Down-modulation of chemokine receptor cartilage expression in inflammatory arthritis

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Objective. To evaluate in vivo expression of chemokine receptors in cartilage tissue samples from healthy and diseased joints.

Methods. Presence and distribution of several chemokine receptors in cartilage samples from patients with osteoarthritis (OA) or inflammatory arthritis (IA) and from multi-organ donors were assessed by immunohistochemistry. The expression of messenger RNA (mRNA) for chemokine receptors was also analysed by reverse transcriptase-polymerase chain reaction (RT-PCR).

Results. Normal and OA-affected cartilage showed a moderate to high expression of chemokine receptors, while staining of IA samples ranged from low to absent. Differences between OA and IA samples were present for all receptors but CCR2 and CXCR4. Moreover, mRNAs for CCR1, CCR5 and CXCR1 were found both in normal and pathological chondrocytes, suggesting that chemokine receptor down-modulation seen in IA samples could be a post-transcriptional event.

Conclusion. Data on normal and pathological chondrocytes underline the role of chemokines in cartilage homeostasis and suggest an imbalance towards catabolic processes in inflammatory conditions.

Key words: Cartilage, Chemokine receptors, Osteoarthritis, Inflammatory arthritis.

Cartilage development and homeostasis in the mature organism is the result of a continuous balance between catabolic and anabolic activities of chondrocytes. They are pushed into the catabolic pathway primarily by pro-inflammatory stimuli, while growth factors are mainly responsible for the anabolic activities of these cells [1]. Besides cytokines and growth factors, chondrocyte function is also influenced by chemotactic cytokines belonging to the chemokine superfamily. Chemokines are divided into two main subfamilies according to the position of the first two cysteine residues: in the CC chemokines the first two cysteines are adjacent, in the CXC chemokines these residues are separated by one amino acid. The main feature of chemokines is the regulation of leucocyte traffic [2], but there is strong evidence about their involvement in cartilage homeostasis. In fact, it has been shown that chondrocytes from healthy and diseased cartilage produce interleukin-8 (IL-8), growth-related gene product (GRO-α), monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein (MIP-1α) and regulated upon activation normal T cells expressed and secreted (RANTES) [3]. Moreover, it has been recently reported that chondrocytes respond to CC and CXC chemokines by releasing matrix metalloproteinase-3 and N-acetyl-β-D-glucosaminidase, thus contributing to the cartilage matrix catabolism [4]. Chemokine effect on target cells is mediated by a family of 7-transmembrane G-protein-coupled receptors, now including 20 members: 5 CXC receptors, 11 CC receptors, plus XCR1, CX3CR1, Duffy and D6 [5]. Most receptors recognize more than one chemokine, and several chemokines bind to more than one receptor, indicating redundancy and versatility of the system, but CC- and CXC-chemokine receptors only recognize members of the corresponding subfamily [2]. Chemokine receptors are widely expressed...
on human leucocytes, having a regulatory effect on their maturation and traffic, and are thought to be implicated in several disease states including allergy, psoriasis, atherosclerosis, and malaria [6]. Chemokine receptors are also present in other cell lines [7, 8], and have been reported to be expressed in human chondrocytes [4].

Following this evidence, we have studied the expression of several chemokine receptors in chondrocytes from healthy and diseased cartilage.

