actors in pannus formation, and the subsequent destruction of cartilage and bone in the joint. One hallmark of the disease is the hyperplasia of these FLS,

Abbreviations:

RA	rheumatoid arthritis
OA	osteoarthritis
FLS	fibroblast-like synoviocytes

which proliferate in an anchorage-independent manner and lack contact inhibition, making it of prime interest to better characterize fibroblast-like synoviocytes at the transcript levels. Most of the studies have addressed issues regarding the response of these cells to different stimuli, mainly proinflammatory cytokines, while very few data focused on the extensive molecular characterization of FLS [9]. Taking advantage of microarrays allowing a full genome analysis of human genes, we compared the transcriptional profile in cultured FLS from patients with RA and patients with osteoarthritis (OA). Over 54,000 transcripts were examined. When focusing our analysis on the cytokines and chemokines, which are the main actors in RA, we noticed that on comparing RA-FLS to OA-FLS, contrary to expectation, these cells had almost the same pattern of gene expression for the cytokines/chemokines and their respective receptors. Indeed, a differential expression pattern was observed for only a very limited number of cytokine/chemokine genes. Among the 171 genes closely examined by mean of 241 probes, only four were differentially expressed, namely IL-32, CCL2, PF4F1 and the recently cloned, bone morphogenic protein GDF10 (BMP-3b) [10, 11]. The IL-32 cytokine gene was the most prominently differentially expressed ($p < 0.0073$), suggesting that it could have a pivotal role in rheumatoid arthritis.

PATIENTS AND METHODS

Patients and FLS cultures

Eight patients with RA, who fulfilled the RA criteria of the American College of Rheumatology [12] and nine patients with OA were included in this study. Tissues were obtained from informed patients who underwent remedial synovectomy, or arthroplasty of the knee. Synovial cells were obtained as previously described [8]. Briefly, freshly collected synovial tissues were finely minced, and treated with collagenase dispase for 2 hours at 37°C. FLS were allowed to adhere to tissue culture plates, and nonadherent cells were removed. FLS were grown to subconfluence (70 %) in culture flasks containing complete medium RPMI 1640 supplemented with 10 % fetal calf serum, 100 µg/mL streptomycin and 500 units/mL of penicillin. All experiments were performed using FLS from the third passage. At this time, there were less than 2 % contaminating lymphocytes, natural killer cells, and macrophages.

RNA preparation

Total RNA was extracted from cultured cells in RLT® RNA extraction buffer (Qiagen, Rneasy kit), and treated with DNase I to eliminate genomic DNA contamination. The integrity and purity of the total RNA, and of the cRNA, were analysed twice using a Bioanalyser 2100, and RNA kit 6000 LabChip (Agilent Technologies). Only total RNA with a 28S/18S ratio > 1.7 was used. cRNA concentrations were measured using NanoDrop (NanoDrop Technologies).

cRNA synthesis and probe array hybridization

cRNA synthesis was performed with 3 µg of total RNA using the GeneChip Expression 3′ Amplification One-Cycle Target Labelling and Control Reagents, hybridized onto the human genome U133 Plus 2.0 (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's protocol (GeneChip® Expression Analysis Technical Manual, Rev.5, Affymetrix Inc., 2004). Briefly, total RNA was first reverse transcribed using a T7-Oligo(dT) promoter primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA was purified and served as a template in the subsequent *in vitro* transcription (IVT) reaction. The IVT reaction was carried out in the presence of T7 RNA polymerase, and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labelling. The biotinylated cRNA targets were then cleaned up, fragmented, and hybridized to GeneChip expression arrays. After washing and staining using the Affymetrix fluidics station 450 (Affymetrix, Inc.), the probe arrays were scanned into the Affymetrix GeneChip Scanner 3000.

Gene expression patterns of FLS

Gene expression profiles were assessed by microarrays using the GeneChip Human Genome U133A Plus 2.0 (Affymetrix, Santa Clara, CA, USA). Gene expression from cultured FLS from eight patients with RA and nine patients with OA was assessed. We selected the results of a total of 241 probes representing 171 different cytokines or chemokines and their respective receptors, for further analysis. Genes whose expression varied at least 1.6-fold between FLS from both diseases and with a p value of less than 0.05 were selected.

