Interleukin-10 and interleukin-10 receptor in human osteoarthritic and healthy chondrocytes

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Abstract

Objective

To evaluate the expression of interleukin-10 (IL-10) and interleukin-10 receptor (IL-10R) on chondrocytes from healthy, osteoarthritic, and foetal cartilage from human subjects.

Methods

Articular cartilage was obtained from 12 patients with osteoarthritis (OA) undergoing surgical knee replacement. Chondrocytes were isolated from the two zones of cartilage showing macroscopically and histologically the lowest (MIN) and highest (MAX) extent of osteoarthritic damage. Additional specimens of cartilage were obtained from 3 healthy donors and 3 human foetuses.

IL-10 mRNA expression was determined by a reverse transcriptase-polymerase chain reaction (RT-PCR). For detection of intracellular IL-10 protein, chondrocytes were permeabilized and then incubated with R-phycoerythrin (PE) conjugated rat anti-human IL-10 mAb. Cell surface IL-10R was detected by incubation with biotinylated recombinant human IL-10; after washing, bound IL-10 was revealed by fluorescein (FITC) conjugated streptavidin. Positive chondrocytes were analysed by flowcytometry.

Results

IL-10 mRNA expression was higher in osteoarthrtic than in normal chondrocytes. IL-10 protein intracellular levels were significantly higher in MAX than in MIN osteoarthritic cartilage or in healthy cartilage. Cell surface IL-10R was expressed on osteoarthritic chondrocytes with no difference in the degree of cartilage damage. The highest levels of IL-10 protein and IL-10R were found in foetal cartilage.

Conclusion

Human chondrocytes synthesise IL-10 and express on their surface IL-10R. Since IL-10 inhibits IL-1 and TNF- expression, its upregulation in osteoarthritic chondrocytes may counteract the detrimental effects of these catabolic cytokines. However, the functions of IL-10 in cartilage may go beyond those activities established in the immunological setting. The high levels of IL-10 and IL-10R in foetal cartilage, an active growing tissue, suggest that IL-10 may play a role in controlling chondrocyte metabolism under physiological conditions.

Key words

IL-10, IL-10R, cartilage, foetal cartilage.

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This work was partially supported by CNR Italy (grant no. 97.4164.04).

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Received on July 11, 2000; accepted in revised form on November 21, 2000.

Introduction

Under physiological conditions, human adult articular cartilage is a tissue with low metabolic activity, with synthesis and degradation processes in perfect equilibrium. In the initial stage of osteoarthritis (OA) this fine balance breaks down and chondrocyte metabolism increases, perhaps in an attempt to restore normal homoeostasis (1). Chondrocyte activation leads to an increased production of an array of cytokines and growth factors that stimulate both new cartilage formation and degradation of extracellular matrix (ECM) components, the final effect being the derangement of the ECM framework and cartilage failure (2).

The effect of some of these mediators on cartilage is well established. IL-1 and TNF- are catabolic cytokines, as they have been shown to inhibit proteoglycan synthesis and to promote metalloprotease production by chondrocytes (3). On the contrary, growth factors such as IGF-1, bFGF, TGF- and BMP family members stimulate the deposition of new cartilage matrix (4, 5).

The role of interleukin-10 (IL-10) on cartilage metabolism is less well defined (6, 7). IL-10 is a pleiotropic cytokine encoded by human chromosome 1 (8) that acts on a variety of cell types, exerting either suppressive or stimulatory effects. IL-10 downregulates IL-2 and IFN-gamma synthesis by Th1 T cells (9), reduces the antigen-presenting ability of monocytes (10), and inhibits the production of IL-1, IL-6, IL-8, and TNF- by monocytes and macrophages (11). Furthermore, IL-10 upregulates IL-1 receptor antagonist production by monocytes (12). In addition to these inibitory effects, IL-10 has been shown to have stimulatory functions, as it induces the differentiation of B cells and immunoglobulin secretion (13). IL-10 binds a specific cell surface receptor (IL-10R), which is a tetrameric complex made up of two IL-10R1 and two IL-10R2 chains (14) and is encoded by chromosome 11 (15).

Few studies have explored the effect of IL-10 on chondrocytes. IL-10 can prevent cartilage destruction induced by IL-1 and TNF- (14) and directly stimulate the proteoglycan synthesis of articular cartilage explants (17). The expression of IL-10R on articular chondrocytes has been never investigated so far.

