A Simple Method for Clinical Assay of Superoxide Dismutase

Yi Sun, Larry W. Oberley, and Ying Li

This assay for superoxide dismutase (SOD, EC 1.15.1.1) activity involves inhibition of nitroblue tetrazolium reduction, with xanthine–xanthine oxidase used as a superoxide generator. By using a reaction terminator, we can determine 40 samples within 55 min. One unit of activity of pure bovine liver Cu,ZnSOD and chicken liver MnSOD was expressed by 30 ng and 500 ng of protein, respectively. The mean concentrations of Cu,ZnSOD as measured by this method in blood from normal adults were 242 (SEM 4) mg/L in erythrocytes, 548 (SEM 20) μg/L in serum, and 173 (SEM 11) μg/L in plasma. The Cu,ZnSOD concentrations in serum and plasma of patients with cancer of the large intestine tended to be less and greater than these values, respectively, but statistically significantly so.

Additional Keyphrases: large-bowel cancer • reference interval

Superoxide dismutase (SOD, EC 1.15.1.1, superoxide:superoxide oxidoreductase) activities in various diseases appear to be of clinical interest. There are two main forms of SOD in cells. One form, found primarily in the cytoplasm, contains Cu and Zn (Cu,ZnSOD). The other form, found predominantly in mitochondria, contains Mn (MnSOD). The specific activity of Cu,ZnSOD is increased in erythrocytes from patients with Down's syndrome (1), 2 or uremia (3) and in serum of patients with renal failure and liver diseases (4). On the other hand, Cu,ZnSOD activity is low in the erythrocytes of patients with Fanconi's anemia (5, 6), sickle cell anemia (7), Duchenne muscular dystrophy (8), or idiopathic pulmonary hemosiderosis (9). There is increased MnSOD activity and decreased Cu,ZnSOD activity in plasma of patients with alcoholic injuries to the liver (10). The MnSOD and total SOD activities of polymorphonuclear leukocytes is decreased in patients with ankylosing spondylitis and rheumatoid arthritis, but Cu,ZnSOD activity is increased significantly in ankylosing spondylitis (11, 12).

Evidently, a rapid and sensitive SOD assay in blood would be useful for routine tests in the clinic. Here we report a very simple, convenient, and sensitive SOD assay, based on the method of Yamanaka et al. (13). We used this assay to measure Cu,ZnSOD activities in blood from normal adults and from patients with cancer of the large intestine.

Materials and Methods

Samples

All samples were from the first and second attached hospital to Zhejiang Medical University in the People's Republic of China. Erythrocytes and plasma of normal adults were obtained from 44 blood donors (ages 18 to 46 years old, 22 of whom were men). Serum was sampled from 38 subjects (ages 21 to 68 years, 30 of whom were men) who agreed to undergo a general survey of health.

Blood samples were also obtained from patients (ages 38 to 76 years) with cancer of the large intestine diagnosed both clinically and pathologically. Patients in the post-operation group had undergone radical surgery for this cancer four to 35 days before sample collection.

Reagents

Standard SOD solution. The stock solution consisted of 4 mg of Cu,ZnSOD from porcine erythrocytes (Shoucho Biochemical Reagent Co., Shoucho, China) dissolved in 50 mL of doubly distilled water, or 8 mg of bovine liver Cu,ZnSOD (Diagnostic Data, Mountain View, CA) dissolved in 8 mL of isotonic saline. This was refrigerated until use. Before use in the assay, the stored solution was diluted to 600 μg/L with doubly distilled water.

To prepare standard MnSOD, we used chicken-liver MnSOD, purified by the method of Weiniger and Fridovich (14); its protein concentration was 1.1 g/L. We diluted this standard 10-fold just before use.

Xanthine oxidase solution (15). We diluted 20 μL of xanthine oxidase (1 kU/g; concentration as supplied, 20 U per 1.2 mL; Boehringer Mannheim GmbH, Mannheim, F.R.G.) to 2.0 mL with ice-cold 2 mol/L ammonium sulfate, freshly prepared. The final concentration of xanthine oxidase was then 167 U/L.

SOD assay reagent. For a 40-tube assay, combine the following reagents in a 200-mL beaker and mix well: 40 mL of 0.3 mmol/L xanthine solution (Sigma Chemical Co., St. Louis, MO), 20 mL of 0.6 mmol/L EDTA solution, 20 mL of 150 μmol/L nitroblue tetrazolium solution (NB; Dongfong Biochemical Reagent Factory, Shanghai, China, or Sigma Chemical Co., St. Louis, MO), 12 mL of 400 mmol/L Na2CO3 solution, and 6 mL of bovine serum albumin (1 g/L; Serva Biochemica, F.R.G.).

