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ORIGINAL ARTICLE

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The levels of leukemia inhibitory factor in synovial tissues of patients with rheumatoid arthritis: inflammation and other proinflammatory cytokines

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Abstract To clarify the effect of leukemia inhibitory factor (LIF) on the destruction of rheumatoid arthritis (RA) joints, we investigated the production of LIF and the expression of LIF mRNA in synovial tissues from patients with RA and osteoarthritis (OA). Synovial fluids from RA were used to measure the LIF concentrations using enzyme-linked immunosorbent assay (ELISA). Immunohistochemistory and RT-PCR were used to examine the expression of LIF by synovial cells. LIF mRNA was detected in all cases in RA synovial cells. Although LIF protein was detected only in 20 cases (19%) in RA synovial fluids, LIF concentration in the synovial fluids significantly correlated with the peripheral leukocyte count ($P < 0.001$) and C-reactive protein (CRP) ($P < 0.01$). Moreover, levels of IL-1β, IL-6, and IL-8, but not TNF-α, were significantly correlated with LIF in the RA synovial fluids. LIF production was promoted by IL-1β and TNF-α stimulation; in contrast, IL-1 ra and IL-4 were found to markedly decrease LIF production by cultured synovial cells. LIF appeared to be a cytokine produced by RA synovium leading to a proinflammatory secretion profile. Moreover, IL-4 and IL-1 ra may represent attenuated activity for reducing the effect of the destruction of joints by LIF.

Key words Cytokine · Leukemia inhibitory factor (LIF) · Osteoarthritis (OA) · Rheumatoid arthritis (RA) · Synovitis

Introduction

Rheumatoid arthritis (RA) is a chronic progressive inflammatory disease characterized by persistent synovitis, which leads to joint destruction and disability. The etiology of chronic arthritis has not been identified. However, the main pathological characteristics of rheumatoid joints include neovascularization and proliferation of synovial tissue, which produces proinflammatory cytokines. In recent years, cytokine has been suggested as a molecule inducing these pathohistological changes. Although cytokine was originally reported as a physiological substance playing an essential role in the maintenance of homeostasis of the living body, cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-8 are produced excessively by fibroblasts, macrophages, and neutrophils in pathological conditions.[1–4] TNF-α and IL-1β are mainly related to joint destruction,[5–7] inducing degradation of proteoglycan[8,9] and resorption of bone.[10,11] It has been strongly suggested that other cytokines, such as IL-6 and leukemia inhibitory factor (LIF), are also related to the pathology of joint destruction.[12,13]

LIF is a glycoprotein consisting of 179 amino acids originally defined by its ability to induce the terminal differentiation of murine M1 myeloid leukemia cells, resulting in the inhibition of their growth.[14] LIF has a diverse range of activities, including the stimulation of platelet production,[15] the inhibitory effect of lipoprotein lipase activity,[16] and regulation of the differentiation of nerve cells.[17] The receptors of LIF exist in monocytes, macrophages, lymphocytes, osteoblasts, fibroblasts, nerve cells, embryonic stem cells, hepatic cells, and lipocytes.[18–21] Moreover, LIF is one of the IL-6 family of cytokines including IL-6, oncostatin-M, ciliary neutrophic factor, and IL-11 on the basis of an overlapping spectra of biological activity and the common use of the gp 130 receptor component in each of their receptor complexes.[22]

LIF also plays an important role in the induction of acute-phase protein synthesis,[23] in the regulation of both bone formation and bone resorption,[24] and in the degradation of proteoglycan.[25] LIF has been detected at high levels in synovial fluids of 35% from patients with RA.[26] Furthermore, the expression of LIF in human synovial fibroblasts was described by Hamilton et al.[27] These findings of the present study or previous findings suggested that LIF was

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one of the participants in joint destruction as a proinflammatory and chondrolytic cytokine. Therefore, to study the effect of LIF on the destruction of RA joints, we investigated the production of LIF and the expressions of LIF mRNA in synovial tissues from patients with RA and compared them with those of osteoarthritis (OA). In addition, we examined the correlation between LIF concentrations in synovial fluids and clinical parameters of disease activity. Finally, we intended to observe the influences on the production of LIF by other cytokines such as TNF-α, IL-1β, IL-6, IL-8, or IL-4, and IL-1 receptor antagonist (IL-1 ra).

Materials and methods

Reagents

All recombinant cytokines such as TNF-α, IL-1β, IL-4, and IL-1 ra were supplied by R&D Systems (Minneapolis, MN, USA).

Synovial fluid samples

One-hundred-one patients with RA and 26 control patients with OA were diagnosed according to the American College of Rheumatology criteria.[28] Synovial fluids were collected during diagnostic or therapeutic arthrocenthesis of the knee (108 knees in 101 cases with RA or 26 knees in 26 cases with OA). All synovial samples were collected under sterile conditions, and cellular component were removed immediately after centrifugation. Supernatants were treated with 200 U/ml of hyaluronidase (Mochida Seiyaku, Tokyo, Japan) at 37°C for 20 min and stored at −80°C.

