Superoxide Scavenging Activity of Leukocytes in Rheumatoid Arthritis and Behçet’s Diseases

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(Received May 24, 1990; Accepted May 24, 1990)

Superoxide radical anion (O$_2^-$) has been suggested to mediate tissue damage associated with chronic inflammatory disorders such as rheumatoid arthritis (RA) and Behçet’s disease (BD). Despite that protection from O$_2^-$ is provided by superoxide dismutase (SOD), there are controversial results about SOD-like activity of polymorphonuclear cells (PMN) in RA and there are no reports about it in BD. In addition, colchicine, a potent inhibitor of phagocytosis, has been used for the treatment of BD, no report about its effect on SOD-like activity of PMN is available.

The present study investigates the superoxide scavenging activity (SSA) of PMN and mononuclear cells (MNC) in both RA and BD, and it examines the effects of colchicine on SSA of PMN in BD using the electron paramagnetic resonance/spin trapping method.

The SSA of PMN, but not MNC, was significantly lower in patients with either RA or BD, as compared with that in healthy controls. The SSA of PMN in both RA and BD showed a strong negative correlation with erythrocyte sedimentation rates and C-reactive protein levels. Colchicine treatment increased the SSA of PMN toward normal level in BD patients, and prevented the decrease of SSA in PMN obtained from healthy adults after the stimulation with opsonized zymosan in vitro.

Our results suggest that the decrease of SSA in PMN is not disease specific and it is probably the results of the increased amount of O$_2^-$ generated by these cells. Colchicine may prevent the decrease of SSA in PMN by blocking the phagocytosis and indirectly the O$_2^-$ generation by these cells.

(Key Words: Behçet’s disease, colchicine, electron spin resonance, leukocyte, rheumatoid arthritis, superoxide dismutase)

INTRODUCTION

Oxygen free radicals including superoxide radicals (O$_2^-$) are probably involved in the pathogenesis of chronic inflammatory disorders, such as rheumatoid arthritis (RA) and Behçet’s disease (BD) (1). The functions of PMN, such as chemotaxis, phagocytosis and O$_2^-$ generation have been shown to be increased in BD (7).

Protection from O$_2^-$ is provided by superoxide dismutase (SOD) inside of cells, and it has been shown to be effective in the treatment of both RA and BD. To elucidate this role of SOD in inflammations, SOD-like activity of PMN have been also measured in RA (8, 9, 13), but controversial data have been reported. Surprisingly, there is no data about SOD-like activity of PMN in BD.

Colchicine, an inhibitor of phagocytosis, is widely used in Japan for the treatment of BD with excellent effects. Despite that, the effect of colchicine on SOD-like activity of PMN is still unknown.

In this study we investigated the superoxide scavenging activity (SSA) of PMN and mononuclear cells (MNC) in patients with either RA or BD, to elucidate whether it has correlation to the activity of diseases. In addition, the effects of colchicine on SSA of PMN were studied to understand the possible mechanism of its effect on BD.

We employed the specific electron paramag-
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Magnet resonance (EPR)/spin trapping method to detect free radicals.

MATERIALS AND METHODS

Subjects

Forty patients with RA and twenty BD patients were studied. RA patients were classified by the criteria of the American College of Rheumatology (2). Ten of the patients had stage I disease, and 10, 10 and 10 patients had stage II, III and IV diseases, respectively. BD patients were classified as complete and incomplete types according to the revised criteria of the Japanese Research Committee of BD (6). Disease of BD patients were considered active when patients showed two symptoms or more during one month before the present study. Treatment of patients: RA patients were treated only with non-steroidal anti-inflammatory drugs (NSAID), and with NSAID plus prednisolone (PSL; 5–10 mg/day). BD patients were treated with only colchicine at a concentration of 0.25–1.00 mg/day, with only NSAID, with colchicine and NSAID, or they were received no drug.

Clinical activity and the following laboratory parameters were assessed in each blood samples: total protein, albumin, hemoglobin, erythrocyte sedimentation rate (ESR), C-reactive protein levels (CPR) and absolute number of PMN. ESR greater than 20 mm/hour and CPR levels above 0.08 mg/ml were considered as abnormal.

Twenty healthy adults, 9 females and 11 males, were used as controls. None of them were taking any drug. The preliminary studies disclosed that SSA of the samples did not vary with sexes or ages of the controls.

