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IL-17, IL-1β and TNF-α stimulate VEGF production by dedifferentiated chondrocytes[1]

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Summary

Objective: To verify the involvement of proinflammatory cytokines IL-17, IL-1β and tumor necrosis factor α (TNF-α) in cartilage vascularization by stimulating the production of vascular endothelial growth factor (VEGF) by chondrocytes isolated from patients with osteoarthritis (OA), in comparison with patients with rheumatoid arthritis (RA) and patients with femoral or humeral neck fracture (FP).

Design: Chondrocytes isolated from patients with OA were maintained in monolayer culture for several passages. Chondrocyte dedifferentiation was monitored by the synthesis of cathepsin B by these cells. Chondrocytes freshly isolated at each subculture (subcultures 1–3) were stimulated with IL-17, IL-1β or TNF-α. Supernatants were collected, immunoassayed for the production of VEGF and cathepsin B and assayed as the source of VEGF on the VEGF sensible ECV304 cell line. The cells were used to quantify intracellular cathepsin B enzymatic activity.

Results: In differentiated conditions IL-1β and TNF-α, but not IL-17, can inhibit the spontaneous secretion of VEGF by human OA, RA and FP chondrocytes, and IL-17 can restore the decrease in VEGF secretion caused by TNF-α. IL-17, together with IL-1β and TNF-α, can enhance VEGF secretion to various extents by dedifferentiated OA chondrocytes. This change in effect with respect to primary culture was observable for all cytokines at the beginning of dedifferentiation, when the production of VEGF by chondrocytes had dramatically fallen and the cathepsin B synthesis had increased. The amount of VEGF induced by cytokines on dedifferentiated chondrocytes never reached the amount of VEGF produced by differentiated chondrocytes. VEGF produced by chondrocytes stimulated the ECV304 cell line proliferation.

Conclusions: These results indicate that dedifferentiated OA chondrocytes secrete VEGF after stimulation with proinflammatory cytokines. This event may be responsible for neovascularization found in OA cartilage.

Key words: Proinflammatory cytokines, VEGF, Osteoarthritis, Dedifferentiated chondrocytes.

Introduction

Osteoarthritis (OA) is a multifactorial disease of unknown origin, associated with structural changes in all articular tissues. It is characterized by synovial fibrosis and cartilage erosion that induce subchondral bone damages[1]. OA is also characterized by an evident inflammation: synovial hypertrophy, proliferation of synovial lining cells, presence of infiltrate in sublining tissue[2]. The presence of inflammation in OA, that in some cases resembles that found in rheumatoid arthritis (RA)[3,4], has been associated with the progression of OA[5]. Cartilage is normally an avascular tissue, but OA damaged cartilage presents vascularized zones. Angiogenesis is a process stimulated in chronic joint inflammation[6], and among factors able to promote endothelial cell proliferation and migration, vascular endothelial growth factor (VEGF) is one of the most important up-regulators of vasculogenesis[7]. VEGF is a 46–48 kDa glycosylated homodimeric polypeptide, which shows several variants. The main ones include 121, 145, 165, 189 and 206 amino acids. These isoforms present different abilities to bind to extracellular matrix proteoglycans and to VEGF receptors[8]. The expression of VEGF has been demonstrated in synovial membrane of OA patients[9,10], although the serum VEGF concentration was normal[11]. It is debated whether VEGF is expressed by normal adult cartilage, but chondrocytes are a source of VEGF in OA cartilage[12–14]. Some physical and chemical factors that can stimulate the secretion of VEGF in inflamed tissues are hypoxia[6], prostaglandin E (PGE) 1 and 2[15], proinflammatory cytokines such as IL-1β and IL-6[16,17].

IL-17, IL-1β and tumor necrosis factor α (TNF-α) are proinflammatory cytokines over-expressed in articular OA tissues that contribute to the pathogenesis of OA[2,5,18,19]. It is known that IL-1β and TNF-α can induce chondrocyte dedifferentiation[20,21] and OA chondrocytes express some dedifferentiation markers, such as types I and III collagen and cathepsin B[21–23]. So, the model of "*in vitro*" dedifferentiating chondrocytes can be important to study the release of growth factors influencing cartilage neovascularization.

The aim of our study was to verify whether the proinflammatory cytokines IL-17, IL-1β and TNF-α were

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able to modulate VEGF secretion by human chondrocytes at different differentiation stages.

Our results indicated that IL-17, together with IL-1β and TNF-α, can enhance VEGF secretion to various degrees by dedifferentiated chondrocytes. In differentiated conditions IL-1β and TNF-α, but not IL-17, can inhibit the spontaneous secretion of VEGF by human chondrocytes and IL-17 can restore the decrease in VEGF secretion caused by TNF-α.

Materials and methods

TISSUE SAMPLES AND PATIENTS

The study was performed on 17 patients with OA (mean age 64 years, range 44–75) undergoing knee (14 cases) or hip (three cases) replacement surgery. Diagnosis of OA was based on clinical, radiological and laboratory parameters[24]. All patients involved in the study showed grade III–IV radiological features according to the Kellgren and Lawrence grading system[25]. Five patients with RA (mean age 57 years, range 24–74) undergoing knee (three cases) or hip (two cases) replacement surgery were used for comparison. Diagnosis of RA was made according to American College of Rheumatology criteria[26]. Besides, human cartilage was obtained from femoral (four cases) or humeral (one case) heads removed after fracture in five patients with no history or articular morphological alterations of OA (FP) (mean age 70 years, range 43–87). The study was approved by the ethical committee of Istituti ortopedici Rizzoli and informed consent was obtained from the patients.

CHONDROCYTE ISOLATION AND CULTURE CONDITIONS

Articular cartilage tissue was removed from the medial condyles or tibial plateau. As expected, the cartilage samples were macroscopically different and erosion areas were present. During tissue sampling, regions with evident signs of erosion were avoided. The remaining fragments of cartilage tissue from each patient were pooled together before digestion. Chondrocytes were isolated by sequential enzymatic digestion of cartilage as previously described[19]. Chondrocytes obtained were resuspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 4 mM glutamine (Sigma, St. Louis, MO, USA), 200 μg/ml gentamycin (Flow Laboratories, Biaggio, Switzerland) and 10% heat inactivated fetal calf serum (FCS) (Gibco BRL) (complete medium), seeded in 96-well flat-bottom microplates at a density of 1×10^5 and 27×10^5 cells/cm^2 in 0.2 ml of complete DMEM and in T 75 culture flask (1×10^5 cells/cm^2) and maintained at 37°C in 5% CO_2 humidified atmosphere. When chondrocytes seeded in T 75 culture flask were confluent (after 10–15 days of culture), they were trypsinized (0.05% trypsin/0.5 mM EDTA, Sigma), washed and split 1:3 to obtain subsequent passages. Under these conditions of culture each subculture represents a 1.5 cell doubling[27–29]. Chondrocytes freshly isolated and at the different passages were used for experiments. Cell viability, determined by the eosin dye exclusion test, was always higher than 95%.

