

Correlation of Intra-Articular Ankle Pathology With Cytokine Biomarkers and Matrix Degradation Products

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ABSTRACT

Background: Articular cartilage degeneration is mediated by inflammatory cytokines and fragments of structural matrix proteins. Few studies have examined the role of these biomarkers in intra-articular pathology of the ankle. **Methods:** Four groups of patients with increasing ankle pathology were enrolled. Group 1 included controls with no pain who underwent unrelated forefoot surgery. Group 2 included patients undergoing arthroscopy with intraoperative mild chondrosis. Group 3 included patients undergoing arthroscopy with moderate/severe chondrosis, osteochondral lesions, impingement, or loose bodies. Group 4 included positive controls with severe arthrosis undergoing ankle arthrodesis/arthroplasty. Ankle fluid was obtained by intra-articular aspiration and was assayed for IL-6, IFN- γ , MCP, MIP-1 β , and fibronectin-aggrecan complex (FAC), a matrix-degradation marker. There were 36 patients total, 21 males and 15 females with a mean age 45 years (± 16 ; range 18 to 76) and a mean VAS for pain of 4.7 (± 3.5 ; range 0 to 9). In groups 1 through 4, there were 11, 6, 15 and 4 patients respectively. **Results:** The mean values of MCP-1 were 49.8 (± 8.0) for minimal pathology and 133.9 (± 33.0) for substantial pathology (pg/ml). The mean values of the FAC were 2.83 (± 1.16) for minimal pathology and 9.62 (± 2.23) for substantial pathology (optical density at 450nm). The groups differed

significantly in age, preoperative VAS, FAC, IL-6, and MCP-1 ($p < 0.05$). **Conclusion:** There are differences in FAC and MCP-1 with increasing grades of severity of intra-articular pathology. **Clinical Relevance:** These tests may play a role in determining the necessity for arthroscopy or intra-articular procedures in equivocal candidates.

Level of Evidence: Diagnostic level of evidence II

Key Words: Biomarker; Ankle Arthroscopy; Pain; Fibronectin; Aggrecan; Cytokines

INTRODUCTION

The ankle is a synovial hinge joint that is relatively resistant to the development of degenerative joint disease and osteoarthritis compared to other major weightbearing synovial joints such as the knee and hip. Only 10% of cases of ankle arthritis are thought to be from primary osteoarthritis.³ Osteoarthritis of the ankle joint, however, is a common sequela of ankle trauma, most frequently caused by rotational ankle fractures.³ The reasons for this are incompletely understood, though there is some evidence of biomechanical, biochemical and cellular properties,¹⁴ specifically of the articular cartilage.

Protein biomarkers associated with osteoarthritis may be useful as diagnostic tools, prognostic indicators, and therapeutic targets. Inflammatory cytokines, matrix degradation products, and proteases have all been implicated in the pathophysiology of synovial joint degeneration, which involves complex signaling among cartilage, synovium and bone.⁷ Immunoreactivity to specific cytokines –interferon-gamma (IFN- γ), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1 beta (MIP-1 β)—has recently been observed in painful knees with meniscal pathology.⁵ There is also evidence that inflammatory cytokines are associated with fibronectin and its fragments,¹⁰ which in turn are associated with aggrecan and its fragments,⁹ in the pathophysiology of degenerative

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Table 1: Frequency distribution of patients among surgical groups with age and preoperative VAS as mean (\pm standard deviation). There are significant differences in age and VAS ($p < 0.001$).

Group	Description	Number	Age	VAS
1	Negative control	11	52 (± 11)	—
2	Arthroscopy—minimal pathology	6	29 (± 11)	6.0 (± 2.0)
3	Arthroscopy—substantial pathology	15	41 (± 14)	7.2 (± 1.4)
4	Positive control	4	67 (± 6)	6.5 (± 1.9)
Total		36	45 (± 16)	4.7 (± 3.5)

joint disease. In addition, it has been observed that fragmentation patterns of aggrecan differ between acute injury and chronic degeneration relative to healthy controls.¹⁹ These results demonstrate the possibility of correlating pathology with the analysis of protein biomarkers, but they have been validated only in the knee.

In this study, we endeavored to quantify in symptomatic ankle joints markers of the inflammatory cascade previously identified as strongly correlated with knee pathology.⁵ We hypothesized that some markers would be correlated with the severity of intra-articular pathology of the ankle.