Materials and methods

Cartilage samples and chondrocyte culture

For immunohistochemistry, cartilage biopsy specimens were obtained during knee arthroscopy of 37 patients. Seventeen (7 men, 10 women) had been diagnosed as having osteoarthritis (OA) based on clinical, laboratory and radiological findings (mean age 62 yr, range 50–72). Twenty patients (11 men, 9 women) had inflammatory arthritis (IA) [either rheumatoid arthritis (RA) meeting the criteria of the American Rheumatism Association (formerly, the American Rheumatism Association) [9] (n = 7) or psoriatic arthritis (PsA) (n = 13)] (mean age of IA patients 54 yr, range 24–73). With the patient under local anaesthesia, a Hamou–Storz microarthroscope (Carl Storz, Tuttinglen, Germany) was introduced into the joint cavity of the knee, with the knee kept at 30° flexion. Cartilage biopsy samples were obtained from the medial condyle in non-weight-bearing areas, as previously described [10], and immediately processed by freezing in liquid nitrogen after OCT (Tissue-Tek, Sakura, Torrance, CA, USA) embedding. Cryostat sections (4 μm thick) were obtained from the cartilage specimens, air-dried, fixed in acetone at 4°C for 10 min and stored at −80°C until analysis. Articular cartilage samples were also obtained from five multi-organ donors (mean age 42 yr, range 25–58) at the time of organ explantation, considered as normal cartilage on the basis of morphological examination, and used both for immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR).

For RT-PCR, articular cartilage samples were also obtained from two patients with OA and two with RA, undergoing hip or knee joint replacement. Tissues were finely minced, and chondrocytes were isolated by sequential enzymatic digestion at 37°C; 0.1% hyaluronidase for 30 min, 0.5% pronase for 1 h, and 0.2% collagenase for 1 h (all from Sigma, St. Louis, MO, USA) were used, diluted in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 25 mM HEPES (Sigma), 100 IU/ml penicillin (Biological Industries, Kibbutz Beit Haemek, Israel), 100 mg/ml streptomycin (Biological Industries), 50 mg/ml gentamicin (Flow, Bioggio, Switzerland) and 2.5 mg/ml amphotericin B (Biological Industries). The isolated chondrocytes were filtered with 100-μm and 70-μm nylon meshes, washed in DMEM, gently centrifuged at 1000 g at 4°C and stored dry at −80°C until processing. RNA extraction was done according to the TRIzol protocol (Gibco BRL), and the extracted RNA was evaluated with a spectrophotometer for quantity and purity.

Informed consent was obtained from each patient. The study was approved by the ethics committees of the Rizzoli Orthopaedic Institute.

Immunohistochemistry analysis

Rehydrated serial sections were incubated overnight at 4°C with monoclonal antibodies (mAb) anti-hCCR1, 2, 3, 5 (R&D Systems, Minneapolis, MN, USA) at a concentration of 20 μg/ml, and with mAb anti-hCXCR1, 2, 3, 4 (R&D Systems) at a concentration of 50 μg/ml. The reacting antibodies were detected using a biotin-labelled goat anti-mouse antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at a concentration of 2.5 μg/ml followed by a streptavidin-AP conjugate (Boehringer Mannheim, GmbH, Germany) at a concentration of 0.5 IU/ml. The reactions were developed using a new fuchsin substrate solution (Dako, Glostrup, Denmark) supplemented with 5 mM levamisole to block endogenous alkaline phosphatase. After nuclear counterstaining with haematoxylin, sections were mounted in glycerol gel and stored for subsequent analysis. At microscopic evaluation after staining, a few sections were judged not suitable for analysis due to gross tissue damage or paucity of tissue and were not used for subsequent quantification. Negative staining control experiments were performed according to the above-described procedure, with the omission of the primary antibody. Specificity control was assessed using mouse IgG1, 2A, 2B isotype control (R&D Systems) at an appropriate dilution. Positive chondrocytes were manually counted by an evaluator blinded to diagnosis. Results were expressed as percentage of positive chondrocytes on the total number of chondrocytes.