Culture of synovial cells

Human synovium was obtained at artificial knee joint replacement with informed consent. Synovial tissue was dissected from the fibrous capsule and later minced. The cells were isolated using enzymatic digestion by 0.25% collagenase (Nitta Gelatin, Osaka, Japan), followed by plating at 5×10^5 cells/well in 24 well dishes (Coster). The synovial cells obtained were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin, and 50 mg/ml streptomycin (Gibco). The culture media were removed from cells by centrifugation after culturing for 24 or 48 h, and after 120 or 168 h, according to the experiments. Supernatants were stored until measurement at −80°C.

Measurement of LIF and other cytokines

LIF concentrations were measured in triplicate by the quantitative sandwich enzyme-linked immunosorbent assay (ELISA), using a commercially available kit (Amersham, Buckinghamshire, UK). The concentrations of TNF-α, IL-1β, IL-6, and IL-8 were determined using a double-ligand immunoassay. Wells of microtiter plates coated with monoclonal mouse antihuman TNF-α antibodies (Pharmigen, San Diego, CA, USA), mouse antihuman IL-1β antibodies (R&D Systems), and mouse antihuman IL-6 or IL-8 antibodies (Biosource, Camarillo, CA, USA) were incubated with test samples and recombinant TNF-α, IL-1β, IL-6, and IL-8 standards. Bound TNF-α was detected with biotinized mouse anti-TNF-α antibodies (Pharmigen) using the immunoperoxidase method. Bound IL-1β, IL-6, and IL-8 were detected with rabbit anti-human IL-1β, IL-6, and IL-8 antibodies (Endogen, Cambridge, MA, USA), respectively, as secondary antibodies conjugated to alkaline phosphatase using the substrate *P*-nitrophenylphosphate.

Extraction of RNA and reverse transcription-polymerase chain reaction method

Total cellular RNA was extracted from synovial tissues with RA and OA using the acid guanidium thiocyanate phenol chloroform method.[29] First, strand cDNA was synthesized from approximately 1 mg total RNA containing the random 9-mers as primers using a Takara RNA kit (Takara Shuzo, Shiga, Japan) in 20 ml reaction buffer and used as a template for polymerase chain reaction (PCR). The sequences of oligonucleotide primers used for PCR amplification were as follows: LIF 5′-GTCTTGGGGCAGGAGTTGT and LIF 3′-CTCCCCTGGGCTGTGTAAT, defining a 216-bp fragment. The sense and antisense primers utilized were previously reported by Moreau et al.[30] PCR was carried out under the following conditions using Program Temp Control System PC-700 (ASTEC, Fukuoka, Japan): denaturing (94°C, 40 s), annealing (64°C, 1 min), and extension (72°C, 1 min), 35 cycles. After electrophoresis using 3% agarose containing ethidium bromide, amplified PCR products were detected by ultraviolet irradiation. To assess the reproducibility and avoid bias in the reverse transcription (RT)-PCR analysis, cells from synovial culture were processed in parallel or subjected to 5- to 15-fold serial dilutions of the cDNA samples to PCR. In addition, samples yielding a PCR product were subjected to a second round of amplification to confirm that PCR results on the same samples were reproducible.

Immunohistochemical staining

Synovial tissues was cut into 5 × 5 × 5 mm, placed in culture plates, and then cultured in DMEM supplemented with 2 ng/ml IL-1β and 10% FBS (Gibco, NY, USA) for 48 h. The medium was removed and the explants washed three times with DMEM. The frozen explants were prepared by embedding in OCT compound (Miles, Elkhurt, USA) by immersion in methylbutane. Five-micrometer serial sections were cut on a cryostat, placed on glass slides, and fixed in acetone. After blocking endogenous peroxidase, serial sections were stained with the immunoperoxidase method using rabbit antihuman LIF antibody (Genzyme, Cambridge, MA, USA) as a primary antibody and

Vecstatin ABC Peroxidase Elite Rabbit IgG kit (Vector, Burlingame, CA, USA) as a secondary antibody. The counterstaining of tissue sections was carried out using hematoxylin and eosin (H&E). In control sections, the normal rabbit serum was used as the primary antibody.

Statistics

To establish a statistical significance of observed differences, the Mann–Whitney *U* test for unpaired samples and Wilcoxon signed-rank test for paired samples were used. Values were expressed as the mean ±SD. Spermann's order correlation coefficient was used as the correlation coefficient (*r*). Less than 5% of the probability rate was defined as statistically significant.

