CONCISE REPORTS

Examination of synovial fluid and serum hyaluronidase activity as a joint marker in rheumatoid arthritis and osteoarthritis patients (by zymography)

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Abstract

Objective—Hyaluronic acid (HA) is an important joint marker and the substrate for hyaluronidase (HAase). Synovial fluid (SF) and serum HAase were measured to investigate the potential use of HAase as a joint marker in rheumatoid arthritis (RA) and osteoarthritis (OA) patients.

Methods—The subjects were 39 patients with RA and 42 patients with OA. HAase activity was measured by zymography and its relation with various parameters examined statistically.

Results—In RA SF a positive correlation ($r=0.458$, $p=0.0186$) was found between SF HAase activity and the concentration of serum C reactive protein. A positive correlation ($r=0.45$, $p=0.024$) was also found between SF HAase activity and platelet count in the RA group. Serum HAase activity in the RA group was significantly higher than in the OA group ($p<0.0001$) and normal controls ($p<0.0001$).

Conclusion—The results suggest that SF HAase activity could be used as a marker of synovial inflammation.

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Hyaluronic acid (HA) is a high molecular weight polysaccharide that is widely distributed in the body. The highest concentrations are found in connective tissue such as synovial membrane and synovial fluid (SF). There have been many reports on the use of HA as a joint marker,¹⁻⁴ but there have been none investigating the use of HAase as such a marker. In 1984, Fiszler-Szafarz measured the HAase activity in serum from healthy human beings by zymography.⁵ In this study, we used zymography to measure HAase activity in serum and SF from rheumatoid arthritis (RA) and osteoarthritis (OA) patients, to examine the potential of HAase as a joint marker.

Methods

PATIENTS

The subjects were 39 patients with RA (mean age 58.3 years) and 42 patients with OA (mean age 65.5 years). We measured HAase activity in

Figure 1 (A) The relation between SF HAase activity and CRP in RA patients was positively correlated ($r=0.458$, $p=0.0186$). (B) The relation between SF HAase activity and platelet count in RA patients was also positively correlated ($r=0.45$, $p=0.024$). (C) A standard curve for SF HAase activity in OA patients. Each square shows the average HAase activity in the same three samples, and the bar shows the standard error. The HAase bands were scanned with an image analyser and HAase activity was expressed numerically as a volume. HAase activity (10 000) corresponds to the activity of 2.7 μ units of hyaluronidase SD from streptococcus dysgalactiae at pH 4.0 (optimum pH 5.8-6.6).

26 serum and 26 SF samples from patients with RA (both were measured in 13 patients) and in 24 serum and 34 SF samples from

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patients with OA (both were measured in 16 patients). SF from the knee joint was collected in tubes without additives (mean volume 20.0 ml in RA, and 18.9 ml in OA), centrifuged, and then stored at -70°C until analysis. Stringing of SF was assessed by measuring the maximum length of the string formed from 10 μl SF held between the thumb and the index finger. Blood was collected in tubes without additives, centrifuged, and the serum was stored at -70°C until analysis. Control serum samples were collected from 40 healthy volunteers (mean age 64.9 years).

The diagnosis of RA was based on the American College of Rheumatology (ACR) criteria. The degree of joint destruction was classified from Stage I to Stage IV using Steinbrocker's criteria, and the degree of functional capacity was classified from Class I to Class IV based on the system used by the ACR. The diagnosis of OA was also based on the ACR criteria, and radiographs were scored according to the Kellgren and Lawrence scale, which characterises knee with OA (Grade 0 to Grade 4).^{6,7} For the HAase substrate, we used cockscomb HA that was donated by Seikagaku Kogyo Corporation (Tokyo).

ZYMOGRAPHY

HA was copolymerised in 10% polyacrylamide gel to a final concentration of 0.17 mg/ml. Samples of SF (1 μl) and serum (1 μl) were diluted in sample buffer, and gels were run at 20 mA per gel for 90 minutes. After the run, the gels were washed with a 2.5% solution of TritonX-100 for one hour. The gels were incubated at pH 4.0 in 0.1 M formate buffer containing 0.03 M NaCl at 37°C for 20 hours. Then the gels were treated with 0.1 mg/ml Pronase solution (20 mM TRIS-HCL, pH 8.0) for two hours at 37°C . The gels were washed with 20% ethanol-10% acetic acid for 20 minutes. Then the gels were treated with 0.5% Alcian blue in 20% ethanol-10% acetic acid for one hour to stain the HA, and then decolour-

ised in 20% ethanol-10% acetic acid for two hours. The region of the gel where HA had been degraded by HAase did not stain, and HAase activity could be detected as unstained bands at a molecular weight of 65 KDa. The gels were then stained with 0.04% Coomassie blue in 20% methanol-10% acetic acid for 30 minutes, and subsequently decolourised in 20% methanol-10% acetic acid for 90 minutes. Unstained bands from non-enzymatic proteins were stained with Coomassie blue and appeared as dark blue bands.^{8,9}

MEASUREMENT OF HYALURONIDASE ACTIVITY

The bands of HAase activity were scanned with a Bioprofil Version 6 (Bilber Lourmat Co Ltd) image analyser, and their surface area and colour intensity were analysed. The HAase activity of each sample was expressed numerically as a volume.