MATERIALS AND METHODS

Subjects

Institutional Review Board (IRB) approval was obtained for the study of inflammatory cytokines and structural matrix protein fragments in ankle joint synovial fluid. Patients were recruited from the private practice of a board certified fellowship trained orthopaedic surgeon specializing in foot and ankle surgery from June 2009 through May 2010. Patients had to be older than 18 years of age and have failed greater than 3 months of nonoperative treatment. Patients were selected based upon diagnoses of various intra-articular ankle conditions that included: osteochondral lesions of the talus, intra-articular loose bodies, soft tissue or bony impingement with synovitis, ankle instability with joint line tenderness, and severe arthrosis. Exclusion criteria were age less than 18 years, the presence of inflammatory arthritis, crystalline arthropathies, or other rheumatologic diseases. Control patients had a history negative for specific ankle pain or trauma.

Four groups of patients with increasing degree of ankle pathology were enrolled. Group 1 included negative controls with a visual analog scale of 0 for ankle pain who were undergoing unrelated forefoot surgery. Group 2 included patients undergoing arthroscopy with intraoperative findings of mild chondrosis and/or synovitis. Group 3 included patients undergoing arthroscopy with intraoperative findings of moderate to severe chondrosis, osteochondral lesions of the talus, anterior impingement, or loose bodies. Group 4 included positive controls with severe arthrosis undergoing

ankle arthrodesis or arthroplasty. Severity of intraoperative findings was graded by arthroscopic visualization or direct inspection at arthrotomy.

Demographic data including age, preoperative ankle pain on a 10-point visual analog scale (VAS), preoperative MRI findings, diagnosis, as well as intraoperative findings were recorded for all patients. Severity of intra-articular pathology was graded and recorded. There were 36 patients total, 21 males and 15 females with a mean age 45 years (± 16 ; range 18 to 76) and a mean pre-procedure VAS for ankle pain of 4.7 (± 3.5 ; range 0 to 9). Table 1 illustrates the frequency distribution of patients among surgical groups 1 through 4 and includes age and preoperative VAS which differed significantly ($p < 0.001$) among groups by analysis of variance (ANOVA).

Sample acquisition, storage and preparation

Synovial fluid from the ankle of all subjects was collected by needle aspiration at the start of the procedure using sterile technique. An 18- or 21-gauge needle was introduced anteromedially and approximately 0.5 to 1 cc of synovial fluid was obtained. The fluid was immediately placed in a sterile polypropylene microtube and frozen at -80°C until the time of sample analysis.⁵ At the time of analysis, each patient sample was thawed to room temperature, treated with 5 mg/ml hyaluronidase, clarified by centrifugation at $\bullet 5000\text{ g}$, and filtered using $0.45\ \mu\text{m}$ low protein binding filtration. The collected filtrate was immediately assayed as described below.

ELISA analysis

A heterogeneous, enzyme-linked immuno-sorbent assay (ELISA) detecting a protein complex of fibronectin and the aggrecan G3 domain was developed and validated on a prior series of patients.¹⁹ Briefly, an anti-aggrecan G3 domain antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in phosphate-buffered saline (PBS)/tween 20/thimerosal was used to coat a 96-well microplate. The plate was treated with bovine serum albumin (BSA) in the same buffer overnight at 4°C to block excess binding sites, then washed six times with a PBS/tween 20/thimerosal solution. The centrifuged and

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filtered sample was aliquoted at three serial dilutions in triplicate into the microplate and incubated for one hour to facilitate binding of the complex to the immobilized antibody. After washing six times with the wash buffer, anti-fibronectin antibody labeled with horseradish peroxidase (HRP) (US Biological, Swampscott, MA) was added and incubated for 1 hour. After six washes, the TMB substrate was added and the reaction product measured by optical density (OD) at 450 nm wavelength. Human fibronectin (BD Biosciences, San Jose, CA, USA) at 10 $\mu\text{g/ml}$ concentration was used as a negative control. A multiplexed bead assay for inflammatory cytokines interleukin-6 (IL-6), monocyte chemotactic protein (MCP-1), macrophage inflammatory protein 1 beta (MIP-1 β) and interferon gamma (IFN- γ), and was conducted as previously described.

