Concentration and Localization of YKL-40 in Hip Joint Diseases

MASASHI KAWASAKI, YUKIHARU HASEGAWA, SEIJI KONDO, and HISASHI IWATA

ABSTRACT. ***Objective.*** YKL-40 is a major secretory protein from human chondrocytes and synovial fibroblasts. We evaluated the concentrations and localization of YKL-40 in hip joint diseases, and analyzed the possibility of YKL-40 as a new inflammatory joint marker.

Methods. The concentration of YKL-40 in synovial fluid (SF) was measured by a sandwich-type ELISA. SF samples were collected from 19 hips with osteoarthritis (OA) of the hip joint, 21 hips with osteonecrosis of the femoral head (ONFH), and 5 hips with failed total hip arthroplasty (failed THA). In all cases of failed THA, cartilage tissue in hip joints was removed completely during the previous THA. The localization of YKL-40 was determined through immunohistochemical analysis using a specific antibody.

Results. The mean SF concentration of YKL-40 was significantly higher in ONFH and failed THA than in OA. Comparison by OA grade was not significantly different. In staging of ONFH, Ficat stage III with collapsed femoral head showed significantly higher YKL-40 concentrations than the other stages. Immunohistochemical studies showed that YKL-40 was localized in chondrocytes in the superficial and middle layers of the cartilage. In the synovium, YKL-40 was localized in fibroblasts and macrophages.

Conclusion. YKL-40 reflects the degree of inflammation rather than cartilage metabolism. YKL-40 may be a useful inflammatory marker of hip joint diseases. (J Rheumatol 2001;28:341–5)

Key Indexing Terms:
YKL-40 OSTEOARTHRITIS OSTEONECROSIS
FAILED TOTAL HIP ARTHROPLASTY INFLAMMATION MACROPHAGES

YKL-40 (HC-gp39) is a 40 kDa glycoprotein originally identified in the whey secretions of nonlactating cows[1]. It is secreted in large amounts by the human osteosarcoma cell line MG-63[2], human synovial cells[3], and human cartilage cells[4,5]. This protein was called YKL-40 according to the one-letter code for its first 3 N-terminal amino acids (tyrosine, lysine, and leucine) and its apparent molecular weight of 40 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis[2]. Hakala, *et al*[5] referred to it as human cartilage glycoprotein-39 (HCgp-39), based on an apparent weight of 39 kDa. YKL-40 mRNA is expressed strongly in chondrocytes and liver, weakly in brain, kidney and placenta, and in undetectable amounts in heart, lung, skeletal muscle, pancreas, mononuclear cells and skin fibroblasts[5] by Northern blotting and reverse transcription-polymerase chain reaction analyses. In addition, YKL-40 mRNA is undetectable in normal newborn or adult human cartilage, but high levels were detected in synovial and cartilage specimens obtained from patients with rheumatoid arthritis (RA)[5]. The amino acid sequence of YKL-40 shows some homology to the bacterial chitinase protein family, which has no chitinase activity[4,5], but does bind chitin[6].

It was recently reported that YKL-40 is expressed *in vitro* by activated macrophages[6-8], in lipid laden macrophages that accumulate in various organs in Gaucher's disease[9,10], and in macrophages in atherosclerotic plaque[11], and that it is excytosed by activation from specific granules of neutrophils[12]. Boot, *et al*[11] noted that YKL-40 may affect cell adhesion and migration during the tissue remodeling processes that take place during atherogenesis in atherosclerosis.

Recent clinical reports have indicated that the serum levels of YKL-40 are increased in diseases including osteoarthritis[4,13,14], rheumatoid arthritis[4,13,15], breast cancer[16], and liver cirrhosis[17]. Johansen, *et al*[14] described levels of YKL-40 in OA synovial fluid (SF), and reported that YKL-40 levels of severe synovial inflammation were significantly higher than those of light or moderate synovitis. In an immunohistochemical study of bone and joint diseases, Volk, *et al*[18] suggested that YKL-40 may be important in cartilage remodeling/degradation of osteoarthritic changes. However, the clinical significance of YKL-40 has not yet been clarified.

