Tissue Levels of Leukemia Inhibitory Factor Vary by Osteoarthritis Grade

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The objective of this study was to observe the expression of leukemia inhibitory factor (LIF) in animals and in different clinical grades of patient osteoarthritic tissues. Thirty-five rabbits were used in a Colombo model of experimental osteoarthritis (OA). Five rabbits each were sacrificed on postoperative days 3, 7, 14, 28, 42, 56, and 84. Immunohistochemistry analysis for LIF expression and distribution in the cartilage and synovium of animals was performed at these times. Sixty-seven samples of human articular tissue were obtained from patients with different grades of OA according to symptoms and radiographic inspection. The mRNA expression of LIF was determined by reverse transcription polymerase chain reaction, and LIF protein was determined by enzyme-linked immunosorbent assay (ELISA). The results showed a slight expression of LIF in normal cartilage tissue but less in synovium tissue; however, the expression of LIF was marked in synovial lining cells and superficial and middle-layer cartilage in animal OA \( (P<0.05) \). Leukemia inhibitory factor mRNA was expressed at the highest level in moderate degrading subchondral bone, and LIF was expressed at the highest level in seriously degrading articular cartilage tissue. These results were similar to those found with ELISA. This study suggests that LIF in OA articular tissues varies by clinical symptoms and grade. It plays an important role in the pathogenesis of OA.
Leukemia inhibitory factor (LIF) is a multifunctional cytokine that belongs to the family of hemopoietic growth factors. It plays a role in growth promotion and differentiation, regulates calcium and bone metabolism, induces acute-phase protein synthesis, and causes cachexia in organisms with neoplastic disorders.\(^2\)\(^3\) Leukemia inhibitory factor also plays an important role in the regulation of bone formation and bone resorption and in the degradation of proteoglycan.\(^2\)\(^4\) It has been detected at high levels in the synovial fluid of patients with rheumatoid arthritis. Immunohistochemical staining has shown that LIF is expressed in hypertrophic chondrocytes and vascular sprouts of cartilage and bone during rat and human osteogenesis.\(^5\) It is produced by joint tissue cells. The induction of LIF by factors that are present during joint inflammation and the effects of LIF on cartilage and synovium cells suggest that LIF is a mediator that can contribute to the pathogenesis of arthritis. It is one of the participants in joint destruction as a proinflammatory and chondrolytic cytokine.\(^1\)\(^6\)

However, few reports are available on the expression of LIF in articular tissue and its potential effects on clinical grade. This study investigated the expression of LIF in animals and in different grades of human osteoarthritic articular tissues according to clinical symptoms and radiographic inspection.

**MATERIALS AND METHODS**

**Animal Study**

Thirty-five 8-month-old Japanese rabbits were used in a Colombo model of experimental OA. Surgery was performed in the right knee and a sham surgery in the left knee of each rabbit. Five rabbits each were sacrificed on postoperative days 3, 7, 14, 28, 42, 56, and 84. Cartilage and synovium were collected from each knee. The articular cartilage tissue was fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) at 4°C and decalcified with 15% ethylenediaminetetraacetic acid in 0.01 M phosphate-buffered saline. After dehydration, the specimens were embedded in paraffin. Five-micron sections were cut sagittally and mounted on silanized glass slides for histology and immunohistochemistry.

**Immunohistochemical Study**

The immunohistochemical procedure has been described previously.\(^7\) The immunohistochemical SP method was used to detect the expression of LIF protein in cartilage tissue with goat anti-LIF. Phosphate-buffered saline substituting primary antibody was used as a blank control.

**Patients and Sample Preparation**

Sixty-seven samples of articular tissue (cartilage, subchondral bone, and synovium) were obtained from 32 patient donors 50 to 76 years old. Twenty patients with OA underwent hip and knee joint synovectomy or replacement surgery. Normal joint tissues were from 12 patients with serious traumatic and destructive extremity injuries (control group). The patients were grouped according to clinical symptoms and radiographic inspection. The slight group had no clinical history. Ten patients with Kellgren-Lawrence grade II comprised the moderate group. Ten patients with Kellgren-Lawrence grade III-IV comprised the severe group. The OA diagnosis was made in all human cartilage, subchondral bone, and synovium tissue samples according to clinical symptoms, surgeries performed, and the results of histological studies. Each tissue sample was cut into 2 parts, with 1 part being stored at -80°C until used for RNA extraction and enzyme-linked immunosorbent assay (ELISA) and the other part being immediately embedded in paraffin wax.

