Volume 282, number 1, 115–118 FEBS 09627 April 1991

ADONIS 001457939100336K

Detection of heterogeneity of Cu, Zn-superoxide dismutase with monoclonal antibodies and the establishment of a highly sensitive fluorescence sandwich enzyme-linked immunosorbent assay

Kumeo Ono[1], Satsuki Kimura[2], Minoru Nakano[2] and Takuji Naruse[1]

[1]*The Third Department of Internal Medicine, Gunma University, Maebashi, Gunma* and [2]*College of Medical Care and Technology, Gunma University, Maebashi, Gunma, Japan*

Received 21 November 1990; revised version received 13 February 1991

Murine monoclonal antibodies directed against a native form of Cu, Zn-superoxide dismutase (SOD) were produced by immunizing SOD purified from human erythrocytes. The monoclonal antibodies able to bind SOD were further screened for their ability to absorb SOD activity using anti-mouse IgG conjugated iron beads as solid supports in magnetic separation. This new screening method revealed the heterogeneity of native SOD in the reactivity with the antibodies. One monoclonal antibody succesfully absorbed the entire activity of SOD detected by an inhibition assay of cypridina luciferin analog (MCLA)-dependent chemiluminescence induced by superoxide anion production, while others absorbed only a part of the SOD activity. The evidence that all of the latter antibodies failed to react with recombinant artificial SOD free of charge isomers suggested correlation of the heterogeneity with the presence of charge isomeric forms. The former antibody was further used to establish a fluorescence sandwich enzyme immunoassay, and this assay provided a very sensitive detection limit as low as 100 pg/ml.

Cu, Zn-superoxide dismutase; Monoclonal antibody; Immunoassay

1. INTRODUCTION

Superoxide dismutases (SOD) are metalloenzymes which catalyze the dismutation of superoxide anions, yielding H_2O_2 and O_2. Recent clinical research has increased interest in the possible involvement of these enzymes in the pathophysiology of human disease [1,2]. Several methods for determining Cu, Zn-SOD and Mn-SOD have been reported and most of the early ones were based on the enzymatic activities of scavenging superoxide [3–9]. Recently a specific and sensitive method using cypridina luciferin analogs as chemiluminescence probes has been reported [10]. However, several factors are still known to interfere with the measurement of the enzymatic activity of SOD in biological fluids or tissues [11,12], and these disadvantages of enzymatic assays spurred the development of immunological methods [13–18]. In these experiments the production of monoclonal antibodies (mAbs) against SOD is essential for sandwich immunoassay [16–18]. Although the immunological asssays using mAbs provided a solution for the problem of the enzymatic assays, it was suggested that the amount of SOD represented with antigenecity is not necessarily parallel with the enzymatic activity of SOD [14,19]. To evaluate the relationship between values obtained from the two independent systems, we first employed an absorption study with mAbs of enzymatic activity measured by the chemiluminescence method in the screening of mAb production against native Cu, Zn-SOD. This enabled us to separate mAbs into two groups, one for reacting with whole active SOD and the other for reacting with only part of it. The former type of antibody (Ab) was further used to establish a fluorescence sandwich enzyme immunoassay in anticipation of a more sensitive assay giving resuls with better correlation with the enzymatic activity.

Correspondence address: M. Nakano, College of Medical Care and Technology, Gunma University, Maebashi, Gunma 371, Japan

Abbreviations: SOD, superoxide dismutase; Ab, antibody; mAb, monoclonal antibody; MCLA, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one; ELISA, enzyme linked immunosorbent assay.

2. MATERIALS AND METHODS

2.1. *Production of monoclonal anti-native human Cu, Zn-SOD antibodies*

Cu, Zn-SOD purified from human erythrocytes (Sigma 3190 U/mg of protein) was used to immunize Balb/c mice. Beginning 4 weeks after the initial immunization with 100 μg of SOD in complete Freund's adjuvant (Difco Laboratories, Detroit, USA), a booster injection of 50 μg of SOD in incomplete adjuvant was given every 2 weeks. Four days after the 4th booster without adjuvant, the spleen cells were harvested and fused to the hypoxanthine-aminopterin-thymidine sensitive SP2/O non-secretory murine myeloma cell line according to the method described by Oi and Herzenberg [20]. The Abs in culture supernatants were screened for their ability to bind SOD by an enzyme linked immunosorbent assay (ELISA) in which

SOD was used at a concentration of 50 μg/ml in 0.1 M carbonate buffer pH 8.3 for coating 96-well flat-bottom EIA plates (Flow Laboratories, USA). The bound Abs were identified using biotinylated anti-mouse IgG developed with biotin-avidin complex conjugated with peroxidase (Vectastain ABC kit, Vector Laboratories, USA). The substrate reaction with hydrogen peroxide and o-phenylendiamine dihydrochloride (Sigma, USA) was performed for 20 min at room temperature. The Abs with binding ability to SOD were further screened for their ability to absorb SOD activity measured by an inhibition assay of chemiluminescence induced by superoxide anion generation [14] as described below. After a second screening, cells secreting Ab capable of absorbing SOD activity were cloned by limiting dilution. The cell clones were enlarged and were injected into pristane-primed Balb/c mice for Ab production in ascitic fluid. Abs from the ascitic fluid were purified by ammonium sulfate precipitation followed by affinity purification on protein A agarose or ion-exchange purification.