Microarray analysis

The raw expression data were processed with GC-RMA File preprocessor in GeneSpring. The GC-RMA algorithm used the probe data stored in the Affymetrix CEL files. Raw data processing, normalization and data analysis were performed with Genespring 7.2. The "Per Gene: Normalize the median" GeneSpring normalization was performed to ensure that the expression value for each gene across the different conditions was centred to 1. This ensures that genes that do not change across conditions have a normalized expression value of 1, allowing for easy visual detection of differentially expressed genes. Clusterings were performed in GeneSpring, using the "condition tree" and "gene tree" with "Standard Correlation". The one-way analysis of variance (ANOVA) test was used to detect differential gene expression between biological conditions. The gene selections were performed using p values produced by the ANOVA test and then by mean ratios (RA/OA).

RESULTS AND DISCUSSION

A total of seventeen fibroblast-like synoviocyte (FLS) cultures (eight from patients with RA and nine from patients with OA) were analysed by microarray. Only tissues from patients with a confirmed diagnosis and without any other associated disease were used. Focusing on expression data for 171 cytokine/chemokine-related genes, we found that only a small number of these genes were differ-

entially expressed *(table 1)* (full set of data for the 241 probes are available upon request to the corresponding author). Hierarchical clustering of the samples using all 241 probes did not allow correct classification (not shown). Although expression of several cytokines has been described in RA synovium, many of those reported results are either controversial, or have not been confirmed. Herein, our results show that cultured OA-FLS and RA-FLS expressed very similar patterns of cytokine and chemokine genes, as well as their respective receptor genes, at least when they were not triggered by additional stimuli. This result could reflect an intrinsic similarity between RA-FLS and OA-FLS. Interestingly, the four genes differentially expressed when comparing RA-FLS and OA-FLS, belonged to three main pathophysiological pathways that have been implicated in the diseases: chemotactism, bone erosion, and inflammation. Furthermore, three out of these four genes have not been the focus of extensive studies either in RA, or in OA, so far. Among these, IL-32 is of particular interest *(table 1*, *figure 1)*. This cytokine was recently cloned by Kim *et al*. [13], who also demonstrated the induction of TNF-α by recombinant IL-32 in differentiated macrophages. As TNF-α, is a crucial, deleterious mediator in RA, as established by the very effective therapeutic effects obtained upon blocking TNF-α activity in RA patients [14, 15] and in other autoimmune/inflammatory diseases, IL-32 might have an important role in rheumatoid arthritis. Several cytokines such as IL-18, IL-1β, IL-12 and IFN-γ were reported to induce IL-32 expression [13]. However, we found no correlation between the expression of any of these cytokines or any other cytokine examined in the present study, and expression of IL-32. It is thus possible that RA-FLS have the intrinsic ability to express this cytokine. As underlined by Kim *et al*. [13], although several immune regulatory molecules have been discovered during the past decade, many phenomena remain unexplained in the understanding of immune regulation. The apparent ability of RA-FLS to constitutively express this new cytokine may to help unravel the complexity of cytokine biology in RA.

In this study, we also observed that the CCL2/MCP-1 gene was expressed more strongly in RA-FLS than in OA-FLS. Work on genetically modified mice have placed CCL2 in a central position in monocyte trafficking and activation [16, 17]. Our results showing that CCL2 gene expression is higher in RA-FLS than in OA-FLS is in agreement with previous studies reporting that synovial fibroblasts may represent the source of CCL2 *in vivo* [18]. Furthermore, we found that expression of both IL-6 and its receptor sub-unit gp130 genes, was slightly higher in RA-FLS than in OA-FLS, although the difference observed did not reach a significance. These results validate further the importance of the IL-6 and CCL2 targeted pathways in RA, and highlight the pivotal role of FLS in this process.

In contrast to CCL2, we found that PF4V1, another chemokine, demonstrated a weaker expression in RA-FLS

Table 1
Set of genes with increased or decreased mRNA expression ratio

Symbol	Aliases	Genomic location	Gene	Affymetrix probes	OA	RA	RA/OA	P
IL-32	NK4	16p13.3	natural killer cell transcript 4	203828_s_at	0.65	2.536	3.85	0.00733
PF4V1	CXCL4V1	4q13.3	platelet factor 4 variant 1	207815_at	1.264	0.784	0.62	0.0218
CCL2	MCP-1	17q12	chemokine (C-C motif) ligand 2	216598_s_at	0.77	1.904	2.5	0.0297
GDF10	BMP-3b	10q11.22	growth differentiation factor 10	206159_at	0.614	2.234	3.70	0.0398