**Histology**

Specimens were fixed in 10% neutral buffered formalin for 7 days, decalcified in 0.25 M ethylenediaminetetraacetic acid at 4°C in phosphate-buffered saline (pH 7.5), dehydrated in graded alcohols, and embedded in paraffin wax. Seven-micron–thick sections were cut sagittally, stained with hematoxylin and eosin, and examined microscopically. Histological studies were used to identify clinical diagnosis and group.

**Extraction of RNA and Reverse Transcription Polymerase Chain Reaction**

Total RNA was extracted directly from cartilage, subchondral bone, and synovium (1 to 2 mm) using a modification of the magnetic beads procedure for the parts stored at -80°C.\(^8\) Templates for the gene were obtained by reverse transcription polymerase chain reaction (RT-PCR) using RNA extracted from tissue samples. The LIF gene, including primer sequences from 5’ to 3’ for polymerase chain reaction, was shown as:

Forward: 5’-CAGCACCACTGAATCA CAGATC-3’

Reverse: 5’-AGTATGAAACATCCCCA CAGGG-3’

Reverse transcription reactions used SuperScript II (Invitrogen, Burlington, Ontario, Canada). The complementary cDNA products obtained were subjected to polymerase chain reaction as previously described\(^9\) to obtain the different products at 527 bp. The amplified products were electrophoresed in a 1.0% agarose gel with a 1-kb DNA ladder as a size marker. The mRNA expression of LIF was measured with SPSS version 11.0 statistical software (SPSS Inc, Chicago, Illinois).\(^9\)

**Enzyme-linked Immunosorbent Assay**

Antigens were dissolved in 0.25 M Tris saline solution and azide (1 g of tissue per 4 mL of Tris solution), then centrifuged at 8000 rpm for 10 minutes at 4°C. The aqueous supernatant was coated at a concentration of 3 μg/mL in 96-well enzyme immunoassay plates by passive adsorp-
tion for 2 hours at 37°C. The plates were washed 4 times with Tris saline–azide buffer (100 µL of enzyme immunoassay buffer per well) for 1 hour at 37°C. Next, alkaline phosphatase substrate was added and the plates were incubated at 37°C until optimal color developed (1 hour). The color was quantified by measuring the absorption at 492 nm using the multiscan method.

**Statistical Analysis**
Statistical analysis was performed with SPSS version 11.0 statistical software (SPSS Inc), followed by Student’s t test. A P value less than .05 was considered significant.

**RESULTS**

**Immunohistochemistry Analysis of Animal Leukemia Inhibitory Factor**
A slight expression of LIF was seen in normal cartilage tissue; there was no expression in normal synovium tissue. However, LIF expression was marked in synovial lining cells and superficial and middle-layer cartilage in animal OA. The expression of LIF was highest at 7 and 28 days, then declined and remained at a stable level (Figure 1). This expression was significantly different from that observed in the control group (P<.05).

**mRNA Expression of Leukemia Inhibitory Factor in Normal Human Articular Tissues**
Low levels of LIF mRNA were expressed in normal human articular tissues, and there was no significant difference (P=.225) between anatomical locations (Figure 2B). Enzyme-linked immunosorbent assay also showed results similar to those with RT-PCR (Figure 2C).

**mRNA Expression of Leukemia Inhibitory Factor in Moderate Osteoarthritis**
Reverse transcription polymerase chain reaction was performed to determine the mRNA expression of LIF in moderate OA. It was expressed at its highest level in degrading subchondral bone. Leukemia inhibitory factor mRNA expression was also high in cartilage and increased in synovium with degenerative processes (Figure 2B).

**mRNA Expression of Leukemia Inhibitory Factor in Severe Osteoarthritis**
The message level for LIF was expressed in all substrates and remained high. Leukemia inhibitory factor was expressed at the highest level in seriously degrading articular cartilage tissue followed by synovium and then by subchondral bone (Figure 2B).

**Leukemia Inhibitory Factor in Patients’ Protein**
Enzyme-linked immunosorbent assay and RT-PCR yielded similar results when
analyzing LIF content in patients’ protein (Figure 2C).

**DISCUSSION**

Osteoarthritis is a degenerative disease characterized by gradual loss of articular cartilage. It is a debilitating, progressive disease of diarthrodial joints associated with the aging process. Repetitive mechanical injury, including abnormal joint anatomy, appears to play a central role. Osteoarthritis is characterized by an imbalance between chondrocyte anabolism and catabolism. Disruption of chondrocyte homeostasis primarily affects the cartilage extracellular matrix, which is responsible for the biomechanical properties of the tissue. Recent evidence has implicated cytokines, of which interleukin-1, -6, and -17 and tumor necrosis factor-α seem most involved in the OA process of cartilage destruction. The primary role of these cytokines is to modulate the expression of matrix metalloproteinases and cartilage extracellular matrix proteins.

