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OSTEOARTHRITIS and CARTILAGE

Effects of exogenous IL-1β, TNFα, IL-6, IL-8 and LIF on cytokine production by human articular chondrocytes

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Summary

Cytokines are potent regulators of the chondrocyte functions. Some of them are produced by chondrocytes and interact to regulate cartilage metabolism. In this study, we investigated the production of interleukin-1β (IL-1β), IL-6, IL-8 and leukemia inhibitory factor (LIF) by human chondrocytes and examined the modulation of their secretion by exogenous cytokines. Human articular chondrocytes were isolated from their extracellular matrix by a triple successive enzymatic digestion of the cartilage. Subsequently, chondrocytes were stimulated by increased amounts of human recombinant cytokines [IL-1β, tumour necrosis factor α (TNFα), IL-8, LIF, IL-6]. IL-1β, IL-6, IL-8 and LIF were assayed into culture media and inside cell extracts by specific enzyme amplified sensitivity immunoassays (EASIAs). Under these experimental conditions, we have identified various interactions between cytokines. IL-β and TNFα highly stimulated IL-6, LIF and IL-8 productions. IL-6 decreased IL-8 synthesis and increased LIF production. IL-8 slightly enhanced IL-6 production. Finally, LIF stimulated IL-1β, IL-6 and IL-8 productions. Using neutralizing antibodies against IL-1, we demonstrated that the effects of LIF were secondary to the stimulation by LIF of IL-1β production by the chondrocytes. In conclusion, chondrocytes secrete a variety of immunocompetent cytokines including IL-1β, IL-6, IL-8 and LIF that can interact to regulate chondrocytes metabolism. These results also define new biological activities of LIF and IL-6, and raise questions concerning their role in the pathogenesis of joint diseases.

Key words: Chondrocytes, Arthritis, Leukemia inhibitory factor, Cytokines.

Introduction

In view of their presence in synovial fluid, various cytokines such as interleukin (IL-1β), tumour necrosis factor (TNFα), IL-6, IL-8 and leukemia inhibitory factor (LIF) [1, 2, 3] are likely to be involved in the pathogenesis of destructive joint disorders. Cytokines are produced transiently in response to an activating signal and all cells, including chondrocytes, can produce some cytokines. These cytokines produced by chondrocytes may exert their effects on neighbouring cells or tissues, as a paracrine manner or on producing cells themselves, as an autocrine manner. TNFα and IL-1β are identified as potent activators of chondrocytes playing a pivotal role in the etiology of cartilage degeneration. In response to these cytokines, chondrocytes start secreting neutral metalloproteinases [4, 5] and active oxygen species [6, 7] which are efficient factors in cartilage matrix destruction. Moreover, these cytokines inhibit proteoglycan [8] and collagen syntheses [9], markedly reducing cartilage repair capacities. Both cytokines may also stimulate bone resorption [10] and induce a transient inflammation and leukocyte infiltration of the synovial membrane [11]. Currently, it is suggested that TNF and IL-1 do not act in isolation and that their effects are often the result of an interaction of various cytokines including IL-6, IL-8 and LIF [12].

IL-6 is a potent B-cell growth factor and differentiation factor [13]. Therefore, through augmentation of rheumatoid factor and other autoantibodies in the synovium, it could indirectly exacerbate inflammation. Moreover, it also induces acute phase proteins synthesis such as C-reactive protein (CRP) and serum amyloid A (SAA) [14]. The effects of IL-6 on chondrocytes are still unclear. No effect [15], slight enhancement [16] as well as

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depression [17] of proteoglycan synthesis has been reported. Production of tissue inhibitor of metalloproteinases (TIMP) [18] and transforming growth factor (TGFβ) [19] is stimulated by IL-6. In this role, IL-6 could be a moderator of the cartilage resorption processes. Because chondrocytes have been shown to proliferate in response to IL-6 [20], IL-6 could also be involved in cartilage early repair reaction occurring in osteoarthritis.

IL-8 is one of the most important chemotactic factors for neutrophils and T lymphocytes [21]. IL-8 also induces neutrophil degranulation, and thus may participate in cartilage degradation [22]. No direct effects of IL-8 on chondrocytes metabolism have been described.

Currently, the role played by LIF in the pathogenesis of joint diseases is not well understood. LIF was initially described on the basis of its ability to induce differentiation of murine myeloid leukemic cells [23]. It inhibits differentiation of pluripotent embryonic stem cells [24], induces the synthesis of hepatic acute phase proteins [25], promotes neuronal differentiation [26], inhibits lipoprotein lipase [27], increases neuronal substance P expression [28] and regulates bone metabolism [29, 30]. The role played by LIF in the homeostasis of cartilage remains unclear.

It is now recognized that cytokines exhibit both pleiotropy and redundancy and that differing cytokines exhibit both synergically and antagonistically. Some recent papers have reported that IL-1β stimulates IL-6, IL-8, and LIF productions [31, 32]. Whether these cytokines are capable of feed back control of cytokines released remains largely uninvestigated. Therefore, we decided to systematically research interactions existing between those cytokines produced by chondrocytes themselves.