In this study, we evaluated the expression of IL-10 and IL-10R in human articular chondrocytes from healthy donors and OA patients in relation to the degree of cartilage destruction. Human foetal cartilage was also studied in order to explore the putative role of IL-10 signalling in non-damaged, developing cartilage.

Patients and methods

Cartilage specimens

Articular cartilage was obtained from 12 OA patients with "genu varum" (9 females, 3 males, age 54-72 years) undergoing surgical knee replacement. The joint surface was subdivided into two zones showing the lowest (MIN) and highest (MAX) degree of osteoarthritic damage, and samples were taken from each zone. The articular cartilage in the MIN zone resembled normal cartilage with a translucent, smooth, intact surface; in the MAX zone the cartilage surface was yellowish, softened and fibrillated.

Macroscopic findings were validated by histological studies performed on full-thickness specimens biopsied from each zone and stained with safranin-O, toluidine blue, and hematoxylin-eosin (Fig. 1). The degree of microscopic cartilage damage was evaluated using the Mankin grading scheme (1).

Healthy knee cartilage from 3 human donors (all males, age 18-33 years) and costal cartilage from 3 human foetuses (13 weeks old) were also studied. Intracellular expression of type I and type II collagen in foetal costal chondrocytes was evaluated by flowcytometry (anti-human type I and type II collagen mAbs, Chemicon, Temecula, CA).

Chondrocyte isolation

Chondrocytes were isolated as described elsewhere (18). Briefly, chondrocytes were released from the cartilage matrix by hyaluronidase (0.2%, 30 min., 37°C, Sigma), pronase (0.25%, 90 min., 37°C, Sigma) and collagenase (0.2%, 3 hours, 37°C,Sigma) enzymatic digestion. More than 95% of the

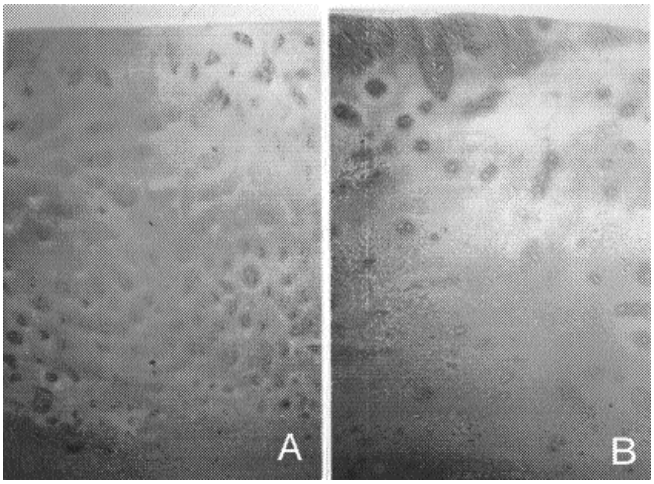

Fig. 1. Photomicrographs of safranin-O-stained histological sections of zones with macroscopically defined minumum (**a**) and maximum (**b**) damage in typical osteoarthritic cartilage (original magnification x 60). In (**a**) adiffuse staining of the cartilage is present, while in (**b**) there is an evident hypocellularity together with a severe reduction of the safranin-O-staining, which in some areas is almost absent.

chondrocytes were viable (Trypan blue exclusion test) after their isolation.

IL-10 reverse transcriptase-polymerase chain reaction (RT-PCR)

Human articular chondrocytes were obtained from the knee joints of 2 healthy donors and 2 osteoarthritic patients. Total RNA was extracted and purified using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. cDNAs were obtained by reverse transcription of 1 g of total RNA using Thermoscript (Life Technologies) with oligo(dT) primer, and equalized for the expression of the housekeeping gene -actin. cDNAs were added to the following PCR mixture: 0.5 UTaq polymerase (Eurogentech), 0.2 mrnol/L dNTPs, 2.5 mg/ml specific primers, 1.5 mmol/L Mg/Cl_2. A negative control (water) was included for every PCR analysis. The PCR reaction was carried out in a Perkin Elmer thermal cycler 9600. After 1 minute denaturation at 95°C, cycles (19 for -actin and 35 for IL-10) were 10 seconds at 94°C, 10 seconds at 60°C, and 30 seconds at 72°C. Cycling was followed by 10 min elongation at 72°C. Primers for -actin were: sense primer 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'; reverse primer 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'. Primers for IL-10 were: sense primer 5'-ACCAAGACCCAGACATCAAG-3'; reverse primer 5'-GAGGTACAATAAGGTTTCTCAAG-3' (19). PCR products were electrophoresed in 1.5% agarose gel in TBE (Tris-borate/EDTA) electrophoresis buffer, stained with ethidium bromide, visualized by UV transillumination, and analysed by densitometry. Amplification products were 352 bp for IL-10 and 661 bp for -actin (Fig. 2).