Results

LIF concentrations in synovial fluid

LIF in 20 cases (19%) with RA in synovial fluid was detected at a mean concentration of 15.7 ± 25.2 pg/ml whereas all cases with OA were below the limit of detection of the assay (Fig. 1). To determine whether the detection of LIF in synovial fluid correlated with clinical manifestations of RA, LIF concentrations were evaluated in relation to blood parameters of disease activity at the time of synovial fluid collection. LIF concentration in the synovial fluid correlated significantly with peripheral leukocyte count ($n = 87$, $r = 0.385$, $P < 0.001$) and C-reactive protein (CRP) ($n = 92$, $r = 0.276$, $P < 0.01$) (Fig. 2A,B), but not with the erythrocyte sedimentation rate ($n = 90$, $r = 0.04$, $P = 0.70$) (data not shown). The correlations between LIF concentrations and other cytokines mentioned were examined in synovial fluids of each RA patient. Significant differences were between LIF levels and IL-1β ($n = 60$, $r = 0.299$, $P < 0.0001$), IL-6 ($n = 96$, $r = 0.335$, $P < 0.01$), and IL-8 ($n = 96$, $r = 0.498$, $P < 0.0001$) levels (Fig. 2C–E). No significant differences in LIF or TNF-α were found.

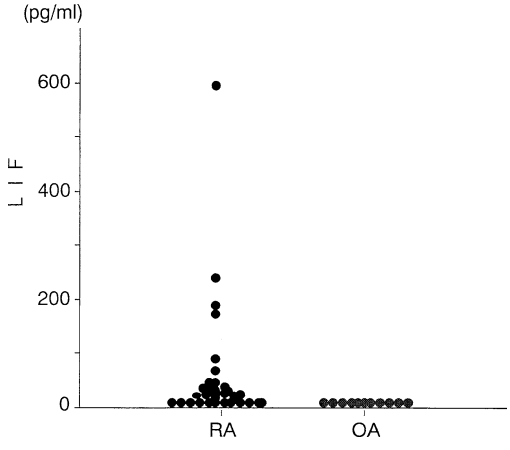

Fig. 1. Levels of leukemia inhibitory factor (LIF) in synovial fluid from patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Detectable levels were seen in 20 of 108 samples of fluid from patients with RA; levels in all patients with OA were below the limit of detection of the assay. Mean ± SEM concentration of LIF in synovial fluid from patients with RA was 15.7 ± 25.2 pg/ml

Expression of LIF mRNA in synovial cells with RA and OA

The 216-bp band showing LIF mRNA in synovial cells in all RA and OA cases was detected using the RT-PCR method (Fig. 3).

Immunohistochemical staining in RA synovial tissues

When localization of LIF-produced cells in RA synovial tissues was studied by immunohistochemical staining, LIF was found to be localized in most of the infiltrative sites of cells. Furthermore, LIF-positive cells were observed in the synovial lining cells and sometimes in endothelial cells. The staining pattern of LIF was intracellular (Fig. 4A–C). The precise phenotype of synovial lining cells responsible for expressing LIF was not determined; both type A and type B synoviocytes are capable of secreting LIF. The fact that cultured synovial fibroblasts produced LIF suggests that type B cells are responsible, although it does not preclude the possibilty that macrophages also participate in vivo.

Study of LIF production in synovial cells in vitro

The LIF concentrations in the supernatants of 5×10^5 synovial cells obtained from patients with RA and OA were 509.3 ± 124.2 pg/ml and 196.7 ± 20.1 pg/ml after 24 h culture, and 1899.5 ± 283.2 pg/ml and 509.3 ± 124.2 pg/ml after 48 h respectively, and significantly higher in synovial cells with RA than those with OA (Fig. 5).

Time course of analysis of LIF, TNF-α, IL-1β, IL-6, and IL-8 production in cultured synovial cells

The changes in LIF production in supernatants of RA synovial cells at the first, second, fifth, and seventh days after culture were compared with those of TNF-α, IL-1β, IL-6, and IL-8. The maximal production of TNF-α and IL-1β was found on the first day; production of TNF-α decreased rapidly with time, but that of IL-1β decreased more gradually. The maximal peak of the production of IL-8 was found at the fifth day, although that of IL-6 and LIF increased with time, and production of LIF increased most gradually (Fig. 6). The values presented here were calculated from the ratio with the maximum amount of each cytokine as 1 (TNF-α, 107.9 pg/ml; IL-1β, 33.7 pg/ml; LIF, 8.28 ng/ml; Il-6, 98.0 mg/ml; Il-8, 902 ng/ml).