Data analysis

Data was analyzed by analysis of variance (ANOVA), t-tests, and by binary logistic regression with backward stepwise elimination using a Wald statistic. For $n = 36$ samples, the power of t-tests is 83% for an alpha value of 0.05 and a very large effect size (Cohen's $d = 1.0$).

RESULTS

Figures 1 to 3 illustrate the results. FAC ($p=0.033$), IL-6 ($p=0.013$), and MCP-1 (0.019) were significantly different among surgical groups by ANOVA. MIP-1 β ($p=0.72$) and IFN- γ ($p = 0.86$) were not significantly different among surgical groups. Controlling for age, IL-6 ($p = 0.017$) and MCP-1 ($p = 0.038$) were significantly different among surgical groups, and FAC ($p = 0.067$) bordered on significance by ANOVA.

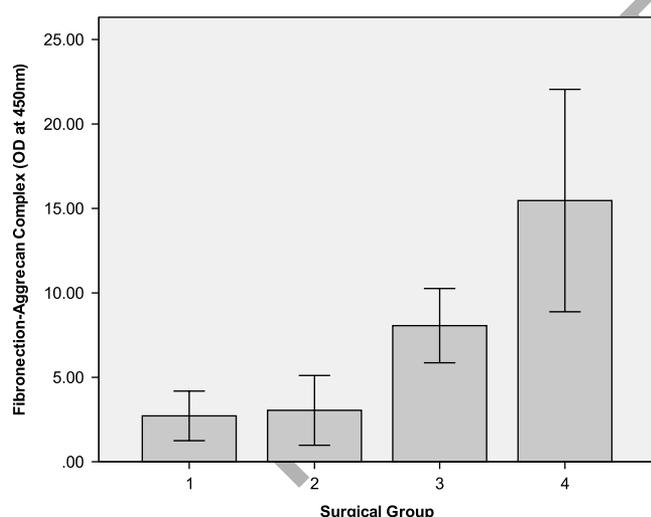


Fig. 1: Concentration of fibronectin-aggrecan complex (FAC) in ankle synovial aspirate by surgical group. The differences are statistically significant ($p=0.033$). The error bars represent the standard error of the mean (SEM).

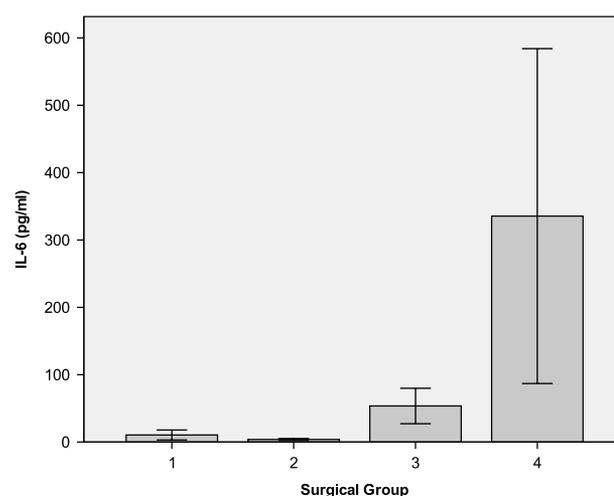


Fig. 2: Concentration of interleukin-6 (IL-6) in ankle synovial aspirate by surgical group. The differences are statistically significant ($p=0.013$). The error bars represent the standard error of the mean (SEM).

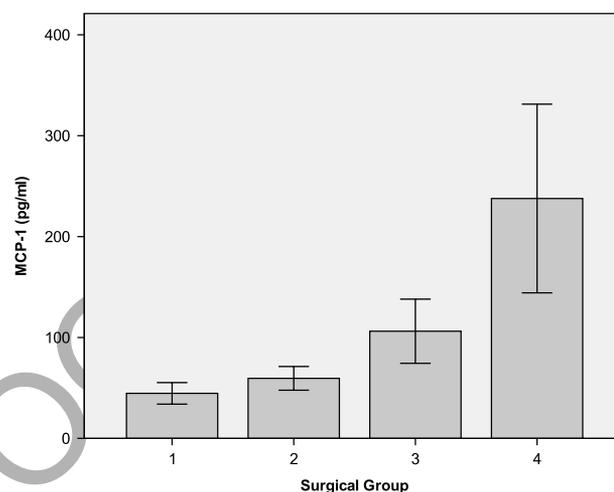


Fig. 3: Concentration of monocyte chemotactic protein-1 (MCP-1) in ankle synovial aspirate by surgical group. The differences are statistically significant ($p=0.019$). The error bars represent the standard error of the mean (SEM).