2.2. *Absorption of SOD activity with mAbs using a magnetic separation system*

In order to accomplish efficient and rapid absorption of SOD with the Abs, iron-beads were used as solid supports in magnetic separation (BioMag, Advanced Magnetics, USA). 100 μl of each culture supernatant containing Ab were added to each well of a round-bottomed 96-well plate and incubated with approximately 5×10^7 iron particles conjugated with anti-mouse IgG with mixing at 15 min intervals. After 45 min incubation, the plate was placed in a strong magnetic field for 3 min to separate free Abs and iron-bead-bound Abs. After removing free Abs by washing with phosphate buffered saline, 100 μl of SOD at a concentration of 1 μg/ml was mixed with the Ab bound with beads and incubated for 1 h with mixing at 15 min intervals. After separation in a magnetic field for 3 min, the supernatant, freed from complexes of SOD-Ab bound to beads, was harvested and residual SOD activity in the supernatant was measured by the chemiluminescence assay.

2.3. *Chemiluminescence assay for determining SOD enzymatic activity*

An inhibition assay for superoxide anion induced chemiluminescence was performed to measure SOD activity. MCLA was used as a chemiluminescence probe as described previously [10]. Reaction mixtures were made by a 100-fold dilution to give a 10 ng/ml concentration of SOD for an unabsorbed sample. SOD activity was expressed in ng/ml of the reaction mixture estimated from the standard curve of SOD (Sigma, 3190 U/mg of protein, Cat.No.S 7886), ranged from 0.01 to 10 ng/ml.

2.4. *Competitive inhibition asssay for detecting SOD specific for respective mAbs*

50 μl of each mAb (1 μg/ml) were mixed and incubated with 50 μl of sample SOD (adjusted to 0.75 μg/ml before absorption) for 30 min. 50 μl of the mixture were added to a plate coated with SOD and incubated for 30 min. The Ab binding to the SOD-coated plate was identified with biotin anti-mouse IgG Ab followed by addition of the biotin-avidin complex conjugated with peroxidase as described above. The reactivity of sample SOD with the respective Ab was expressed by % inhibition of the Ab binding to SOD on the plate by the following calculation:

$$\% \text{ inhibition of Ab binding to SOD} = \frac{a-b}{a} \times 100$$

in which a is absorbance at 492 nm of control Ab binding without inhibitor and b is absorbance at 492 nm of the binding with sample SOD as inhibitor.

2.5. *Reagents*

Polyclonal Ab against Cu,Zn-SOD was purchased from the Binding Site Ltd. (Birmingham, England) and was biotinylated in carbonate buffer at pH 8.2 for 4 h at room temperature according to the method of Jackson et al. [21].

Recombinant human Cu,Zn-SOD with the same amino acid sequence as native SOD or with Cys-111 replaced by Ser were kindly provided by Nippon Kayaku Co. and Toyo Jyozo Co., respectively.

2.6. *Fluorescence sandwich enzyme immunoassay procedure*

A 96-well polystyrene plate (B. plate, Dynatech Laboratories Inc., USA) was coated with 50 μl (10 μg/ml of 0.1 M carbonate buffer, pH 8.3) of mAb, named 15, and left overnight at 4°C. Unbound sites were saturated by incubation with phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA, EIA grade, Ciba Chikusan Kogyo, Japan) for at least 30 min at room temperature. After washing, 50 μl of various dilutions of SOD (Sigma) were added and incubation followed for 30 min at room temperature. The plates were washed and incubated with biotinylated polyclonal anti-SOD Ab for 30 min. They were then washed again and incubated with 50 μl of 1.0 U of β galactosidase-avidin (Vector Laboratories, USA)/ml of PBS-BSA-Tween for 30 min. After washing, 100 μl of 40 μg of 4-methylumbelliferryl-β-d-galactoside (Sigma)/ml of 0.01 M phosphate buffer at pH 7.2 was added and incubated for 30 min at room temperature. Absorbance was measured using a fluorescence microplate reader (Microfluore, Dynatech Laboratories Inc., USA) calibrated for excitation at 365 nm emission at 450 nm.

