Erythrocyte Superoxide Dismutase in Various Hematological Diseases

Fumio OMATA, Hiroe NAKAZAWA, Minoru NAKANO and Shigeru ARIMORI

Department of Internal Medicine, School of Medicine, Tokai University (Received May 24, 1990; Accepted May 24, 1990)

We measured superoxide scavenging activity (SSA) of erythrocytes with the recently developed chemiluminescence method by Nakano et al in Down syndrome and various hematological diseases. Hematological disorders were aplastic anemia, myelodysplastic syndrome, multiple myeloma, malignant lymphoma and chronic myelogenous leukemia. The SSA of erythrocytes was 1.7 times higher in Down syndrome, which was consistent with values reported in the previous publications. The erythrocyte SSA in patients of multiple myeloma treated with interferon-alpha was higher than that in healthy volunteers. The erythrocyte SSA in myelodysplastic syndrome, malignant lymphoma and chronic myelogenous leukemia did not differ from that in healthy volunteers. The mean value of erythrocyte SSA in aplastic anemia also remained within normal range. However, when an individual's hemoglobin concentration was compared with his/her own erythrocyte SSA, there was a clear correlation between them. Namely erythrocyte SSA increased when anemia was severe. There was no correlation between erythrocyte SOD activity and age.

(Key words: erythrocyte, superoxide dismutase, hematological diseases, interferon-alpha)

INTRODUCTION

Recent evidence suggested that levels of superoxide dismutase (SOD) in tissues or in circulating cells are influenced by the pathological conditions of various diseases. Thus several studies were performed to investigate the value of erythrocytes SOD in various hematological diseases in order to elucidate the underlying mechanisms. However among these reports the SOD value of erythrocytes remained controversial. Regarding Fanconi’s anemia, several studies presented decreased erythrocyte SSA (9, 16, 20, 25) while one paper showed no significant difference (23). Concerning acute leukemia and lymphoma, some papers presented decreased erythrocyte SSA (1, 12, 18, 21, 22) though other papers showed increased erythrocyte SSA (5).

One of the reasons for these opposing results appeared to depend on incompleteness in the method of SSA measurement. With a recent establishment of very stable and comparatively direct method of SSA using MCLA\textsuperscript{a}, we intended to perform this study to show SSA of erythrocytes in various hematological diseases. In this study, we measured the erythrocyte SSA in the patients with aplastic anemia, myelodysplastic syndrome, multiple myeloma, chronic myelogenous leukemia and malignant lymphoma. We also measured the erythrocyte SOD in patients with Down syndrome by the same method as comparison since the levels of SOD in Down syndrome were well established (4).

MATERIALS AND METHOD

Blood samples
We used only 0.4 ml of whole blood which was drawn into a tube with EDTA for the purpose of routine complete blood count examination. Samples were taken from the patients with hematological disease in ward and out patient clinic. These patients consisted of six
aplastic anemia, three myelodysplastic syndrome, three multiple myeloma, five malignant lymphoma and two chronic myelogenous leukemia. Clinical characteristics were shown in table 1a-c. We also took samples from eight normal volunteers and eight Down syndrome individuals.

Preparation of erythrocyte lysates

Erythrocyte lysates were obtained by the procedure described by Oyanagui (17). The Tsuchihashi reagent treated sample was diluted with water as needed. (Ethanol and chloroform removed the hemoglobin from lysed erythrocyte preparaion.)

Reagents

The 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazol [1,2-alpyrazin -3-one hydrochloride (MCLA) was obtained from Gunma University (6, 15). Each compound was dissolved to a concentration of 50 µg/ml in double distilled water. The solution was stored in 1.0 ml aliquots at -20°C, until needed. MCLA concentration was based upon E430nm = 9600 M⁻¹cm⁻¹ and E410nm = 8900 M⁻¹cm⁻¹, respectively. Hypoxanthine was a product of Wako pure chemicals and was used without further purification.

