Flow cytometric analysis of intracellular chemokines in chondrocytes in vivo: constitutive expression and enhancement in osteoarthritis and rheumatoid arthritis

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Abstract Chemokines play a key role in modulating leukocyte functions at sites of inflammation. To assess chondrocyte contribution to the chemotactic environment of inflamed joints the intracellular content of CC and CXC chemokines was investigated. IL-8, GROα, MCP-1, RANTES, MIP-1α and MIP-1β expression was evaluated by flow cytometric analysis and RT-PCR in chondrocytes isolated from cartilage specimens obtained from patients with osteoarthritis and rheumatoid arthritis and multiorgan donors as normal controls. All the chemokines except RANTES were found in normal chondrocytes, with different degrees of staining intensity. In osteoarthritis and rheumatoid arthritis patients, an enhancement of IL-8, GROα, MIP-1α and MIP-1β was observed.

Key words: Chemokine; Chondrocyte; Flow cytometry; Osteoarthritis; Rheumatoid arthritis

1. Introduction

The role of chemokines as second step mediators of local inflammation has recently gained increasing evidence [1,2]. The ability of chondrocytes to express in vitro some members of this expanding family of molecules has been reported. These cells release interleukin-8 (IL-8) when stimulated with interleukin-1 (IL-1), tumor necrosis factor α (TNFα), or lipopolysaccharide (LPS) [3–5]. IL-8 was localised in vivo in cartilage derived from rheumatoid arthritis (RA) and osteoarthritis (OA) patients with a distribution resulting in a chemotactic gradient favouring localisation of neutrophils to the joint lumen: an average 20% positive cells in joint surface in both diseases [6]. IL-1β induction of GROα in normal chondrocytes was described by Recklies and Golds [5] who found that these cells produce all the members of the GRO family when cultured in monolayer, while only mRNA for GROγ was detectable in cartilage explants.

These data were recently confirmed by Heller et al. [7] who, using a cDNA microarray of human proinflammatory genes, found in primary chondrocytes low constitutive levels of RANTES and a strong upregulation of IL-8 and GROα upon activation with phorbol myristate acetate and IL-1.

Among the CC chemokines, the induction of MCP-1 by IL-1, TNFα, LPS, platelet-derived growth factor and transforming growth factor β in cultured chondrocytes and in organ cultures was reported by Villiger et al. [8]. In addition, the temporal kinetics and zonal distribution details of IL-1 effects across the various cartilage layers were investigated by in situ hybridisation on cartilage organ cultures.

Overall, most of these reports are focused on the expression of the prototypic members of the CC and CXC chemokine subfamilies upon stimulation with proinflammatory activators, and a wide analysis of the chondrocyte chemokine profile in vivo is lacking.

The aim of the present study was therefore to investigate intracellular chemokine expression in freshly isolated chondrocytes derived from healthy subjects and patients with OA and RA, to disclose a disease-related chemokine profile in vivo. Freshly isolated chondrocytes were used, to avoid the possibility of altering the phenotypic pattern of proteins in cultured cells. The staining of the cytoplasmic chemokines was performed in suspension, followed by flow cytometric analysis, thus exploiting the great analysis potential of this technology and the superior sensitivity of immunofluorescence particularly when performed on intact cells. A parallel assessment of the expression of these chemokines at the RNA level was also undertaken, by RT-PCR.

Chondrocytes have been repeatedly likened to cells of the monocyte-macrophage lineage both at the functional [9,10] and at the phenotypic level [11]. We included in our analysis several chemokines produced by monocytes upon stimulation, belonging to either the CXC (IL-8 and GROα) or the CC family (MCP-1, RANTES, MIP-1α, MIP-1β).

2. Materials and methods

2.1. Cell isolation

Chondrocytes were isolated from specimens obtained from eight OA patients (mean age 62.5, range 23–76) and five RA patients (mean age 63.4, range 53–74) undergoing joint replacement and from cartilage obtained immediately post mortem from two healthy multiorgan donors (age 24 and 57). Informed consent from the patients and approval by the ethical committee of the hospital were obtained.

Cells were isolated by sequential enzymatic digestion as previously described [12].

After isolation, cell count and viability were assessed and the cells were fixed with filtered, freshly prepared 4% paraformaldehyde in PBS for 2 h at room temperature (RT), then washed twice with PBS and kept at 4°C in DEPC-treated PBS at a concentration of 4×10^6/ml until used.