To assess whether YKL-40 reflects predominantly cartilage remodeling/degradation or the degree of inflammation, we analyzed the concentrations and localization of YKL-40 in various hip joint diseases: OA of hip joints, osteonecrosis of the femoral head (ONFH), and failed total hip arthroplasty (failed THA) by ELISA and immunohistochemical techniques.

From the Department of Orthopaedic Surgery, Nagoya University School of Medicine, Nagoya, Japan.

M. Kawasaki, MD; Y. Hasegawa, MD, Associate Professor; S. Kondo, MD, Orthopaedic Surgeon; H. Iwata, MD, Professor and Chairman, Department of Orthopaedic Surgery.

Address reprint requests to Dr. M. Kawasaki, Department of Orthopaedic Surgery, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan.

Submitted February 8, 2000 revision accepted August 10, 2000.

MATERIALS AND METHODS

Clinical diagnoses were confirmed mainly by radiography and/or magnetic resonance imaging (MRI). All the OA cases were diagnosed radiographically. According to Kellgren-Lawrence grade[19], degrees of OA were classified radiographically as follows: grade 0 showed no features of OA, grade 1 showed minute osteophytes, grade 2 showed definite osteophytes and unimpaired joint space, grade 3 showed moderate diminution of the joint space, and grade 4 showed that the joint space was greatly impaired with sclerosis of subchondral bone. All the ONFH cases had characteristic findings on MRI, which shows a band-like zone of low signal intensity within the femoral head on T1 weighted images, which surrounds a high signal intensity line on T2 weighted images[20]. According to Ficat stage[21], degrees of ONFH were classified as follows: stage I showed normal on standard anteroposterior and lateral radiographs, stage II showed continuing flattening of the femoral head or sequestrum formation, stage III showed broken contour of the femoral head, but a normal joint space, and stage IV showed progressive loss of articular cartilage and the development of acetabular osteophytes. Failed THA was defined as migration or a change in the position of the components or cement[22,23] on both the acetabular side and femur side radiographically, associated with pain.

YKL-40 assay. Patients and collection of SF. SF samples were collected during surgery from 19 patients with OA of primary hip dysplasia, from 21 patients with ONFH, and from 5 patients with failed THA. Before capsulotomy, SF was aspirated using a 10 ml syringe and an 18 G needle, avoiding contamination by blood. The SF was immediately centrifuged at 3000 rpm for 20 min. All samples were frozen and stored at –80°C until analysis. The average age of the patients with OA was 48 years (range 16 to 78), patients with ONFH 46 years (27–68), and failed THA patients 63 years (50–79). Grades of OA were grade 2: 5 patients, grade 3: 9 patients, and grade 4: 5 patients. Rotational acetabular osteotomies were chosen for 12 cases and THA for 7 cases. Stages of ONFH were stage II: 5 patients, stage III: 11 patients, and stage IV: 5 patients. Patients with ONFH were treated with transtrochanteric rotational osteotomy[24] in 13 cases and THA in 8 cases. The osteonecrosis was steroid induced in 8 cases, due to alcohol abuse in 9 cases, and idiopathic in 4 cases. All the failed THA cases were treated with revision surgery with cementing.

Detection of YKL-40. Concentrations of YKL-40 in SF were determined using a sandwich ELISA kit (Metra Biosystems Inc., USA) according to Harvey, *et al*[13].

Immunohistochemical investigation. Patients and samples. Samples of cartilage, synovial membrane, and pseudocapsule tissue were obtained from OA, ONFH, and failed THA cases during surgery.