A model was built to discriminate minimal pathology (Groups 1 and 2) from substantial pathology (Groups 3 and 4) by means of molecular markers. FAC ($p = 0.014$) and MCP-1 ($p = 0.025$) were significantly different between groups with minimal and substantial pathology by ANOVA. The mean values of the inflammatory marker MCP-1 in pg/ml were 49.8 (± 8.0) for minimal pathology and 133.9 (± 33.0) substantial pathology. The mean values of FAC in optical density at 450 nm were 2.83 (± 1.16) for minimal pathology and 9.62 (± 2.23) for substantial pathology. The levels of FAC and MCP-1 were used to discriminate between minimal and substantial pathology by binary logistic regression. The resulting model was 82% specific, 63% sensitive, and 72% accurate in predicting the presence of substantial intra-articular pathology.

DISCUSSION

Inflammatory cytokines, matrix degradation products, and proteases have been implicated in the pathophysiology of joint degeneration.⁷ The ankle has been a relatively untapped resource for understanding inflammatory conditions, in part because of its surprising resistance to the development of osteoarthritis despite its weight bearing function. In the present study, we sought to correlate increasing severity of intra-articular ankle pathology with inflammatory markers and matrix proteins previously studied in the knee.⁵ The main finding is that FAC, IL-6 and MCP-1 are significantly increased with increasing degree of pathology.

Candidate biomarkers have been identified in the pathophysiology of degenerative joint diseases. Lavage fluid from knee joints with painful meniscal injury demonstrates greater immunoreactivity to interferon gamma (IFN- γ), interleukin-6 (IL-6), macrophage inflammatory protein-1 beta (MIP-1 β), and monocyte chemoattractant protein-1 (MCP-1) when compared to fluid from non-painful knees.⁵ Inflammatory cytokines have a complex relationship with fibronectin and its fragments¹⁰ and aggrecan and its fragments⁹ in degenerative joints disease as well.

Fibronectin and its fragments (FN-f) are implicated in degeneration of the synovial joints¹¹ and intervertebral discs¹ are associated with the release of cytokines in chondrolysis.¹⁰ They also cause the cleavage of aggrecan in cartilage degeneration.⁹ Potential cytokine mediators of cartilage inflammation and destruction induced by FN-f have been investigated.¹⁷ The ability of FN-f to stimulate chondrocyte expression of multiple proinflammatory cytokines and chemokines suggests that damage to the cartilage matrix is capable of inducing a proinflammatory state. This milieu is responsible for further progressive matrix destruction, which also includes the chemoattraction of inflammatory cells.¹⁷ FN-f that binds to integrin stimulates chondrocyte-mediated cartilage destruction and may play an important role in the progression of arthritis. Therefore, Fn-f itself may be pathogenic, in addition to being secondary degradation products of other pathogenic processes.¹¹

Aggrecan is a cartilage proteoglycan that, together with type II collagen, is a major extracellular structural component of articular cartilage.²⁰ Degradation of aggrecan by aggrecanases is thought to be an important event in cartilage degeneration,² and numerous specific aggrecan fragments have been purified from synovial fluid.¹⁸ Aggrecan cleavage is induced by fibronectin fragments,⁹ and certain aggrecan fragments are markers of joint degeneration which differ among inflammatory, traumatic, acute and chronic disease states.¹⁵ In our study, we found the presence of a fibronectin-aggrecan complex to be a significant predictor of intra-articular ankle pathology.

Recently cytokines have been identified in predicting outcomes of epidural interventions for lumbar disc syndromes⁴ as have degradation products of cartilage matrix

proteins. Additionally, high levels of specific cytokines and a fibronectin-aggrecan complex (FAC) have been identified and correlated with intra-articular knee pathology such as symptomatic meniscal tears.⁵ Indeed, there is mounting evidence for corroboration between the complex interactions of both structural proteins and cytokines for initiating chondrolysis and joint destruction. However, a thorough understanding by which cartilage degradation is initiated continues to elude us. Further research into which of these cytokines or proteins precedes the other likely will provide further insight.

