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ORIGINAL ARTICLE

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Multivariate analysis of biochemical markers in synovial fluid from the shoulder joint for diagnosis of rotator cuff tears

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Abstract Multivariate discriminant analysis allowed definition of cytokine, matrix metalloproteinases (MMP), and tissue inhibitors of metalloproteinases (TIMP) levels in synovial fluid (SF) of patients with rotator cuff tears and other shoulder lesions. We analyzed SF aspirated from the glenohumeral joints of 17 patients with rotator cuff tears; SF from nine patients with other shoulder lesions was used to characterize a non-rotator-cuff-tear (NRCT) group. Discriminant analysis demonstrated statistically significant differences in (1) the determination of whether rotator cuff tear patients are separable from the NRCT group using the influential functions, the most influential of which were interleukin 1-beta, MMP-2, and MMP-13, and (2) the assessment of whether full-thickness rotator cuff tears are distinguishable from partial tears using identical influential functions. The most influential function in the latter analysis was MMP-13. Both interleukin 1-beta and MMP-13 might be biochemical markers of impending rotator cuff tears.

Keywords Cytokines · Matrix metalloproteinases · Multivariate discriminate analysis · Rotator cuff tears · Synovial fluid

Introduction

Rotator cuff tears are a common problem that causes shoulder pain, especially among people beyond middle age [1, 2, 3, 4]. Neer [5] reported that 95% of rotator cuff tears were initiated by impingement wear rather than circulatory impairment or trauma. Areas of hypovascularization in the rotator cuff were found to be associated with aging. These areas were shown to coincide with common sites of degeneration noted in studies of the arterial supply to the human rotator cuff [3]. Degenerative changes occur first in the avascular zone, where the tendon of the supraspinatus near its insertion and the superior portion of the insertion of the infraspinatus are most involved [6]. Not surprisingly, rotator cuff tears are commonly observed in these areas.

Recently, cytokines such as interleukin 1-beta (IL-1β) were observed in glenohumeral synovium after rotator cuff perforation, and they indicate worsened inflammation in rotator cuff diseases except in cases involving mechanical or circulatory problems [7]. Matrix metalloproteinases (MMP) are considered to be important for attributing proteolytic activity as the primary cause of the pathologic destruction of cartilage [8, 9, 10, 11].

Tendon tissue cells derived from the shoulders are capable of producing MMP and tissue inhibitors of metalloproteinases (TIMP) [12]. Furthermore, MMP levels in the synovial fluid (SF) in the glenohumeral joint have been reported to be associated with the size of the involved area in rotator cuff tears [13]. However, few studies have demonstrated that these biochemical markers in the SF are more closely related to rotator cuff tears than to other shoulder lesions. They interact in the same SF. Therefore, the multiplicity of statistical analyses should be considered when we study several biochemical markers in the SF from patients with different diseases. To satisfy that requirement, we used multivariate discriminant analysis to evaluate cytokine, MMP, and TIMP levels in glenohumeral joint SF from patients with rotator cuff tears and other shoulder lesions.

Materials and methods

Patients

The Human Ethics Review Committee of our institution approved the protocol of this study. The aims and

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methods of the study were explained to all the patients; all gave their informed consent. We studied 17 patients with rotator cuff tears, including six with partial-thickness tears and 11 with full-thickness tears. Each patient had undergone rotator cuff surgery at the Department of Orthopaedic Surgery of Gunma University Hospital in Gunma, Japan, between May 2001 and December 2001. Additionally, nine patients with other shoulder lesions were recruited into a non-rotator-cuff-tear control group (NRTG). Table 1 summarizes the patients' clinical characteristics.

Among those with rotator cuff tears, 15 were male and two were female. Their mean age was 61 years (range 46–80). Definite diagnosis was made for each of the patients based on findings during surgery. Those with full-thickness tears (mean age 62 years) were subdivided into three groups according to the tear size: four had large tears (defined as more than 5 cm wide), seven had medium tears (3–5 cm wide), but none had small tears (less than 3 cm wide). Of the patients with partial-thickness tears (mean age 59 years), four had bursal-side and two had joint-side tears.

All patients received nonsteroidal anti-inflammatory drugs. None had been treated with intra-articular injection of steroids or hyaluronan for at least 6 months prior to this study. No cartilage destruction of the shoulder joint was observed in any patient. The mean duration between the start of symptoms attributable to the rotator cuff tear and surgery was 5 months (range 1–24).