Materials and methods

SHORT-TERM SUSPENSION CULTURE OF HUMAN CHONDROCYTES

Chondrocytes were cultured for a short period in order to maintain their phenotype. In this method, we used cartilage obtained from the knee joints of normal young adults shortly after death. Avoiding the calcified layer, cartilage slices were excised from the superficial and medium layers of cartilage, cut into small fragments and submitted to enzymatic digestion. Cartilage fragments were sequentially treated by hyaluronidase, pronase and collagenase. Cartilage pieces were incubated with hyaluronidase dissolved in Dulbecco's Modified Eagle's Medium (DMEM, ICN Biomedical) (0.5 mg/ml-3 g cartilage per 10 ml enzyme solution) for 30 min at 37°C and constantly agitated (200 rpm). Cartilage fragments were then put into pronase solution (1 mg/ml in DMEM-3 g per 10 ml enzymatic solution) and incubated 1 h at 37°C and subsequently re-incubated, under constant agitation (200 rpm), with collagenase (1 mg/ml-3 g per 10 ml enzyme solution) dissolved in DMEM containing 1% Ultroser G (Gibco, Gent, Belgium) for 20 h at 37°C. Ultroser G is a serum substitute which replaces fetal calf serum in *in vitro* culture. The biological activity of the reconstituted solution is fivefold that of fetal calf serum. Cells were then filtered through 25 μm nylon mesh, washed three times, counted (ranged among 1–1.5 10^6 cells/ml) and seeded in 10 ml polypropylene Falcon flasks. Chondrocytes were cultured in DMEM containing 1% Ultroser G. Cells were maintained in suspension by constant agitation on a giratory shaker (100 rpm) in an atmosphere of 95% air 5% CO_2. Cells and supernatant were separated by centrifugation (1000 rpm, 5 min). The cell viability, estimated by trypan blue incorporation, was 95%. Culture media were directly assayed for cytokines. Cell pellets were washed twice with HBSS (Hank's Buffer Saline Solution, Gibco, Merelbeeke, Belgium) and then homogenized in PBS pH 7.5 by ultrasonic dissociation (10 s pulses, power 50 W/cm^2) at 4°C.

TREATMENTS

Human recombinant IL-1β was purchased from Boehringer (Boehringer Mannheim, Mannheim, Germany) and used at concentrations ranging from 50 to 2500 units per ml (specific activity about 5×10^7 U/mg) corresponding respectively to 0.58×10^{-10}–2.9×10^{-9} M. According to the manufacturer specifications, IL-1β preparation was free of endotoxin.

Human recombinant TNFα was purchased from Boehringer (Boehringer Mannheim, Mannheim, Germany) and tested at concentrations from 2–100 ng/ml corresponding to 1.17×10^{-10}–5.9×10^{-9} M. The specific activity was superior at 1×10^8. One unit was defined as the amount of TNFα that was required to mediate half-maximal cytoxicity with WEHI 164 cells in the presence of actinomycin. Endotoxin, evaluated by Limulus Amebocyte Lysate test (LAL-test) was <10 EU/ml.

Human recombinant IL-6 was kindly provided by SANDOZ (Basel, Switzerland) and used at concentrations of 100, 1000 and 10 000 U/ml which corresponded, respectively, to 2, 20 and 200 ng/ml. Specific activity was 52×10^6 U/mg. According to

the manufacturer specifications, endotoxin was not detectable in the IL-6 preparation.

Human recombinant IL-8 (SANDOZ, Basel, Switzerland) was used at concentrations ranged among 0.8–80 ng/ml corresponding to 1×10^{-10}–1×10^{-8} M. The absence of endotoxin was verified by LAL-test.

Human recombinant LIF was obtained from PeProtech (Rocky Hill, U.S.A.) and added to the culture medium at concentrations from 8–800 U/ml. Specific activity corresponded at 4×10^7 U/mg. These concentrations corresponded respectively at 10^{-11}–10^{-9} M. The preparation was endotoxin free.

Rabbit antiserum (purified IgGs) neutralizing IL-1β and α activities was purchased from Calbiochem (La Jolia, U.S.A.). One milligram of purified IgG neutralized 10 000 U of IL-1β. Antibodies were added into culture medium at concentrations of 10 and 50 μg/ml.

Purified polyclonal antibodies neutralizing LIF activities were a gift of Dr A. Godard (University of Nantes, France). Ten micrograms of these antibodies neutralized 2.5 ng of LIF.

Three culture flasks were used for each concentration of cytokine as well as for the corresponding controls. Each culture was run with chondrocytes from the knee joint of a single patient.

IMMUNOASSAYS FOR CYTOKINES AND DNA ASSAY

IL-1β, IL-6, IL-8 and LIF were assayed in the culture medium and in the corresponding cellular phase by specific EASIA (Enzyme Amplified Sensitivity Immunoassay) kindly provided by Medgenix (Fleurus, Belgium). Results were expressed in pg/ml. The methods used, type of tracer, sensitivity, precision, reproducibility and accuracy have been previously described [33]. Chondrocyte DNA content was measured according to the fluorimetric method of Labarca and Paigen [34].

CALCULATION AND STATISTICAL ANALYSIS

The results were expressed as amounts of cytokines measured in the culture medium per μg of DNA and as cellular phase content per μg of DNA. Total cytokine productions were also calculated by adding the amount found in the culture medium and within the corresponding cellular phase. The mean $\pm$ S.D. of each variable was calculated. Comparison of mean values was performed using the unpaired Student's t-test, with a limit of signification at $P < 0.05$. Production curves for each experimental condition were compared by means of Zerbe's randomization test [35]. This test is based on an approximate F test with degree of freedom approximation that depends on both observations and time interval considered. Differences were considered statistically significant when $P < 0.05$.