Preparation of samples (16). We heparinized whole blood obtained by venipuncture, centrifuged (3000 rpm, 10 min, 0–4 °C), and carefully separated the plasma. We lysed 0.1 mL of erythrocytes with 0.9 mL of ice-cold water (4 °C), then removed hemoglobin (and MnSOD in plasma and serum) by adding 0.3 mL of chlorofome and 0.5 mL of ethanol and vigorously vortex-mixing for 1 min. We centrifuged the mixture at 18 000 × g for 60 min. The supernatant fluid is diluted by a factor of 100, and 0.5 mL of the diluted solution is used to assay Cu,ZnSOD activities as described below. We treated the samples of plasma and serum with chlorofome and ethanol also. We used for SOD assay 0.5 mL of the supernate after centrifugation at 18 000 × g for 60 min.

Assay

Add 2.45 mL of the SOD assay reagent to each of 40 tubes, then add 0.5 mL of pure Cu,ZnSOD (0–270 ng), MnSOD (0–5130 ng), or blood fraction to each tube. When MnSOD is to be measured, add NaCN (final concentration, 5 mmol/L) to the assay tube and pre-incubate for 30 min to inhibit Cu,ZnSOD. The final volume of the reaction system is 3.0
ml and it contains, per liter, 0.1 mmol of xanthine, 0.1 mmol of EDTA, 50 mg of bovine serum albumin, 25 μmol of NBT, 9.9 mmol of xanthine oxidase, and 40 mmol of Na2CO3 (pH 10.2). Place a rack of 40 tubes into a water bath adjusted to 25 °C. Add 50 μL of xanthine oxidase solution to each tube at 30-s intervals. Incubate each tube for 20 min (the time needed to add xanthine oxidase to the 40 tubes), then terminate the reaction by adding 1 mL of 0.8 mmol/L CuCl2 solution to every 30 s (the interval between adding xanthine oxidase to the last tube and adding CuCl2 to the first tube was 30 s). In this way, a 40-tube assay can be done within 40 min, plus 15 min for spectrophotometrically reading the absorbance of each sample. The production of formazan is determined at 560 nm. Under these conditions, the absorbance at 560 nm of the blank tube is about 0.25. The percent inhibition is calculated as below:

\[
\text{% inhibition} = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100\%
\]

Draw the standard inhibition curve with the x-axis being the protein concentration and the y-axis the values of percent inhibition. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. Calculate the Cu,ZnSOD activity by comparison with the standard curve. To avoid determinate error, we constructed a standard curve for each assay run, using porcine erythrocyte SOD as standard.

Statistical Methods

Significance was tested by analysis of variance. We compared means by the Newman–Keuls procedure, with \( P < 0.05 \) indicating significance.

Results

Figure 1 shows the standard inhibition curve obtained with pure bovine-liver Cu,ZnSOD (Diagnostic Data). The percentage inhibition of the rate of NBT reduction, followed at 560 nm, is presented as a function of the concentration of SOD. The amount of pure bovine liver Cu,ZnSOD needed for 50% inhibition of NBT (i.e., 1 unit) is about 30 ng per tube, i.e., 10 ng/mL. For porcine erythrocyte Cu,ZnSOD, however, 1 unit is equal to 60 ng per tube (20 ng/mL). The maximum inhibition by SOD was about 90%, because xanthine oxidase can transfer electrons directly to NBT without involving O2− as an electron-carrying intermediate (17). The inset in Figure 1 shows the same data plotted on probit paper; the x-axis shows the log of SOD concentration. A linear response is obtained over most of the curve, showing that logit analysis can be used with our assay, as has been reported (18, 19) for other SOD assays.

In order to validate our assay for MnSOD, we measured chicken-liver MnSOD. Figure 2 shows the standard inhibition curve for MnSOD. One unit of activity was expressed by about 500 ng of protein (i.e., 500 ng per tube, 167 ng/mL). Even though less sensitivity is seen with MnSOD, our assay is sensitive enough to measure both Cu,ZnSOD and MnSOD. The inset again shows that the probit vs log dose curve is linear, except at very high percentage inhibitions.

Table 1 shows the Cu,ZnSOD concentrations in blood of normal adults and patients with cancer of the large intestine. No statistically significant difference was found among the three groups.

Discussion

SOD is thought to play a very important role in protecting living cells against toxic oxygen derivatives. The enzyme catalyzes the dismutation of two superoxide radicals (O2−) into O2 and H2O2 (20). Many methods have been devised to measure SOD activity. We modified the method of Yamanaka et al. (13) as follows: (a) We increased the concentration of xanthine oxidase to 9.9 mmol/L to keep the absorbance at 560 nm equal to 0.25 in the blank tube. The accuracy of an absorbance measurement is higher at the higher absorbance. (b) We increased the CuCl2 concentration to 0.8

**Table 1. Cu,ZnSOD Concentrations in Blood from Normal Adults and Patients with Cancer of the Large Intestine**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Erythrocytes ng/mL</th>
<th>Serum μg/L</th>
<th>Plasma μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal adults</td>
<td>240 ± 9 (9)</td>
<td>246 ± 10 (9)</td>
<td>242 ± 4 (44)</td>
</tr>
<tr>
<td>Before operation</td>
<td>490 ± 27 (11)</td>
<td>473 ± 30 (6)</td>
<td>548 ± 20 (38)</td>
</tr>
<tr>
<td>After operation*</td>
<td>222 ± 17 (9)</td>
<td>206 ± 20 (9)</td>
<td>173 ± 11 (44)</td>
</tr>
</tbody>
</table>

*Patients had undergone radical surgery for large-bowel cancer four to 35 days earlier.