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2.2. Chemokine staining and flow cytometric analysis

The monoclonal antibodies (mAbs) against human chemokines were: anti-IL-8 (PeproTech, Rocky Hill, NJ, USA), anti-GROα (R&D Systems, Minneapolis, MN, USA), anti-MCP-1 (PeproTech), anti-RANTES (R&D Systems), anti-MIP-1α (R&D Systems) and anti-MIP-1β (R&D Systems). For labeling experiments 10^5 cells were processed in 0.5 ml tubes, washing at $3200 \times g$ between each step.

Aliquots of 25 μl from cell stock containing this amount of cells were centrifuged with an equivalent volume of saponin buffer (0.1% saponin and 0.1 M HEPES buffer in HBSS). After a permeabilisation step (10 min at RT soaking in 0.1% saponin and 0.01 M HEPES buffer in HBSS) the cells were pelleted and incubated with 10 μg/ml of the various mAbs in saponin buffer for 30 min at RT. After washing with saponin buffer, a goat anti-mouse FITC conjugate (Becton Dickinson, San Jose, CA, USA) at 12.5 μg/ml was added and incubated for 20 min at RT in the dark. After washing the cells were analysed on a FACStar Plus cytometer (Becton Dickinson). For comparison between experiments, fluorescein quantitative standard beads (Flow Cytometry Standards, Research Triangle Park, NC, USA) were used to quantify the fluorescence signal, constructing a standard curve of fluorescence to represent the $\log_{10}$ of the fluorescence intensity in molecules of equivalent soluble fluorochrome (MESF) as a function of the mean channel value. In cases of bimodal distribution of the fluorescence signal, the mean channel value of the more representative portion of the sample was taken into account. The experimental calibration curve was $y = 0.004x + 2.71$ (Pearson coefficient = 0.999) obtained from the following data: fluorescence intensity in MESF/mean channel 0/17.9; 2500/176.5; 6500/270.8; 19 000/395.5; 55 000/499.4; 150 000/621.3.

2.3. Immunohistochemistry

Cryostat sections of 5 μm thick were fixed with absolute acetone for 10 min at 4°C and then air dried. After a rehydration step with 0.1% saponin in Tris-buffered saline pH 7.4 (TBS), blocking of non-specific binding was performed by incubating the sections for 30 min in PBS with 2% bovine serum albumin. All washes between the various steps were performed in TBS with 0.1% saponin at RT. Anti-chemokine mAbs were incubated overnight at RT at the following concentrations: 5 μg/ml (anti-IL-8), 10 μg/ml (anti-MIP-1α) and 20 μg/ml (anti-GROα). After washing a biotinylated goat anti-mouse antiserum (Kirkegaard and Perry labs, Gaithersburg, MD, USA) at 1.5 μg/ml was incubated for 1 h at RT followed by alkaline phosphatase streptavidin conjugate diluted 1:2000 (Boehringer, Mannheim, Germany). New fuchsin (Dako, Glostrup, Denmark) in the presence of 5 mM levamisole (Dako) to block endogenous alkaline phosphatase activity, was used as substrate to detect chemokine positive cells.

2.4. RNA extraction and RT-PCR

Soon after isolation the cells were gently pelleted at $1000 \times g$ at 4°C and stored dry at −80°C until processing.

RNA extraction was done according to the TRIzol protocol (Gibco BRL, Grand Island, NY, USA) and the extracted RNA was evaluated with the spectrophotometer for quantity and purity. 1 μg of each sample was reverse transcribed for 1 h at 42°C with 2.5 μM Oligo-dT and 250 U of MuMLV RNase H− (Promega, Madison, WI, USA), according to the manufacturer's instructions. 50 ng per tube of the various cDNAs were used in the subsequent RT-PCR performed with 2.5 U Taq with equimolar concentration of Taq Start antibody (Clontech, Palo Alto, CA, USA) at pH 8.3 and 1.5 mM magnesium, 200 μM dNTPs and 1 μM concentration for the primers. The thermocycling profile comprised four cycles at 94°C 10 s, 56°C 10 s, 72°C 10 s and 31 cycles at 90°C 10 s, 60°C 10 s, 72°C 10 s. Primer sequences were such as to amplify respectively: a 219 bp product for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as an internal quality standard (upstream primer corresponding to nt 771–788 and downstream primer complementary to nt 971–989 of M17851); a 289 bp product for IL-8 (upstream primer corresponding to nt 771–788 and downstream primer complementary to nt 971–989 of M17851), a 164 bp product for GROα (upstream primer corresponding to nt 154–172 and downstream primer complementary to nt 297–317 of X12510), a 353 bp product for MCP-1 (upstream primer corresponding to nt 24–47 and downstream primer complementary to nt 351–377 of X14768), a 275 bp product for RANTES (upstream primer corresponding to nt 27–48 and downstream primer complementary to nt 282–302 of M21121), a 300 bp product for MIP-1α (upstream primer corresponding to nt 79–97 and downstream primer complementary to nt 361–379 of M23542) and a 286 bp product for MIP-1β (upstream primer corresponding to nt 106–127 and downstream primer complementary to nt 370–392 of J04130).