All patients gave informed consent for the collection of SF. It was aspirated from their glenohumeral joints during surgery before the joint was opened ($n = 2$). When it was impossible to aspirate the crude SF, 20 ml of saline solution was injected into the glenohumeral joint; then the mixture of SF and saline was aspirated and reinjected a few times. The SF samples which contained blood resulting from active bleeding or large amounts of debris during the sampling procedure were excluded from the study. The samples in which recovery was less than 50% of the injected volume were also excluded, based on findings of previous studies [14, 15, 16]. The remaining SF samples were then collected ($n = 15$) and centrifuged at 2,000 *G* for 5 min immediately after aspiration to remove cells. Then the samples were stored at −80°C until assay was performed.

Of the nine patients in the non-rotator-cuff-tear group (NRCT), we collected SF from two with impingement syndrome, four with superior labrum anterior-to-posterior (SLAP) lesions (often found in baseball players), and three with anterior instability of the shoulder as a result of trauma, using normal saline injection methods similar to those described above. Definite diagnosis was made for each patient based on findings during surgery. The mean age of these patients was 25 years (range 17–60). The mean duration between the start of symptoms attributable to the shoulder lesion and surgery was 31 months (range 12–84). Treatments were similar to those of patients with rotator cuff tears.

Measurement of MMP, TIMP, and cytokines

Levels of MMP-1, MMP-2, MMP-3, MMP-8, MMP-13, and TIMP-1 in the glenohumeral joint SF were measured by the corresponding one-step sandwich enzyme immunoassay systems using commercially available kits (Fuji, Toyama, Japan) according to the instructions provided by the manufacturer. Concentrations of interleukin 1-beta (IL-1β) and tumor necrosis factor alpha (TNF-α) in the SF were also determined with the respective human enzyme-linked immunosorbent assay kits (BioSource, Camarillo, Calif., USA), respectively. The SF concentrations of MMP, TIMP, and cytokines were calculated using the calcium concentrations of the glenohumeral joint SF.

Statistical analysis

Independent analysis of the marker molecules between the two groups was performed using Student's or Welch's *t*-test, depending on the equality or nonequality of variance in data. Furthermore, we applied a discriminant analysis for the whole comparison of these marker molecules between the two groups. The statistical significance of the mean age of subjects was analyzed by Mann-Whitney U test.

Results

The concentrations of MMP-1, MMP-2, MMP-3, MMP-8, MMP-13, TIMP-1, and IL-1β in the SF from patients with rotator cuff tears were (mean ± SD) 23.4 ± 15.3, 40.3 ± 21.8, 772.5 ± 385.1, 2.1 ± 1.3, 0.1 ± 0.2, 153.9 ± 76.0 ng Ca/ml, and 7.1 ± 6.9 pg Ca/ml, respectively. Those in the NRCT group were 6.5 ± 4.0, 9.7 ± 4.8, 458.1 ± 344.0, 1.7 ± 0.4, 0, 80.4 ± 21.3 ng Ca/ml, and 1.7 ± 2.0 pg Ca/ml, respectively (Table 2).

Table 1 Patient characteristics

Group	Rotator cuff tear ($n = 17$)	Non-rotator-cuff-tear ($n = 9$)
Mean age in years (range)	61 (46–80)	25 (17–60)
Males/females	15/2	7/2
Shoulder lesions	11 Full thickness (4 large, 7 medium), 6 partial thickness (4 bursal-side, 2 joint-side)	2 Impingement syndrome, 4 SLAP lesion, 3 anterior shoulder instability

Table 2 MMP, TIMP, and cytokine levels in SF from shoulder joints with rotator cuff tears and other lesions. Levels were calculated using the respective calcium concentration. The data show means ± SD

Group	MMP-1[a]	MMP-2[a]	MMP-3[a]	MMP-8[a]	MMP-13[a]	TIMP-1[a]	IL-β[b]
Rotator cuff tear	23.4 ± 15.3	40.3 ± 21.8	772.5 ± 385.1	2.1 ± 1.3	0.1 ± 0.2	153.9 ± 76.0	7.1 ± 6.9
Non-rotator-cuff-tear[c]	6.5 ± 4.0	9.7 ± 4.8	458.1 ± 344	1.7 ± 0.4	0	80.4 ± 21.3	1.7 ± 2.0
P value	0.0188	0.0019	0.0002	0.4462	0.03789	0.1257	0.1008