CHONDROCYTE STIMULATION

All experiments were performed in a 96-well flat-bottom microplate in 0.2 ml of complete culture medium using chondrocytes, either freshly isolated, between the first and third subcultures (primary culture and subcultures 1–3, seeded at 2×10^5 cells/cm^2), or as primary culture which had begun to actively proliferate (proliferating primary culture, seeded at 1×10^5 cells/cm^2). After overnight, or in proliferating primary culture, 0.1 ml was substituted with 0.1 ml of complete medium with or without stimuli: 50 ng/ml recombinant human (rh) IL-17 (IL-17) (R&D Systems, Minneapolis, MN, USA), 100 U/ml rhIL-1β (IL-1β; specific activity 5×10^7 U/mg) (Boehringer, Mannheim, Germany), 100 U/ml rhTNF-α (TNF-α; specific activity 1×10^8 U/mg) (Boehringer) (final concentration). Chondrocytes were also stimulated with IL-17/IL-1β or IL-17/TNF-α, as described for each experiment. After an incubation of 72 h, supernatants were collected and maintained at −80°C until their use. Previous experiments had shown that these stimulation conditions were adequate to detect soluble VEGF produced by chondrocytes at all used subcultures; chondrocytes at subculture 3, maintained without stimuli, needed at least 48 h to secrete detectable amounts of VEGF by an enzyme-linked immunosorbent assay (ELISA). Bovine serum albumin in PBS added to the complete medium at the same concentration of the cytokine preparation used to stimulate the chondrocyte cultures did not affect the secretion of VEGF by chondrocytes in non-stimulated conditions.

CELL LINE CULTURE AND PROLIFERATION ASSAY

A human umbilical cord transformed endothelial cell line, ECV304 (American Type Culture Collection, Rockville, MD, USA), which is known to be stimulated to proliferate in the presence of VEGF[30], was maintained in culture in medium 199 supplemented with 4 mM glutamine (Sigma), 200 μg/ml gentamycin (Flow Laboratories) and 10% heat inactivated FCS (Gibco BRL) (growth medium). For proliferation assay, ECV304 was seeded in 96-well flat-bottom microplates in 0.2 ml of growth medium at 1.25×10^3 cells/well. After 8 h of incubation, the growth medium was substituted with 0.2 ml of medium 199 supplemented with 2% FCS to reduce the normal high proliferation speed and to highlight the stimulating effect of VEGF better. After further 14 h, 5 ng/ml recombinant $VEGF_{165}$ (rVEGF) (R&D Systems) in complete DMEM or supernatants from 72 h stimulated OA chondrocytes as conditioned medium (CCM), both 1:2 diluted with serum-free medium 199, were used to substitute the culture medium. At the end of 10 h incubation, the cells, pulsed with 0.5 μCi per well of [methyl-^{3}H]-thymidine (ICN Biomedicals, Irvine, CA, USA) in the last 4 h, were collected onto glass-fiber filters, using an automatic cell collector (Filtermate 196, Packard Instrument Company, Meriden, CT, USA) and cpm values from cultures were counted with a liquid scintillation counter (Topcount B99020, Packard Instrument Company). The cell number per well, rVEGF concentration, culture mediums and incubation times were based on results obtained in previous experiments performed to assess the optimal conditions to detect the CCM effect on ECV304 cell line proliferation. The specificity of the VEGF proliferative response was evaluated by incubating the ECV304 cell line for 1 h at 37°C with the indicated concentrations of anti-VEGF receptor 2 antibody (anti-VEGFR-2) (R&D Systems) before the addition of rVEGF or culture supernatants (CCM). Each assay was conducted in triplicate.

ELISA ASSAY FOR VEGF

VEGF secreted by non-stimulated and stimulated chondrocytes was determined in the supernatant of 72 h culture

by an ELISA Quantikine® for human VEGF (R&D Systems), following the manufacturer's recommended protocol. Quantikine® recognizes $VEGF_{121}$, $VEGF_{165}$ and naturally occurring human VEGF; so, most of the VEGF detected in our samples was $VEGF_{121}$, the secreted isoform, soluble and not binding heparan sulfate or extracellular matrix[8]. To better compare the VEGF secretion by chondrocytes at different culture passages, for each sample we normalized the amount of VEGF to 10^5 cells in view of the fact that chondrocytes in primary culture did not show evident proliferating activity during 72 h of culture; on the contrary, chondrocytes in proliferating primary culture were seeded at lower density, but received the stimuli after various proliferation cycles and subcultures 1−3 were stimulated being in the logarithmic growth phase, until reaching confluence within the 72 h of stimulation.

To determine the number of cells present in each well evaluated for VEGF release, a PicoGreen® dsDNA Quantitation kit (Molecular Probes, Eugene, OR, USA) for double strand DNA assay was used. After collecting supernatants, adherent cells were lysed by adding 100 μl lysant buffer (cell-lysis buffer of CyQUANT® Cell Proliferation Assay Kit, Molecular Probes) to each well and then 100 μl of working solution of the PicoGreen reagent was added. After 5 min incubation at room temperature in the dark, the fluorescence of the samples was determined using a Spectramax Gemini dual-scanning microplate spectrofluorimeter (Molecular Device, Sunnyvale, CA, USA) (480 nm excitation, 540 nm emission and 515 nm cut-off), in the well-scan mode. The fluorescence of each sample was compared with the fluorescence of a curve obtained with 1:2 (scalar) dilutions of chondrocytes from 0.5×10^6 to 0.016×10^6 or with a curve of standard DNA.