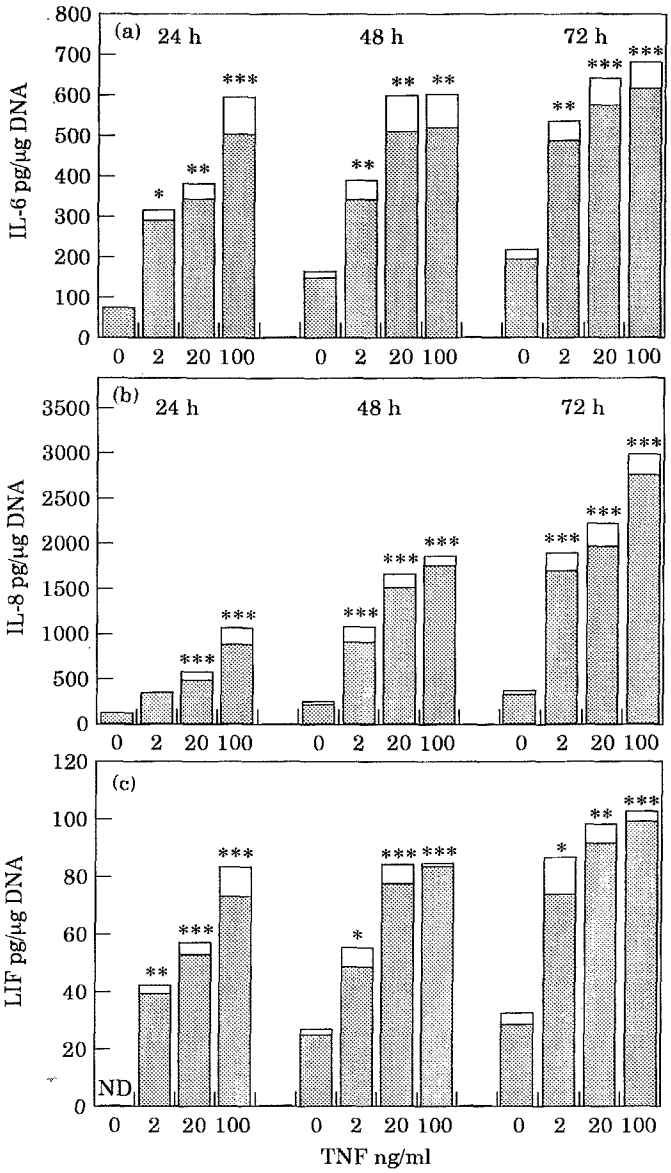

FIG. 1. Effect of human recombinant tumour necrosis factor-α (TNFα) on IL-6 (a), IL-8 (b) and LIF (c) amounts produced and released into culture medium by human chondrocytes. Cytokines levels were measured after 24, 48 and 72 h of treatment with increased amounts of TNFα. The data are expressed as amounts of cytokines found into culture per μg of DNA and presented as the mean and standard deviation (S.D.) of triplicate cultures. Statistical significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Results

EFFECT OF TNFα ON CYTOKINE PRODUCTION

Cytokines released into culture medium

Il-6, IL-8 and LIF amounts released in the culture medium by unstimulated chondrocytes increased progressively as a function of the culture period. Nevertheless, analysis of the time courses suggested that a high ratio of the IL-6 and IL-8 production occurred in first 24 h of the culture. When chondrocytes were cultured in the presence of TNFα, regardless of the TNFα dose (2, 20 and 100 ng/ml) and culture period (24, 48 or 72 h) [Fig. 1(a), (b), (c)] cytokine amounts measured in culture media were significantly higher than control values ($0.05 < P < 0.001$). This stimulating effect of TNFα was dose-dependent for IL-8 production ($0.95 < P < 0.98$; $P < 0.01$) whichever the culture period considered [Fig. 1(b)]. Concerning IL-6 production, the stimulation was only dose-dependent after 24 and 48 h of culture ($0.65 < r < 0.84$; $0.05 < P < 0.001$). As shown by Fig. 1(c), unstimulated chondrocytes did not produce detectable amounts of LIF during the first 24 h of culture. Nevertheless, LIF production was significantly increased by TNFα.

Cytokines content inside cell pellets

Table I shows amounts of cytokine measured in the cellular phase as a function of the culture time. It appears that cytokine levels present in the cellular phase reached a maximum after 24 h and then decreased as a function of culture period. We observed also that cytokine levels in the cellular phase were lower than amounts found in the culture medium. TNFα significantly increased the level of IL-8 content in the cellular phase. Responses were dose-dependent at each culture time studied ($0.95 < r < 0.98$; $0.01 < P < 0.001$). IL-6 content was also enhanced by TNFα ($P < 0.01 < P < 0.05$). In our culture and assay conditions, LIF was not detectable in the cellular phase.

EFFECT OF IL-1β ON CYTOKINE PRODUCTION

Fig. 2 shows the total IL-6, IL-8 and LIF production (sum of cytokine amounts released in the culture medium and within the corresponding cellular phase) after 72 h of culture with or without IL-1β. Total cytokine productions were nearly equal to the cytokine amounts released into culture medium after 72 h. Less than 5% of the total cytokine production was present in the cellular phase at day 3. IL-1β induced a highly significant increase of the total IL-6, IL-8 and LIF production ($P < 0.001$; Fig. 2). Responses were dose-dependent for IL-8 ($r = 0.90$) and LIF ($r = 0.71$) productions.

EFFECT OF IL-6 ON CYTOKINE PRODUCTION

During the first 48 h of incubation, IL-6 did not significantly affect the total IL-8 production by human chondrocytes. When culture time was prolonged until 72 h, IL-6 decreased IL-8 production whatever the concentration studied (2, 20 or 200 ng/ml; $0.001 < P < 0.01$; Fig. 3). IL-6 also enhanced LIF amounts secreted and released into culture medium by chondrocytes (Fig. 4). After 72 h of culture, the effect was significant at each concentration of IL-6 tested ($0.01 < P < 0.05$).

