Correlation Between Inflammatory Cells and Sulfated Glycosaminoglycan Concentration in Synovial Fluid of Subjects with Secondary Knee Osteoarthritis

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ABSTRACT. ***Objective.*** To analyze the presence of inflammatory cells in the synovial fluid (SF) of subjects with osteoarthritis (OA) and correlate them with the concentration of sulfated glycosaminoglycan (GAG).
Methods. The degree of chondral injury from 20 subjects (17 men, 3 women, mean age 48.06 ± 14.35 yrs) was evaluated macroscopically by means of arthroscopy and graded according to the International Cartilage Repair Society classification. All subjects presented anterior cruciate ligament (ACL) injuries, associated or not to menisci injuries. SF was aspirated and centrifuged. Sulfated GAG concentration was quantified by dimethylene blue staining. Manual morphometry was used to determine the inflammatory cell count (mononuclear and polymorphonuclear) by optical microscopy, after staining with May-Grünwald-Giemsa. The time of injury and degree of chondral injury were considered, and correlations among the variables were obtained by Spearman nonparametric correlation test.
Results. There was no significant correlation between the amount of mononuclear cells and the GAG concentration. There was a significant positive correlation between the polymorphonuclear cells and the GAG concentration, and a significant negative correlation between the degree of chondral injury and the GAG concentration.
Conclusion. Polymorphonuclear cells may be either a cause or an effect in the metabolic and biochemical processes occurring in chondral injuries. The correlations support the hypothesis that inflammatory cells have a significant role in the progression and chronicity of chondral injury in secondary OA. (First Release April 15 2008; J Rheumatol 2008;35:1096–101)

Key Indexing Terms:
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Osteoarthritis (OA), the most common form of synovial joint arthritis, is the cumulative result of biological and mechanical events that generate an unbalanced situation between synthesis and degradation in joint tissues[1,2]. The disease, which frequently affects knee joints, may be secondary to meniscus and ligament injuries, and, in this case, can affect young, adult, and elderly subjects.

OA is a complex process including multiple changes in joint components, such as cell and extracellular matrix[1], and is defined as a progressive degenerative disease rather than an inflammatory disease. However, the existence and relevance of inflammatory changes in the synovial membrane have been described by many investigators[2-6]. It is supposed that synovitis in arthritic joints has a role in pain, joint inflammation, and cartilage degeneration[2,7,8], thus being a determinant of OA pathophysiology[3,6]. As degradation of cartilage begins, synovial and inflammatory cells act as phagocytes, engulfing tissue breakdown products released into the synovial fluid (SF), and secrete proinflammatory cytokines such as interleukin 1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α), the latter being principally involved in the onset of OA[1,5]. These cytokines are responsible for increased levels of nitric oxide and prostaglandins, described as mediators for inflammation and cartilage destruction[2,9-11]. The presence of these components in joint fluid stimulates the proteolytic action of the inflammatory cells, resulting in release of larger fragments into the SF, closing a cycle that can perpetuate the OA process and progression of the disease[12,13].

Chondrocytes are able to synthesize a variety of proteolytic enzymes, such as matrix metalloprotease (MMP). The

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proteolytic cascade responsible for degradation involves collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2:A, MMP-9:B), and stromelysins 1 (MMP-3), 2 (MMP-10) and 3 (MMP-11)[14-16]. The rise in proteolytic activity is considered to be the primary cause of pathological cartilage destruction in OA, especially that caused by members of the metalloprotease family, which are directly involved in extracellular matrix breakdown[17]. They also have an important role in inflammatory processes to fight injury and maintain tissue turnover[18]. Polymorphonuclear (PMN) cells are sources of powerful oxidants and proteolytic enzymes, including elastase and active metalloprotease, that are capable of degrading intact cartilage[19].

Moore, *et al*[20] reported the relationship between PMN cells and extracellular matrix glycosaminoglycans (GAG), when they identified the presence of a neutrophilic infiltrate after intradermal injection of GAG in rats. Thus the products of the breakdown of the cartilaginous matrix by PMN cell enzymes are liberated into the SF and may be chemotactic to the PMN leukocytes themselves, thus perpetuating joint tissue injuries[20]. As well, the mononuclear cells (MNC) have an injurious effect in joint cartilage. Immunohistochemical studies have confirmed that synovial tissue from patients with early OA is characterized by MNC infiltration and production of proinflammatory cytokines and mediators of joint damage[3]. In addition, blood vessel formation and expression of proinflammatory mediators and nuclear transcription factors are overexpressed in early OA[3].