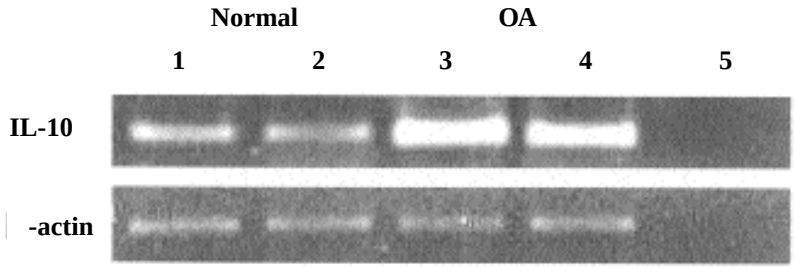

Fig. 2. Interleukin-10 (IL-10) and -actin mRNA expression in normal (lanes 1 and 2) and osteoarthritic (OA) (lanes 3 and 4) human articular chondrocytes. Negative control is in lane 5. Amplification products were 352 bp for IL-10 and 661 bp for -actin. IL-10 mRNA is expressed by chondrocytes at higher levels in OA than in normal cartilage.

Intracellular IL-10 detection

Freshly isolated chondrocytes were resuspended in PBS containing 0.1% sodium azide and 0.2% bovine serum albumin, and blocked by incubating with 2% normal human serum (Advanced Protein Products, UK). After fixation with paraformaldehyde and permeabilization with saponin (Fix & Perm Cell Permeabilization Kit, CaltagLab, Burlingame, CA), chondrocytes were incubated for 20 min at 4°C with 5 ml of R-phycoerythrin (PE) conjugated rat anti-human IL-10 mAb (Pharmingen, San Diego, CA). Control samples were incubated with rat IgG1-FITC/IgG2-PE (DAKO, Denmark). Stained cells were analysed on a FACScan (Lysis 2, Becton Dickinson, Mountain View, CA). The FACS setting was identical throughout the study.

IL-10 receptor (IL-10R) expression

Freshly isolated chondrocytes were incubated with biotinylated recombinant human IL-10 (rhIL-10) (Genzyme, Cambridge, MA) ovenight at room temperature and, after washing, bound IL-10 was detected by incubation with fluorescein (FITC) conjugated streptavidin for 20 min at 4°C. Positive chondrocytes were analysed by flowcytometry.

Statistical analysis

Results are shown as the mean ± 1 SD (standard deviation). The statistical difference among the distinct specimens of cartilage was assessed using a two-way ANOVA with LSD (least square differences) range test. The significance level was set at $p < 0.05$.

Results

Interleukin-10 (IL-10) mRNA expression

Human articular chondrocytes express IL-10 mRNA. Expression levels were higher in OA cartilage than in healthy cartilage as analysed by semi-quantitative RT-PCR (Fig. 2).

Interleukin-10 (IL-10) expression

Intracellular IL-10 protein was detected in all the chondrocyte specimens and expressed both as a percentage of positive chondrocytes and as the mean

