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

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). Immunohistochemical 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-1ra 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 secretory profile. Moreover, IL-4 and IL-1ra 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 pathophysiological 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 proteoglycan8,9 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
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. Synovial fluids were collected during diagnostic or therapeutic arthrocentesis 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 was removed immediately after centrifugation. Supernatants were treated with 200 U/ml of hyaluronidase (Mochida Seiyaku, Shiga, 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 × 10⁵ 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 anti-human TNF-α antibodies (Pharmergin, San Diego, CA, USA), mouse anti-human IL-1β antibodies (R&D Systems), and mouse anti-human 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. 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, Tokyo, 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'-GTCTTGGGGAGAGTTGT and LIF 3'-CTCCCCGTGGGCTGTGTAAT, defining a 216-bp fragment. The sense and antisense primers utilized were previously reported by Moreau et al. 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 gel 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 were 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, Elkhart, 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 anti-human LIF antibody (Genzyme, Cambridge, MA, USA) as a primary antibody and