Separation of PMN and MNC

Cells were separated by the modified method of Boyum et al. (3). Both PMN and PMN were adjusted to 1.5–3×10⁷/ml in phosphate bufferes saline (PBS⁻) and kept at −80°C until measurement. Purity (average ± one standard deviation) or MNC was 96±2%, and that of PMN was 97±2%. After stimulation with opsonized zymosan (OZ), viability of PMN was greater than 97%.

Measurement of superoxide scavenging activity (SSA)

The EPR/ spin trapping technique, which was described by Hiramatsu et al. (4), was used for the measurement of SSA. SSA was calculated by the inhibition rate of O₂⁻ generated by the hypoxanthine-xanthine oxidase system (H/XO) by samples. A standard curve of DMPO-O₂⁻ using different concentrations of SOD (0–20 U/ml) was made (Fig. 1). The SSA in test samples was determined by adding 50 µl of lysed cell suspension (1.5–3×10⁷ cell/ml) to the H/XO system instead of SOD.

EPR measurements were made on a JEOL (JES-FE 2X6, Tokyo, Japan). Conditions of EPR spectrometry for measurement of SSA were as follows: magnetic field 335±5 mT, power 0.8 mW, response 0.3 seconds, modulation 0.125 mT, temperature; room temperature 23 ±1°C, amplitude 2.0×10³, sweep time; 2 minutes, using a magnetic field modulation frequency of 1000 kHz.

Protein content of cell suspensions was determined by the micro-adaptation of the Lowry method (5). Mean activity of each sample was obtained from three measurements.

For statistical analysis Student's t-test was employed.

Effect of colchicine on SSA of PMN

To test the effect of colchicine on SSA in vitro, PMN were separated from five healthy subjects (2×10⁷ cells/ml), and were preincubated for 45 minutes in Hank's balanced salt solution (HBSS) at 37°C in the presence or absence of colchicine (2–200 μg/ml). After the preincubation time was completed, colchicine was washed out twice with PBS⁻. After that OZ (1.1 mg/ml) was added to the PMN, and cells were incubated for further 15 or 30 minutes. The reaction was stopped at low temperature (−80°C), and the samples were kept at −80°C until SSA measurement were performed. Our control studies showed that neither colchicine nor OZ did interfere with the O₂⁻ generating system for measurement of SSA.

RESULTS

The superoxide scavenging activity (SSA) of PMN was about half in both RA and BD as compared with that in healthy controls. There was no difference in SSA of MNC between either RA or BD as compared with that in healthy controls.
Fig. 1 Inhibition of low magnetic field of the DMPO-O$_2^-$ adduct by superoxide dismutase (SOD): The low magnetic field peak of DMPO-O$_2^-$ spin adduct generated by the H/ XO system is shown. The first peak (1) is the manganese-oxide peak as an internal standard. The second peak (2) is the low magnetic field peak observed of the DMPO-O$_2^-$ spectra. The amount of generated O$_2$ radical is calculated by comparing this DMPO-O$_2^-$ peak with the MnO$-$ peak. Superoxide dismutase (SOD) added to the system decrease the EPR intensity of the peak in a dose dependent manner.

There was no correlation between the SSA of PMN, and clinical classification of RA patients. SSA of PMN in BD was lower in subgroup of complete type as compared with subgroup of incomplete type (Figure 2), however this was statistically not significant. There was no difference in SSA of PMN between subgroups of active- and inactive patients in BD classified by clinical symptoms.

When the laboratory parameters were considered, the SSA of PMN both in RA and BD was significantly lower in the patients with elevated erythrocyte sedimentation rate (ESR), and SSA was lower in BD with positive CRP test than those with normal test results (Table 1). Absolute numbers of PMN were not correlated with the SSA of PMN either in RA or BD. There were no correlation between SSA of PMN and other laboratory paramenters, such as total protein, albumin or hemoglobin.

The intervention studies of colchicine, NSAID, prednisolone (PSL) and combination of them showed that SSA in PMN was significantly higher in the BD patients receiving colchicine than those without the drug. There were no significant difference in SSA of PMN between BD patients taking NSAID and those without the drug. This observation suggest that only colchicine is the one responsible for the increase of SSA in PMN. We could not make such comparisons in RA patients, since all RA patients were taking NSAID and they were not treated with colchicine. On the other hand, there was no significant difference in SSA of RA patients treated with NSAID alone and those treated with the combination of NSAID and PSL.