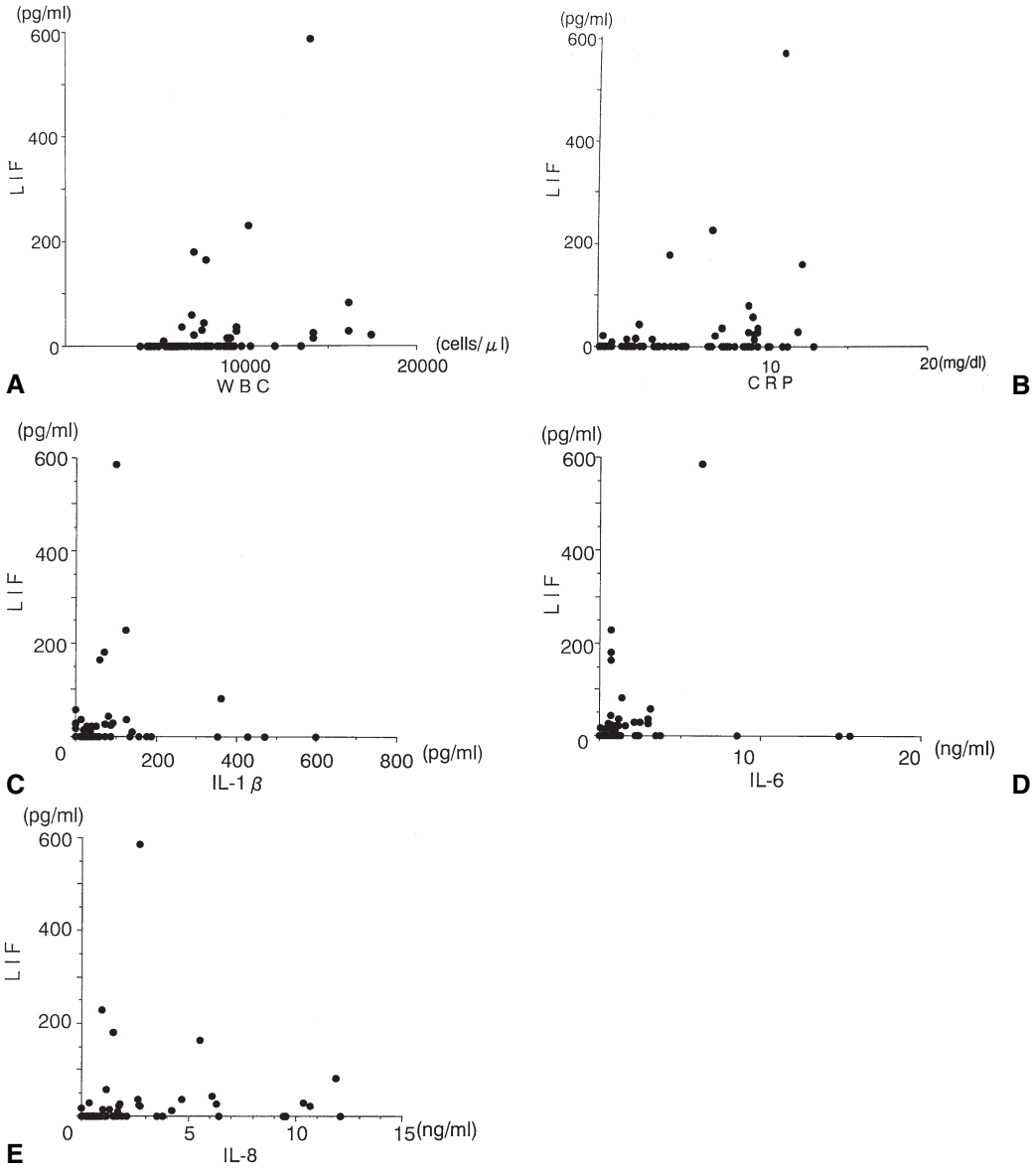

Fig. 2. **A** Correlation between synovial fluid levels of LIF and serum levels of white blood cell counts in patients with RA ($n = 87$, $r = 0.385$, $P < 0.001$). **B** Correlation between synovial fluid levels of LIF and serum levels of C-reactive protein (CRP) in patients with RA ($n = 92$, $r = 0.276$, $P < 0.01$). **C** Correlation between synovial fluid levels of LIF and IL-β in patients with RA ($n = 96$, $r = 0.299$, $P < 0.01$). **D** Correlation between synovial fluid levels of LIF and IL-6 in patients with RA ($n = 96$, $r = 0.335$, $P < 0.01$). **E** Correlation between synovial fluid levels of LIF and IL-8 in patients with RA ($n = 96$, $r = 0.498$, $P < 0.01$)