Variations in the cellular and humoral composition of the SF reflect changes in the synovial tissues[21]. During the destructive process in the extracellular matrix, there is an increase in its concentration due to the presence of macromolecules resulting from the breakdown of the cartilage, such as proteins, proteoglycan fragments, GAG, collagen, inflammatory cells, and free chondrocytes or their cellular components[22-25].

The concentration of sulfated GAG in synovial liquid has been investigated in various joint diseases, being considered as joint markers that can reflect the metabolism of the joint tissue under both normal and pathological conditions, in which its concentration is modified[26]. However, few studies have related the presence of leukocytes in the SF with cartilaginous matrix products, particularly in OA. In synovitis, PMN cells are found in both the SF and the synovial membrane, and may adhere to the joint surface and thus attack the cartilage directly[27]. Nevertheless, the presence of inflammatory cells in ligamentous knee injuries should be restricted to the post-trauma acute period. Therefore, because of the injurious and amplifying role of inflammation, there are many reasons to study the behavior of PMN and MNC associated with products of cartilaginous matrix breakdown such as GAG.

Our aim was to analyze the existence of inflammation in SF from the presence of inflammatory cells (MNC and PMN), and correlate this with a biological marker of cartilaginous metabolism, in this case the concentration of sulfated GAG, taking into account the time of injury and the degree of chondral injury of knees in secondary OA caused by ACL injury.

MATERIALS AND METHODS

Subjects. Forty-four subjects of both sexes took part in this study. Twenty-four were excluded as follows: they presented with systemic, cardiorespiratory, or rheumatic conditions; absence of SF during collection; the presence of osteomyoarticular disease in the healthy lower limb as reported by the subject; had undergone joint infiltration; had a limited range of motion between 0˚ and 90˚; or had a time from injury < 4 weeks or > 14 months.

According to the criteria, 20 subjects were selected (17 men, 3 women, average age 48.06 ± 14.35 yrs). The subjects presented 2, 3, and 4 degrees of chondral injury, according to the International Cartilage Repair Society (ICRS) classification[28].

All the subjects presented chondral injuries, secondary OA, due to trauma involving the ACL, associated or not with the menisci, with an average time from injury of 118.23 ± 109.37 days. The subjects underwent clinical and imaging evaluation and surgery was ordered by the physician responsible for the arthroscopic repair of the injured structure.

The subjects had used nonsteroidal antiinflammatory drugs, but discontinued them for 2 to 3 weeks prior to their SF puncture, and no subject reported recurrent trauma during the previous month.

Evaluation record forms were filled in for all the subjects prior to data collection to assure that the inclusion and exclusion criteria were met. The aims and procedures of the study were explained to the subjects and they gave signed informed consent as required by the 196/96 resolution of the National Health Committee. The study was approved by the Ethics Committee for Research with Human Beings of the Federal University of São Carlos (National Health Committee protocol no. 143/04).

Synovial fluid aspiration. SF was collected from the injured knee by means of punctures prior to surgery. The anterolateral face of the injured knee was first shaved and anesthetized. A needle was inserted into the lateral suprapatellar knee region in order to collect fluid from the subquadricipital bursa.

All fluids were quantified for volume and divided into 3 samples for storage at –70°C until analysis.

Macroscopic evaluation of joint cartilage. After puncture for SF, the same surgeon described the macroscopic aspects of the joint surface, trochlea, patella, tibial and femoral condyles, medial and lateral menisci, and the ACL during the arthroscopic procedure.

To determine the degrees of tissue injury of the joint cartilage, an evaluation record was used, based on the ICRS histological classification[28].

Scoring of the degree of tissue destruction was defined using the "maximum score" pattern, represented by the greatest degree of cartilage injury (0 to 4 scale) found in any one of the femoral or tibial condyles, patella, or trochlear regions.

To analyze the results of the macroscopic evaluation of the joint cartilage, the highest score found for each subject was used, not taking into account the area size or number of foci.

Evaluation of sulfated GAG. The sulfated GAG concentration in SF was evaluated by means of absorbance analysis after staining with dimethylmethylene blue (DMMB), using the method described by Farndale, *et al*[29]. The spectrophotometric method associated with the dimethylmethylene reagent was chosen for simplicity and specificity for measurement of sulfated GAG in tissues and body fluids.