CATHEPSIN B ASSAY

Cathepsin B secreted by chondrocytes was evaluated in the supernatant of primary and proliferating primary cultures by a specific ELISA test, following the manufacturer's recommended protocol (Human Cathepsin B ELISA test, KRKA, Novo Mesto, Slovenia). The detection limit was 0.03 nmol/l. The amount of intracellular cathepsin B present in chondrocytes at the end of 72 h of culture was determined on primary and proliferating primary cultures for each patient, at basal condition. Cathepsin B assay was performed following the method described by Zwicky and Baici[31], with minor modifications. Briefly, at the end of the culture, after collecting supernatant, the cell layer was washed twice with physiological solution and then the plate was frozen at −80°C until the cathepsin B assay. Then, 0.2 ml of DMEM without phenol red was added to each well and the plates were sonicated in a cool water bath (five cycles of 2 s, 5 s intervals). The suspension was centrifuged at 2000*g* for 15 min at 4°C and 10 μl of supernatant was incubated with 200 μl of 0.5 mM *N*-carbobenzoxy-L-arginyl-L-arginyl-7-(4-methyl)-coumarylamide (Z-arg-arg-Nmec; Bachem, Budendorf, Switzerland) in 100 mM sodium phosphate buffer pH 6 containing 2 mM EDTA and 2 mM dithiothreitol for 6 h at 25°C in the dark under gentle shaking. The reaction was stopped by adding 5 μl of 68 mM iodoacetic acid. The fluorescence of each sample evaluated in triplicate was determined using a Spectramax Gemini dual-scanning microplate spectrofluorimeter (Molecular Device, Sunnyvale, CA, USA) (383 nm excitation, 455 nm emission and 435 nm cut-off), in the end point mode, and compared with 5 mM 7-amino-4-methyl-coumarin (Bachem) used as fluorimetric standard. Cathepsin B activity was expressed as biological units (U) for 10^5 cells. One biological unit corresponded to the amount of enzyme necessary to hydrolyze 1 nmol of substrate.

STATISTICAL ANALYSIS

Parametric and nonparametric tests were used for statistical analysis of the results; differences between groups were compared by the Student's *t*-test or by the Mann−Whitney *U* test and when the Friedman ANOVA test for multiple comparison was significant, paired data were analyzed by the Wilcoxon matched pairs test; correlations were analyzed by the Spearman test.

Results

VEGF SECRETION BY CHONDROCYTES

Chondrocytes from all subjects constitutively released VEGF immediately after isolation (primary culture), but the level of VEGF released by OA or RA chondrocytes was higher than that released by FP chondrocytes (Fig. 1). VEGF secretion by OA and RA samples was not statistically different. Chondrocytes from OA patients were also evaluated for VEGF secretion at different subcultures. Eight to 25 days (mean time ± SD, 12.0 ± 4.9 days) were necessary to observe an active proliferation of chondrocytes seeded at 1×10^5 cells/cm^2 after isolation, depending on individual variability. At this time (proliferating primary culture), cultures were stimulated for 72 h. Interestingly, we verified that a strong decrease of VEGF secretion was obtained by proliferating primary culture from all subjects evaluated, compared to cells in primary culture, also when the beginning of proliferation by primary culture was reached after only 8 days of culture. The effect of the subculturing was a progressive decrease of VEGF secretion

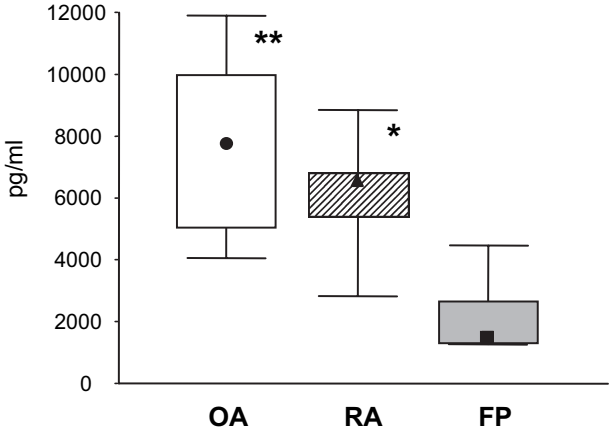

Fig. 1. Production of VEGF by freshly isolated chondrocytes. Data are represented from chondrocytes of 15 OA subjects, five RA subjects and five subjects with femoral or humeral neck fracture (FP). Results are expressed as pg/ml of VEGF secreted by chondrocytes seeded at 2×10^5 cells/cm^2 and not showing any proliferation at the end of 72 h culture. The boxes show 25th and 75th percentiles, the symbols the medians, and the vertical lines below and above the boxes 10th and 90th percentiles. Statistical comparison was carried out using the Mann−Whitney *U* test. $^*P<0.05$ and $^{**}P<0.01$ vs FP subjects.

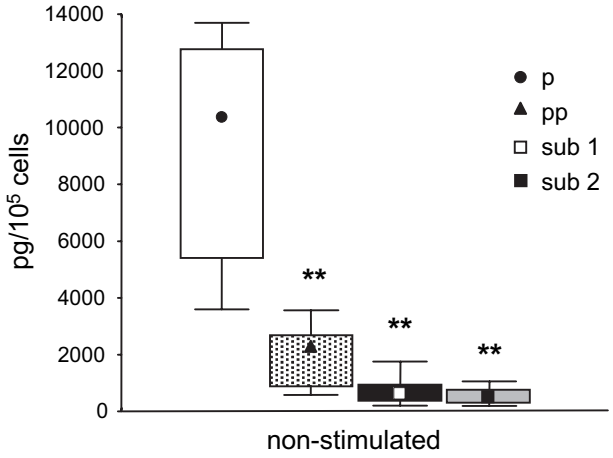

Fig. 2. Effect of subculture on VEGF production by OA chondrocytes. Data are represented from chondrocytes of 15 subjects in primary culture (p) and in proliferating primary culture (pp), 13 subjects in subcultures 1 and 2 (sub 1 and sub 2). Results are expressed as pg of VEGF secreted by 10^5 cells, on the basis of the number of cells present in each well at the end of 72 h of culture in non-stimulated conditions. The boxes show 25th and 75th percentiles, the symbols the medians, and the vertical lines below and above the boxes 10th and 90th percentiles. Statistical comparison was carried out using the Wilcoxon matched pairs test. $^{**}P<0.01$ vs p.

from primary culture to subculture 3 (Spearman rank order correlation $r = -0.831$; $P<0.01$) (Fig. 2).