STATISTICAL ANALYSIS

In RA and OA patients, we examined the relation between HAase activity and the following items: duration of illness, age of patient, and volume and stringing of SF. We also examined the relation between HAase activity and joint score, Lansbury index, haemoglobin content, C reactive protein (CRP), erythrocyte sedimentation rate (ESR), platelet count, rheumatoid factor, and immune globulin (Ig A, Ig M, and Ig G) values in RA patients.

The correlations between HAase activity and each parameter were tested using Pearson correlation coefficients. The Mann-Whitney test was used to test for significant differences in HAase activity between each of the groups, between each of the stages and classes within the RA group, and between each of the grades within the OA group. Differences were considered significant when $p < 0.05$.

Results

In RA SF, we found a positive correlation between SF HAase activity and serum CRP ($r = 0.458$, $p = 0.0186$; fig 1(A)). A positive correlation was found between SF HAase activity and platelet count ($r = 0.45$, $p = 0.024$; fig 1(B)). HAase activity was not correlated with any other parameter, and there were no significant differences in HAase activity between any of the stages or classes.

In OA SF, HAase activity was not correlated with any of the parameters. Comparing grades, HAase activity in Grade 0 was significantly higher than in Grade 1 ($p = 0.03$) and Grade 3 ($p = 0.015$), and HAase activity in Grade 2 was higher than in Grade 3 ($p = 0.033$). There was no significant difference in SF HAase activity between the RA and OA groups.

In the serum from RA patients, no correlation was found between HAase activity and any of the parameters, and there was no significant difference in HAase activity between any of the stages or classes. In the serum from OA patients, no correlation was found between HAase activity and any of the parameters, and there were no significant differences in HAase activity between any of the grades. Serum

Figure 2 Comparison of the serum HAase activity in the RA group, OA group, and normal controls. Serum HAase activity in the RA group was significantly higher than in both the OA group and the controls, but there was no significant difference between the OA group and the controls. (* $p < 0.0001$, † $p < 0.0001$, ‡ $p = 0.62$).

HAase activity in the RA group was significantly higher than in both the OA group ($p < 0.0001$) and normal controls ($p < 0.0001$) (fig 2).

In the RA and OA patients, no correlation was found between serum HAase activity and SF HAase activity.

Discussion

CRP, ESR, and platelet count are parameters associated with disease activity in RA. We found a direct relation between SF HAase activity and CRP ($r = 0.458$, $p = 0.0186$). Hutchinson *et al*¹⁰ reported a direct relation between the activity of RA and platelet count. We also found a direct relation between platelet count and SF HAase activity ($r = 0.45$, $p = 0.024$). These results suggest that SF HAase activity reflects the activity of RA. The pathogenesis of RA involves the destruction of articular cartilage, accompanied by inflammation. In this study, we found no significant differences in SF HAase activity between any of the stages (each stage reflects a different degree of destruction of articular cartilage) in RA patients. Therefore, SF HAase activity may indicate synovial inflammation in general.

The pathogenesis of OA involves destruction of cartilage and bony remodelling, even though early OA shows histological synovitis.¹¹ Spector *et al* reported that CRP concentrations are significantly increased in early OA patients. They suggested that low grade inflammation might be a significant aspect of early OA.¹² In our study, we cannot deny the possibility of the inclusion of patients with a microcrystal synovitis in OA patients. However, we believe early OA patients have a low grade synovitis. Therefore, SF HAase activity is similar in RA and OA, and early OA patients have more SF HAase activity than those with more advanced disease (usually SF stringing is poorer).

A number of studies have shown that serum HA is increased in both OA and RA compared with normal controls.¹³ Goldberg reported that serum HA was sevenfold higher in RA and twofold higher in OA when compared with controls.¹⁴ In our study, serum HAase activity in RA was significantly higher than in both OA ($p < 0.0001$) and controls ($p < 0.0001$), while there was no significant difference in serum HAase activity between OA and controls. If we consider that the serum HAase activity varies

inversely with the serum HA, then the serum HAase activity in RA ought to be lower than that in OA and the controls. The metabolism of HA and HAase in vivo is not well understood.¹⁵ In our study, the optimum pH of HAase was 4.0, and no HAase activity could be detected at pH 5.0–7.0. However, systemic arterial pH is maintained between 7.35 and 7.45, so serum HAase may not hydrolyse serum HA in vivo. We believe that serum HAase activity is not always correlated with serum HA, and that serum HAase activity may reflect some inflammation.

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