SF samples were centrifuged at 14,000 g for 15 min in a model 5415D Eppendorf centrifuge; 100 µl aliquots of the sample supernatants were then digested in 100 µl 0.4 M acetate buffer, pH 5.8, containing 10 mM EDTA, 20 mM of N-acetylcysteine, and papain added immediately before use in the proportion of 300 µg per 1 ml SF. After incubation in a double-boiler at 60°C for 24 h, iodoacetic acid was added to a final concentration of 10 mM,

for a total of 22.2 µl, then centrifuged 4 min at 12,000 g. One unit of streptomyces hyaluronidase was then added to the supernatant. After incubation in a double-boiler at 37°C for 12 h, 100 µl were removed. When the sample was ready for measurement, DMMB reagent solution (2.5 ml) was added to the material and the absorbance was read at λ = 526 nm in a spectrophotometer after a few seconds.

Chondroitin sulfate solutions of known concentrations were used to determine the concentrations of sulfated GAG.

Cytology. Stored SF was prepared for cytologic analysis after thawing at room temperature. The material (100 µl sample from each subject) was processed in a Fanem® Citospin-248® cytocentrifuge for 5 min at 1500 rpm. The material was then dry-fixed and stained by the special May-Grünwald-Giemsa procedure[27,30].

The numbers of PMN and MNC in SF samples were determined by manual counting via optical microscopy. Two evaluators carried out the slide analysis using magnification ×40 with an Axiolab light microscope (Zeiss).

Calculation of the number of fields/slide for each sample was based on the Bernoulli distribution statistical method, in which the total number of slide fields for each subject and the number of inflammatory cells present in normal and inflamed SF were taken into account. It was decided that at least 30 fields be sampled for each subject for subsequent reading under the light microscope. Thirty-two fields/subject were analyzed, for a total of 1216 fields evaluated. This result presented a margin of error of ± 3 percentage points, with 95% of confidence.

Statistical analysis. Analysis was by use of Statistica for Windows (version 6.0). The tests for normality (Shapiro Wilk's W test) and homogeneity (Levene) were applied to the variables, and nonparametric tests were indicated for data analysis. To evaluate correlation between results obtained by the 2 observers, the nonparametric Spearman test with R = 0.8 and p = 0.05 was applied. This same test was applied to determine the relationships among GAG concentrations, quantity of inflammatory cells, degree of OA, and time after injury.

For all statistical analyses, a significance level of 5% was considered (p = 0.05).

RESULTS

All the subjects presented degrees of chondral injury between grade 2 and 4. Seventeen of them had meniscal tears and 11 had ACL injury associated with the cartilage injury. The average amount of SF aspirated was 5.9 ± 5.4 ml.

The SF samples presented a citric yellow coloration. In some cases there were streaks of blood present due to sample contamination by the needle. There was considerable variation of number of inflammatory cells per ml SF, from 117.2 to 25,359.4 MNC cells/ml and from 0 to 421.9 PMN cells/ml.

Sulfated GAG concentrations varied between 25.2 µg/ml and 314.8 µg/ml. Table 1 shows values for the variables studied.

As shown in Table 2, PMN and MNC cells had a positive moderate correlation (r = 0.82, p = 0.0001). There was no significant correlation between the MNC cells and GAG concentrations. However, a significant positive correlation between PMN cells and GAG concentrations was found (r = 0.60, p = 0.01; Figure 1). A significant negative correlation (r = –0.62, p = 0.003) was identified between the GAG concentration and the degree of chondral injury (Figure 2).

No correlation was found between the degree of chondral injury or time from injury and the measures of MNC or PMN cells.

DISCUSSION

Normal SF contains only about 10 to 20 inflammatory cells/ml, with a predominance of MNC cells, only 10% of the total being PMN cells[31]. In the SF samples from joints showing chondral injury evaluated in our study it can be considered that the concentrations of cells, 117.2 to 25,359.4 MNC/ml and 0 to 421.9 PMN/ml, are characteristic of an inflammatory joint process.