DIFFERENTIATION DEGREE OF CULTURED OA CHONDROCYTES

The differentiated morphology shown by primary culture of OA chondrocytes (rounded cells, presence of cell clusters, typical for freshly isolated chondrocytes and cultured for 4 days) was lost when cells began to proliferate to reach confluence and assumed the appearance of polygonal cells, uniformly distributed into the culture plate (proliferating primary culture). In subcultures 1–3 chondrocytes had become fibroblast-like cells. To establish whether the different VEGF production by chondrocytes in primary and proliferating primary cultures was due to the degree of chondrocyte differentiation, we compared the cathepsin B synthesis with VEGF production by chondrocytes in these two culture conditions, cathepsin B being a marker of chondrocyte dedifferentiation[23]. We verified that the effect of few culture days was evident also in cathepsin B synthesis by chondrocytes, because the activity present in the intracellular pool of and the protein released into the supernatant by chondrocytes in primary culture considerably increased when cells were in proliferating primary culture (Fig. 3), while VEGF production decreased from primary to proliferating primary culture.

DIFFERENT EFFECT OF CYTOKINES ON VEGF SECRETION BY CULTURED OA CHONDROCYTES

The ability of primary culture of OA chondrocytes to release VEGF was not affected by IL-17, but when chondrocytes were stimulated by IL-1β or TNF-α the release of VEGF decreased compared to non-stimulated controls or IL-17-stimulated cells. IL-1β and TNF-α had a similar effect [Fig. 4(a)]. On the contrary, IL-17, IL-1β and TNF-α stimulated VEGF secretion in proliferating primary culture and subcultures 1 and 2, when chondrocytes were tending to lose their ability to secrete VEGF [Fig. 4(b–d)]. Most of the cartilage tissue (14/17) was obtained during knee surgery. We did not detect any difference in VEGF production or cytokine effect between hip (three cases) and knee (14 cases) cartilage samples. IL-1β stimulated the secretion of higher amounts of VEGF compared to IL-17 in subcultures 1 and 2, and compared to TNF-α in proliferating primary culture, subcultures 1 and 2 [Fig. 4(b–d)]. Only three subjects were evaluated through to subculture 3 and at this passage their chondrocytes showed the same trend: the basal secretion of VEGF was lower than that at subculture 2 and was increased by IL-17, IL-1β and TNF-α (data not shown). The effect of the proliferation in monolayers was also a progressive decrease of VEGF secretion by stimulated chondrocytes from primary culture to subculture 3 (Spearman correlation: $r = -0.783$ and $P<0.01$, $r = -0.478$ and $P<0.01$, $r = -0.579$ and $P<0.01$, for IL-17, IL-1β and TNF-α stimulation, respectively). These cytokines, although increased the VEGF secretion by cultured chondrocytes, did not restore the high VEGF secretion shown by non-stimulated chondrocytes in primary culture [Fig 4(a–d)].

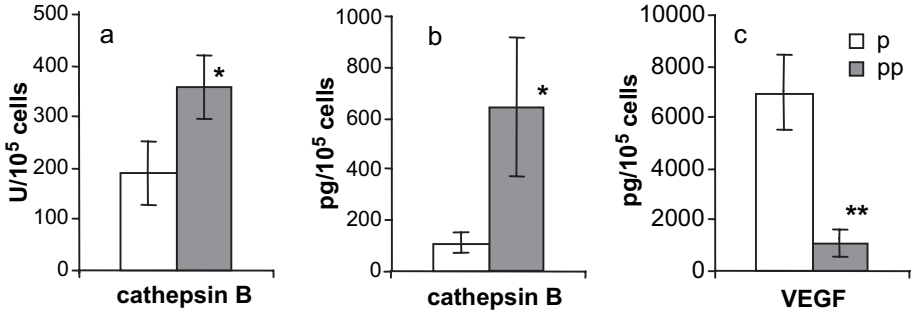

Fig. 3. Metabolic differences between primary culture (p) and proliferating primary culture (pp) of chondrocytes. Chondrocytes from seven OA subjects had been evaluated for their cathepsin B synthesis and VEGF production. To compare the activity of chondrocytes at p and pp, which presented samples with evident differences in cell numbers, we normalized each result to 10^5 cells. (a) Cathepsin B as intracellular enzymatic activity at the end of 72 h culture is shown and expressed as biological units (U, amount of enzyme necessary to hydrolyze 1 nmol of substrate); (b) cathepsin B released in the supernatant of 72 h culture; (c) VEGF released in the supernatant of 72 h culture. Statistical comparison was made using the Wilcoxon matched pairs test. $^{*}P<0.05$ and $^{**}P<0.01$ vs p.

