Superoxide dismutase isoenzymes of the synovial fluid in rheumatoid arthritis and in reactive arthritides

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SUMMARY The activity of superoxide dismutase isoenzymes was determined in knee joint synovial fluid from 21 patients with rheumatoid arthritis, nine patients with reactive arthritides, and from 17 patients before arthroscopy or arthrotomy for suspected meniscal or ligament injury (controls). Extracellular superoxide dismutase was the major isoenzyme and accounted for about 80% of the total superoxide dismutase activity in the controls. The pattern of the superoxide dismutase isoenzymes was significantly different in rheumatoid arthritis, extracellular (EC) superoxide dismutase being half, CuZn superoxide dismutase double, and the total superoxide dismutase activity a third lower than the activity in the synovial fluid of the controls. The superoxide dismutase activities were similar in synovial fluid from the controls and from the patients with reactive arthritides. The total superoxide dismutase activity was almost three times higher in control synovial fluid than in normal human plasma, but 300 times lower than in human tissues.

Key words: oxy radicals.

There is evidence to suggest that toxic oxygen reduction products are involved in the pathogenesis of inflammatory joint disease. Arthritic joints contain large amounts of granulocytes and monocytes, which have the potential to produce superoxide radicals and hydrogen peroxide upon activation.1 The toxicity of these intermediates is greatly amplified by iron through hydroxyl radical formation.2 Moreover, there is an increased content of iron in synovial fluid (SF) from patients with rheumatoid arthritis (RA).3 4 and parenteral desferroxamine has been shown to reduce the activity of experimental inflammatory joint disease.5 Exposure of human IgG in vitro to oxy radicals results in the formation of fluorescent aggregates. Similar aggregates can be isolated from fresh SF from RA patients.6 Exposure to superoxide radical and hydrogen peroxide in vitro leads to degradation of hyaluronic acid,7 collagen,8 and also cartilage constituents.9 10

Since it has been shown that superoxide dismutase (SOD; EC 1.15.1.1) in the medium protects against such degradation and that intra-articular injections of SOD ameliorate the symptoms in several types of joint disease,11 we decided to assess the basal protection against superoxide radicals in SF. The present paper reports the results of determinations of the level of SOD isoenzymes in SF from patients with different types of inflammatory joint disease and in non-inflammatory SF obtained at surgical procedures. The analyses were made with a direct, highly specific assay for SOD.

Patients and methods

KNEE JOINT SF

All SFs were collected during routine needle aspiration of knee joints before local corticosteroid injections (patients), or before surgical intervention (controls) with a sterile disposable syringe and without using anaesthetics. The fluids were centrifuged at 3000 g for 15 minutes, and the supernatants frozen and stored at −80°C until analysed.

PATIENTS

Twenty one patients with definite or classic,12 rheumatoid factor positive, and erosive RA were
included. Their mean age was 52 years and their mean disease duration 14 years. Nine patients with reactive arthropathies (ReA) to gastrointestinal or to lower urinary tract infections were also included in the study. Their mean age was 28 years and their disease duration less than one year, except for one case with exacerbations during a 10 year period. For comparison, SF from four patients with ankylosing spondylitis, two with psoriatic arthropathy, two with gout, and two with pyrophosphate arthropathy were included in the study.

**Controls**

Control SF specimens were obtained from 17 patients (mean age 33 years) immediately before arthroscopy or arthrotomy for previous trauma to menisci or ligaments of their knee joints. None of these patients had a history suggestive of inflammatory arthropathy, and at the time of aspiration of SF none of them had swollen joints or a raised sedimentation rate. The SF from all these patients was clear, highly viscous, and of low volume.

**Chemicals**

Con A-Sepharose was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ovine testis hyaluronidase 1000 U/mg was a product of Boehringer Mannheim GmbH, FRG.