To test the possible effect colchicine and that of O$_2^-$ generation on SSA, PMN were preincubated with different concentrations of colchicine. SSA of PMN decreased significantly after the stimulation with OZ. The preincubation of PMN with colchicine prevented the decrease of SSA in a dose dependent manner.

DISCUSSION

Our results demonstrate that the SSA of PMN in both RA and BD is approximately half of the SSA obtained from the healthy subjects. It has been shown that the PMN in both RA and BD are more active and the generation of superoxide radicals increases as well (1, 12),
Table 1. Correlation between SSA of PMN and erythrocyte sedimentation rates (ESR) or C-reactive protein levels (CRP)

<table>
<thead>
<tr>
<th></th>
<th>Normal ESR</th>
<th>Elevated ESR</th>
<th>CRP (-)</th>
<th>CRP (+)</th>
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<tbody>
<tr>
<td>rheumatoid arthritis</td>
<td>2.54±1.30</td>
<td>1.59±1.10*</td>
<td>—</td>
<td>1.93±0.98</td>
</tr>
<tr>
<td>(n=12)</td>
<td>(n=28)</td>
<td></td>
<td></td>
<td>(n=40)</td>
</tr>
<tr>
<td>Behçet's disease</td>
<td>2.56±1.22</td>
<td>1.24±1.24*</td>
<td>2.48±1.51**</td>
<td>1.34±0.97</td>
</tr>
<tr>
<td>(n=15)</td>
<td>(n=8)</td>
<td>(n=13)</td>
<td></td>
<td>(n=10)</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD (U/mg protein).
* p value between elevated ESR and normal ESR groups is less than 0.05
** p value between CRP (+) and CRP (-) group is less than 0.02
Number of cases are shown in parentheses.

therefore, two possibilities might be considered. First, the activity of SOD is lower, the SOD diminution favors the increase of intracellular $O_2^-$ production. The deficient SSA observed may be attributed to problems either in gene expressions of SOD or dysfunctions in the induction mechanisms in both diseases. No such a change have been found in RA, although it have not been examined in BD. Against this hypothesis is also the fact that the productions of two types of SOD (Mn-SOD, Cu/Zn-SOD) are independent, and their corresponding genes are located on chromosomes 21 and 6, respectively. Genetic factors could likely be excluded by our finding that the activity remained unaltered in MNC. Certainly, dysfunctions in the induction mechanisms occurring only in PMN, can not be excluded by our results, all the other PMN-functions were not deficient, but rather enhanced both in RA and BD.

The second possibility is, that oxidative species such as superoxide radicals $O_2^-$, hydroxyl radicals (OH) or hydrogen peroxide (H$_2$O$_2$) may inactivate SOD. If this is the case, the
primary cause of decreased SOD activity is due to the increase of $O_2^-$ generation itself. Hydrogen peroxide (10) and radiation induced radicals (11) have been shown to inactivate SOD in cell free systems. We observed the decrease in SSA after the exposure of hyman recombinant SOD against $O_2^-$ generated by the H/XO system (unpublished observation). Furthermore, we have shown in this study that the SSA of PMN decreases after stimulation of PMN with opsonized zymosan.

We hypothesized that the induction of SOD by $O_2^-$ radicals may not be sufficient compared to the amount of $O_2^-$ generated by PMN in either RA or BD. Conversely $O_2^-$, $H_2O_2$ or both may inactivate SOD inside the cells.

The lack of significant correlation between disease subtypes or clinical activities determined by symptoms and the SSA of PMN in both diseases is difficult to explain. As the classification of RA patients considered, it depends on severity of joint destructions which should not follow the actual activity of the diseases. However, the findings that the SSA of PMN is significantly lower in the patients with elevated erythrocyte sedimentation rates (ESR) in both RA and BD, or positive CRP tests in BD, suggest that (1) the decrease in SSA of PMN is not disease specific, (2) and SSA of PMN has negative correlation to the severity of actual inflammation. Accepting the "inactivation theory of SOD" this observation also suggest that PMN were activated in vivo and active PMN (with possible higher $O_2^-$ generation rate) have lower SSA.

Colchicine had a protective effect against the decrease in SSA of PMN in our results. We used OZ in this study, and OZ stimulates the $O_2^-$ generation by stimulating the phagocytosis itself. Since it have been shown that colchicine blocks phagocytosis, we hypothesized that colchicine prevented the decrease in SSA of PMN by blocking the $O_2^-$ generation of the cells indirectly.

REFERENCES
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