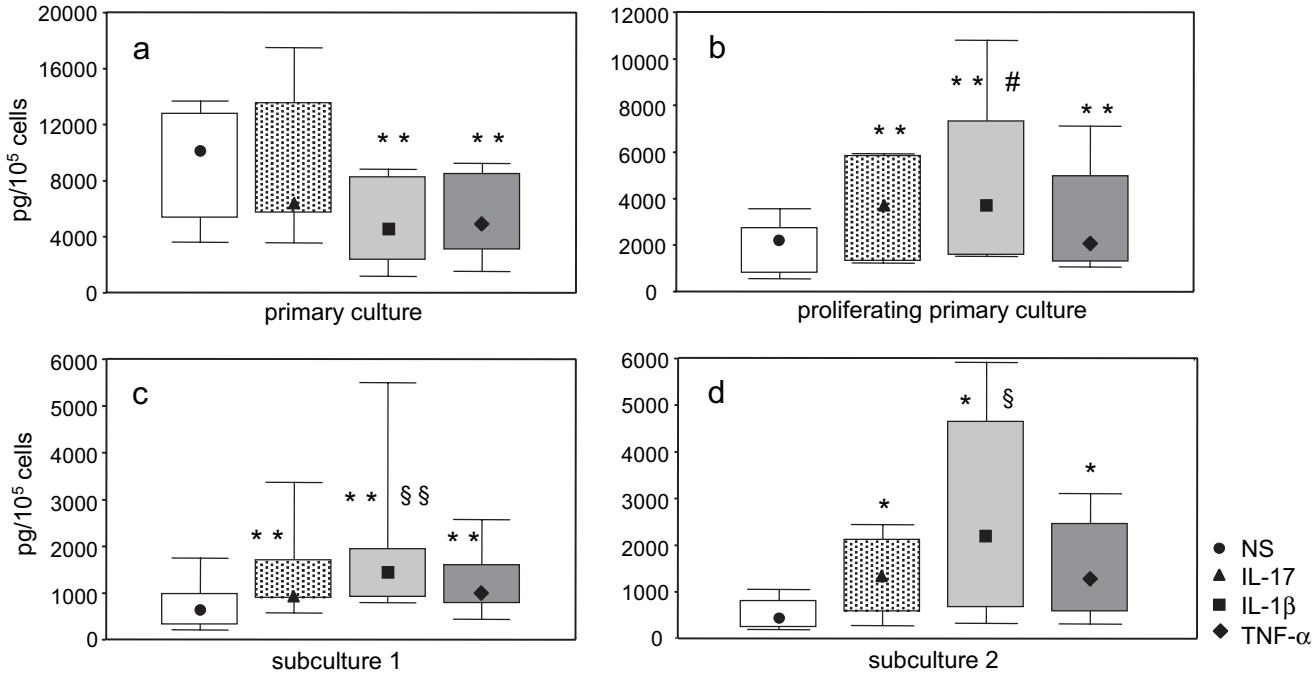

Fig. 4. Effect of cytokines and passages in culture on VEGF production by OA chondrocytes. Data are represented from 15 subjects in primary culture and in proliferating primary culture and 13 subjects in subcultures 1 and 2. Results are expressed as pg of VEGF secreted by 10^5 cells incubated for 72 h in non-stimulated conditions (NS) or with IL-17 (50 ng/ml), IL-1β (100 U/ml) or TNF-α (100 U/ml). The boxes show 25th and 75th percentiles, the symbols the medians, and the vertical lines below and above the boxes 10th and 90th percentiles. Statistical comparison was made using the Wilcoxon matched pairs test. (a) $^{**}P<0.01$ vs NS and IL-17-stimulated chondrocytes; (b) $^{**}P<0.01$ vs NS; $^{\#}P<0.05$ vs TNF-α-stimulated chondrocytes; (c) $^{**}P<0.01$ vs NS; $^{\S\S}P<0.01$ vs IL-17- and TNF-α-stimulated chondrocytes; (d) $^{*}P<0.05$ vs NS; $^{\S}P<0.05$ vs IL-17- and TNF-α-stimulated chondrocytes.

EFFECT OF IL-17 ON VEGF SECRETION STIMULATED BY IL-1β OR TNF-α ON OA CHONDROCYTES

We also investigated the combined effect of IL-17 with IL-1β or TNF-α on VEGF secretion. Chondrocytes from five OA patients were utilized to control the additional effect of IL-17 on primary and proliferating primary cultures and subculture 1 stimulated with IL-1β or TNF-α. Three of the five OA patients were also utilized in subculture 2. Although IL-17 did not affect the release of VEGF by primary cultures of chondrocytes, when freshly isolated OA chondrocytes were stimulated by TNF-α, the additional presence of IL-17 was able to induce a secretion of VEGF as high as that constitutively released by chondrocytes and the inhibiting effect of TNF-α was eluded. On the contrary, IL-17 did not restore the release of VEGF decreased by IL-1β compared with non-stimulated or IL-17-stimulated chondrocytes [Fig. 5(a)]. When we used OA chondrocytes in proliferating primary culture and subcultures 1 and 2, we did not obtain any additional effect of IL-17 on cultures stimulated by IL-1β or TNF-α [Fig. 5(b–d)].

BIOLOGICAL EFFECT OF VEGF RELEASED BY CHONDROCYTES

To verify if IL-17, IL-1β and TNF-α could cause neovascularization by increasing VEGF released by OA chondrocytes, the biological effect of the CCM, containing VEGF, on the proliferation of the EVC304 cell line was evaluated by [methyl-^{3}H]-thymidine incorporation and compared with the effect of rVEGF.

The effect of rVEGF was dose-dependent on 1–25 ng/ml in all evaluated cell numbers per well (ranging $0.312–2.5\times10^3$ cell/well) (data not shown). At a density of 1.25×10^3 cells/well the pre-incubation with anti-VEGFR-2 antibody (100–1000 ng/ml) completely blocked the 42% increase of [methyl-^{3}H]-thymidine incorporation by ECV304 cell line stimulated by 5 ng/ml rVEGF. On the contrary, 50 ng/ml anti-VEGFR-2 antibody was not able to completely block the effect of rVEGF ($P<0.05$ vs non-stimulated control) [Fig. 6(a)]. CCM obtained by 72 h incubated chondrocytes in the absence of stimuli or presence of IL-17, IL-1β or TNF-α were chosen among chondrocyte supernatants containing different VEGF amounts ranging from 0.54 to 12.8 ng/ml, that corresponded to 0.27–6.4 ng/ml when diluted in proliferation tests. The ECV304 cell line incubated with CCM showed a higher increase in proliferation than that induced by 5 ng/ml rVEGF (44%), compared with the proliferation in the presence of medium alone [Fig. 6(b)]. Neutralization of VEGFR-2 with specific antibodies decreased but did not completely inhibit the proliferation induced by CCM, that remained higher than the one obtained by spontaneously proliferating ECV304 cell line ($P<0.05$ at least). Thus, the CCM effect seems to be not only dependent on VEGF, but also on other growth factors which could interfere with VEGF-induced chondrocyte proliferation. As a control, the EVC304 cell line was also incubated in the presence of IL-17, IL-1β or TNF-α at the concentrations same as those used to obtain CCM, and the incorporation of [methyl-^{3}H]-thymidine was comparable to that obtained at basal conditions. This finding allowed us to exclude the hypothesis that the higher ECV304 cell line proliferation obtained by CCM, compared with the culture stimulated by rVEGF or maintained without stimuli, was due to IL-17, IL-1β or TNF-α present in CCM.