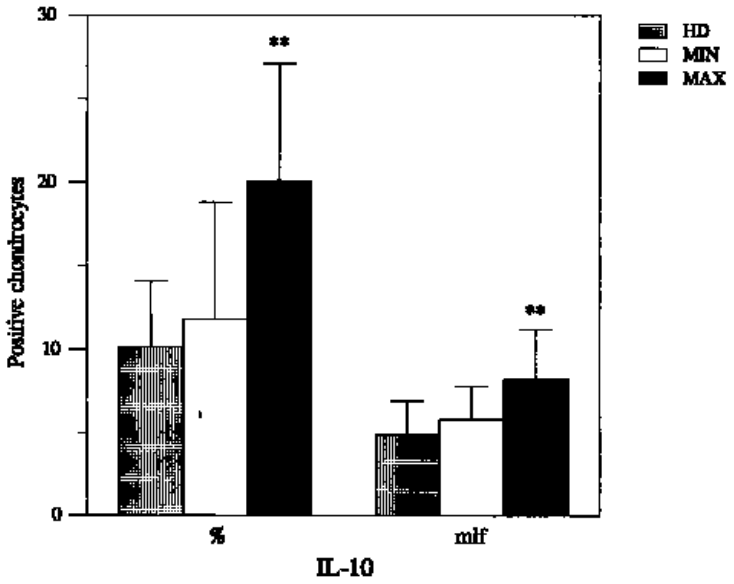

Fig. 3. Percentage (%) and mean fluorescence intensity (mif) of chondrocytes expressing intracellular IL-10 protein in healthy cartilage (HD), in the lowest (MIN), and highest (MAX) damaged zones of osteoarthritic cartilage. Values are expressed as means ± 1 SD.

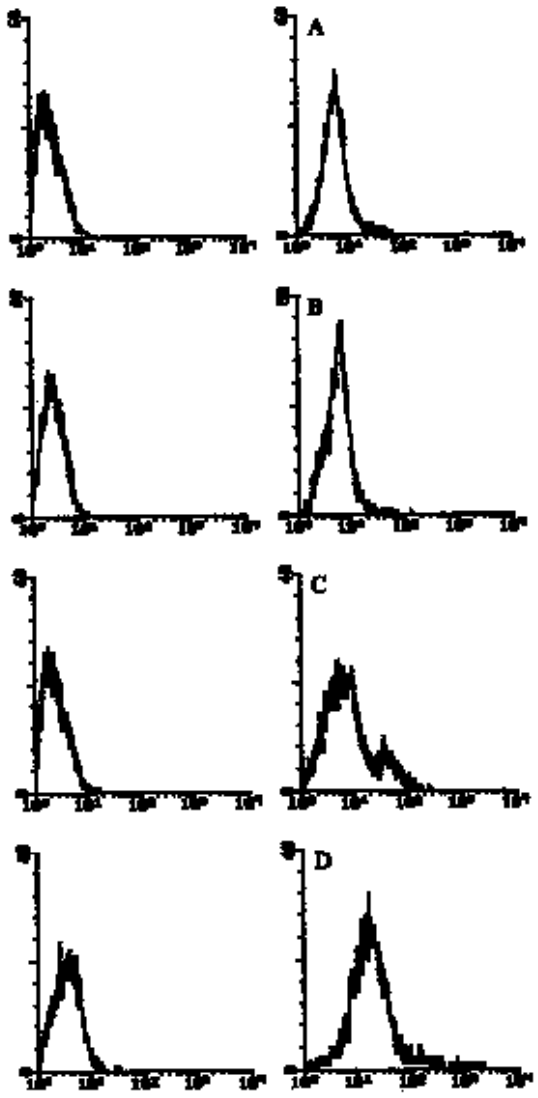

Fig. 4. Fluorescence intensity for intracellular IL-10 from healthy cartilage (**panel A**), from osteoarthritic cartilage with minimal (**panel B**) and maximum (**panel C**) anatomic damage, and from a foetal cartilage (**panel D**). Negative control histograms (a non-binding mAb) are shown on the left. The longitudinal axis shows the percentage of chondrocytes positive for intracellular IL-10 and the horizontal axis the mean channel fluorescence. IL-10 protein expression is definitely low in normal adult and in minimally damaged osteoarthritic cartilage and progressively increases in maximally damaged osteoarthritic and in foetal cartilage.

intensity of fluorescence (mif) (Fig. 3). The percentage of IL-10 positive chondrocytes was significantly higher in MAX (20.1% ± 7) than in either MIN osteoarthritic articular cartilage (11.8% ±7, $p < 0.01$) or in healthy articular cartilage (10.1 % ± 4, $p < 0.01$). No significant difference between healthy chondrocytes and MIN osteoarthritic chondrocytes was found. An identical pattern of expression was found when intracellular IL-10 was evaluated as mif, which is a measure of the density of the antigen per cell. Chondrocyte IL-10 mif was significantly higher in MAX (8.2 ± 3) than in both MIN osteoarthritic cartilage (5.8 ± 2, $p < 0.01$) and healthy chondrocytes (4.9 ± 2, $p < 0.01$). Once again, IL-10 mif did not differ between healthy cartilage and MIN osteoarthritic cartilage.

In Figure 4 representative histograms of the fluorescence intensity for intracellular IL-10 expressed by chondrocytes from healthy, osteoarthritic, and foetal cartilage are shown.