Immunohistochemical staining of YKL-40. The tissue specimens were harvested in 5 mm cubes. The samples were fixed immediately in 20% acid formalin, embedded in paraffin, and sliced at 5 µm thickness. A conventional avidin/biotinylated horseradish peroxidase staining technique (ABC complex) for polyclonal antibodies was used and included the following steps: (1) washing tissue sections for 15 min with 4°C phosphate buffered saline (PBS); (2) preincubation for 15 min at 20°C with 10% normal bovine serum (BSA); (3) incubation for 2 h at 20°C with an affinity purified rabbit polyclonal IgG against human YKL-40 (Metra Biosystems) used at a protein concentration of 0.0104 g/l diluted in PBS with 1% BSA. Nonimmune rabbit polyclonal IgG (Metra Biosystems) was used as a control at the same IgG concentration of 0.0104 g/l diluted in PBS with 1% BSA; (4) washing of tissue sections for 15 min with 4°C PBS; (5) incubation for 10 min with a swine-anti-rabbit immunoglobulin IgG (H+L) (Wako, Osaka, Japan) used at a dilution of 1:20 in PBS; (6) washing for 15 min with 4°C PBS. Antibody binding was visualized by incubation for 5 min with ABC complex and staining for 10 minutes with DAB (diaminobenzidine) as a substrate. Next, to confirm the localization of YKL-40 in macrophages, immuno-double staining (YKL-40 and CD68) was performed. Tissue macrophages were identified using anti-human macrophage antibody: a mouse-CD68 KP-1 (Dako). Additional immunohistochemical detection for CD68 was performed as described, and stained for 10 min with 4-chloro-1-naphol as substrate. The color of YKL-40 showed as brown, and that of CD68 dark blue. All slides were observed by light microscopy.

Statistical analysis. Analysis of variance was performed using a Statview statistical program (Abacus Concepts, Berkeley, CA, USA) on a Macintosh computer. Differences of YKL-40 concentrations in SF between various hip joint diseases (OA, ONFH, and failed THA), according to OA grade and ONFH stage were calculated by nonparametric Mann-Whitney U test and Kruskal-Wallis test for unpaired differences. A p value was corrected by Bonferroni method. A p value < 0.05 was considered statistically significant.

RESULTS

Concentrations of YKL-40. The level of YKL-40 (median, range) in SF was significantly higher in ONFH than in OA (ONFH median 2224 ng/ml, range 810-5391; OA median 886 ng/ml, range 154-4728; $p = 0.0015$). The level of YKL-40 in SF was significantly higher in failed THA than in OA (failed THA median 3265 ng/ml, range 2436-4009; OA median 886 ng/ml, range 154-4728; $p = 0.015$). No significant differences in YKL-40 levels in SF were observed between ONFH and failed THA (Table 1).

Differences in the level of YKL-40 (median, range) in SF in different grades of OA were not significant (grade 2, median 1321 ng/ml, range 240-1772; grade 3, median 822 ng/ml, range 154-1333; grade 4, median 876 ng/ml, range 480-4728) (Figure 1). In stage III ONFH, YKL-40 in SF was significantly higher than in the other stages (stage II, median 1116 ng/ml, range 810-2052; stage III median 4224 ng/ml, range 2395-

Table 1. Concentration of YKL-40 in synovial fluid in osteoarthritis of the hip (OA), osteonecrosis of the femoral head (ONFH), and failed total hip arthroplasty (failed THA).

Hip Joint Disease	n	YKL-40, ng/ml, median (range)	p
OA	19	886 (154–4728)	
ONFH	21	2224 (810–5391)	0.0015 vs OA
Failed THA	5	3265 (2436–4009)	0.015 vs OA

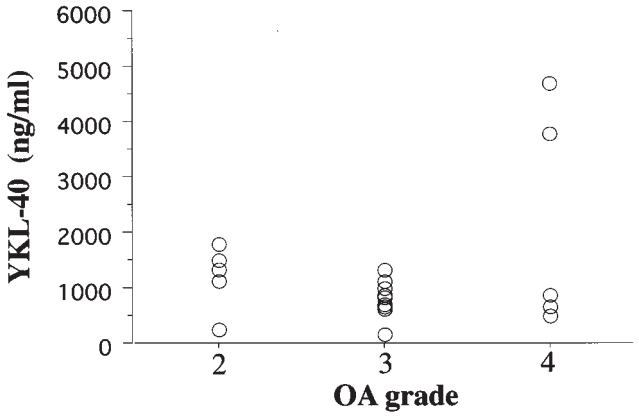

Figure 1. Comparison of YKL-40 values in synovial fluid in OA by OA grades. Symbols denote concentrations of YKL-40. No significant differences were noted according to OA grade.

5391; stage IV, median 1261 ng/ml, range 1143-2010; p = 0.0054) (Figure 2).

Localization of YKL-40 by immunohistochemical study. In OA cartilage, YKL-40 was found in chondrocytes, where there was intracellular staining in the superficial and middle layers. In synovial membrane in OA, YKL-40 was localized in fibroblasts (Figure 3A).