3. Results

3.1. FACS analysis

Chemokines were detected in vivo by intracellular staining of chondrocytes isolated from eight OA patients, five RA patients and two multiorgan donors (MD). Flow cytometric analysis indicated differential magnitudes of the signal for the different chemokines tested, as assessed by evaluation of the increments over control fluorescence intensity (Fig. 1). It is interesting to point out that the mean values of fluorescence increments obtained in the three groups under study were strictly correlated (Kendall $Tau = 0.52$, $P = 0.006$) with the mean values of corresponding chemokine concentration (ng/ml) in the supernatant of unstimulated chondrocytes obtained from the same groups (data not shown, obtained from two

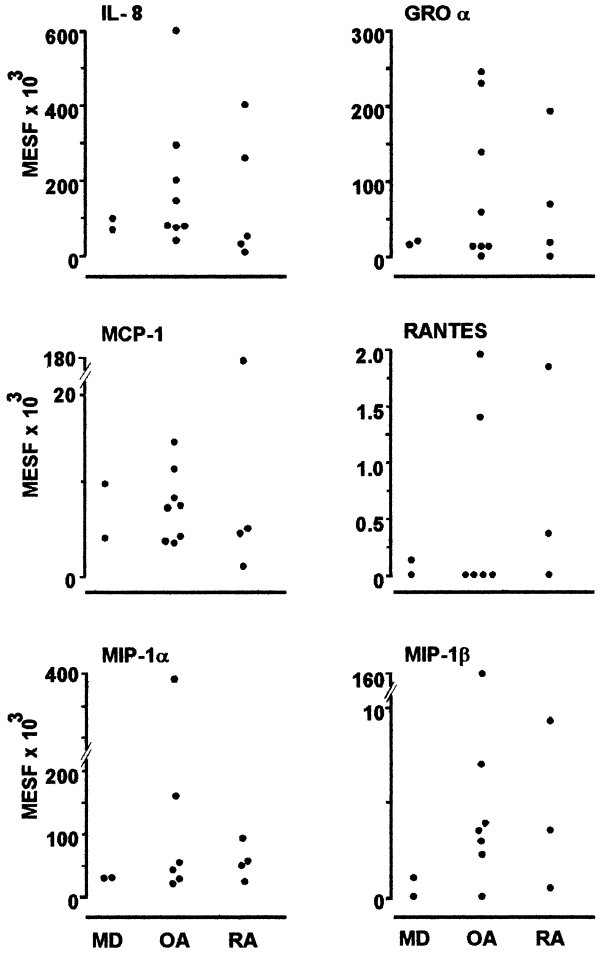

Fig. 1. Indirect immunofluorescence staining of intracellular chemokines: fluorescence signal of labeled chondrocytes obtained from multiorgan donors (MD), osteoarthritis patients (OA), and rheumatoid arthritis patients (RA). Results, expressed in molecules of equivalent soluble fluorochrome (MESF), report increments over basal fluorescence intensity of control cells (cells incubated with the fluorescein-conjugated goat anti-mouse immunoglobulins) and indicate an enhanced chondrocyte content of IL-8, GROα, MIP-1α and MIP-1β in OA and RA patients.