Table I.
IL-6 and IL-8 levels measured inside chondrocytes after 24, 48 and 72 h of culture with or without increased amounts of TNFα

Culture duration and parameters study	Control	TNFα 2 ng/ml	TNFα 20 ng/ml	TNFα 100 ng/ml
IL-6 pg/μg DNA				
24 h	12.1 ± 0.85	12.6 ± 0.85	16.4 ± 2.64	21.3 ± 3.05*
48 h	4.35 ± 0.57	7.91 ± 1.12*	11.4 ± 1.92*	10.2 ± 1.2*
72 h	3.03 ± 0.27	4.91 ± 0.66*	5.63 ± 0.53*	5.81 ± 0.3**
IL-8 pg/μg DNA				
24 h	17.9 ± 3.9	42 ± 3.5**	65 ± 11**	114 ± 14**
48 h	15.6 ± 2.0	39 ± 5.0**	62.5 ± 5.8***	110 ± 7.6**
72 h	14 ± 1.0	35 ± 1.4***	60 ± 8.6**	76 ± 10**

Values are the means of three separate determinations and are expressed as amounts of cytokines present inside cells per μg of DNA. *Statistically significant difference compared with the controls *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

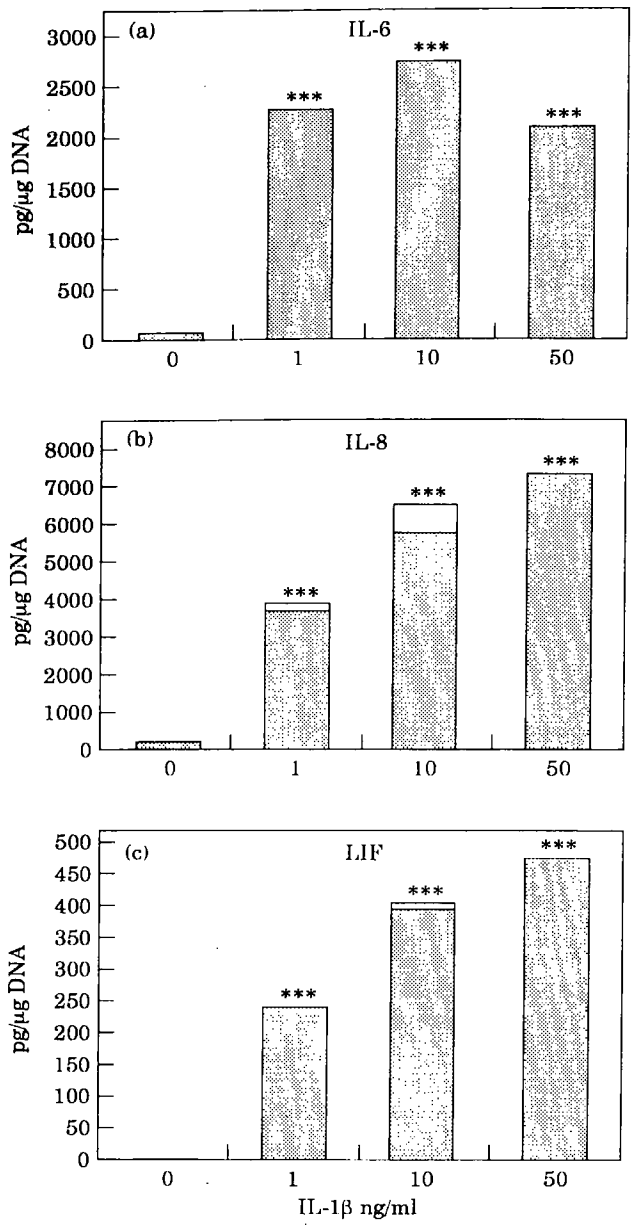

FIG. 2. Total amounts (sum of the amount found within culture medium and inside cellular phase) of (a) IL-6, (b) IL-8 and (c) LIF produced by human chondrocytes cultured for 72 h in the absence or presence of increased doses of IL-1β. Results are expressed as mean values of three cultures from the same donor. Responses are significant ***$P < 0.001$.

EFFECT OF IL-8 ON CYTOKINE PRODUCTION

At the concentration of 80 ng/ml, IL-8 slightly increased the amount of IL-6 released in the culture medium ($P < 0.05$; Table II). The effect was present after 48 and 72 h of culture. IL-8 had no significant effect on IL-6 content in the cellular phase. However, IL-8 had no effect on LIF production either in the cellular phase or in the culture medium (data not shown).

EFFECT OF LIF ON CYTOKINE PRODUCTION

The time course analysis of IL-6 production shows that the IL-6 released in the culture medium continuously increased as a function of the culture period with or without the addition of LIF (Fig. 5). However, the curves obtained for the highest concentrations of LIF (2 and 20 ng/ml) were significantly higher than the control curve showing a stimulation. No effect was observed at the concentration of 0.2 ng/ml. The amount of IL-6 found in cellular phase rapidly increased during the first 24 h of culture and progressively decreased as a function of culture duration. LIF increased significantly the IL-6 content at the doses of 2 and 20 ng/ml (Fig. 5).

LIF also stimulated total IL-8 production (Fig. 6). In the presence of 20 ng/ml of LIF, stimulation was already significant after 12 h. In our culture and assay conditions, unstimulated chondrocytes did not spontaneously produce IL-1β. Interestingly, the production of IL-1β rapidly increased with the addition of LIF. These results suggested that LIF effects on IL-6 and IL-8 productions could be secondarily mediated by IL-1β. To verify this hypothesis, we simultaneously cultured chondrocytes with LIF and antibodies neutralizing IL-1α and β. Surprisingly, 5 and 10 μg of neutralizing antibodies added into culture medium inhibited the spontaneous production of IL-6. Neutralizing IL-1α and β activities antibodies, totally inhibited the stimulating effect of LIF on IL-6 production (Fig. 7). Similar results were obtained on IL-8 production (data not shown).