Enzymes

Superoxide dismutase (SOD) from bovine erythrocytes and xanthine oxidase (XOD, grade II) were obtained from Sigma. The SOD proved to have an activity of 3200-3700 units/mg of protein, in agreement with the supplier’s assay of >3000 units/mg protein.

Chemiluminescence method

The standard reaction mixture contained 2 × 10⁻⁷M MCLA, 5 × 10⁻⁵M hypoxanthine, 6.5 units of XOD, SOD (0-20 ng/ml) and 50 mM Tris-HCl buffer containing 0.1 mM EDTA at pH 7.8, in a total volume of 3.0 ml. The chemiluminescence measurement was initiated by the addition of MCLA to the reaction mixture and XOD was added 2 minutes later. Chemiluminescence was then measured with a Luminescence Reader (Aloka, BLR-102) AT 25°C (14).

MCLA dependent luminescence and erythrocyte DOD measurement.

A typical time course of luminescence intensity in the MCLA containing system was shown in (Fig. 1). When XOD was added to the MCLA containing system, the luminescence rapidly increased reaching a maximum at 2 min after the addition of XOD and remained constant for an additional 3 min. This was inhibited by SOD in a dose dependent manner when it was added to the system prior to the reaction. Although a non-specific background luminescence was always present and constant for 10 min after the addition of MCLA and its consistency was not significantly affected by SOD. Thus, the XOD induced luminescence (a), expressed in terms of light intensity (counts/min), was calculated by subtraction of the non-specific light intensity at 5 min after the addition of MCLA from the light intensity at 3 min after the addition of XOD. The same experiments, except that SOD or sample were present, were carried out and XOD induced luminescence (c) was counted in the same manner as in the absence of SOD or samples. The percent of SOD-dependent inhibition on the XOD-induced luminescence (a) was calculated from equation 1, and was plotted against various SOD concentrations to obtain the standard curve.

\[
\% \text{inhibition} = \frac{a - c}{a} \times 100 \quad \text{Equation 1}
\]

From this standard curve, the SOD activity of erythrocyte lysates was obtained (Fig. 1).

RESULTS

The mean value of the erythrocyte SSA in normal volunteers was 0.9 ± 0.52 ng/10⁵ RBCs. The mean value of the erythrocyte SSA in Down syndrome was 1.5 ± 0.54 ng/10⁵ RBCs, which was 1.7 times higher than that in normal controls and agreed with that in previous reports. The erythrocyte SSA in aplastic anemia and myelodysplastic syndrome were not statistically different from those of normal controls. However there was an inverse correlation between the erythrocyte SSA and the hemoglobin concentration. Erythrocyte SSA increased as the hemoglobin decreased (Fig. 3). There was no correlation between erythrocyte SSA and hemoglobin values in patients with anemia due to other causes. The SOD activity in multiple myeloma was higher than that of normal con-
controls. In patients with malignant lymphoma and chronic myelogenous leukemia, there was no difference statistically in the SOD activity compared with that of normal controls. (Fig. 2a-b). We also compared the erythrocyte SSA with age, but there was no correlation between the erythrocyte SSA and patient age (Fig. 4). Even when this relationship was evaluated in each disease group, there was no correlation between them.

DISCUSSION

The MCLA dependent chemiluminescence method was shown to have 60 times higher sensitivity than the cytochrome C method (13). The MCLA was also demonstrated to be more specific to superoxide than other chemiluminescent probes such as luminol. Therefore we employed this method and showed that MCLA dependent chemiluminescence in the standard reaction mixture was stable and that the SSA of a blood sample could be measured in a systematic manner.

We could not find any abnormal change of erythrocyte SOD in both idiopathic aplastic anemia and myelodysplastic syndrome. As to the inverse relationship between the erythrocyte SOD and the hemoglobin concentration which was observed only in aplastic anemia, it appeared to be interesting to elucidate the underlying mechanism in the future.