In cartilage in ONFH, YKL-40 was found in chondrocytes in the superficial and middle layers. Chondrocyte staining was observed intracellularly (Figure 3B). In synovial membranes in ONFH, YKL-40 was localized in fibroblasts and macrophages (Figure 3C). YKL-40 and CD68 were observed in the cytoplasm of macrophages.

In pseudocapsule specimens from failed THA, many macrophages were observed. YKL-40 was localized in macrophages and fibroblasts. YKL-40 and CD68 were coexpressed in the cytoplasm of macrophages (Figure 3D).

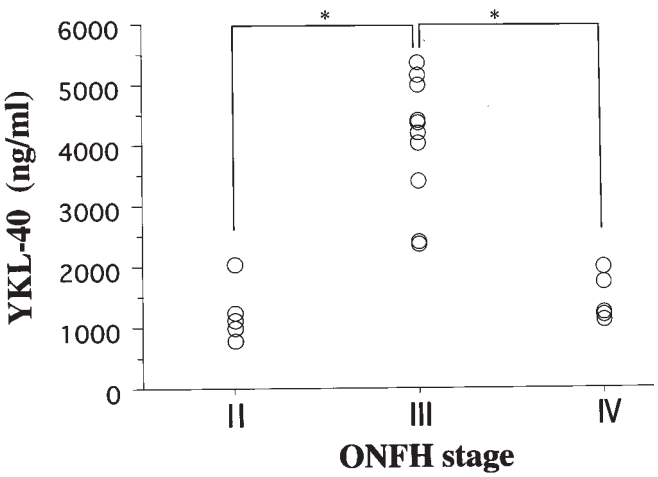

Figure 2. Comparison of YKL-40 values in synovial fluid in ONFH by ONFH stages. Symbols denote concentrations of YKL-40. ONFH stage III showed significantly higher values than the other stages. *p = 0.0054.