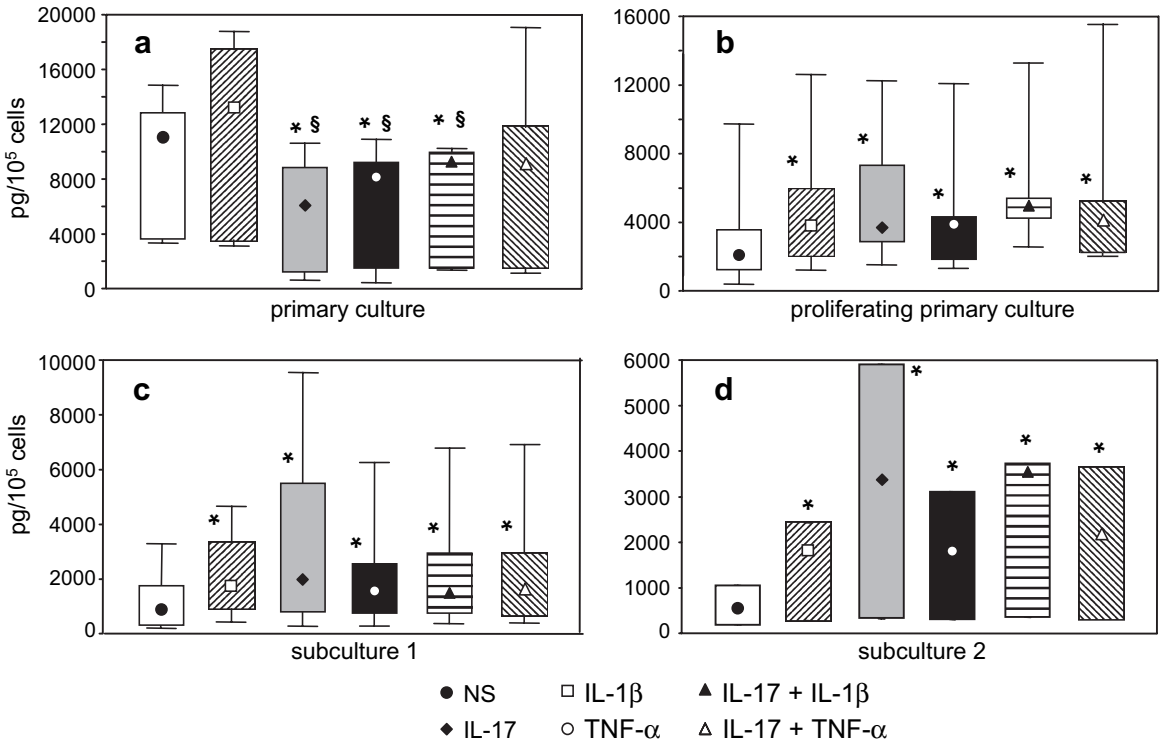

Fig. 5. Effect of IL-17 on IL-1β- or TNF-α-stimulated VEGF secretion by OA chondrocytes. Chondrocytes from five OA subjects in primary culture (a), proliferating primary culture (b) and subculture 1 (c) and from three subjects in subculture 2 (d) had been evaluated. Results are expressed as pg of VEGF secreted by 10^5 cells incubated for 72 h in non-stimulated conditions (NS) or with IL-17 (50 ng/ml), IL-1β (100 U/ml), TNF-α (100 U/ml), IL-17/IL-1β and IL-17/TNF-α. The boxes show 25th and 75th percentiles, the symbols the medians, and the vertical lines below and above the boxes 10th and 90th percentiles. Statistical comparison was made using the Wilcoxon matched pairs test. *$P < 0.05$ vs NS and §$P < 0.05$ vs IL-17-stimulated chondrocytes.

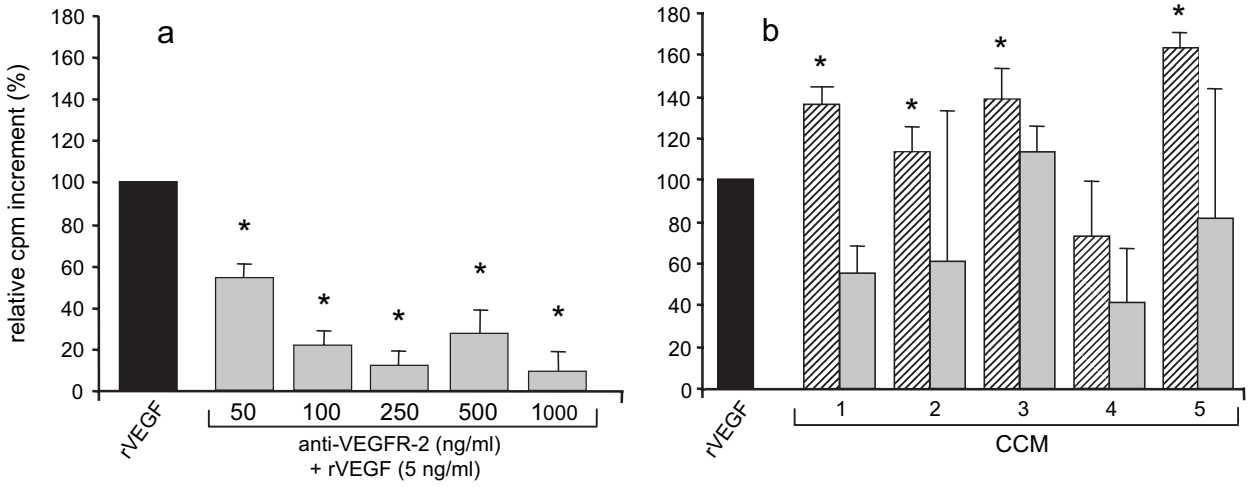

Fig. 6. Effect of rVEGF and CCM on the proliferation of ECV304 cell line. Results are expressed as relative cpm increment, referred to the percent increment of rVEGF-dependent proliferation considered as 100%. (a) Inhibition of rVEGF-stimulated ECV304 proliferation. ECV304 cell line received either rVEGF alone (black bar) or different concentrations of anti-VEGFR-2 antibody (ranging 50–1000 ng/ml) followed by rVEGF (gray bars) as previously described in Materials and Methods. Percentage means of rVEGF-dependent increment ± SD of triplicate cultures are represented and are representative of two independent experiments. *$P < 0.05$ vs rVEGF (Student's *t*-test). (b) Effect of CCM on ECV304 cell line proliferation. ECV304 cell line received rVEGF alone (black bar) or CCM containing VEGF at known concentrations determined by ELISA (hatched bars) (sample 1: 5 ng/ml VEGF; sample 2: 6.05 ng/ml VEGF; sample 3: 0.27 ng/ml VEGF; sample 4: 1.05 ng/ml VEGF; sample 5: 0.73 ng/ml VEGF). For inhibition assay, the ECV304 cell line was pre-incubated with 200 ng/ml anti-VEGFR-2 and then stimulated with CCM (gray bars). Experiments were performed in triplicate and the CCM-dependent increment of proliferation is expressed as mean percentage of rVEGF-dependent increment ± SD and calculated as follows: [(CCM-stimulated cpm − non-stimulated cpm)/(rVEGF-stimulated cpm − non-stimulated cpm)] × 100. *$P < 0.05$ vs VEGF (Student's *t*-test); CCM-stimulated ECV304 cell line: anti-VEGFR-2 pretreated vs non-pretreated samples, $P < 0.05$ (Wilcoxon matched pairs test).