3. RESULTS AND DISCUSSION

Eight hybrid cell lines secreting Abs that react with plates coated with SOD were obtained from two fusions after a first screening using ELISA. These cell lines were then examined for whether their Abs could absorb SOD activity measured by an inhibition assay utilizing MCLA-dependent chemiluminescence. This absorption was accomplished efficiently using anti-mouse IgG conjugated iron-beads as solid supports in magnetic seperation. An Ab, named 15 could absorb the entire SOD enzymatic activity, three Abs named F3.4, 13 and 14 could absorb part of the SOD activity, and the other Abs failed to absorb any. The cell lines with Ab of absorbing ability were cloned by a limiting dilution. Purified mAbs were tested by a quantitative absorption assay as shown in Fig. 1. Each Ab showed absorption of SOD

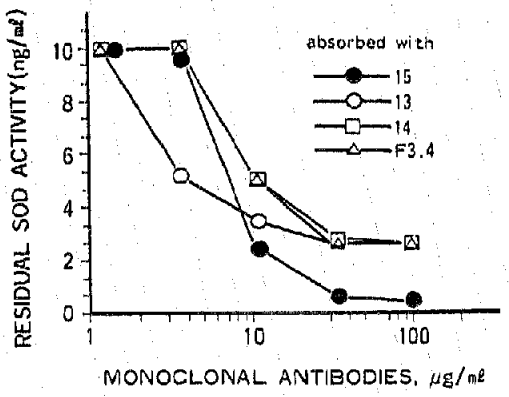

Fig. 1. Quantitative absorption of SOD enzymatic activity with mAbs. 100 μl of 1 μg/ml SOD was incubated with 5×10^7 anti mouse IgG conjugated iron particles preincubated with 100 μl of varous concentrations of mAbs: 15 (●), 13 (○), 14 (□), or F3.4 (△). After separation of free SOD from Ab-bound SOD on a magnetic field, residual activity of free SOD was measured by an inhibition assay utilizing MCLA-dependent chemiluminescence. Reaction mixtures of sample SOD for chemiluminescence assay were made by a 100-fold dilution to give a 10 ng/ml concentration for an unabsorbed sample. SOD activity was expressed by ng/ml estimated from a standard curve of SOD.

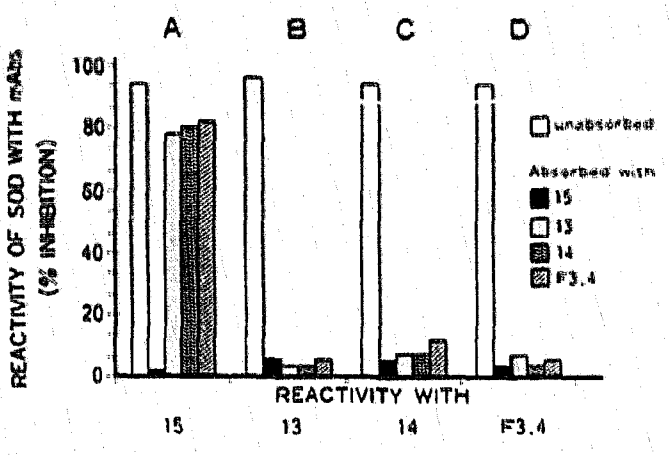

Fig. 2. Competitive inhibition assay for detecting SOD specific for respective mAbs. SOD reactive with respective mAbs was measured by a competitive inhibition assay with sample SOD in solution on binding of each mAbs to SOD. On a plate 50 μl of 1 μg/ml mAbs, 15(A), 13(B), 14(C), or F3.4(D) was incubated with 50 μl of 0.75 μg/ml SOD unabsorbed or absorbed with the mAbs for 30 min and the mixture was then added to the plate coated with SOD. After 30 min incubation, the Ab binding to SOD coated on the plate was identified with biotin anti-mouse IgG developed with the avidin–peroxidase complex. Reactivity of sample SOD with each Ab was expressed as % inhibition of the Ab binding to SOD. The absorbance at 492 nm of control Ab binding without inhibitor SOD was 222, 182, 173 and 168 for Ab 15, 13, 14 and F3.4, respectively.