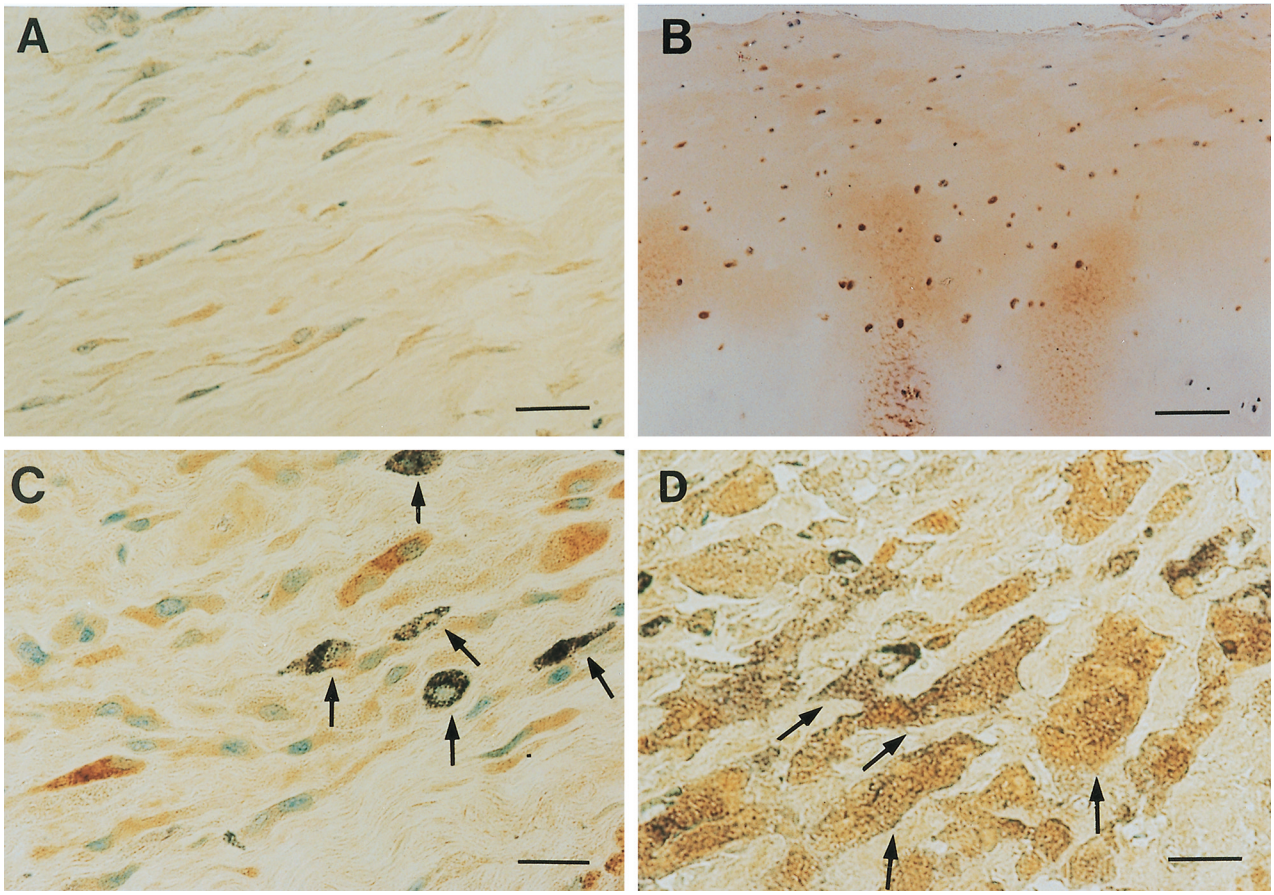

Figure 3. Immunohistochemical observations. A. Immuno-double staining (YKL-40 and CD68) in synovial membrane of OA. YKL-40 (brown) is shown in fibroblasts (×400, bar = 25 µm). B. Cartilage of ONFH. YKL-40 (brown) is shown in chondrocytes in the superficial and middle layers. Chondrocyte staining is observed intracellularly (×100, bar = 100 µm). C. Immuno-double staining (YKL-40 and CD68) in synovial membrane of ONFH. YKL-40 (brown) is shown in fibroblasts and macrophages. Both YKL-40 (brown) and CD68 (dark blue) are visible in the cytoplasm of macrophages (arrows) (×400, bar = 25 µm). D. Synovial tissue of pseudocapsule (nucleus staining not performed). Immuno-double staining for YKL-40 (brown) and CD68 (dark blue) shows coexpression of both antigens in the cytoplasm of macrophages (arrows) (×400, bar = 25 µm).

DISCUSSION

It has been reported that the concentration of YKL-40 in serum in OA and RA is significantly higher than that in healthy adults, and it is reflected by degradation of cartilage and inflammation of synovium[4,13,15]. In this study, the concentration of YKL-40 in SF in failed THA was significantly higher than in OA. As the cartilage tissue in failed THA has been completely removed during previous THA, YKL-40 is affected by inflammation rather than degradation of cartilage tissue.

Iwase, *et al*[25] reported that the concentration of matrix metalloproteinase-3 (MMP-3)[26,27] in ONFH is higher than that in OA, and it reflects the degree of inflammation. Comparing the YKL-40 levels in SF according to ONFH stage, YKL-40 concentrations in Ficat stage III joints, where collapse of the joint surface is more apparent, were significantly higher than in the other stages. We infer that collapse promotes inflammatory synovitis and may induce MMP-3 and YKL-40 production. In contrast, differences in the levels of YKL-40 according to OA grade were not significant. This is consistent with the report of Harvey, *et al*[13], that suggested that YKL-40 is not useful as an OA marker. Because YKL-40 in failed THA, in which cartilage had been completely removed, was much higher than in OA, in which some cartilage was still present, we surmise that YKL-40 is produced more abundantly from synovium than from chondrocytes and does not have specificity as a marker of cartilage metabolism in arthritis. Two cases of OA grade 4 showed a high value of YKL-40 in comparison with the other cases. We macroscopically observed that the degree of synovial inflammation was more severe than in the other cases. Johansen, *et al*[14] suggested that levels of YKL-40 in the presence of severe synovial inflammation were higher than in light or moderate inflammation.