Discussion

VEGF, an important contributor of synovial and cartilage vascularization, is active during embryogenic development and its maximal production has been ascertained as occurring immediately before the cartilage ossification and bone formation[32]. The production of VEGF seems to be only a peculiarity of hypertrophic chondrocytes from growth plate of fetal and neonatal long bone, normally absent in resting or proliferating chondrocytes[32–34]. The presence of VEGF in normal adult cartilage is not easily determinable, but it had been shown that OA chondrocytes can produce high amounts of VEGF, and VEGF positive chondrocytes have been found in OA cartilage in relation to the stage of the disease[12–14].

In this study we have shown that chondrocytes isolated from cartilage of OA subjects are able to secrete VEGF as well as chondrocytes from RA subjects. Inflammation seems to play an important role in activating the VEGF secretion by chondrocytes, because when we analyzed chondrocytes from non-inflammatory pathology, we found lower amounts of VEGF in comparison with OA and RA chondrocytes. The higher vascularization in articular RA tissues in comparison with articular tissues from OA patients could be dependent on a higher secretion of other angiogenesis factors by RA rather than OA chondrocytes, or on a higher secretion of VEGF by RA rather than OA synovial cells, also involved in articular pathological modifications. The VEGF secretion strongly decreased in about 12 days of culture, and continued to decrease until the third passage in culture (which was reached in about 50 days) without disappearing. Other authors have shown that in human articular chondrocytes the effect of a 42-day culture was a complete loss of expression of VEGF gene[35]. On the contrary, Carlevaro *et al.* demonstrated that culture in suspension in adequate medium led to the condition of differentiated chondrocytes, which maintain their ability to synthesize high amounts of VEGF[33]. During the course of OA the cartilage presents areas of matrix degradation and remodeling where chondrocytes assume some characteristics generally attributed to dedifferentiated chondrocytes[36,37]. For this reason, when we harvested cartilage to obtain isolated chondrocytes, we avoided the region of cartilage which presented major morphological anomalies. This method selects a chondrocyte enriched population from areas of cartilage that are scarcely affected by major OA pathological modifications. When the chondrocytes were isolated from these areas, they maintained their round morphology for some days, then, cultured in monolayers, they began to lose their typical morphology, proliferate and dedifferentiate into fibroblast-like cells[27]. It is known that synovial fibroblasts can be stimulated to produce VEGF by IL-1β and TNF-α[16,38]. At this stage, we found that the strong decrease of VEGF release matched an increase of cathepsin B synthesis, which represents a ''dedifferentiated'' phenotype of chondrocytes[23,39,40]. On the basis of our results, we suppose that freshly isolated chondrocytes are representative of the phenotype of chondrocytes present in cartilage regions with pathological modifications, but not expressing a dedifferentiated feature. However, our data have been obtained by an ''*in vitro*'' model and should be confirmed by analyzing the VEGF production from areas of chondrocytes showing dedifferentiation markers in cartilage samples from OA patients.

The differentiation status can influence the sensitivity of chondrocytes to proinflammatory cytokines. IL-1β and TNF-α inhibited VEGF production by freshly isolated human chondrocytes, while IL-17 was ineffective. On the contrary, when chondrocytes dedifferentiate and proliferate, IL-17, IL-1β and TNF-α can increase the progressively reduced VEGF production. We previously found that IL-1β and TNF-α are strongly expressed in OA cartilage, together with IL-17 receptor[18,19]. Furthermore IL-17 can also contribute to the production of inflammatory chemokines by human chondrocytes[19].

In our model IL-17 modulated only the TNF-α ability to decrease the VEGF secretion by freshly isolated chondrocytes, while it seemed not to affect either IL-1β decreased or IL-1β and TNF-α increased VEGF secretion in primary cultures or proliferating cultures and subcultures, respectively. The linkage of cytokines to their receptor expressed on cell surface can stimulate different cell activation pathways. The different effect of IL-17 on IL-1β- and TNF-α-dependent VEGF secretion by chondrocytes at passage 0–2 could be due to the utilization by chondrocytes of activation pathways peculiar to each cell differentiation status and differently interfering each other.

When the ECV304 cell line was stimulated by CCM containing known VEGF concentrations, the increase of cell proliferation was higher than that obtained with rVEGF and not VEGF dose-dependent. This finding is not surprising, because chondrocyte supernatants can also contain other angiogenesis factors[41,42]. The partial inhibition of the stimulating effect of CCM on the ECV304 cell line by VEGFR-2 neutralization seems to confirm the presence of more factors stimulating ECV304 proliferation.

These proinflammatory cytokines present in the articular microenvironment can contribute to neovascular formation in the cartilage areas where proliferating chondrocytes could assume a feature of hypertrophic/dedifferentiated. The infiltration of cartilage by neovessels in the areas of chondrocytes proliferation can contribute to altering the characteristics of resistance depending on a normal cartilage architecture.

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References

1. Poole AR. An introduction to the pathophysiology of osteoarthritis. Front Biosci 1999;4:d662–70.
2. Martel-Pelletier J, Alaaeddine N, Pelletier JP. Cytokines and their role in the pathophysiology of osteoarthritis. Front Biosci 1999;4:d694–703.
3. Haraoui B, Pelletier JP, Cloutier JM, Faure MP, Martel-Pelletier J. Synovial membrane histology and immunopathology in rheumatoid arthritis and osteoarthritis. *In vivo* effects of antirheumatic drugs. Arthritis Rheum 1991;34:153–63.
4. Farahat MN, Yanni G, Poston R, Panayi GS. Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. Ann Rheum Dis 1993;52:870–5.
5. Pelletier JP, Martel-Pelletier J, Abramson SB. Osteoarthritis, an inflammatory disease. Potential implication for the selection of new therapeutic targets. Arthritis Rheum 2001;44:1237–47.