As Jansson LT suggested, oxygen stress was increased in iron deficiency anemia which was experimentally produced in rats and resulted in the compensatory increase in erythrocyte SOD (7), this correlation might imply that the oxygen stress...
Fig. 2a  Erythrocyte SOD activity of normal volunteers, Down syndrome, aplastic anemia and myelodysplastic syndrome. Mean for Down syndrome significantly higher than mean for normal controls (p<0.05; Student's t test). Mean (—) and S.D. (—) shown.

Fig. 2b  Erythrocyte SOD activity of normal volunteers, multiple myeloma, lymphoma and chronic myelogenous leukemia. Mean for Multiple myeloma was significantly higher (p<0.005; Student's t test) than mean for normal control.
Fig. 3  Correlation between erythrocyte SOD and hemoglobin concentration in aplastic anemia. Erythrocyte SOD activities showed negative correlation with hemoglobin concentration.

Fig. 4  No correlation between erythrocyte SOD activity and age. There was no correlation between erythrocyte SOD activity and ageing.
### Table 1a Clinical Characteristics of the Patients with Aplastic Anemia and Myelodysplastic Syndrome

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Disease Duration (Month)</th>
<th>Previous Treatment</th>
<th>Bone Marrow ANC (X10^3) Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>F</td>
<td>12yr</td>
<td>P, Oxy</td>
<td>24.8 AA</td>
</tr>
<tr>
<td>60</td>
<td>F</td>
<td>12</td>
<td>P, Flu Vit D₃</td>
<td>1.7 AA</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>2yr</td>
<td>P, Oxy</td>
<td>0.9 AA</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>15yr</td>
<td>P, Oxy, CycloA Flu, ALG</td>
<td>8.8 AA</td>
</tr>
<tr>
<td>57</td>
<td>F</td>
<td>30yr</td>
<td>P, Oxy, ALG</td>
<td>1.6 AA</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>1</td>
<td>P, Oxy</td>
<td>3.4 AA</td>
</tr>
<tr>
<td>56</td>
<td>F</td>
<td>5yr</td>
<td>P, Oxy, U, Flu</td>
<td>5.9 RA</td>
</tr>
<tr>
<td>51</td>
<td>M</td>
<td>6</td>
<td>Oxy, U, Flu</td>
<td>28.1 RAEB</td>
</tr>
<tr>
<td>69</td>
<td>M</td>
<td>2</td>
<td>C</td>
<td>32.9 CMML</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; M, male; yr, years; ANC, all nucleat cell count; P, prednisolon; Oxy, oxy-methone; Flu, fluoxymesterone; CycloA, cyclosporineA; ALG, antilymphocyte globulin; U, ubenimex; C, carboquone; AA, aplastic anemia; RA, refractory anemia; RAEB, refractory anemia with excess of blast; CMML, chronic myelomonocytic leukemia.

### Table 1b Clinical Characteristics of the Patients with Malignant Lymphoma

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Disease Duration (Month)</th>
<th>Stage</th>
<th>Previous Treatment</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>F</td>
<td>6</td>
<td>III</td>
<td>CHOP, CMOPP</td>
<td>HD</td>
</tr>
<tr>
<td>36</td>
<td>M</td>
<td>28</td>
<td>III</td>
<td>Combination chemotherapy</td>
<td>NHD</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>62</td>
<td>IV</td>
<td>Combination chemotherapy</td>
<td>NHD</td>
</tr>
<tr>
<td>54</td>
<td>M</td>
<td>4</td>
<td>IV</td>
<td>CHOP</td>
<td>NHD</td>
</tr>
<tr>
<td>63</td>
<td>M</td>
<td>7</td>
<td>III</td>
<td>Combination chemotherapy</td>
<td>NHD with stomatocytosis</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; M, male; CHOP, cyclophosphamide doxorubicin vincristine prednisolon; CMOPP, cyclophosphamide vincristine procarbazine prednisolone; HD, Hodgkin's disease; NHD, Non Hodgkin's disease.