[a]In ng Ca/ml
[b]In pg Ca/ml
[c]Including impingement syndrome, SLAP lesion, and anterior shoulder instability

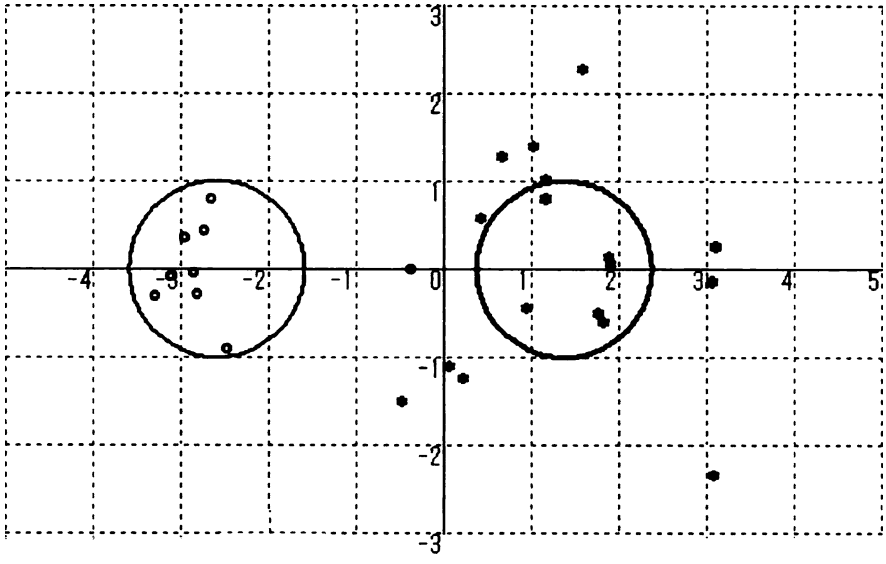

Fig. 1 Canonical coordinates for rotator cuff tears and other shoulder lesions. Patients with rotator cuff tears (*asterisks*) and other shoulder lesions (*open circles*) were distinguished completely by discriminant analysis

MMP-13 in the NRCT group and TNF-α in all patients could not be measured because of the low concentrations or even absence of these substances.

To assess whether patients with rotator cuff tears could be distinguished by the above factors from the NRCT group, discriminant analysis demonstrated a statistically significant difference, with F = 13.4022 and degrees of freedom (df) = 5, 20 ($\alpha = 0.05$) (Fig. 1). The most influential function of this analysis was IL-1β (partial F 2.485), followed by MMP-2 (partial F 2.269), and then MMP-13 (partial F 1.1032) (Table 3). In the SF from patients with rotator cuff tears, the concentrations of MMP-1, MMP-2, MMP-3, MMP-8, MMP-13, TIMP-1, and IL-1β from the complete tears were (mean ± SD) 25.4 ± 18.4, 43.3 ± 21.7, 674.5 ± 423.2, 1.6 ± 1.1, 0.1 ± 0.1, 157.6 ± 100.6 ng Ca/ml, and 4.3 ± 4.3 pg Ca/ml, respectively, whereas the respective concentrations among the SF samples from partial tears were (mean ± SD) 19.7 ± 10.0, 34.7 ± 21.8, 952 ± 301.7, 3.1 ± 1.2, 0.2 ± 0.3, 147.2 ± 35.1 ng/Ca, and 12.1 ± 9.1 pg Ca/ml, respectively (Table 4).

Table 3 Discriminant analysis of rotator cuff tears and other shoulder lesions

Parameter	Partial F value
IL-1β	2.485
MMP-2	2.269
MMP-13	1.1032

In the assessment of whether full- and partial-thickness rotator cuff tears could be separated, discriminant analysis demonstrated a statistically significant difference, with F = 3.2178 and df = 5, 11 ($\alpha = 0.05$) (Fig. 2). The most influential function of this analysis was MMP-13 (partial F 5.4622), followed by TIMP-1 (partial F 3.438), MMP-3 (partial F 3.3435), MMP-8 (partial F 1.8171), and IL-1β (partial F 0.9183) (Table 5).