According to Yanni, *et al*[32] there is a correlation between the number of synovial membrane macrophages and the degree of cartilage erosion. The macrophages would be responsible for amplifying the chronic inflammation through the release of cytokines by the inflammatory cells[32,33]. The release of cytokines such as IL-1, IL-8, and TNF-α by macrophages during the inflammatory process stimulates leukocytes to pass through the endothelial wall and participate actively in angiogenesis. The release of IL-1, IL-6, and TNF-α by synovial macrophages also stimulates the release of collagenase and elastase, which have an important role in joint tissue destruction[34]. Benito, *et al*[3] found an increase in the amount of MNC infiltrate in the early stages of OA, a phase in which other investigators have described a greater GAG concentration[35-37]. However, in our samples, there was no significant correlation between MNC and the GAG concentration, although the latter did show a significant correlation with PMN, and PMN presented a strong correlation with MNC.

Moore, *et al*[20] described one possible mechanism for the influx of inflammatory cells; their studies showed that GAG may be chemotactic to neutrophils. Once these fragments are in contact with joint cartilage, the PMN would stimulate greater matrix breakdown through the release of neutrophilic enzymes and stimulation of metalloprotease activity, which leads to greater tearing of cartilage components. These degraded components would be released into the SF and then engulfed by inflammatory cells acting as phago-

Table 1. Mean (± SD) for study variables.

Age, yrs	Time from Injury, days	GAG, µg/ml	MNC, no. of cells/ml	PMN, no. of cells/ml
48.06 ± 14.35	118.23 ± 109.37	124.96 ± 75.82	5255.9 ± 8374.9	152.34 ± 122.74

GAG: glycosaminoglycans, MNC: mononuclear cells, PMN: polymorphonuclear cells.

Table 2. Correlations among the study variables (r value).

	MNC	PMN	GAG	Time from Injury	Degree of Chondral Injury
MNC	1.000	0.826**	0.335	–0.146	–0.188
PMN	0.826**	1.000	0.602*	–0.192	–0.255
GAG	0.335	0.602*	1.000	–0.167	–0.626**
Time from injury	–0.146	–0.192	–0.167	1.000	0.456
Degree of chondral injury	–0.188	–0.255	–0.626**	0.456	1.000

* $p \leq 0.05$; ** $p \leq 0.001$. For definitions see Table 1.

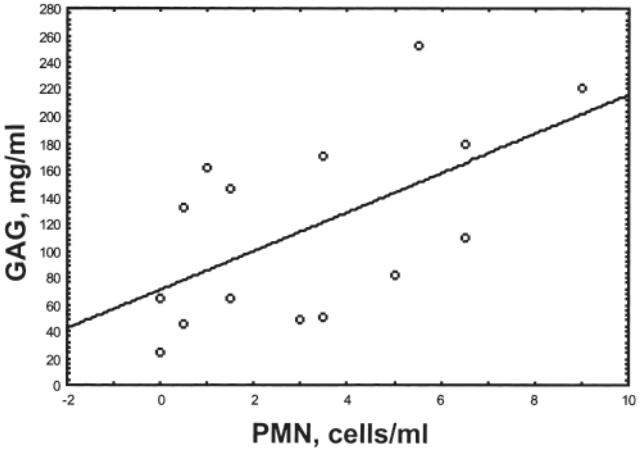

Figure 1. Number of polymorphonuclear cells (PMN) in relation to sulfated glycosaminoglycan (GAG) concentration (r = 0.60, p = 0.01).

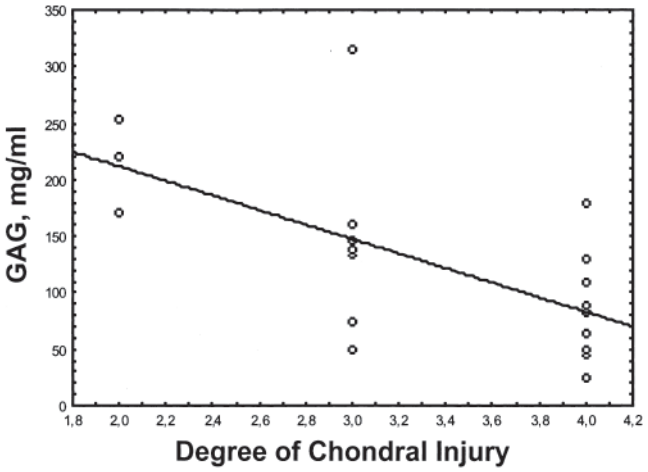

Figure 2. Sulfated glycosaminoglycan (GAG) concentration in relation to the degree of chondral injury in subjects with cartilage injury classified as International Cartilage Repair Society (ICRS) grade 2 to 4 (r = –0.62, p = 0.003).

cytes, which would thus secrete more cytokines and inflammatory mediators, perpetuating the OA process[19,20,38-40].