Studies showed that YKL-40 was not expressed in normal adult human cartilage or noninflamed synovium *in vitro*, but it was observed in chondrocytes and fibroblasts from RA and OA *in vitro*[5]. Johansen, *et al*[17] reported that YKL-40 was found in pericellular and perivenular fibrosis in fibrotic septa along the sinusoids in liver cirrhosis. In our study, YKL-40 was localized in the cytoplasm of chondrocytes, fibroblasts, and macrophages. A negative result of YKL-40 in the intercellular matrix would not exclude the presence of antigen, because it might have been diluted or bound in a way preventing its detection. In addition, we found that YKL-40 was localized in chondrocytes in the superficial and middle layers. These results are consistent with the report of Volk, *et al*[18], which showed that YKL-40 tended to be localized in most chondrocytes in lateral areas of the femoral head, known to be associated with biomechanical load. These results suggested YKL-40 in chondrocytes may have some function in cartilage metabolism turnover.

Studies showed that YKL-40 is expressed in macrophages in various tissues[6-11] associated with the maintenance of inflammation[28], and is secreted abundantly from macrophages *in vitro*[6-8]. In this immunohistochemical study of synovial membrane, YKL-40 was localized in fibroblasts and macrophages in synovial membrane. These results suggest that YKL-40 is potentially involved in inflammation. Therefore, we thought that macrophage derived YKL-40 may play a significant role in revealing the degree of inflammation in hip joint diseases.

As well, previous studies reported that in failed THA, macrophages are activated around wear particles in the bone-cement interface, and secrete many kinds of inflammatory cytokines[28], which appear to be the main cause of loosening of the prosthesis[29,30]. We surmise that in failed THA, wear particles activate macrophages and enhance the production of YKL-40. However, to clarify the regulation and function of YKL-40, further investigations are required.

We determined the concentration and localization of YKL-40 in various hip joint diseases. YKL-40 was localized not only in cartilage tissue but also in fibroblasts and macrophages. The concentration of YKL-40 in SF in ONFH and failed THA was higher than that in OA. As the level of YKL-40 is high in failed THA, which lacks cartilage tissue, and differences in the levels of YKL-40 according to OA grade were not significant, YKL-40 concentration is more appropriately used as an inflammatory marker than as a marker of cartilage metabolism in arthritis. Moreover, we found that it is possible to use it as a joint marker to analyze the state of synovitis in ONFH, especially Ficat stage III.

ACKNOWLEDGMENT

We are grateful to I. Imamura, Product Planning and Development Department of TFB, Incorporated, Tokyo, Japan, for providing the YKL-40 kit and primary antibody, which was an affinity purified rabbit polyclonal IgG against human YKL-40.

REFERENCES

1. Rejman JJ, Hurley WL. Isolation and characterization of a novel 39 kilodalton whey protein from bovine mammary secretions collected during the nonlactating period. Biochem Biophys Res Commun 1988;150:329-34.
2. Johansen JS, Williamson MK, Rice JS, Price PA. Identification of proteins secreted by human osteoblastic cells in culture. J Bone Miner Res 1992;7:501-12.
3. Nyirkos P, Golds EE. Human synovial cells secrete a 39 kDa protein similar to a bovine mammary protein expressed during the non-lactating period. Biochem J 1990;268:265-8.
4. Johansen JS, Jensen HS, Price PA. A new biochemical marker for joint injury. Analysis of YKL-40 in serum and synovial fluid. Br J Rheumatol 1993;32:949-55.
5. Hakala BE, White C, Recklies AD. Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. J Biol Chem 1993;268:25803-10.
6. Renkema GH, Boot RG, Au FL, et al. Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family of 18 glycosyl hydrolases secreted by human macrophages. Eur J Biochem 1998;251:504-9.
7. Kirkpatrick RB, Emery JG, Connor JR, Dodds R, Lysko PG, Rosenberg M. Induction and expression of human cartilage glycoprotein 39 in rheumatoid inflammatory and peripheral blood monocyte-derived macrophages. Exp Cell Res 1997;237:46-54.