EFFECT OF POLYCLONAL ANTIBODIES (PAB LIF) NEUTRALIZING LIF ACTIVITY ON CYTOKINE PRODUCTION

When human articular chondrocytes were cultured for 72 h in the presence of antibodies neutralizing LIF activity, IL-6 production was significantly decreased (Table III). On the other hand, IL-8 produced by chondrocytes was slightly decreased as compared with the controls. In the presence of IL-1β (17 ng/ml), the cytokines production was markedly enhanced ($0.01 < P < 0.001$). When IL-1β and LIF Pab were simultaneously added in the culture medium, the stimulating effect of IL-1β was not significantly modified. These data suggest that LIF had no effect upon the IL-1β activity.

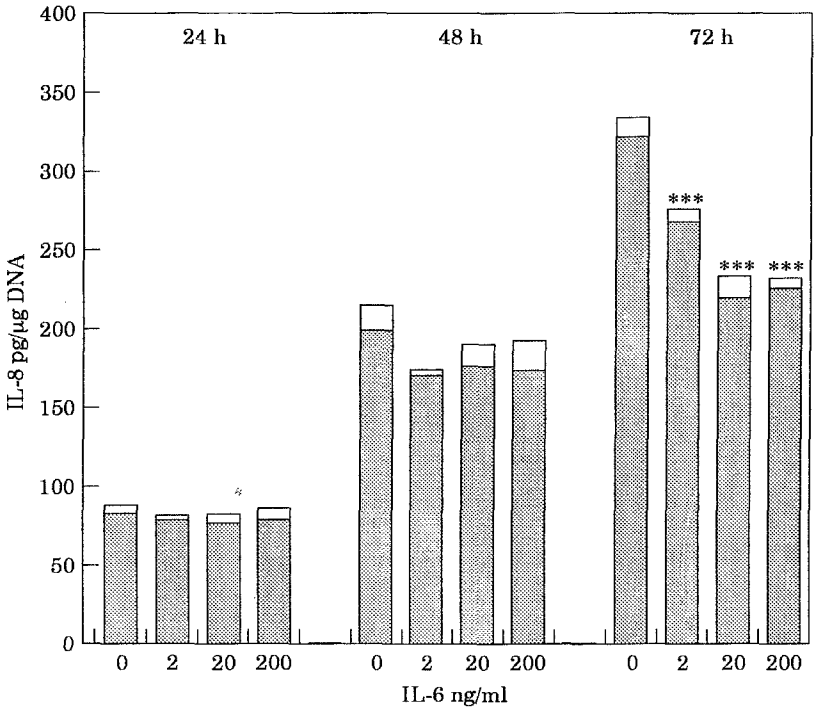

FIG. 3. Human chondrocytes were incubated for the time specified in the absence or presence of varying concentrations of human recombinant interleukin(IL-6). At the end of the period, supernatant and cells were separated by centrifugation and were assayed for interleukin (IL-8) by a specific EASIA. The values correspond to the total amount of IL-8 (sum of the amount found inside cellular phase and within culture medium) produced by chondrocytes. After 72 h of incubation, IL-6 significantly decreased IL-8 production as compared with the control values. Responses are significant with a $***P < 0.001$.

Discussion

The data presented in this paper show that freshly isolated human chondrocytes can spontaneously secrete IL-6, IL-8 and LIF. It is now accepted that these cytokines mediate a variety of activities on immunocompetent cells and connective tissues. Nevertheless, regulator effects of these cytokines on the other cytokine production are still obscure and not well documented. In the present work, we have identified some regulator pathways existing between IL-1β, IL-6, IL-8 and LIF. These regulator pathways may play an important part in the maintenance of the dynamic equilibrium between synthesis and degradation in cartilage as well as in the pathogenesis of joint disease.

We have demonstrated that IL-1β and TNFα are potent stimulators of IL-6, IL-8 and LIF production. The presence of high levels of these cytokines in the synovial fluid of patients with rheumatoid arthritis (RA) suggests that they may participate in the joint destruction in RA [1, 2, 3]. Under our culture conditions, the levels of IL-1β that induced IL-6 and IL-8 were in the same range of concentrations as those found in RA synovial fluid. This finding suggests that stimulated chondrocytes are an important source of cytokines that may contribute, at least partially, to the enhancement of the cytokine levels observed in joint diseases. Interestingly, our results also provide evidence that chondrocytes could be an important reservoir of IL-8. This finding suggests those chondrocytes have the potential to initiate and propagate inflammation through their ability to rapidly release in the extracellular environment a high level of the potent neutrophils-activating cytokine. By this way, chondrocytes may act on neighbouring tissues and participate in the development of synoviotis and bone remodelling present in RA. Although the role played by IL-1β and TNFα in the pathogenesis of joint diseases is clearly demonstrated, the actions of IL-6, IL-8 and LIF require further examination.

Because equally high amounts were found in the supernatant of chondrocytes culture stimulated with IL-1β, we tested human recombinant IL-6 at concentrations ranging from 2–200 ng/ml. In this concentration range, IL-6 induced a depression of IL-8 production and a stimulation of LIF synthesis. In light of the biological functions of IL-8, the inhibition of this cytokine suggests a negative feedback regulation to limit inflammatory reaction