### Table 1c Clinical Characteristics of the Patients with Myeloproliferative Disorders and Multiple Myeloma

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Disease Duration (Month)</th>
<th>Previous Treatment</th>
<th>Bone Marrow and other feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>F</td>
<td>54</td>
<td>VPD, Busulfan</td>
<td>CML Ph-1 (+) in chronic phase</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>2</td>
<td>VPD</td>
<td>CML Ph-1 (+) in blast crisis</td>
</tr>
<tr>
<td>58</td>
<td>M</td>
<td>7</td>
<td>Combination chemotherapy, Interferon-alpha</td>
<td>Multiple Myeloma (Ig A K) under Hemodialysis</td>
</tr>
<tr>
<td>53</td>
<td>M</td>
<td>4</td>
<td>Melphalan Prednisolone, Interferon-alpha</td>
<td>Multiple Myeloma (Ig A 1)</td>
</tr>
<tr>
<td>63</td>
<td>M</td>
<td>10</td>
<td>Melphalan Interferon-alpha</td>
<td>Multiple Myeloma (Ig G K)</td>
</tr>
</tbody>
</table>

Abbreviations: VPD, vincristine prednisolone doxorubicin.
increased as the hemoglobin concentration decreased in idiopathic aplastic anemia. The conclusion remained to be determined at this stage.

We could find significantly increased erythrocyte SOD in patients with multiple myeloma. We found that all three patients were treated with interferon-alpha as was shown in table 1c. Our results could no be compared with others because there are no reports yet which measured erythrocyte SOD in multiple myeloma. The effect of interferon-alpha on erythrocyte SSA was not investigated either. One simple explanation for the elevated erythrocyte SSA in multiple myeloma was that multiple myeloma itself could be associated with the increased erythrocyte SOD activity. However we found another plausible mechanism for it since both genes of interferon-alpha and Cu-Zn-SOD were located on the 21th chromosome. Therefore the increase in erythrocyte SSA might be caused by the interaction between exogenously administered interferon-alpha and Cu-Zn SOD.

Additionally the influence of hemodialysis had to be considered since Vanella A et al reported the significantly increased erythrocyte SOD activity after hemodialysis (24). However this factor could be eliminated because there was only one patient who was treated with hemodialysis.

We could not observe any significant change of erythrocyte SOD in malignant lymphoma although there were controversial reports which described increased erythrocyte SOD (5) and decreased one (1, 21, 22). It seemed reasonable to reserve the conclusion until we could accumulate more samples.

In two cases of chronic myelogenous leukemia; one was in the chronic phase, the other in blast crisis, we found lower erythrocyte SOD activity which was compatible with the previous results which showed decreased erythrocyte SOD in acute leukemia (12, 18, 21, 22). However the number of patients was too small to have a statistically conclusive results.

Regarding the relationship between the erythrocyte SOD activity and ageing, the previous reports also remained controversial; Jozwiak Z et al reported that erythrocyte superoxide dismutase activity decreased slightly with advanced age (10) while Dubinina EE showed that superoxide dismutase plasma and erythrocytes in newborn children had higher activity than that in adults (3). On the other hand, Lipecka K et al and Ripalda MJ et al denied the correlation between the erythrocyte SOD activity and ageing (11, 19). In this study we could not find any correlation between the erythrocyte SOD ageing.

ACKNOWLEDGEMENTS

We thank Hirokazu Kimura and Satsuki Kimura in Gunma University for their kind instruction and advice.

REFERENCES

14) Nakano M, Sugioka K, Ushijima Y, Goto T: Chemiluminescence Probe with Cypridina Luciferin Analog. 2-Methyl-6-phenyl-3,7-dihydroimidazol[1,2-a]pyrazin-3-one, for Estimating the Ability of Human
Granulocytes to Generate O$_2^\cdot$.