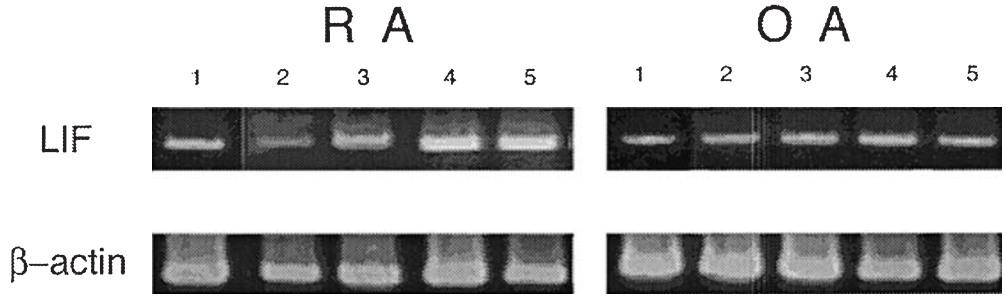

Fig. 3. RT-PCR analysis of the expression of LIF mRNA in RA (*left*) and OA (*right*) synovial tissues. An *Hae*III-digested *f* X174 marker was used in each run. PCR products were separated on 3% agarose gels, stained with ethidium bromide, and visualized under ultraviolet

Effect of TNF-α on stimulation of LIF production in cultured synovial cells

LIF concentration in the supernatants of RA synovial cells was 421.2 ± 71.1 pg/ml in control, 465.7 ± 71.5 pg/ml by stimulation of TNF-α (0.5 ng/ml), and 541.7 ± 84.1 pg/ml by TNF-α (5 ng/ml) during 24 h cultivation. During 48 h cultivation, LIF concentrations were 1892.0 ± 213.4 pg/ml in control, 2166.2 ± 55.4 pg/ml by stimulation of TNF-α (0.5 ng/ml), and 2458.2 ± 68.9 pg/ml by TNF-α (5 ng/ml). LIF production in RA synovial cells was significantly promoted by TNF-α ($P < 0.05$) (Fig. 7A).

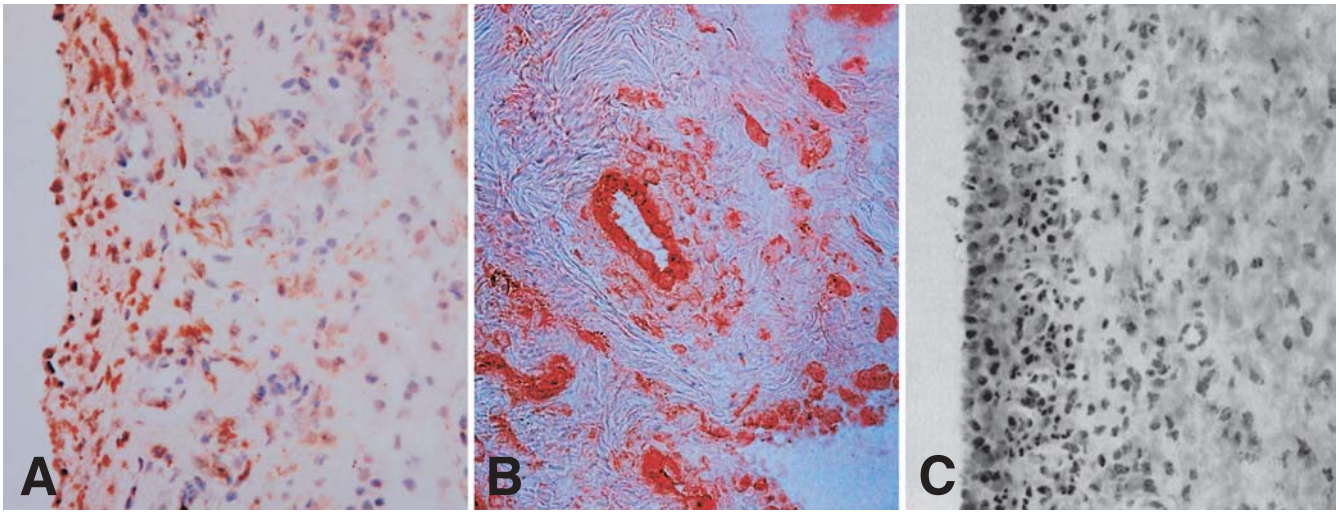

Fig. 4. Immunohistochemical staining with antihuman LIF antibody in RA synovial tissue. **A** Representative sections of RA synovium, showing LIF expression. The perinuclear staining pattern was found in the infiltrative sites of inflammatory cells. Localization of LIF was found in the synovial lining cell layer. Hematoxylin and eosin counterstain. ×200. **B** LIF-positive cells were also sometimes observed in the endothelial cells of blood vessels. No counter stain. ×200. **C** In negative control sections, the normal rabbit serum failed to stain any cells. H and E counterstain. ×200