**SOD analysis**

SOD was determined in terms of its ability to catalyse the disproportionation of superoxide radicals in alkaline aqueous solution. The disproportionation was directly studied in a spectrophotometer, essentially as previously described except that all isoenzymes were assayed at pH 9.5-0. Cyanide (3 mM) was used to distinguish between the cyanide sensitive isoenzymes CuZn SOD and EC SOD and the cyanide resistant isoenzyme Mn SOD. In order to separate CuZn and EC SOD a Con A-Sepharose column was used as described below. One unit in the assay is defined as the activity that brings about a decay in superoxide radical concentration at a rate of 0.1/s in 3 ml buffer. It corresponds to 8.3 ng human CuZn SOD, 8.8 ng human EC SOD, and 65 ng bovine Mn SOD. Pure human Mn SOD has not been investigated with this assay, but its specific activity is probably similar to that of the bovine enzyme. The xanthine oxidase/cytochrome c assay for SOD14 works at physiological conditions, i.e., at neutral pH and low superoxide radical concentration. When human enzymes are analysed one unit in the present assay corresponds to 0.024 units CuZn SOD, 0.024 units EC SOD, and 0.24 units Mn SOD, respectively in the ‘xanthine oxidase’ assay. The present assay is thus about 10 times more sensitive for CuZn SOD and EC SOD activity than for Mn SOD activity.

**Separation of SOD isoenzymes with Con A-Sepharose**

Unlike CuZn SOD and Mn SOD, EC SOD binds to concanavalin A, probably because of the presence of carbohydrate.15 Before separation, the viscosity of the specimen was reduced by treatment with hyaluronidase (10 units/ml) for 10 min at 37°C. The treatment had no adverse effect on the isoenzymes, and compensation was made for the small SOD activity in the hyaluronidase preparation. SF (0.5 ml) was applied to a 3 ml Con A-Sepahrose column equilibrated with 10 mM potassium phosphate in 120 mM NaCl (pH 6.5). To allow adsorption of the sample along the length of the column the 0.5 ml buffer was added three times at five minute intervals, and then the column was eluted with 2.5 ml buffer. The eluting fluid from the SF and buffer additions

Table 1  **SOD isoenzyme activities in SF and plasma**

<table>
<thead>
<tr>
<th>Isoenzyme and Disease</th>
<th>EC SOD (U/ml)</th>
<th>CuZn SOD (U/ml)</th>
<th>Cyanide resistant SOD (U/ml)</th>
<th>Protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=17)</td>
<td>67.3 (39.4)*</td>
<td>11.1 (7.7)</td>
<td>4.3 (1.2)</td>
<td>25.6 (3.7)</td>
</tr>
<tr>
<td>Rheumatoid arthritis (n=21)</td>
<td>29.1 (9.0)</td>
<td>21.3 (8.9)</td>
<td>3.5 (0.84)</td>
<td>43.3 (9.5)</td>
</tr>
<tr>
<td>Reactive arthritis (n=9)</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.05</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Ankylosing spondylitis (n=4)</td>
<td>63.8 (55.0)</td>
<td>10.2 (3.9)</td>
<td>3.2 (1.0)</td>
<td>45.4 (8.5)</td>
</tr>
<tr>
<td>Gout (n=2)</td>
<td>NS</td>
<td>NS</td>
<td>p&lt;0.05</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Psoriatic arthropathy (n=2)</td>
<td>32.3 (9.5)</td>
<td>15.0 (6.6)</td>
<td>2.5 (0.56)</td>
<td>48.1 (16.4)</td>
</tr>
<tr>
<td>Pyrophosphate arthritis (n=2)</td>
<td>59.113</td>
<td>18 (9.6)</td>
<td>2.6 (3.5)</td>
<td>43.4</td>
</tr>
<tr>
<td>Plasma, healthy controls (n=16)</td>
<td>26.47</td>
<td>7.5 (11)</td>
<td>5.1 (3.8)</td>
<td>49.45</td>
</tr>
<tr>
<td>Plasma, healthy controls (n=16)</td>
<td>33.42</td>
<td>13 (12)</td>
<td>1.6 (3.6)</td>
<td>32.41</td>
</tr>
<tr>
<td>Plasma, healthy controls (n=16)</td>
<td>19.6 (3.0)</td>
<td>2.8 (0.9)</td>
<td>5.4 (0.5)</td>
<td></td>
</tr>
</tbody>
</table>