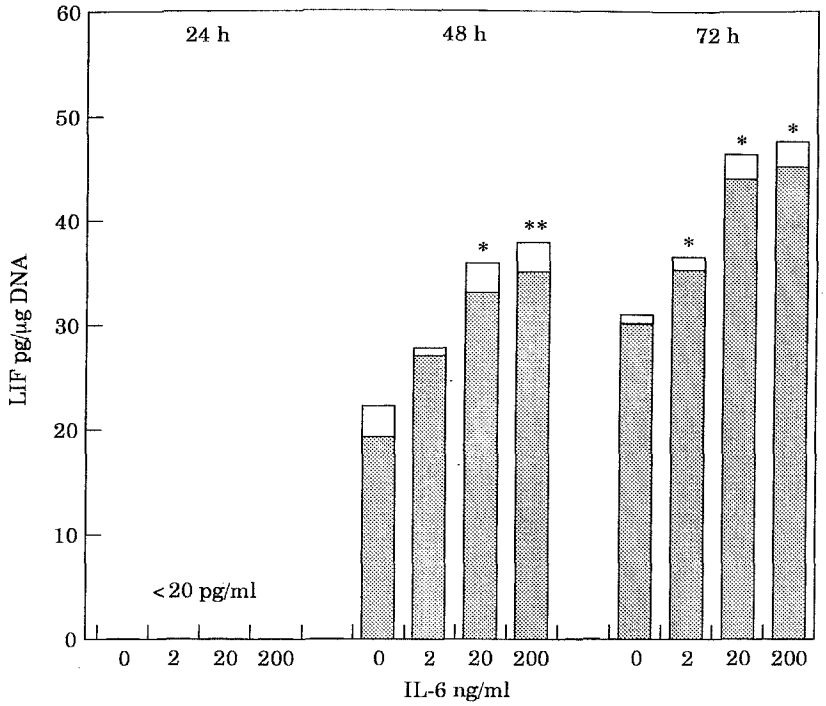

FIG. 4. Leukemia inhibitory factor (LIF) amount released within culture medium as a function of culture duration. Interleukin-6 (IL-6) added into culture medium at concentrations ranging from 2–200 ng/ml increased LIF amount produced by human chondrocytes. Significant effects were observed after 48 h of culture at the concentrations of 20 and 200 ng/ml and for each concentration used after 72 h of culture. Statistical significance: $*P < 0.05$; $**P < 0.01$.

and joint invasion by neutrophils and T lymphocytes. Indeed, it is now well established that IL-8 is one of the most potent chemoattractant factors for these cells [21]. This cytokine also induces neutrophil degranulation and the release of lysosomial enzymes and active oxygen species in the joint cavity [22]. In this way IL-8 may contribute to cartilage degradation. This inhibitory effect of IL-6 on IL-8 production can be included in the series of protective actions recently described for IL-6. In fact, IL-6 stimulates TIMP production by chondrocyte without affecting collagenase activity [36]. Moreover, IL-6 increases TGFβ [19] synthesis and stimulates chondrocytes proliferation [20] but does not modify prostaglandin E_2 (PGE_2), collagen or proteoglycan syntheses [15, 18]. On the other hand, together with the secretion of IL-6, chondrocytes may contribute to the development of autoimmunity directed toward cartilage antigens such as type II collagen. In fact, IL-6 is required for T-cell activation, B-cell maturation and antibody production, including the rheumatoid factor [13, 37]. IL-6 may also contribute to the development of the inflammatory reaction accompanying RA. IL-6 induces acute phase production by hepatocytes [25]. Nevertheless, a recent in vivo

Table II.
IL-6 amounts found in the cultured medium and inside chondrocytes cultures in the absence or in the presence of increase concentrations of IL-8

Culture duration	Control	IL-8 2 ng/ml	IL-8 20 ng/ml	IL-8 100 ng/ml
In culture medium				
24 h	18.2 ± 1.15	14.2 ± 3.2	15.6 ± 5.6	18.6 ± 1.15
48 h	29 ± 2.4	29 ± 1.8	31.6 ± 6	37 ± 1*
72 h	35.3 ± 2.5	33.3 ± 4.16	45 ± 4.2	53.3 ± 7.5*
Inside cell extracts				
24 h	5.1 ± 1	4.9 ± 0.1	6.2 ± 0.9	6.2 ± 0.4
48 h	3.6 ± 0.2	4.4 ± 0.4	4.3 ± 0.7	5.4 ± 0.9
72 h	1.03 ± 0.03	0.99 ± 0.004	2 ± 0.5	1.4 ± 0.19

Data are the mean ± S.D. and are expressed in pg per μg of DNA. $N = 3$ in each group. *Significantly different from the control with a $P < 0.05$.