Discussion

Rotator cuff tears accompany local inflammation that is typically attributable to nutritional and mechanical causes [17, 18]. Furthermore, some massive rotator cuff tears proceed to glenohumeral degenerative arthritis [17]. Cytokines such as IL-1β and TNF-α are produced by osteoarthritic cartilage; they play an important role in local inflammation and pain [10, 15]. Gotoh et al. [7] reported that the glenohumeral synovium in patients with rotator cuff disease produced IL-1β.

Matrix metalloproteinases (MMPs) are also thought to cause cartilage destruction. In particular, MMP-1 and MMP-3, which are produced by synovium and chon-

Table 4 MMP, TIMP, and cytokine levels in SF from shoulder joints with complete and partial rotator cuff tears. Levels were calculated using the respective calcium concentration. The data show means ± SD

	MMP-1[a]	MMP-2[a]	MMP-3[a]	MMP-8[a]	MMP-13[a]	TIMP-1[a]	IL-1β[b]
Complete	25.4 ± 18.4	43.3 ± 21.7	674.5 ± 423.2	1.6 ± 1.1	0.1 ± 0.1	157.6 ± 100.6	4.3 ± 4.3
Partial	19.7 ± 10.0	34.7 ± 21.8	952.0 ± 301.7	3.1 ± 1.2	0.2 ± 0.3	147.2 ± 35.1	12.1 ± 9.1
P value	0.5851	0.5295	0.2703	0.0754	0.3993	0.8512	0.2010

[a]In ng Ca/ml
[b]In pg Ca/ml

drocytes, induce cartilage breakdown [19, 20, 21]. Other MMPs, such as MMP-2, MMP-8, and MMP-13, have also been observed in SF in the knee joints of patients with osteoarthritis; these MMPs also were shown to cause cartilage destruction [11].

When MMPs act in local tissues, tissue inhibitors of matrix metalloproteinases (TIMP) are considered to be important. TIMP-1, which is produced by articular cartilage [22] and shoulder tendon tissues [12], exhibited affinity for MMP-1, MMP-3, and MMP-13 [22]. It balances MMP activity and is present in the SF of patients with osteoarthritis and rotator cuff tears [9, 11, 13].

When we performed the independent analysis in this study, statistically significant differences were found in MMP-1 ($P = 0.0188$), MMP-2 ($P = 0.0019$), MMP-3 ($P = 0.0002$), and MMP-13 ($P = 0.03789$) levels between the rotator cuff tear and NRCT groups (Table 2). No statistically significant difference was observed in the data between the complete and partial tear groups (Table 4). These data are compatible with previous findings regarding MMP [13] but not cytokines [7].

The mean age of subjects in the rotator cuff tear group (61 years) was higher than in the NRCT group (25 years) ($P = 0.0003$), whereas little difference was observable between the groups with full-thickness tears (62 years) and partial tears (59 years) ($P = 0.546$).

Osteoarthritic change with aging might increase the level of MMP [11]. However, a previous animal experimental paper reported that MMP in the SF from adult joints was not related to age, although from juvenile joints it was present at higher levels than in adults [23]. In this study, no cartilage destruction of the shoulder joint was observed in any patient. These facts suggest that age difference between the rotator cuff tear and NRCT groups might have had little effect on the experimental results.

A problem arose when MMP and cytokines were separately analyzed by statistical methods: these macromolecules influenced each other in the SF [18]. Repeated pairwise comparison induces the wrong rejection of a null hypothesis because of the increase in type I familywise error rate. Based on these facts, we applied discriminant analysis for these macromolecules.

When factors that affected rotator cuff tears were analyzed separately from those affecting other shoulder lesions, discriminant analysis allowed a distinction revealed strikingly in canonical coordinates (Fig. 1). Canonical coordinates show the discrimination between two groups based on the parameters and are the criteria demonstrating the distance between groups. The important factors, in descending order, were IL-1β, MMP-2, and MMP-13 (Table 3), although the difference in IL-1β was not statistically significant according to the independent analysis (Table 2). Furthermore, when we analyzed factors that possibly distinguished between complete rotator cuff tears and partial tears, the discriminant analysis allowed such a distinction (Fig. 2). The important factors, in descending order, were MMP-