Leukemia inhibitory factor is a more recently identified cytokine that regulates the function of various cell types. It is involved in osteoarticular tissue metabolism and may participate in osteogenesis. The literature suggests that LIF is involved in the delicate balance between the rate of formation of calcified cartilage and its vascularization for bone development. Some researchers have shown that LIF also enhances the expression of other cytokines, including IL-6, MCP-1, and IL-8, and indirectly stimulates connective tissue cells. Probably more important to connective tissue metabolism are the direct effects of LIF on chondrocytes and synoviocytes. In addition to the induction of other cytokines, LIF stimulates the production of the metalloproteinases stromelysin and collagenase and may inhibit extracellular matrix synthesis. Leukemia inhibitory factor can be characterized as a catabolic cytokine that is qualitatively similar to IL-1 and tumor necrosis factor in its effects on joint tissue cells. In both cell systems, matrix metalloproteinase-1, -3, and -13 were strongly induced by IL-1β, without significant induction of metalloproteinase-2. Interleukin-6 was also found to be upregulated by IL-1β in both cellular models.

In the current study, immunohistochemistry analysis showed that the normal articular tissues, cartilage, subchondral bone, and synovium expressed low levels of LIF mRNA, and there were no significant differences between tissues. However, the expression of LIF was marked in synovial lining cells and superficial and middle-layer cartilage in animal OA.

The highest expression of LIF was detected at 7 and 28 days, then declined and remained at a stable level. It was implied that the early expression of LIF in OA related primarily to trauma, and acute synovial inflammation may stimulate the secretion of LIF by articular chondrocytes. Expression of LIF was different in the early stages; with the development of OA and the destruction of cartilage, LIF expression may be significantly reduced. At 28 days, the serious degeneration of cartilage and the decreased quantity of chondrocytes may have affected the synthesis and expression of LIF, but the structure and function of subchondral bone was not affected. The inflammation may stimulate osteoblasts and other cells to secrete LIF at a high level.

This study’s results were almost the same for the OA patients and for the animals. Reverse transcription polymerase chain reaction analysis was performed to determine the mRNA expression of LIF. The message level was expressed in all substrates and remained at a high level compared with that of normal articular tissues. Leukemia inhibitory factor expression was significantly different in different articular tissues depending on clinical symptoms and OA grade. In early OA, there was joint instability, late OA cytokine expression and reduced quantity, subchondral bone with ivory-like changes, sclerosing bone below possibly appearing cystic, and cytokine easily through hardened bone. In moderate OA, LIF mRNA was expressed at its highest level (P<.05) (A). Analysis of LIF content in protein with enzyme-linked immunosorbent assay showed results similar to those with immunohistochemical staining, histological observation, and reverse transcription polymerase chain reaction measurements (C).

![Figure 2: Leukemia inhibitory factor (LIF) expression was low in normal animal articular tissue. However, in animal osteoarthritis, its expression in cartilage was significantly high at different times, declining to a stable level after 28 days (P<.05) (A). In patients with moderate degrading subchondral bone, LIF mRNA was expressed at its highest level (P<.05) (B). Analysis of LIF content in protein with enzyme-linked immunosorbent assay showed results similar to those with immunohistochemical staining, histological observation, and reverse transcription polymerase chain reaction measurements (C).](image-url)
biomechanical changes before appearing on articular cartilage degeneration, the proinflammatory cytokines first act due to microfractures through the cartilage calcification of articular cartilage. Leukemia inhibitory factor mRNA was expressed at a higher level in cartilage than in synovium. However, with OA development and cartilage destruction, in severe OA, the expression of LIF in subchondral bone declined to its lowest level. Likely late in OA, osteoblasts and other cells repair the subchondral bone, subchondral bone remodeling, and later joint deformation to gradually stimulate cartilage and synovium, increasing the amount of proinflammatory cytokines. Articular tissue destruction was focused in the cartilage and synovium. Leukemia inhibitory factor mRNA was expressed at the highest level in severely degrading articular tissues followed by synovium and then by subchondral bone. Analysis of LIF content with ELISA showed results similar to those with immunohistochemical staining, histological observation, and RT-PCR measurements.

**CONCLUSION**

This study suggests that LIF in OA articular tissues varies by clinical symptoms and grade. This implies that LIF has the potential to propagate inflammatory responses and may be one of the cofactors of joint destruction in different OA grades. Leukemia inhibitory factor may also induce connective tissue degradation and contribute to systemic manifestations of OA.

**REFERENCES**


5. Grimaud E, Blanchard F, Charrier C, Gouin F, Redini F, Heymann D. Leukemia inhibitory factor (LIF) mRNA was expressed at a higher level in cartilage and synovium. Mod Rheumatol. 2003; 13(2):121-216.