8. Krause SW, Rehli M, Kreutz M, Schwartzfischer L, Paulauskis JD, Andreesen R. Differential screening identifies genetic markers of monocyte to macrophage maturation. J Leuk Biol 1996;60:540-5.
9. Hollak CEM, van Weely S, van Oers MHJ, Aerts JMFG. Marked elevation of plasma chitotriosidase activity: A novel hallmark of Gaucher disease. J Clin Invest 1994;93:1288-92.
10. Den Tandt WR, van Hoof F. Marked increase of methylumbelliferyl-tetra-N-acetyl-chitotetraoside hydrolase activity in plasma from Gaucher disease patients. J Inherit Metab Dis 1996;19:344-50.
11. Boot RG, van Achterberg TAE, van Aken BE, et al. Strong induction of members of the chitinase family of proteins in atherosclerosis: chitotriosidase and human cartilage gp-39 expressed in lesion macrophages. Arterioscler Thromb Vasc Biol 1999;19:687-94.
12. Volck B, Price PA, Johansen JS, et al. YKL-40, a mammalian member of the chitinase family, is a matrix protein of specific granules in human neutrophils. Proc Assoc Am Physicians 1998;110:351-60.
13. Harvey S, Weisman M, O'Del J, et al. Chondrex: new marker of joint disease. Clin Chem 1998;443:509-16.
14. Johansen JS, Hvolris J, Hansen M, Backer V, Lorenzen I. Serum YKL-40 levels in healthy children and adults. Comparison with serum and synovial fluid levels of YKL-40 in patients with osteoarthritis or trauma of the knee joint. Br J Rheumatol 1996;35:553-9.
15. Johansen JS, Stoltenberg M, Hansen M, Florescu A, Hoslev-Petersen K, Lorenzen I. Serum YKL-40 concentrations in patients with rheumatoid arthritis: relation to disease activity. Rheumatology 1999;38:618-26.
16. Johansen JS, Cintin C, Jorgensen M, Kamby C, Price PA. Serum YKL-40: A new potential marker of prognosis and location of metastases of patients with recurrent breast cancer. Eur J Cancer 1995;31A:1437-42.
17. Johansen JS, Moller S, Price PA, et al. Plasma YKL-40: A new potential marker of fibrosis in patients with alcoholic cirrhosis? Scand J Gastroenterol 1997;32:582-90.
18. Volk B, Ostergaard K, Johansen JS, Garbarsch, Price PA. The distribution of YKL-40 in osteoarthritic and normal human articular cartilage. Scand J Rheumatol 1999;28:171-9.
19. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957;16:494-501.
20. Mont M, Hungerford DS. Current concepts review. Non-traumatic avascular necrosis of the femoral head. J Bone Joint Surg 1995;77A:459-74.
21. Ficat RP. Idiopathic bone necrosis of the femoral head. Early diagnosis and treatment. J Bone Joint Surg 1985;67B:3-9.
22. Harris WH, McCarthy JC Jr, O'Neill DA. Femoral component loosening using contemporary techniques of femoral cement fixation. J Bone Joint Surg 1982;64A:1063-7.
23. Harris WH, McGann WA. Loosening of the femoral component after use of the medullary-plug cementing technique. J Bone Joint Surg 1986;68A:1064-7.
24. Sugioka Y. Transtrochanteric anterior rotational osteotomy of the femoral head in the treatment of osteonecrosis affecting the hip. A new osteotomy operation. Clin Orthop 1978;295:281-8.
25. Iwase T, Hasegawa Y, Ishiguro N, et al. Synovial fluid cartilage metabolism marker concentrations in osteonecrosis of the femoral head compared with osteoarthritis of the hip. J Rheumatol 1998;25:527-31.
26. Walakovits LA, Moore VL, Bhardwaj N, Gallick GS, Lark MW. Detection of stromelysin and collagenase in synovial fluid from patients with rheumatoid arthritis and posttraumatic knee injury. Arthritis Rheum 1992;35:35-42.
27. Woessner JF Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 1991;5:2145-54.
28. Kunkel SL, Chensue SW, Strieter RM, Lynch JP, Remick DG. Cellular and molecular aspects of granulomatous inflammation. Am J Respir Cell Mol Biol 1989;1:439-47.
29. Kim KJ, Chiba J, Rubash HE. In vivo and in vitro analysis of membranes from hip prostheses inserted without cement. J Bone Joint Surg 1994;76A:172-80.
30. Ishiguro N, Kojima T, Ito T, et al. Macrophage activation and migration in interface tissue around loosening total hip arthroplasty components. J Biomed Mater Res 1997;35:399-406.