Interleukin-10 receptor (IL-10R) expression

The percentage of osteoarthritic chondrocytes bearing on their surface IL-10R is shown in Figure 5. IL-10R expression was 32.5% ± 15 in MAX and 30.2% ±12 in MIN cartilage; the difference was not statistically significant. The fluorescence intensity for IL-10R was also similar in MAX (9.6 ±3) and MIN (9.8 ± 3) osteoarthritic chondrocytes.

Cell surface IL-10R was detected by binding studies using biotin-conjugated rhIL-10. In order to verify the specificity of IL-10 binding, chondrocytes were incubated with increasing amounts of rhIL-10. As shown in Figure 6, IL-10 binding reached a plateau, demonstrating that IL-10 binding on chondrocytes is mediated by specific receptors that undergo saturation with concentrations of IL-10 ranging between 2 and 2.4 g/ ml.

Foetal chondrocytes

Foetal chondrocytes (Fig. 7) expressed high levels of intracellular type II collagen (92 ± 5%) and low levels of type I collagen (7 ± 3), indicating that these

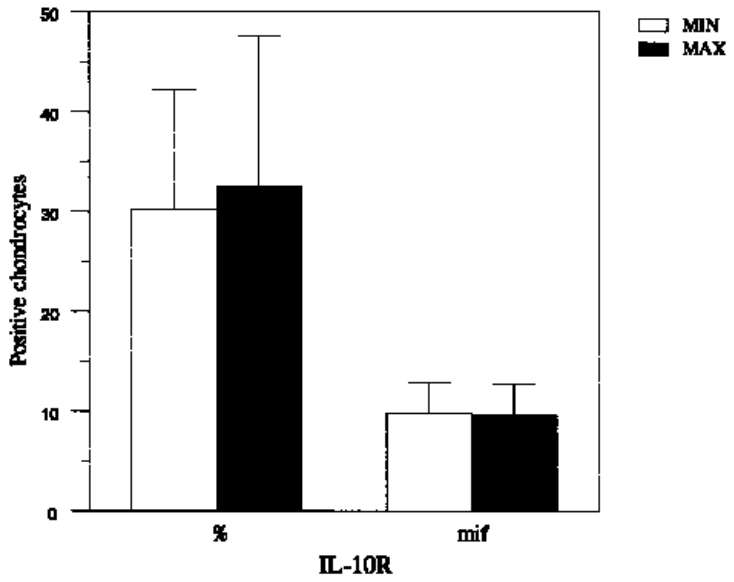

Fig. 5. Percentage (%) and mean fluorescence intensity (mif) of chondrocytes expressing cell surface IL-10R in the lowest (MIN) and highest (MAX) damaged zones of osteoarthritic cartilage. Values are expressed as means ± 1 SD.