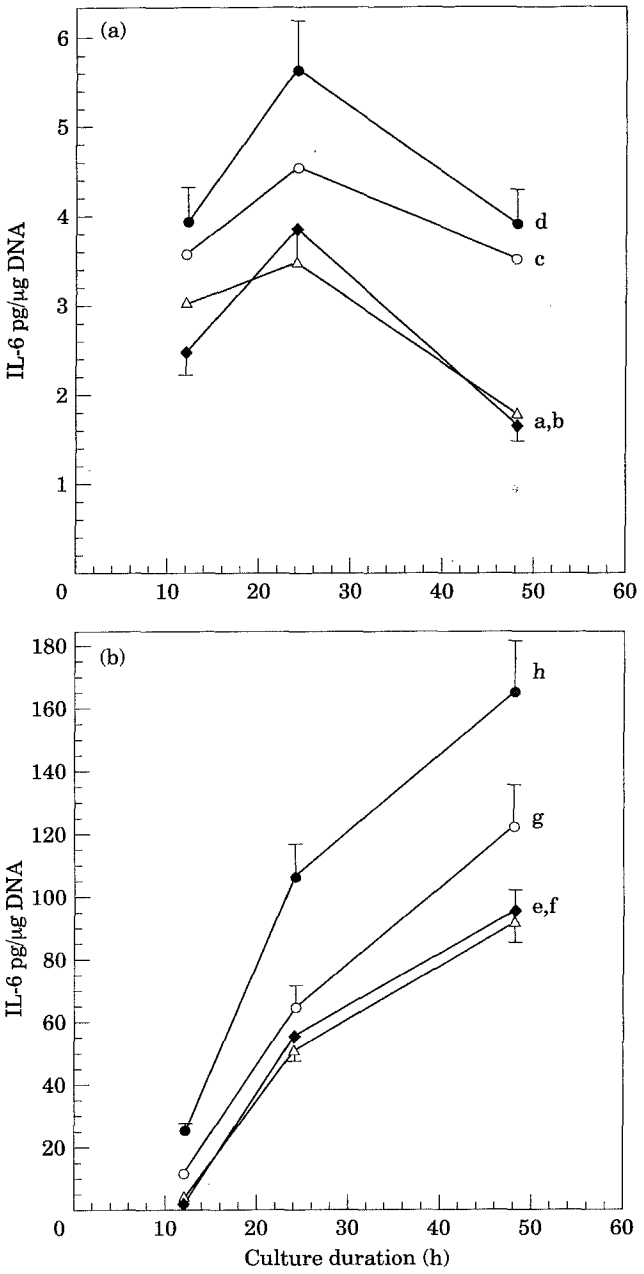

FIG. 5. Time course of interleukin (IL)-6 production by human chondrocytes. Primary chondrocytes were cultured in the absence (-◆-) or presence of 0.2 ng/ml (-△-), 2 ng/ml (-○-) or 20 ng/ml (-●-) of LIF. (a) IL-6 present inside cellular phase. (b) IL-6 released into culture medium. Results are the mean values of triplicate of primary chondrocytes culture in a representative experiment and error bars represent S.D. Differences between curves are tested by Zerbe test. a,b ≠ c: $P < 0.025$; a,b ≠ d: $P < 0.01$; c ≠ d: $P < 0.05$; e,f ≠ g; $P < 0.05$; e,f ≠ h: $P < 0.01$; g ≠ h: $P < 0.01$.

study failed to demonstrate the efficacy of anti-IL-6 treatment in experimental arthritis [38].

In the present study, we have also demonstrated that chondrocytes produced LIF. This production was highly increased by pro-inflammatory cytokines such as IL-1β, TNFα and IL-6. These results are in agreement with previous studies that showed that LIF production was induced not only by pro-inflammatory cytokines but also by growth factors such as TGFβ, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) and basic fibroblast growth factor (bFGF) [39]. The role played by LIF in the development of articular diseases is still obscure. We have previously described that LIF decreased proteoglycans production [40]. Together with the observations of Lotz *et al.* [39] that LIF can induce expression of collagenase and stromelysin by human articular

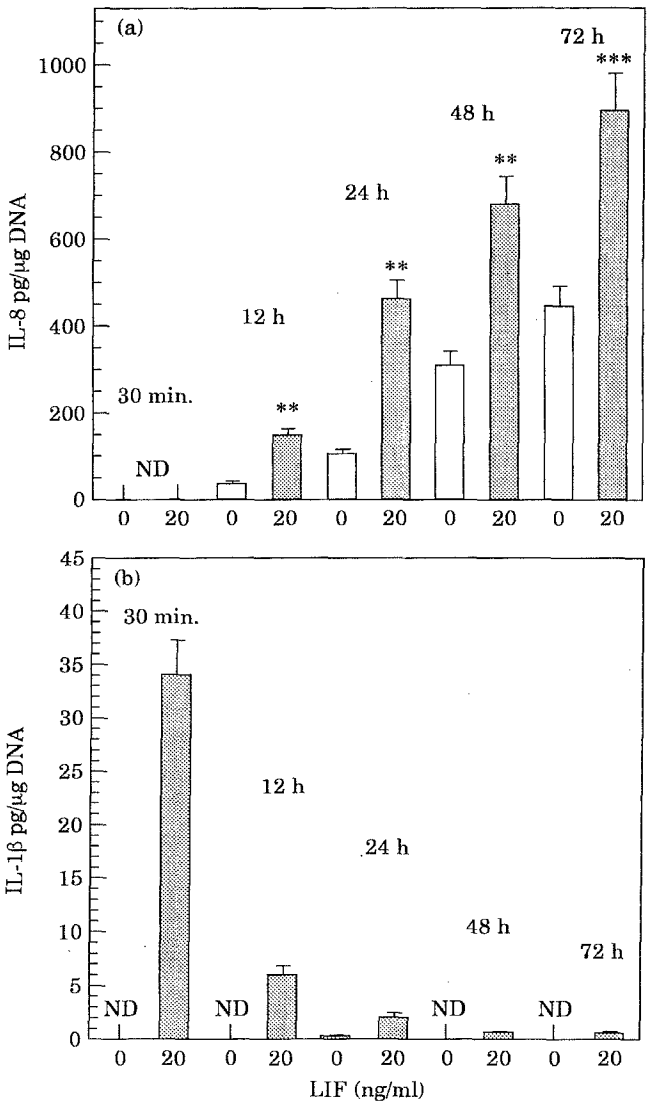

FIG. 6. Effect of leukemia inhibitory factor (LIF) on (a) interleukin (IL-8) and (b) IL-1β released into culture by human chondrocytes. Cytokine levels were assayed by enzyme-amplified sensitivity immunoassay at each time point. Values are the mean and S.D. ($N = 3$). Statistical analysis is performed with unpaired Student's *t*-test for assessing significant differences between controls and treated groups: $^{**}P < 0.01$; $^{***}P < 0.001$.