Mean ± SEM. Numbers in parentheses: numbers of specimens analyzed.
mmol/L to ensure full termination of the reaction. We found that 0.2 mmol/L of CuCl₂ was not concentrated enough. (c) We added xanthine oxidase at 30-s intervals, so we can measure 40 samples within 55 min. Oyanagui (21) compared seven methods for SOD assay and concluded that the nitrite method as improved by him was the best. Our method, however, is superior to the nitrite method in at least two ways: (a) Our assay is simpler and faster. By using a reaction terminator, which stops the reaction with no subsequent change in absorbance within 24 h (data not shown), we can measure 40 samples within 55 min. In Oyanagui’s assay (21), 70 tubes would take 2 h. (b) Our method also is more sensitive (50% inhibition of NBT reduction by bovine-liver Cu,ZnSOD was 10 ng/mL) than Oyanagui’s (60 ng/mL). Oberley and Spitz (22) developed an assay for SOD activity with high sensitivity and accuracy, but it is too time-consuming for routine clinical use. In addition to measurement of Cu,ZnSOD, our assay is also adequate to determine MnSOD, although the sensitivity is not as high (50% inhibition of NBT reduction by MnSOD purified from chicken liver was 167 ng/mL). Our simple, rapid, and sensitive method for both Cu,ZnSOD and MnSOD assay is more suitable for routine clinical use with a large number of samples than are most assay methods.

There are several previous papers on Cu,ZnSOD concentrations in the blood of normal adults. Using the epinephrine oxidation method, Concetti et al. (23) found 6.6 (SD 1.3) mg of Cu,ZnSOD per 100 mL of erythrocytes from healthy human adults. Using autooxidation of pyrogallol for generation of O₂⁻, Minami and Yoshikawa (16) reported the Cu,ZnSOD concentration in 45 blood samples from normal Japanese men to be 32.9 (SD 10.5) µg per mL of blood. The results from the polarographic method were 5.47 (SD 1.03) × 10⁻⁶ g of Cu,ZnSOD per cell or 0.18 (SD 0.03) µg per gram of hemoglobin (24) and 175 (SD 40.5) µg per gram of hemoglobin (25). The results from competition radioimmunoassay and pulse radiolysis were 854 (SD 100) ng of Cu,ZnSOD per mg of hemoglobin (26) and 9 × 10⁻⁶ mol of this enzyme per mg of hemoglobin (27), respectively. Also using riboflavin as the O₂⁻ generator, Winterbourn et al. (28, 29) measured 2900 (SD 330) units of Cu,ZnSOD per mg of hemoglobin and Ueda and Ogata (30) observed a Cu,ZnSOD activity of 12.6 (SD 2.7) units per milligram of hemoglobin or 2082 (SD 698) units per milliliter of whole blood. With ferrocychrome c as the indicator of the reaction, erythrocyte Cu,ZnSOD activity was 13.7 (SD 1.7) units per milligram of hemoglobin or 37.5 (SD 4.4) units per 10⁸ cells (31). Instead of ferrocychrome c, we used NBT as an indicator, and measured a Cu,ZnSOD concentration in erythrocytes of 44 normal adults of 242 (SEM 4) µg per liter of erythrocytes. Furthermore, we measured enzyme activity in serum and plasma and found very low Cu,ZnSOD concentrations 548 (SEM 20) and 173 (SEM 11) µg per liter, respectively. Obviously, different methods and different units show different numerical results.

During the past few years, SOD activity in tumor cells has received increasing attention. Usually, tumor cells have decreased MnSOD activities. Cu,ZnSOD activity also is decreased in many, but not all, tumors (32). Changes in SOD activity of blood in leukemia patients have been reported; erythrocyte SOD decreased in patients with malignant lymphomas and acute myeloid leukemia (33). Total SOD activity was significantly lower in leukemic blasts from five patients with T-cell or B-cell leukemia. MnSOD activity was significantly lower in all types of acute lymphoblastic leukemia. However, both total and MnSOD activities were significantly greater in acute myeloblastic leukemia than in normal polymorphonuclear leukocytes (34). Reportedly, in myelocytic, monocytic, and lymphocytic leukemia cells the Cu,ZnSOD activities are increased as compared with those in the corresponding normal cells (13). In erythrocytes from patients with acute myelogenous leukemia and lymphoproliferative syndromes, the Cu,ZnSOD also is significantly increased (35).

We measured Cu,ZnSOD in blood of patients with cancer of the large intestine before and after treatment by radical surgery. The enzyme activities in serum and plasma in patients had a tendency to decrease and increase, respectively, as compared with normal adults, although the trend was not statistically significant.

We thank Ms. Susan Redfern and Susan Barnett for their help in manuscript preparation. This work was supported in part by NIH grant 1 RO1-CA41267.

References