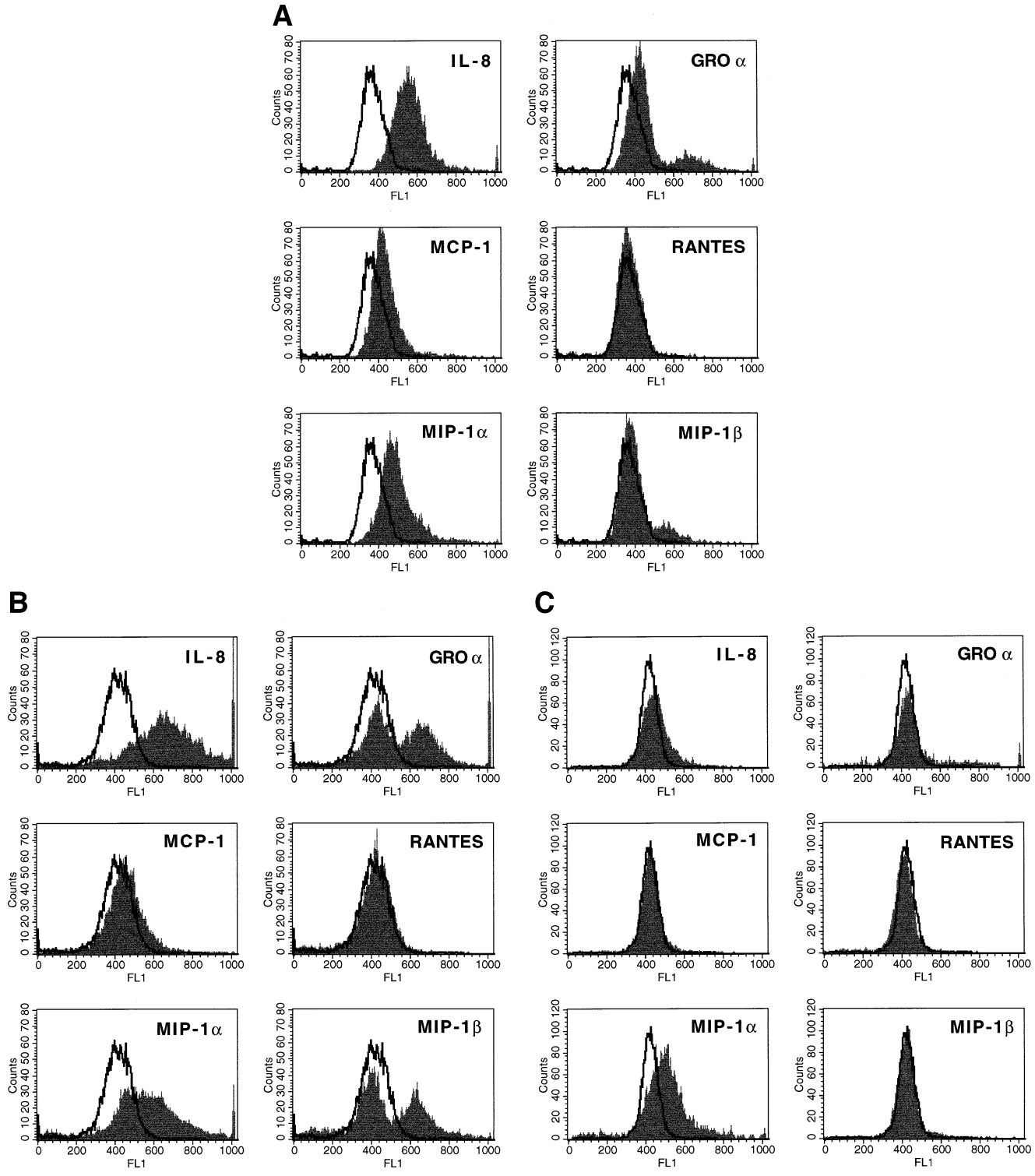

Fig. 2. Flow cytometric data of intracellular chemokine labeling in chondrocytes derived from an organ donor (A), an osteoarthritis patient (B) and a rheumatoid arthritis patient (C). Empty pattern: control cells, incubated with the secondary antibody only; solid pattern: cells incubated with the chemokine-specific monoclonals.

controls, 10 OA and 11 RA cases). OA and RA chondrocytes showed an enhancement of intracellular content of IL-8, GROα, MIP-1α and MIP-1β. Chondrocyte chemokine profiles representative of the two organ donors and of OA patients are shown in Fig. 2A and Fig. 2B respectively.