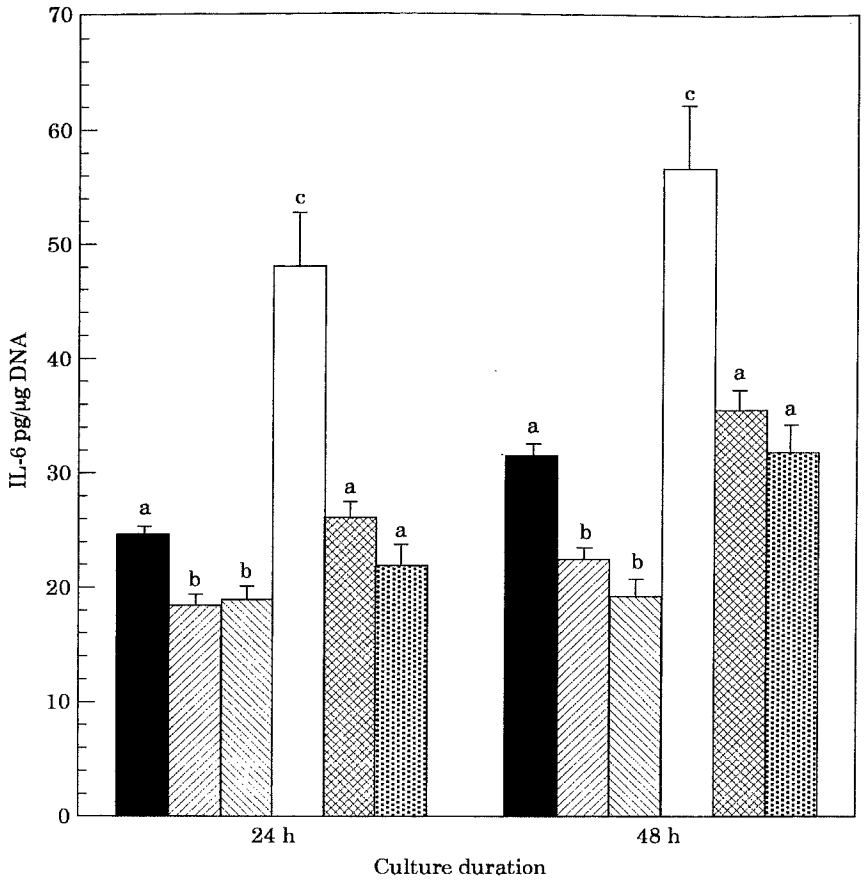

FIG. 7. Leukemia inhibitory factor (LIF) induction of interleukin (IL)-6 is mediated by IL-1. During 48 h, primary chondrocytes cultures were simultaneously incubated with LIF (20 ng/ml) and specific antibodies neutralizing IL-1α and β activities (Pab IL-1), or with LIF (20 ng/ml) or specific neutralizing antibodies only. IL-6 was directly assayed in the culture medium by specific enzyme-amplified sensitivity immunoassay. Results are expressed as mean values from one representative experiment performed in triplicate. Statistical significance: $a \neq b \neq c$ $P < 0.05$. ■ = controls; ▨ = Pab 5 μg/ml; ▧ = Pab 10 μg/ml; □ = LIF 20 ng/ml; ⊞ = LIF 20 ng/ml + Pab 5 μg/mL; ▦ = LIF 20 ng/ml + Pab 10 μg/ml.

chondrocytes without affecting TIMP production, LIF appears to be a potent mediator of cartilage resorption [32]. Furthermore, we have shown that LIF stimulated production of pro-inflammatory cytokines including IL-6, IL-8 and IL-1β. These observations support the notion that LIF may be directly or indirectly involved in the development of cartilage destruction and the process of joint inflammatory events. The finding that LIF induced IL-1β production by chondrocytes suggested that the LIF effect could be mediated indirectly by IL-1. To verify this hypothesis, chondrocytes were

Table III.
Quantitation of cytokines (IL-6, IL-8, LIF) present in free cell supernatants after 48 h of culture with culture medium, purified antibodies neutralizing LIF activities (Pab LIF), IL-1β only or simultaneously with Pab LIF and IL-1β

Parameters studied	Control	Pab LIF 50 μg/ml	IL-1β 17 ng/ml	IL-1β 17 ng/ml Pab LIF 50 μg/ml
IL-6 pg/μg DNA	509 ± 47a	234 ± 5b*	7774 ± 1499c***	8138 ± 272c***
IL-8 pg/μg DNA	2909 ± 113a	2748 ± 137a	11093 ± 129b**	13881 ± 1100c***
LIF pg/μg DNA	890 ± 17.6a	Not detectable	2987 ± 234b**	Not detectable

Letters indicate differences between treated and untreated groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table IV.
The negative and positive effects of the different cytokines on each other

	Cytokines assayed			
Cytokines added	IL-6	IL-8	LIF	IL-1β
IL-1β	+	+	+	ND
TNFα	+	+	+	ND
IL-6	ND	–	+	ND
IL-8	+	ND	0	ND
LIF	+	+	ND	+

+ = stimulation; – = inhibition; 0 = no effect; ND = Not determined.

cultured in the presence of antibodies neutralizing IL-1α and β activities. We observed that LIF-induced IL-6 and IL-8 productions are totally inhibited by antibodies neutralizing IL-1α and IL-1β activities. This finding provides evidence that LIF effects are secondary at the release of IL-1. This hypothesis is supported by the observation that LIF induced a rapid secretion of IL-1β which occurred within 30 min, before the induction of IL-8 and IL-6. Interestingly, the addition of antibodies neutralizing LIF activity in the culture medium decreased significantly the spontaneous IL-6 production but did not modify the effect of IL-1β on this parameter. These results suggest that LIF and IL-1β are not synergic.

In summary (Table IV), IL-1β and TNFα are potent inducers of the production of pro-inflammatory cytokines. These latter cytokines may interact to regulate their productions by chondrocytes. We have clearly identified the following interactions. IL-6 decreases IL-8 production and slightly increases LIF synthesis. IL-8 have a moderate stimulatory effect on IL-6 production but does not effect LIF production. Finally, LIF is a potent stimulant to induce cytokines production, such as IL-1β, IL-6 and IL-8. Nevertheless, these effects are likely to be mediated by, and secondary to, IL-1 secretion. In conclusion, our study suggests that through the production of cytokines, chondrocytes may act on neighbouring connective tissue metabolism and initiate and modulate the inflammation and immune responses. These productions are regulated by a complex interactive network of mediators.

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