*The results are presented as mean (SD). Statistical comparison between controls and the RA and ReA groups was performed with the Wilcoxon rank sum test. The data for gout, psoriatic arthropathy, and pyrophosphate arthritis are presented as patients’ individual results.
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was collected and contained the CuZn SOD and Mn SOD of the sample. The column was washed with 40 ml of the phosphate buffer, and EC SOD was then eluted with 10 ml of 150 mM α-methyl-D-mannoside in 50 mM sodium phosphate (pH 6.5) added in 2 ml aliquots at five minute intervals. The column was regenerated with 10 ml 0.5 M α-methyl-D-mannoside followed by 40 ml 10 mM potassium phosphate in 120 mM NaCl (pH 6.5). The yield of EC SOD from the column, tested with pure EC SOD and with partially purified enzyme, was regularly about 75%. To compensate for this all EC SOD results were multiplied by a correction factor (1/0.75).

The validity of the Con A-Sepharose procedure was checked with antibodies towards CuZn SOD and EC SOD immobilised on CNBr activated Sepharose.16 The antibodies do not cross react.16 The SOD activity eluted with α-methyl-D-mannoside was completely adsorbed by anti-EC SOD and was not affected by anti-CuZn SOD, confirming that this activity is given by EC SOD. Most of the cyanide sensitive SOD activity which did not bind to Con A-Sepharose was adsorbed by anti-CuZn SOD, but about 7% (mean of four experiments) of the SOD activity which did not bind to Con A-Sepharose was adsorbed by anti-EC SOD, indicating that the attachment of EC SOD during the procedure was not fully complete. The figures presented for CuZn SOD will therefore include some EC SOD and will be somewhat too high and those for EC SOD somewhat too low.

Compensation for CuZn SOD contributed by haemolysed erythrocytes

A few of the control SFs were weakly reddish because of the presence of haemoglobin. The haemoglobin probably originated from bleeding caused by the puncture for collection of the fluids. In these samples the haemoglobin content was determined17 and the results used for calculation of CuZn SOD contributed by haemolysed erythrocytes using the mean CuZn SOD activity per milligram haemoglobin of normal erythrocytes.18 The results were subtracted from the CuZn SOD activity found in the fluids.

SOD isoenzymes in human plasma

Plasma was obtained from 16 healthy controls using heparin as anticoagulant. The isoenzyme composition was assessed using antibodies towards CuZn SOD and EC SOD immobilised on Sepharose 4B.16 The plasma specimens were incubated overnight at 4°C with (a) Sepharose 4B (sham incubation), or (b) anti-CuZn SOD-Sepharose 4B, or (c) anti-CuZn SOD-Sepharose 4B+anti-EC SOD-Sepharose 4B. After centrifugation the SOD activity remaining in the supernatants was determined. The CuZn SOD activity was taken as (a) minus (b) and the EC SOD activity as (b) minus (c). The Mn SOD activity was estimated from the cyanide resistant activity of supernatant (c).

Protein analysis

For protein analysis Coomassie brilliant blue G-250 was used.19 Human serum albumin was used for standardisation.

Results

Table 1 shows the results of analysis of SOD activity in the SFs. Data for human plasma are presented for comparison. The major SOD isoenzyme in SF from all groups was EC SOD. The EC SOD activity in SF from the controls and from the ReA patients was not significantly different, but both were higher than the activity in fluids from RA patients. The plasma EC SOD activity was lower than the activity in SF from all groups.

The CuZn SOD activity in SF from controls and from patients with ReA did not differ significantly, but the values were clearly lower than in SF from RA patients. The CuZn SOD activity in SF from all groups was higher than the CuZn SOD activity in normal human plasma (Table 1).