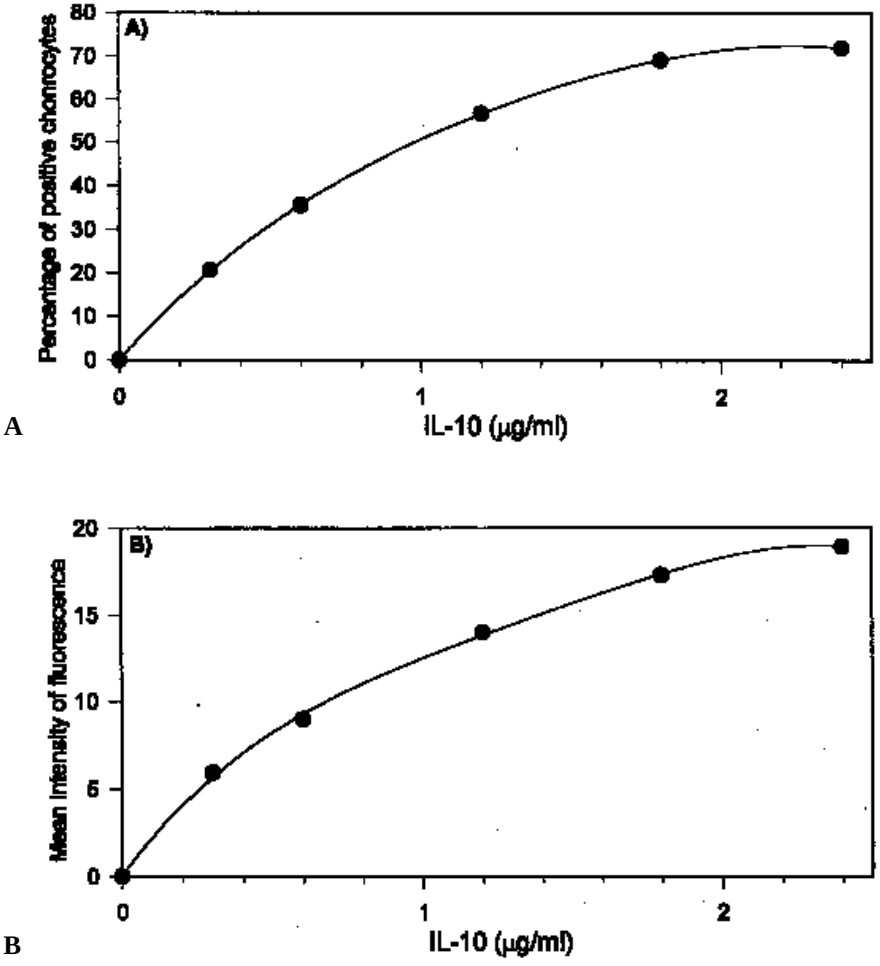

Fig. 6. Percentage (**A**) and fluorescence intensity (**B**) for rhIL-10 of chondrocytes showing the expression of IL-10 receptors that are saturated by increasing amounts of rhIL-10.

cells were already differentiated chondrocytes. Moreover, foetal chondrocytes had the highest levels of intracellular IL-10 protein (33.9% ±8, mif 17.5 ±3) and the highest expression of cell surface IL-10R (54.8% ±15, mif 15.4 ± 4).

Discussion

In this study we have shown that human articular chondrocytes produce IL-10 and express its receptor (IL-10R). By semi-quantitative RT-PCR analysis, IL-10 mRNA expression was demonstrated to be higher in osteoarthritic than in normal cartilage. The levels of intracellular IL-10 protein were low in healthy articular cartilage and progressively increased in osteoarthritic cartilage based on the extent of anatomic damage. The significant increase in IL-10 expression in the osteoarthritic cartilage was expressed in both the percentage of chondrocytes and the fluorescence intensity per cell. The former indicates a recruitment of new chondrocytes producing IL-10 and the latter suggests that an upregulation of IL-10 transcription occurs in cartilage during OA. No regulation of IL-10R expression was found in osteoarthritic chondrocytes.

IL-10 activates a wide range of functional responses in different cell types, inducing either inhibitory or stimulatory effects such as downregulating IL-1 and TNF- synthesis by monocytes (11) or promoting the growth and differentiation of B cells (13). Recently, IL-10 has been detected by immunohistochemistry in osteoarthritic cartilage within and around chondrocytes and its expression was inversely correlated with TNF- immunoreactivity (20). In the latter study, IL-10 expression did not differ between healthy cartilage and macroscopically normal osteoarthritic cartilage, which is consistent with our flowcytometry data which showed similar levels of intracellular IL-10 in healthy chondrocytes and in chondrocytes from osteoarthritic cartilage with minimal anatomic changes. Joosten *et al.* have shown that in murine collagen-induced arthritis (CIA) IL-10 administration can prevent cartilage destruction by reducing IL-1 and

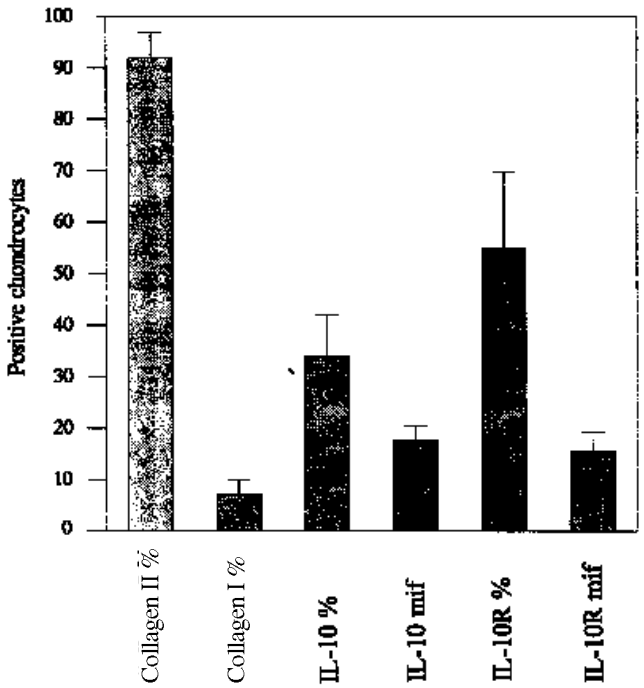

Fig. 6. Percentage (**A**) and fluorescence intensity (**B**) for rhIL-10 of chondrocytes showing the expression of IL-10 receptors that are saturated by increasing amounts of rhIL-10.