Reverse transcriptase-polymerase chain reaction

One microgram of each RNA sample was reverse transcribed for 1 hr at 42°C with 2.5 mM oligo(dT), and 250 IU of Moloney murine leukemia virus Reverse Transcriptase (all from Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. Fifty nanograms per tube of the various complementary DNAs were used in the subsequent PCR reaction, which was performed with 1.25 IU Taq with a 1:28 molar ratio of Taq Start antibody (Sigma), 1.5 mM MgCl2, 0.2 mM dNTPs (all from Applied Biosystems), and 0.5 mM for the primers. The thermocycling profile consisted of 4 cycles at 95°C for 10 s, 55°C for 10 s, and 72°C for 15 s, followed by 40 cycles at 90°C for 10 s, 60°C for 10 s, and 72°C for 15 s. Out of the total number of receptors analysed, three were randomly chosen for RT-PCR. Primers were designed using the PRIME program (Genetics Computer Group, Madison, WI, USA) and sequences were as follows: for CCR1, 5'-CAC-CAC-AGA-GGA-CTA-TGA-CAC-3' (sense) and 5'-GCC-TCA-CAA-AAA-ACT-GAG-3' (antisense), yielding a 197-bp product; for CCR5, 5'-GCT-ATT-TGC-ATG-ATC-3' (sense) and 5'-GGA-CTG-TTA-CCT-CAA-CAC-3' (antisense), yielding a 320-bp product; for CXCR1, 5'-GCC-ATT-TTG-ATT-ACC-3' (sense) and 5'-CTG-GAG-TCA-ATG-TGA-CAG-3' (antisense), yielding a 389-bp product.

Statistical analysis

The Kruskal–Wallis ANOVA test for multiple comparison of unpaired data was used, followed by the Mann–Whitney U-test (to compare variables between groups) when ANOVA was significant. The analyses were performed using CSS Statistical Software (Statsoft Inc., Tulsa, OK, USA).

Results

Figure 1 shows a typical result, comparing an OA sample (A), which is highly positive and an IA sample (B), which is negative. As shown in Figs 2 and 3, the main findings of the study can be summarized as follows. Cartilage samples from multi-organ donors expressed all receptors in a
FIG. 1. A. Cartilage sample from an osteoarthritis (OA) patient stained for CXCR3. B. Cartilage sample from an inflammatory arthritis (IA) patient stained for CXCR3 (original magnification ×125).

FIG. 2. CC chemokine receptor expression by chondrocytes from patients with osteoarthritis (OA), inflammatory arthritis (IA), and from normal donors (DON). Boxes show 25th and 75th percentiles. Squares within boxes show medians. Vertical lines below and above boxes show 10th and 90th percentiles. Statistically significant differences (OA vs IA) are indicated as follows: (▲) $P < 0.005$, (●) $P < 0.01$, (●) $P < 0.05$.

FIG. 3. CXC chemokine receptor expression by chondrocytes from patients with osteoarthritis (OA), inflammatory arthritis (IA), and from normal donors (DON). Boxes show 25th and 75th percentiles. Squares within boxes show medians. Vertical lines below and above boxes show 10th and 90th percentiles. Statistically significant differences (OA vs IA) are indicated as follows: (▲) $P < 0.001$, (●) $P < 0.01$, (●) $P < 0.05$. 
high percentage, usually exceeding OA sample expression. OA-affected chondrocytes showed a moderate to high expression of all receptors, significantly higher than that in IA in all receptors but CCR2 and CXCR4. Finally, we found no difference between RA and PsA patients. In particular, both groups showed a very low expression of CC and CXC receptors, most of the samples analysed being substantially negative. For this specific reason we decided to include the two groups under the same label of inflammatory arthritis.

Moreover, mRNA for CCR1, CCR5 and CXCR1 were found to be present in normal, OA and RA chondrocytes (data not shown).

**Discussion**

The aim of the study was to evaluate the contribution of chemokine receptors in cartilage pathophysiology, by analysing the expression of such receptors in cartilage tissue samples from healthy and diseased joints. We show here a peculiar pattern of expression, characterized by moderate to high staining in OA-affected chondrocytes and in multi-organ donors cartilage, and a low to absent positivity for almost all receptors analysed in IA samples.