6. Walsh DA, Pearson CI. Angiogenesis in the pathogenesis of inflammatory joint and lung diseases. Arthritis Res 2001;3:147–53.
7. Ferrara N. Vascular endothelial growth factor: molecular and biological aspects. Curr Top Microbiol Immunol 1999;237:1–30.
8. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999;13:9–22.
9. Inoue H, Takamori M, Nagata N, Nishikawa T, Oda H, Yamamoto S, *et al*. An investigation of cell proliferation and soluble mediators induced by interleukin 1β in human synovial fibroblasts: comparative response in osteoarthritis and rheumatoid arthritis. Inflamm Res 2001;50:65–72.
10. Wauke K, Nagashima M, Ishiwata T, Asano G, Yoshino S. Expression and localization of vascular endothelial growth factor-C in rheumatoid arthritis synovial tissue. J Rheumatol 2002;29:34–8.
11. Lee SS, Joo YS, Kim WU, Min DJ, Min JK, Park SH, *et al*. Vascular endothelial growth factor levels in the serum and synovial fluid of patients with rheumatoid arthritis. Clin Exp Rheumatol 2001;19:321–4.
12. Pfander D, Kortje D, Zimmermann R, Weseloh G, Kirsch T, Gesslein M, *et al*. Vascular endothelial growth factor in articular cartilage of healthy and osteoarthritic human knee joints. Ann Rheum Dis 2001;60:1070–3.
13. Pufe T, Petersen W, Tillmann B, Mentlein R. The splice variants $VEGF_{121}$ and $VEGF_{189}$ of the angiogenic peptide vascular endothelial growth factor are expressed in osteoarthritic cartilage. Arthritis Rheum 2001;44:1082–8.
14. Enomoto H, Inoki I, Komiya K, Shiomi T, Ikeda E, Obata K, *et al*. Vascular endothelial growth factor isoforms and their receptors are expressed in human osteoarthritic cartilage. Am J Pathol 2003;162: 171–81.
15. Harada S, Nagy JA, Sullivan KA, Thomas KA, Endo N, Rodan GA, *et al*. Induction of vascular endothelial growth factor expression by prostaglandin E2 and E1 in osteoblasts. J Clin Invest 1994;93:2490–6.
16. Jackson JJ, Minton JAL , Ho ML, Wei N, Winkler JD. Expression of vascular endothelial growth factor in synovial fibroblasts is induced by hypoxia and interleukin 1β. J Rheumatol 1997;24:1253–9.
17. Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ. Interleukin 6 induces the expression of vascular endothelial growth factor. J Biol Chem 1996;271: 736–41.
18. Melchiorri C, Meliconi R, Frizziero L, Silvestri T, Pulsatelli L, Mazzetti I, *et al*. Enhanced and coordinated *in vivo* expression of inflammatory cytokines and nitric oxide synthase by chondrocytes from patients with osteoarthritis. Arthritis Rheum 1998;41: 2165–74.
19. Honorati MC, Bovara M, Cattini L, Piacentini A, Facchini A. Contribution of interleukin 17 to human cartilage degradation and synovial inflammation in osteoarthritis. Osteoarthritis Cartilage 2002;10:799–807.
20. Murakami S, Lefebvre V, de Crombrugghe B. Potent inhibition of the master chondrogenic factor *Sox9* gene by interleukin-1 and tumor necrosis factor-α. J Biol Chem 2000;275:3687–92.
21. Sandell L, Aigner T. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. Arthritis Res 2001;3:107–13.
22. Pei M, Yu C, Qu M. Expression of collagen type I, II and III in loose body of osteoarthritis. J Orthop Sci 2000;5: 288–93.
23. Berardi S, Lang A, Kostoulas G, Hörler D, Vilei EM, Baici A. Alternative messenger RNA splicing and enzyme forms of cathepsin B in human osteoarthritic cartilage and cultured chondrocytes. Arthritis Rheum 2001;44:1819–31.
24. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, *et al*. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and therapeutic criteria committee of the American Rheumatism Association. Arthritis Rheum 1986;29:1039–49.
25. Hart DJ, Spector TD. Radiographic criteria for epidemiologic studies of osteoarthritis. J Rheumatol 1995; 22:46–8.
26. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31: 315–24.
27. Blanco FJ, Geng Y, Lotz M. Differentiation-dependent effects of IL-1 and TGF-β on human articular chondrocyte proliferation are related to inducible nitric oxide synthase expression. J Immunol 1995;154: 4018–26.
28. Kolettas E, Buluwela L, Bayliss MT, Muir HI. Expression of cartilage-specific molecules is retained on long-term culture of human articular chondrocytes. J Cell Sci 1995;108:1991–9.
29. Grigolo B, Roseti L, Neri S, Gobbi P, Jensen P, Major EO, *et al*. Human articular chondrocytes immortalized by HPV-16 E6 and E7 genes: maintenance of differentiated phenotype under defined culture conditions. Osteoarthritis Cartilage 2002;10: 879–89.
30. Song HS, Myung JS, Lee YM, Kim WJ, Lee SW, Kim CW, *et al*. Oxygen tension regulates the maturation of the blood–brain barrier. Biochem Biophys Res Commun 2002;290:325–31.
31. Zwicky R, Baici A. Cytoskeletal architecture and cathepsin B trafficking in human articular chondrocytes. Histochem Cell Biol 2000;114:363–72.
32. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 1999;5: 623–8.
33. Carlevaro MF, Cermelli S, Cancedda R, Cancedda Descalzi F. Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci 2000;113:59–69.
34. Petersen W, Tsokos M, Pufe T. Expression of VEGF121 and VEGF165 in hypertrophic chondrocytes of the human growth plate and epiphyseal cartilage. J Anat 2002;201:153–7.
35. Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vécsei V, *et al*. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis Cartilage 2002;10:62–70.
36. Goldwasser M, Astley T, van der Rest M, Glorieux FH. Analysis of the type of collagen present in osteoarthritic human cartilage. Clin Orthop 1982;167: 296–302.

37. Baici A, Lang A, Hörler D, Knöpfel M. Cathepsin B as a marker of the dedifferentiated chondrocyte phenotype. Ann Rheum Dis 1988;47:684–91.
38. Paleolog EM, Young S, Stark AC, McCloskey RV, Feldmann M, Maini RN. Modulation of angiogenic vascular endothelial growth factor by tumor necrosis factor alpha and interleukin-1 in rheumatoid arthritis. Arthritis Rheum 1998;41:1258–65.
39. Baici A, Lang A. Effect of interleukin-1 beta on the production of cathepsin B by rabbit articular chondrocytes. FEBS Lett 1990;277:93–6.
40. Kostoulas G, Lang A, Trueb B, Baici A. Differential expression of mRNAs for endopeptidases in phenotypically modulated (dedifferentiated) human articular chondrocytes. FEBS Lett 1997;412:453–5.
41. Kume K, Satomura K, Nishisho S, Kitaoka E, Yamanouchi K, Tobiume S, *et al*. Potential role of leptin in endochondral ossification. J Histochem Cytochem 2002;50:159–69.
42. Takigawa M, Nakanishi T, Kubota S, Nishida T. Role of CTGF/HCS24/ecogenin in skeletal growth control. J Cell Physiol 2003;194:256–66.