TNF- mRNA expression in articular chondrocytes, whilst neutralizing anti-IL-10 antibodies accelerate the onset and enhance the severity of CIA (16). In addition, IL-10 has been shown to directly stimulate proteoglycan synthesis by human chondrocytes *in vitro* (17), without affecting the production of collagenase 3 (21). Therefore, it is conceivable that raised IL-10 levels in osteoarthritic chondrocytes could counteract the degradative effects of the proinflammatory cytokines TNF- and IL-1, which could induce IL-10 production as in rheumatoid arthritis synovitis (22). However, in human fibroblast cells which, like chondrocytes, have a mesenchymal origin [and it is well known that articular chondrocytes during *in vitro* passaging undergo a dedifferentiation process towards a fibroblast-like phenotype (23)], IL-10 downregulates type I collagen mRNA expression and enhances collagenase and stromelysin gene expression (24). This diversity of response indicates that downstream events following IL-10 binding may vary depending on the cell target phenotype and on the environment of that cell.

We also investigated the expression of IL-10R by binding experiments, using biotin-IL10 labeled with streptavidin-FITC. We have shown that the binding is specific as increasing concentrations of IL-10 lead to saturation of the cell receptor. The level of expression of IL-10R on chondrocytes did not change with the progression of osteoarthritic injury. A similar phenomenon in osteoarthritic chondrocytes has been demonstrated for insulin-like growth factor I (IGF-1) (25) and its receptor (24), since IGF-1 expression increases with cartilage damage, whereas IGFR-1 remains unmodified.

In OA the metabolism of cartilage is enhanced. Cell cycle studies have shown that the proportion of chondrocytes in the S-phase increases with the progression of cartilage injury (18). Furthermore, the expression of nuclear Ki-67 antigen, a marker of proliferative activity, is higher in osteoarthritic cartilage than in healthy cartilage (20). We wondered whether the extent of IL-10 expression might be related to the state of cell activation and whether IL-10 may play a physiological role in chondrocyte metabolism. It has already been shown that IL-10 induces the growth and differentiation of B cells by stimulating DNA replication and immunoglobulin production (13). To address this issue, we evaluated IL-10 and IL-10R expression in foetal cartilage, an example of non-pathologic cartilaginous tissue with high metabolic activity. The chondrocyte phenotype of foetal cells was confirmed by flow cytometry showing high intracellular levels of type II collagen. In all the specimens of foetal chondrocytes, IL-10 protein levels and IL-10R expression were much higher than in healthy and osteoarthritic chondrocytes. Obviously, the presence of IL-10 in foetal chondrocytes leads one to speculate on functions other than those played in osteoarthrtic cartilage. These findings, together with the observation that IL-10 stimulates chondrocytes to produce proteoglycans *in vitro* (17), would suggest that IL-10, beside exerting anti-inflammatory activity, can also act as a "growth factor" on chondrocytes and that it may play a role in the differentiation of these cells. It is conceivable that during cartilage development IL-10 may be involved in tissue differentiation and maturation; its production then decreases in healthy mature cartilage, and again increases in osteoarthritic cartilage when chondrocyte metabolism is enhanced.

Our study provides evidence that chondrocytes synthesise IL-10 and that its production is significantly increased in osteoarthritic cartilage. Its action probably restores normal chondrocyte functions by inhibiting the catabolic effects of IL-1 and TNF- and by directly stimulating the production of ECM components by chondrocytes. The high levels of IL-10 found in normal foetal chondrocytes, cells by definition with high proliferative and metabolic activity, further corroborates the hypothesis that articular chondrocytes during OA undergo a metabolic boost. We have also shown for the first time that human articular chondrocytes express IL-10R. A better knowledge of the role of IL-10 in the pathogenesis of OA might be gained through a deeper understanding the physiological role of this cytokine. This could then allow us to explore novel therapeutic strategies for the treatment of OA.

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