It is noteworthy that in one RA case chondrocytes were nearly devoid of all the chemokines except MIP-1α (Fig. 2C).

3.2. RT-PCR

The overexpression of chemokines in OA and RA compared to normal controls was confirmed also in RT-PCR (Fig. 3), performed in one case for each group.

3.3. Immunohistochemistry

The intense positivity for IL-8 in intact normal cartilage

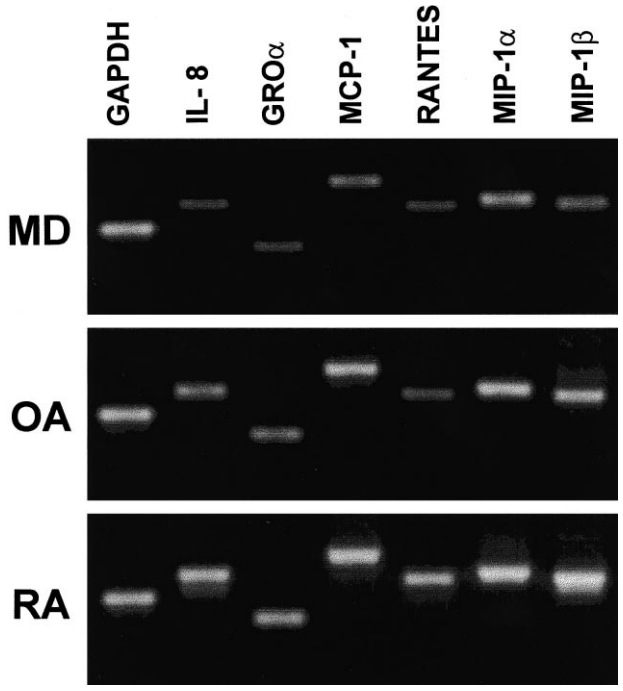

Fig. 3. RT-PCR of IL-8, GROα, MCP-1, RANTES, MIP-1α and MIP-1β starting with 50 ng cDNA from normal chondrocytes (MD), osteoarthritic chondrocytes (OA) and rheumatoid arthritis chondrocytes (RA). GAPDH was used as an internal control to ensure equal loading between samples. Band intensities indicate an enhancement of transcription for most chemokines in OA and RA patients.

was confirmed also in immunohistochemistry performed in one MD case, thus excluding the possibility of the flow cytometric results being an artifact of the isolation process (Fig. 4). Compared to IL-8, signal intensities for GROα and MIP-1α were lower (data not shown), in agreement with the results of flow cytometric analysis (mean values of fluorescence increments for the two MD: IL-8 = 82 514 MESF, GROα = 16 802 MESF, MIP-1α = 28 916 MESF).

4. Discussion

The present study extensively describes the analysis of chondrocyte chemokine profile in vivo. The main finding is that chondrocytes derived from healthy joints store differential amounts of chemokines in intracellular organelles with no need for previous stimulation with proinflammatory cytokines. Chondrocytes could therefore be ready to amplify an inflammatory response because they already contain stored chemokines which can modulate leukocyte responses. The presence of chemokines in normal cartilage might fulfil a physiological role which has yet to be elucidated. In OA and RA, a strong upregulation may occur, possibly due to the effect of IL-1 and TNFα. Our data are not in agreement with the findings of Recklies and Golds [5] and Deleuran et al. [6]: Recklies and Golds monitored the de novo synthesis of IL-8 and of the chemokines of the GRO family in chondrocytes and cartilage explants stimulated with IL-1β and reported that when exposed in their natural environment chondrocytes express IL-8 mRNA and only the GROγ mRNA among the GRO family. In contrast we found expression of GROα in uncultured normal chondrocytes and an enhancement of transcription in the OA and RA patients. Partially accounting for this discrepancy could be the fact that isolation of pure RNA from cartilage is difficult, due to the low cell density and the interfering high glycosaminoglycan content. Second, we designed GROα primers to amplify a small fragment, comprised of the coding sequence, which leads to a more successful amplification than the long fragment used by Recklies and Golds, taking into account the high rate of RNA turnover of inducible proteins. To our knowledge the only report dealing with the in vivo expression of chemokines in chondrocytes was the work of Deleuran et al. who only found IL-8 in nearly 20% of superficial chondrocytes in OA and RA patients. Our flow cytometric and immunohistochemistry data show instead that all chondrocytes are positive, possibly because of the superior sensitivity of immunofluorescence on intact cells and immunohistochemistry coupled with an avidin-biotin detection over the APAAP technique [13]. Thus, although at different levels, all the chemokines we analysed are present in OA and RA chondrocytes, and could therefore contribute to the chemotactic environment of synovial fluid (SF), whose composition may reflect differing pathophysiologic mechanisms operational in these diseases. This could be particularly relevant for osteoarthritis, given the absent or minor role of synovial tissue inflammation. On the other hand, most chemokines have been found at far higher levels in RA SF compared to OA (GROα [14]; MCP-1 [15]; MIP1α [16]), due to the contribution of the inflamed synovial tissue. It has been reported that the combined action of adhesion molecules, chemokines and chemokine receptors provides an address code for leukocyte extravasation and migration into tissue [17]. Therefore, it is conceivable that the different cell composition of SF in OA and RA depends on the different chemokine composition of the SF itself.