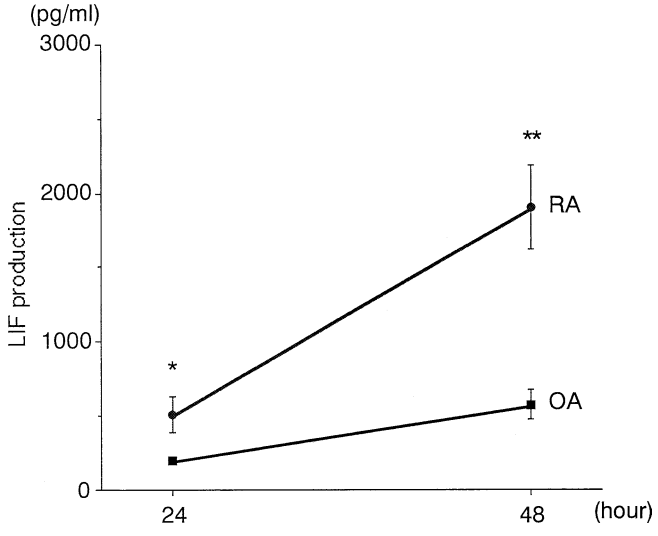

Fig. 5. Leukemia inhibitory factor production by synovial cells from RA and OA at 5×10^5 synovial cells/well, cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After 24 and 48 h, supernatants were measured by sandwich enzyme-linked immunosorbent assay (ELISA)

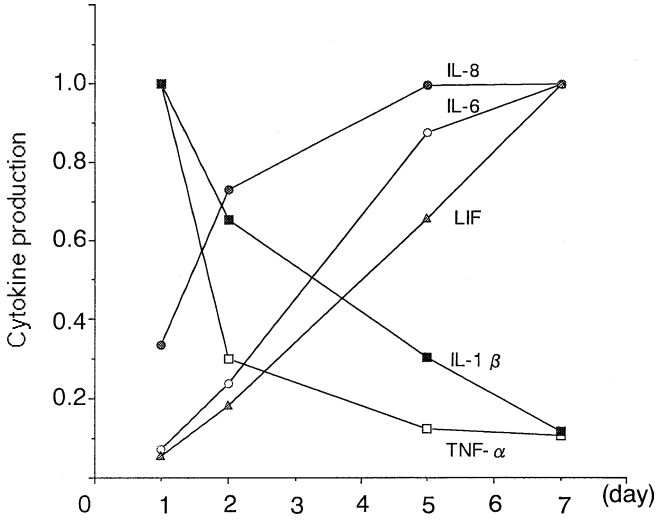

Fig. 6. Time-course analysis of LIF, TNF-α, IL-1β, IL-6, and IL-8 production in cultured RA synovial cells. Each cytokine level was measured at day 1, 2, 5, or 7 after cultivation. Measurements obtained by ELISA during the cultivation periods were estimated from the maximum amount of each cytokine (TNF-α, 107.9 pg/ml; IL-1β, 33.7 pg/ml; LIF, 8.2 ng/ml; IL-6, 98.0 mg/ml; IL-8, 902 ng/ml)

Effect of IL-1β on stimulation of LIF production in cultured synovial cells

LIF production in the supernatants of RA synovial cells was 441.5 ± 54.5 pg/ml in controls, 571.1 ± 82.9 pg/ml by stimulation of IL-1β (0.5 ng/ml), and 657.7 ± 114.6 pg/ml by IL-1β (5 ng/ml) during 24 h cultivation. After 48 h cultivation, LIF concentrations were 2056.5 ± 269.7 pg/ml (control), 2443.8 ± 167.3 pg/ml (0.5 ng/ml IL-1β) and 2734.0 ± 190.0 pg/ml (5 ng/ml IL-1β). From the findings of the present study, it was clear that IL-1β significantly enhanced the production of LIF in RA synovial cells (Fig. 7B).

Effect of IL-1 ra or IL-4 on inhibition of LIF production in cultured synovial cells

LIF production in RA synovial cells was 1982.0 ± 108.4 ng/ml in controls, 1716.9 ± 24.7 pg/ml in IL-1 ra (20 ng/ml), 1348.9 ± 104.3 pg/ml in IL-1 ra (200 ng/ml), 748.9 ± 123.6 pg/ml in IL-4 (1 ng/ml), and 621.5 ± 106.7 pg/ml in IL-4 (10 ng/ml) after 48 h cultivation. These findings show the production of LIF in RA synovial cells was significantly inhibited by IL-1 ra or IL-4 in contrast to the actions of IL-1 or TNF-α. The effect of inhibition of LIF production was more efficiently in 200 ng/ml than 20 ng/ml of IL-1ra, and