This investigation focused solely on the severity of intra-articular pathology and patients were grouped accordingly. The authors feel further investigations and comparisons of primary osteoarthritis versus posttraumatic osteoarthritis may yield additional insights into their causes. Similarly, comparative studies evaluating patients with osteochondral defects versus other types of chondrolytic pathologies may additionally yield potential further therapies in the future. Similarly, there may in fact, be several pathways that lead to chondrolysis via a combination of genetic predisposition, trauma, autoimmune phenomena, or others as yet not identified.

Likely there are similarities between the pathogenesis of articular cartilage degradation of all weightbearing joints; however, the ankle has different mechanical and biological characteristics. The ankle joint has a smaller weightbearing area of articular contact than does the knee or hip.³ At 500N of load, the contact area of the ankle joint averages 350 mm², much less than 1,120 mm² for the knee and 1,100 mm² for the hip.¹³ In addition, articular cartilage of the ankle differs from that of the knee and hip in thickness and tensile properties. The average thickness of ankle articular cartilage ranges from less than 1 mm to slightly less than 2 mm.¹³ Furthermore, compared to the cartilage in the knee, ankle cartilage has a higher content of proteoglycans and water, as well as an increased rate of proteoglycan turnover and synthesis, all of which are responsible for its increased stiffness and reduced permeability. Chondrocytes within ankle cartilage also have a decreased response to catabolic factors such as interleukin-1 and fibronectin fragments, compared to the chondrocytes of knee cartilage.¹² These metabolic properties may protect the ankle articular cartilage from primary osteoarthritis. Additionally, the biochemical milieu of the ankle joint may lend factors which are protective against primary osteoarthritis. For example, a matrix metalloproteinase isolated from chondrocytes, TIMP-3, which is more prevalent in healthy compared to degraded articular cartilage, has been shown to be more abundant in ankle compared with knee cartilage.¹⁶ TIMP-3 levels are also affected by levels of other cytokines, such as TNF-alpha and IL-1beta, showing that there is likely a complex signaling cascade of early cartilage degradation which is not yet fully understood. Future studies may help us better understand which factors protect the ankle from developing primary osteoarthritis, and these could possibly lead to the development of treatment options for the hip and knee joints.

Morphological changes observed in osteoarthritis include cartilage erosion as well as a variable degree of synovial inflammation. Current research attributes these changes to a complex interaction of biochemical factors, including proteolytic enzymes that lead to breakdown of the cartilage macromolecules. Cytokines such as IL-1 and TNF-alpha produced by activated synoviocytes, mononuclear cells, or by articular cartilage itself, significantly up-regulate metalloproteinase (MMP) gene expression.⁶ Cytokines also blunt chondrocyte compensatory synthesis pathways required to restore the integrity of the degraded extracellular matrix (ECM).⁶ Though research focusing on animal models identified TNF-alpha as an important inflammatory cytokine, this has not been borne out in human studies. More cytokine research on human cartilage is needed to enhance our understanding.⁸

Our study had several limitations. Sample acquisition by needle aspiration is a variable clinical procedure that may result in a small volume of aspirate, a bloody aspirate, failure to enter a small joint such as the ankle, or the aspiration of a large effusion with unknown dilution effects on the quantification of candidate biomarkers.²⁰ Additionally, the control arm in this investigation was not a true control, as all had pathology in the ipsilateral limb, which may confound the intra-articular profile. The investigators suggest normal volunteers or perhaps the contralateral limb in future research.

CONCLUSION

The present study detected high levels of MCP-1 and FAC as biomarkers of intra-articular ankle pathology. These results are intriguing as they suggest that in the future, a foot and ankle surgeon may be able to direct operative intervention based on an ankle aspiration which could be performed as a simple office procedure. If analysis of synovial fluid can predict intra-articular pathology, a surgeon would know which cases would require addressing joint problems at the time of surgical intervention. Also, a negative analysis could spare patients the morbidity of an ankle arthroscopy if they have purely extra-articular disease. However, further investigations are necessary to more accurately identify the levels and markers which are clear indicators of the presence or absence of intra-articular pathology. Moreover, future studies are warranted to better evaluate and treat those with concomitant extra-articular and intra-articular pathology. This will require a larger sample size to better identify the levels of MCP-1 and/or FAC necessary to assess for the presence of intra-articular pathology, as well as outcome studies to determine if these levels do indeed predict surgical response to arthroscopic surgery.

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