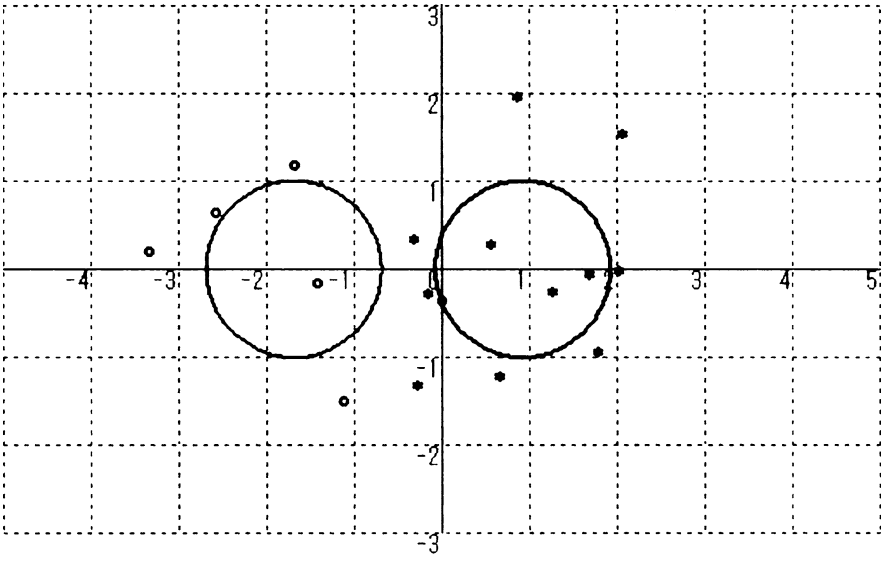

Fig. 2 Canonical coordinates for full-thickness and partial-thickness rotator cuff tears. Patients with full-thickness rotator cuff tears (*asterisks*) were distinguished completely from those with partial-thickness tears (*open circles*) by discriminant analysis

Table 5 Discriminant analysis of full-thickness and partial-thickness rotator cuff tears

Parameter	Partial F value
MMP-13	5.4622
TIMP-1	3.438
MMP-3	3.3435
MMP-8	1.18171
IL-1β	0.9183

13, TIMP-1, MMP-3, MMP-8, and IL-1β (Table 5), although these were not statistically significant differences by independent analysis.

Rotator cuff perforation increases IL-1β production in SF, enhancing inflammatory intensity at the injury site [7]. MMP-2, MMP-8, and MMP-13 in SF contribute to the tissue degeneration [10]. Others such as MMP-1 and MMP-3 in SF significantly correlate with rotator cuff tear size [13]. Considering these reports and our results, MMP-2 and MMP-13 might be important biochemical markers of impending rotator cuff tears followed by the increased production of IL-1β. In addition, MMP-13, MMP-3, and MMP-8 might be factors causing the progression of rotator cuff tears. TIMP-1, which was the second important factor for the distinction between complete and partial rotator cuff tears, might be attributable to the production of MMPs, such as MMP-3 and MMP-13 [22].

The numbers of subjects in each group were small in this study. Greater numbers are necessary for precise statistical analysis. However, until now, no report has evaluated these marker molecules with multivariate analysis. In this sense, the results elucidate rotator cuff tears from a new biochemical perspective.

The number of immune cells in the SF affects the experimental data. However, SF samples containing blood from active bleeding or excessive debris were excluded from the study. Furthermore, the samples were centrifuged immediately after aspiration to remove cells and debris. These procedures reduced the cytokines and MMP produced by the immune cells in the aspirated SF to the minimum.

The SF MMP and cytokine concentrations were calculated using the calcium concentrations of the glenohumeral SF, because the total protein concentration could not be measured in some samples of diluted SF. The volume of normal saline injected was constant for all joints and much greater than that of the original SF. Therefore, the concentration of components in the diluted SF was representative of their amount in the original SF and independent of the amount of aspirate obtained.

Further, in experimental animal models, knee joint fluid volumes were estimated by measuring endogenous calcium concentrations. A statistically significant correlation was observed between knee joint fluid volumes obtained with the intravenous injection of a radioisotope and this method [24]. Considering these facts, the calcium concentration in the diluted joint fluid becomes the new candidate marker of the dilution [24]. The MMP and cytokine concentrations calculated using calcium concentrations in the SF might demonstrate values based on the SF volume.

Regarding discriminant analysis, these results might have differed with different control groups. In this sense, further investigation would be necessary using higher numbers of patients with rotator cuff tears and NRCT patients with other types of shoulder lesions. In any case, discriminant analysis appears elucidate rotator cuff tears from a new, biochemical perspective.

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