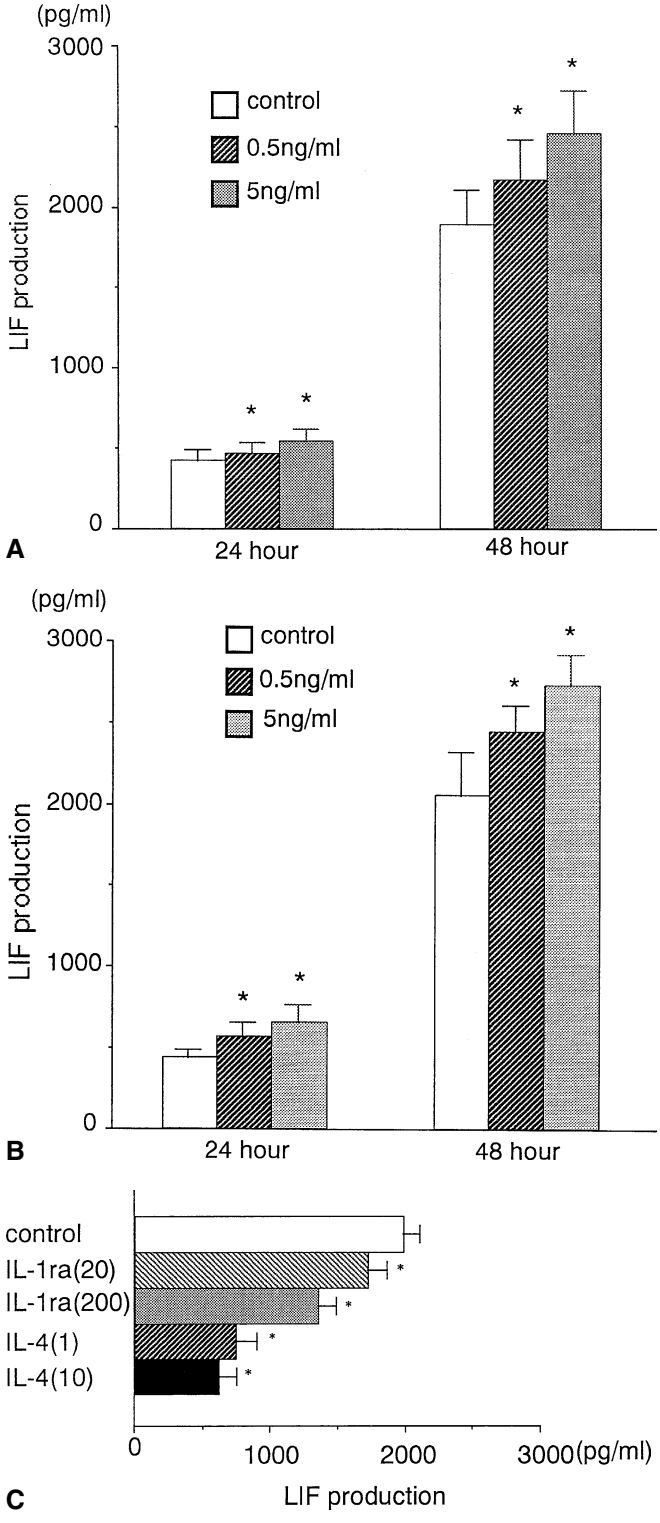

Fig. 7. Effects of cytokines on LIF production. **A** Effect of TNF-α on LIF production. RA synovial cells (5×10^5 cells/well) in 24-well dishes were stimulated with TNF-α at concentrations of 0.5 or 5 ng/ml and then cultured. Supernatants were collected after 24 or 48 h, stored at −80°C, and then analyzed using ELISA (*$P < 0.05$). **B** Effect of IL-1β on LIF production. RA synovial cells at 5×10^5 cells/well were stimulated with IL-1β at 0.5 or 5 ng/ml and cultured. Supernatants were collected after 24 or 48 h, stored at −80°C, and then measured. **C** Effects of IL-1 ra or IL-4 on LIF production. RA synovial cells at 5×10^5 cells/well were stimulated with IL-1 ra at 20 or 200 ng/ml or with IL-4 at 1 or 10 ng/ml. Supernatants were collected after cultivation for 24 or 48 h, stored at −80°C, and then measured

10 ng/ml than 1 ng/ml of IL-4 in a dose-dependent manner, respectively (Fig. 7C).