A high percentage of normal and OA-affected chondrocytes express chemokine receptors. Previous studies from our laboratory have underlined that chondrocytes are indeed a source of chemokines [3], express functionally active receptors and respond to CC and CXC chemokines by releasing matrix metalloproteinase-3 and N-acetyl-β-d-glucosaminidase [4]. Moreover, a recent paper from Yuan et al. [11] confirms the presence of CCR2 and CCR5 in OA and normal chondrocytes by RT-PCR, flow cytometry and immunofluorescence staining. These observations point towards a role of chemokines and their receptors in normal cartilage turnover, suggesting that chemokines may be normally implicated in physiological processes of matrix remodeling. On the other hand, the presence of chemokine receptors in OA samples at levels comparable with those in normal subjects needs to be analysed. It is important to note that in our OA samples such expression is extremely variable, ranging from 0 to 80% of positive cells, while in normal cartilage values have a smaller range. This can be in line with differences in the cartilage inflammation pattern in OA patients. In fact, our OA-affected population is extremely variable in terms of disease duration, severity and use of anti-inflammatory drugs (data not shown). Therefore, one can suggest that, in OA cartilage, chemokine receptor expression can be modulated and diverted from a physiological condition depending on the degree of inflammation. Moreover, for those OA samples showing a similar expression to normal cartilage, it can be suggested that chondrocytes may respond to either an anabolic or catabolic path, depending on ligand dose and time of exposure to the ligand itself. In particular, in OA, pro-inflammatory stimuli derived from both the synovium and the cartilage [12–14] can enhance chondrocyte chemokine production [3] and cause enzymatic tissue degradation [4]. Consequently, it is possible to postulate that in pathological conditions chondrocytes may be pushed by several pro-inflammatory molecules to overwhelm homeostatic processes toward cartilage catabolism. It is worth noting that, in their recent paper, Alaaeddine et al. [15] show no expression of CCR1 in normal cartilage. This is in contrast not only with our results but also with results from Borzi et al. [4] showing expression of this receptor using immunohistochemistry, flow cytometry and RT-PCR. Differences in tissue processing (freezing in liquid nitrogen vs paraffin embedding), antibody concentration or staining detection can deeply affect the sensitivity of the test, and be the cause of such discrepancy.

Furthermore, we show here that chondrocyte expression of chemokine receptors differs between OA and IA patients. Our results confirm the presence of such receptors in normal and disease-affected chondrocytes, but add important information to previous findings [3, 4], introducing the concept of a different role and regulation of the chemokine/receptor system in different pathological conditions. It has been recently demonstrated in neutrophils that tumour necrosis factor (TNF)-α induces down-modulation of IL-8 receptors, which is dramatically inhibited by metalloproteinase inhibitors [16]. Consequently, it can be hypothesized that in the presence of large amounts of pro-inflammatory cytokines, chemokine receptors undergo a proteolytic cleavage with significant loss of staining intensity. This could be the case in IA where cartilage is the target of cytokines (mainly IL-1β and TNF-α) and chemokines produced by the inflamed synovium [14, 17], and produces chemokines itself by consequence of such stimulation [3]. This could result in the production of large quantities of metalloproteinases and subsequent chemokine receptor down-modulation. This hypothesis is also supported by our RT-PCR results, showing the presence of chemokine receptor mRNA in RA chondrocytes, thus suggesting that such down-modulation could be a post-transcriptional event. Furthermore, no significant difference is noted between RA and PsA in any of the receptors analysed, suggesting that, as far the chemokine/receptor system is concerned, the final pathogenetic mechanism is similar in the two inflammatory arthropides.

Finally, it is interesting to note that in all samples analysed, CXCR4 expression is comparable. This receptor seems to be essential for life at least in mice [5], and has biological functions significantly different from those of other chemokine receptors [6]. It may fulfil some essential function or be constitutively expressed and its presence not influenced by molecules produced during joint inflammation. Therefore our results may be consistent with a different role and regulation of CXCR4 compared with the other receptors.
To conclude, in the present study we show different patterns of chemokine receptor expression in healthy and diseased cartilage. On the one hand, the presence of these receptors and their ligands in normal chondrocytes confirms the role of chemokines in cartilage homeostasis. On the other hand, the different receptor expression in pathological samples leads us to postulate that the balance between catabolism and anabolism can be deeply affected in inflammatory conditions, leading to enhanced activation of catabolic processes and consequent cartilage breakdown.

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