The cyanide resistant SOD activity was a little lower in the SFs from patients with arthritis. All or part of the activity was probably given by Mn SOD, but since the activity is very low much fluid has to be added during SOD assay and various types of unspecific scavenging of superoxide radical might have contributed to the measured activity. The difference between the groups is therefore difficult to evaluate.

The total SOD activity of the SFs was two to three times greater than the total SOD activity in human plasma.

A few SF samples were assayed from patients with ankylosing spondylitis, gout, psoriatic arthropathy, and pyrophosphate arthritis (Table 1). The results suggest that no major deviations in activity exist in these groups compared with the controls and the other arthritide groups.

Discussion

The CuZn SOD and Mn SOD are mainly found in cells and tissues, and the minor activity in plasma or other extracellular fluids is probably due to cellular leakage.20 21 The EC SOD activity in tissues is much
lower than the activity of the other isoenzymes, and since it is the major isoenzyme in plasma and lymph an extracellular role for EC SOD has been suggested.20-22 In this study EC SOD was found to be the isoenzyme of highest activity present in non-inflammatory SFs. Moreover, the activity of this isoenzyme and of CuZn SOD was greater in control SFs than in normal human plasma.

The source of EC SOD remains to be identified,20-21 and the significant decrease of this isoenzyme activity in the RA SFs compared with control and ReA SFs is at present difficult to explain. A dilution effect cannot be ruled out, and inactivation by hydrogen peroxide16 from activated granulocytes is another possibility. Nevertheless, a reduction in EC SOD activity would increase the susceptibility of SF and cartilage components in the joints of RA patients to damage by oxy radicals. The mean EC SOD activity in SFs of ReA patients was similar to that of controls.

The isoenzyme of second highest activity in all SFs was CuZn SOD. Its increased activity in rheumatoid SFs suggests an increased leakage from tissue damage and possibly from the haemorrhages in the synovial membranes. The considerable range of CuZn SOD activity in control SFs may partly be due to difficulties in separating cells and debris by centrifugation of the highly viscous normal SFs.

Previous studies of total SOD activity in SF using indirect methods have reported contradictory results. With a xanthine oxidase nitroblue tetrazolium assay no SOD activity was found in SF,23 but analyses with the xanthine oxidase/cytochrome c assay14 have resulted in approximately 10-fold24 and twofold25 higher activities in SF than found by us. Indirect SOD assays are liable to unspecific interferences with the superoxide producing and detecting systems and are relatively unsensitive, which makes the assay of the low SOD activity in SF ambiguous.

With the very sensitive direct method of our study no remaining cyanide sensitive SOD activity was found after exposure of SF to immobilised antibodies towards CuZn SOD and EC SOD (data not presented). This shows the specificity of the method used and probably explains the difference between our results and previous findings.

Although the total SOD activity in SF is about three times higher than that of plasma, the activity is still low. The SOD concentration of human tissues is about 300 times higher than that of SF,22 but the SOD activity in SF is still potentielly important. If the rate of SOD catalysed dismutation in SF is compared with the rate of spontaneous dismutation at pH 7-426 it is found that the SOD catalysed reaction is about 100 times faster at 1 μM superoxide radical and 100 000 times faster at 1 nM superoxide radical. The situation in the joints is of course more complex than that described in the above comparison, with ascorbate and other reductants, ferric iron, and other substances competing for the superoxide radicals. Therapeutic use of 4–8 mg CuZn SOD intra-articularly,21 corresponding to 106 units SOD activity, will considerably increase the SF SOD activity.

We wish to thank Ms Agneta Öberg and Mrs Lena Hedman for skifull technical assistance and Mrs Kristina Lindberg for expert secretarial work. The study was supported by the Swedish Medical Research Council, grant No 04761 and by the Swedish Arthritis Foundation, Stockholm.

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Superoxide dismutase isoenzymes of the synovial fluid in rheumatoid arthritis and in reactive arthritides.
S L Marklund, A Bjelle and L G Elmqvist

*Ann Rheum Dis* 1986 45: 847-851
doi: 10.1136/ard.45.10.847

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