Discussion

Cytokines such as TNF-α and IL-1β are synthesized by inflammatory cells, such as monocyte-macrophages, synoviocytes, and chondrocytes, which are present in increased amounts in inflamed joint tissues. Their role in the tissue destruction of inflammatory joint diseases, such as RA, has been the subject of many studies.[4–11] The evidence that TNF-α or IL-1β is involved in RA pathophysiology has been convincing. However, some doubts concerning the mechanism of joint destruction have been raised. Hollander et al. reported that TNF-α and IL-1 do not mediate all the cartilage changes observed in RA and OA.[31] Furthermore, they proposed a cofactor interacting in a synergistic way with TNF-α and IL-1 in synovial fluid to degrade cartilage tissues. Van de Loo et al. reported that IL-6 is a cofactor in IL-1-induced suppression of proteoglycan synthesis in human cartilage explants.[32] LIF is one of the IL-6 family of cytokines and displays a broad range of in vitro activities, including induction of acute-phase protein synthesis by hepatocytes, bone resorption, stimulation of cartilage proteoglycan resorption, and inhibition of proteoglycan synthesis. Based on these findings, LIF is assumed to have the potential to propagate inflammatory responses. Therefore, it is suggested that LIF may be one of the cofactors of TNF-α and IL-1. Bell and Carroll have suggested that LIF is one of the additional factors that induced cartilage degradation in addition to IL-1 and TNF-α.[13] We demonstrated here that LIF was produced inside the joints and that the amounts of LIF in synovial fluids were significantly correlated with CRP ($P < 0.01$) and with the peripheral leukocyte count ($P < 0.001$). These findings suggest that LIF is one of the factors contributing to joint destruction in accordance with the activities of RA inflammation.

Although a quantity of LIF in synovial fluid was detected in only 19% of cases in the present study with RA, Waring et al. showed that LIF was detected in synovial fluid from 18% (28/152 cases) of patients with inflammatory arthrides including RA.[26] They also reported that the RA patients with LIF-positive synovial fluids had significantly higher peripheral leukocyte and synovial fluid leukocyte counts. We also showed that peripheral leukocyte count and CRP were correlated with LIF concentrations in synovial fluids. In addition, LIF could be responsible for the systemic effect, such as the acute-phase response[23] seen in patients with active RA. In conjunction with the correlation of synovial fluid LIF concentrations with CRP or peripheral leukocyte count, an association of synovial fluid LIF concentrations and objective markers of active RA cannot be concluded from this presentation because the quantity of LIF in detected synovial fluid was low. However, it remains possible that LIF in concentrations below the level of detection with the ELISA system employed might still be involved in the activation of these systemic responses.

None of the patients with OA had detectable synovial fluid LIF in the present study, LIF mRNA in OA synovial tissues was expressed by the RT-PCR method. This discrepancy suggests that OA synovial tissues contains the possible capability of LIF production. Indeed, we demonstrated that LIF production was to be observed in OA synovial cells in vitro, whereas the amount of LIF was low compared with those in RA synovial cells. Furthermore, LIF in RA synovium detected immunohistochemically lymphoid cells, synovial lining cells, endothelial cells, and fibroblasts. Thus, LIF may be produced in OA in the conditions occurring with secondary arthritis.

The present in vitro study demonstrated that the synovial fluid level of LIF correlated positively with the those of IL-1β, IL-6, and IL-8 in RA patients. IL-1β, IL-6, and IL-8 cytokines were mainly produced by monocyte-macrophages, synovial fibroblasts, endothelial cells, and chondrocytes. We demonstrated that LIF was also produced by such cells. Temporal productions for up to 7 days of LIF, TNF-α IL-1β, IL-6, and IL-8 cytokines in cultured RA synovial cells demonstrated that TNF-α and IL-1β levels were maximum at day 1 and rapidly returned to almost normal levels by day 7. However, the amounts of LIF with IL-6 and IL-8 were increased and reached the maximum by day 7, compared with the levels of TNF-α and IL-1β. These findings suggest the existence of two distinct patterns of cytokine production in cultured RA synovial cells. Thus, it was confirmed that LIF was not the primary initial cytokine but was induced during the latter period of inflammation, because cultured human synoviocytes are known to produce LIF when stimulated by IL-1 or TNF-α (REF = 27). Moreover, Villiger et al. reported that LIF induces IL-1β and IL-6 gene expression on human rheumatoid synoviocytes.[33] Therefore, LIF has the potential to propagate inflammatory responses and might be one of the cofactors of joint destruction.

We demonstrated here that IL-4 and IL-1 ra reduced the production of LIF compared with IL-1 and TNF-α. Dechanet et al. reported that IL-4 but not IL-10 inhibited the production of LIF.[34] Recently, Chabaud et al. also confirmed that IL-4 but not IL-10 inhibited the IL-1- and IL-17-induced production of LIF.[35] IL-4, known as a group of Th2 cytokines, was produced at low levels in the lesions of arthritis joints,[36] and IL-1 ra also showed low levels.[37] These findings suggest that IL-4 and IL-1 ra in the synovial compartment may not be sufficient to neutralize IL-1 catabolic functions. Therefore, LIF induced by IL-1 was overproduced and was affected, in part, by the destruction of the joint. Conversely, control of the action of IL-4 and IL-ra may present a target for reducing the effects of proinflammatory cytokines including LIF.

In conclusion, LIF appeared to be a cytokine produced by the RA synovium leading to a proinflammatory secretion profile. Moreover, IL-4 and IL-1 ra may represent attenuating activity for reducing the effect of the destruction of arthritis joints by the secretion of LIF.

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