Our data also showed a negative correlation between the degree of chondral injury and the concentration of sulfated GAG, which means that with lower degrees of cartilage injury, the GAG concentration was higher. Other authors have also found an inverse relationship between the degree of chondral injury and the average GAG concentration in SF, stating that greater degrees of cartilaginous degeneration tended to present a decrease in the proteoglycan concentration. This relationship probably results from the reduction in the amount of cartilaginous tissue in the joint[35,37,41-43].

Stefanie, *et al*[2] evaluated changes in the macroscopic aspects of OA, such as radiographic alterations and clinical signs, comparing the action of inflammatory mediators, and found no correlations between them[2]. Our results showed a correlation between the molecular indicators of cartilage injury (proteoglycan fragments, GAG) and the inflammatory cells present in SF. However, as noted by Stefanie, *et al*[2], morphological alterations such as a decrease in joint space and the presence of osteophytes had already occurred, but in our study these alterations had not yet occurred. Perhaps methods of early diagnosis should be improved and focused on the joint microenvironment, as suggested by Lohmander, *et al*[35,36].

It is known that the presence of PMN cells contributes to degradation of GAG[19], although chemotaxis of the PMN in the joint environment in individuals with chronic ligament injury and who are not in the trauma period is undetermined. In this type of population it was expected that the inflammatory process must exist only after trauma, which was not the case, because the presence of PMN and MNC was observed in the individuals without symptoms who were studied. A possible mechanism for the perpetuation of inflammatory cells is the positive correlation between the GAG and PMN. The instability generated by ligament loss alters the movements between joint surfaces, causing trauma in the cartilage in different areas of the joint surface.

These micro-movements are acknowledged by the chondrocytes, and when these cells sense perturbation in the tissue they alter their metabolic state in order to respond to the mechanical stress[44,45]. This alteration is identified in the SF since it reflects the entire cartilage environment. The presence of proteoglycan fragments and GAG in the joint envi-

ronment results in activation of mechanisms for the absorption/removal of these fragments. Considering that micro-traumas are frequent, an unbalanced situation between GAG synthesis and reabsorption in the joint environment would increase the concentration of GAG, and therefore the mechanism of chemotaxis between the GAG and PMN would be activated and maintain the presence of the inflammatory cells. Moore, *et al*[20] also described this mechanism.

Consequently, if aggression processes in the tissue are maintained, the cartilage starts its degenerative process and therefore its thickness will gradually be lost, leading to OA. Thus in cases of severe chondral injury, such as OA, the decrease in GAG would be directly related to the absence of chondrocytes, which are responsible for synthesis of the extracellular matrix.

Our results verify that inflammation of the SF is of great relevance in the metabolic and biochemical processes occurring in the joint after mechanical trauma. Consequently, primary care in cartilage maintenance, such as the control of joint inflammation, would appear to be instrumental in avoiding perpetuation of the degenerative process[12]. Thus in patients with focal chondral injury resulting from trauma who are symptom-free, the presence of GAG would be an indication of cartilage metabolic activity, and this would enable therapeutic intervention. As a result, on a longterm basis, perpetuation of the presence of GAG in the SF would lead to an amplifying effect of the injury through leukocyte chemotaxis. Therefore, control of micro-traumas and recurrent traumas such as joint sprains would seem to be fundamental to the health of the joint cartilage.

Knowledge of the interactions between inflammation, biomechanics, and the behavior of cartilage biomarkers may lead to understanding of the etiopathogenesis and progression of OA, providing new therapies for this disease.

Our results showed a positive correlation between the increase in concentration of sulfated GAG and the presence of inflammatory cells, mainly PMN, in the joint environment of individuals with chondral injury due to trauma involving the ACL, sometimes associated with the menisci, and who were not in the post-trauma acute stage; our findings suggest that the presence of PMN cells in the joint environment is perpetuated by the chemotactic action of the GAG concentration. The progressive degeneration of the cartilage is amplified by an inflamed environment and by the loss of GAG due to recurrent micro-traumas, which can favor perpetuation of the cycle of inflammation, leading to premature development of secondary OA.

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