The prominent cell type in RA SF is polymorphonuclear cells [18] recalled by a chemotactic activity which is accounted for by IL-8, GROα and ENA78 [14]. Instead, mononuclear cells (MNC) are prevalent in non-inflammatory SF, with

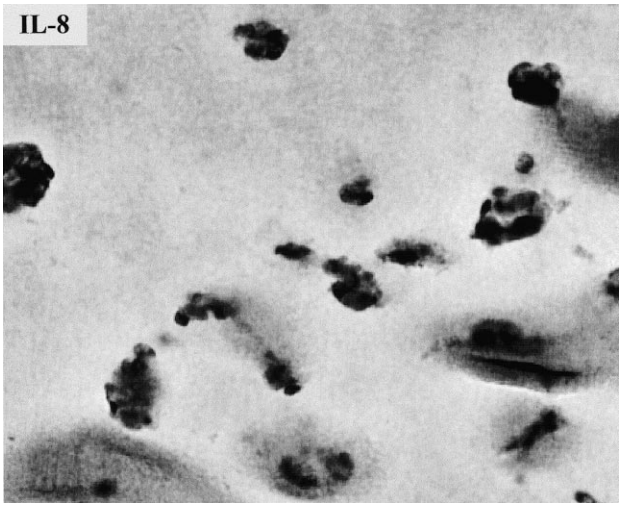

Fig. 4. Immunohistochemistry of a 5 μm thick normal cartilage specimen (multiorgan donor): all chondrocytes stained positive for IL-8 (avidin-biotin technique with streptavidin-alkaline phosphatase, new fuchsin as substrate and hematoxylin counterstaining). The image was rendered in a grey scale by acquisition with a monochrome charge-coupled device camera (Sony, Kangawa-ken, Japan). Magnification ×162.5.

monocytes being the most prominent cell type [19]. It has been reported that a relevant fraction of OA SF chemotactic activity for monocytes and particularly for activated monocytes is represented by MIP-1β, which is present at a far higher concentration compared to RA SF [20]. Our data suggest that chondrocytes might greatly contribute to MIP-1β concentration in SF.

The MNC composition of OA and RA SF has been compared by Fort and coworkers [18]: although RA SF presented a significantly greater number of cells of all types, some significant differences were found in the percentages: CD3+ (T lymphocytes) and CD4+ (T helper cells) percentages were higher in RA, while percentages of CD14+ (i.e. macrophage/monocytes) and CD16/CD56 (i.e. natural killer cells) were higher in OA. Other authors [21] have also reported that the great majority of CD4+ cells are CD45RO+ and CD45RA− in both patient groups. Mast cells also seem to play a relevant role in inflammation and cartilage breakdown in OA: mast cell counts [22] and mast cell activation indices [23] are higher in OA compared with other joint diseases.

Concerning RANTES, the magnitude of the signal for this cytokine was far lower, but a significant increase was observed in some OA and RA patients.

In conclusion, chondrocytes store large amounts of chemotactic factors, which can be further enhanced in some pathological conditions, following exposure to proinflammatory cytokines. The same factors lead to release of these chemoattractants from the cells to the matrix, as could be the case for the patient with RA who appeared to be nearly negative for most chemokines. The proteoglycan-rich matrix, then, could play a key role in conditioning the release of chemokines into synovial fluid, which can be modulated by differences in their affinity for matrix components [5,8].

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