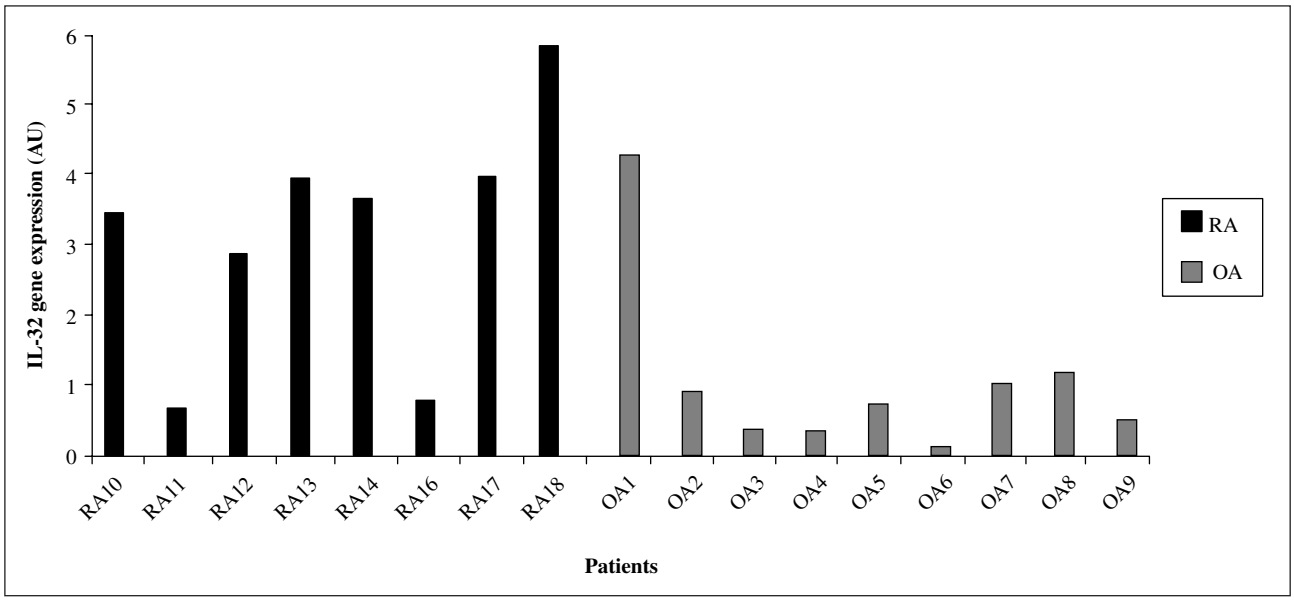

Figure 1
RA-FLS expressed higher level of IL-32 than OA-FLS. Results are arbitrary units (AU) (see Materials and Methods) and expressed as ratios of RA or OA cultured synovial cells. All the experiments were performed using SFs at the third passage in tissue culture; at this time the contaminating cells were less than 2 %. Data are from 8 RA and 9 OA patients.

than in OA-FLS. PF4V1 is the product of the non-allelic variant gene of CXCL4, the first chemokine described to inhibit neovascularization. Although the gene for PF4V1 was cloned in 1990 [19], few data are available on the effect of this chemokine. It was showed that secreted CXCL4, and PF4V1/CXCL4L1, differed in only three amino acids, and that CXCL4L1 was more potent than CXCL4 in inhibiting chemotaxis of human microvascular endothelial cells toward IL-8 or bFGF [20]. Thus, PF4V1/CXCL4L1 appears to be a potent regulator of endothelial cell biology, down-regulating angiogenesis. Synovitis has been recognized for a long time as a hallmark of RA and it is now recognized as a common and important feature of osteoarthritis. As the molecular balance between angiogenic and antiangiogenic factors is disturbed in the joint, new blood vessels are allowed to grow into normally avascular structures, such as articular cartilage. Although in both RA and OA vascular growth is enhanced, higher expression of an inhibitor of angiogenesis by OA-FLS compared to RA-FLS could explain the particularly evident, ongoing neoangiogenic process in RA.

Another of the intriguing results we reported herein is the higher expression of the GDF10/BMP-3b gene in RA-FLS compared to OA-FLS. Indeed, this gene was reported to induce bone tissue and cartilage formation [21], the two main affected tissues in RA. Thus, FLS cells in rheumatoid arthritis could have a dual potential, having the ability of promoting both inflammation (IL-32 and CCL2), and tissue repair (GDF10).

In summary, the present analysis of the expression profiles of the selected set of genes has provided evidence for dysregulated biological pathways, highlighting potentially important chromosomal loci, and revealing novel genes possibly involved in RA. A weak association of CCL2 polymorphism with susceptibility to RA in patients lacking the HLA shared epitope [22] has been reported. It will be interesting to analyse the genetic association of the three new genes reported here, with susceptibility to RA. The differences in gene expression patterns between fibroblast-like synoviocytes from patients with either RA or OA may suggest biological pathways for therapeutic intervention.

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