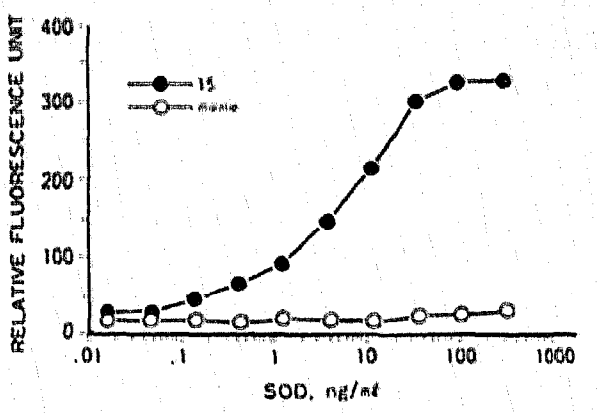

Fig. 4. Standard curve of the fluorescence ELISA for native Cu, Zn-SOD with mAb 15 (●) or without Ab for coating the plate (○). Absorbance of background without SOD was 22.

activity in a dose-dependent fashion, reaching a maximum at a concentration of over 30 μg/ml. Ab 15 exhibited over 95% absorption at these points, while others showed 70–80% absorption, leaving 20–30% of residual SOD activity. This result implies the presence of a fraction of SOD reactive with 15 but not with either F3.4, 13, or 14. This was further confirmed by testing the reactivity of SOD with respective Abs by an ELISA after absorbing SOD activity with each Ab. The amount of SOD reactive with a respective Ab was measured by checking the competitive inhibitory activity of SOD on the binding of the Ab to the SOD-coated plate. In Fig. 2, after absorption by 15, sample SOD lost its inhibitory activity on the binding of all Abs to SOD on the plate (Fig. 2A,B,C,D) but SOD absorbed to 13, 14 or F3.4 still retained inhibitory activity on the binding of 15 (Fig. 2A) to SOD in contrast with the complete loss of such activity on binding of 13, 14 and F3.4 (Fig. 2B,C,D). These results indicate that 13, 14 and F3.4 recognize the same subfraction of SOD, while 15 recognizes fractions, both reactive and unreactive with other Abs. Next, the reactivity of these mAbs with recombinant SOD was examined. Fig. 3 shows the results obtained by the same ELISA used in the first screening of the Abs. Two kinds of recombinant human Cu,Zn-SOD were used to coat the plate, one had the same amino acid sequence as native SOD but the other

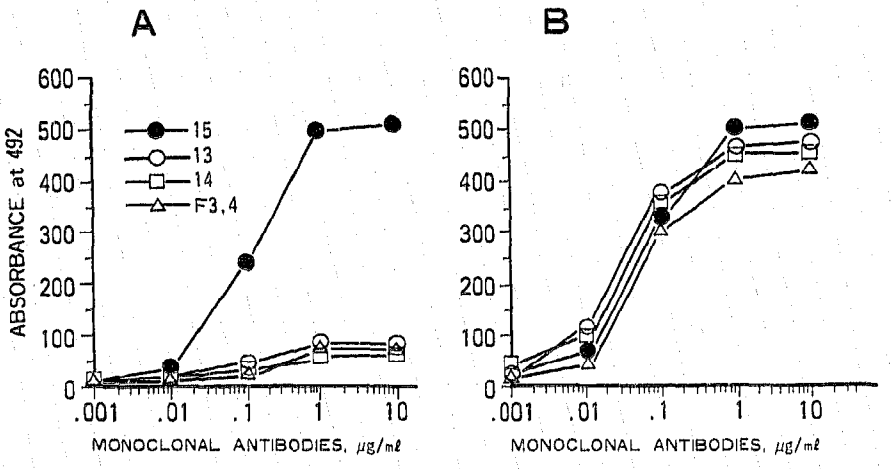

Fig. 3. Differential reactivities of mAbs against artificial recombinant SOD. 50 μl of various concentrations of each mAbs, 15 (●), 13 (○), 14 (□), or F3.4 (△) was incubated on plates coated with artificial recombinant SOD in which Cys-111 residue was replaced by Ser (A) or with recombinant SOD with the native amino acid sequence (B). The bound Ab was identified with biotin-anti-mouse IgG Ab developed with the biotin–avidin complex conjugated with peroxidase.

had a different sequence with Cys-111 replaced by Ser. The latter form of recombinant SOD has been reported to be free of charge isomers [22]. Ab 15 reacted with the latter form of SOD (Fig. 3A), as well as the former type of SOD (Fig. 3B), while other Abs failed to react with the latter form of SOD free of charge isomers (Fig. 3A). These results suggest that F3.4, 13 and 14 may recognize a subfraction of charge isoforms of SOD.

Our present results indicate that only one of the mAbs, 15 is reactive with the entire population of active SOD and other Abs are thus inadequate to identify the entire SOD activity. Therefore, we used 15 to establish a specific and highly sensitive enzyme immunoassay for determining SOD, employing the fluorescence sandwich immunoassay, from which we expected higher sensitivity than the conventional colorimetric assay. Standard curves of SOD purified from human erythrocytes were made by serial 3-fold dilutions as shown in Fig. 4. The SOD was detected above the background level as low as 100 pg/ml and the activity level reached a plateau with increasing concentration at 30 ng/ml. The assay system was specific for 15, since no increase in flurorescence intensity over the background level was detected when the plate was not coated with the Ab. Thus this new fluorescence sandwich enzyme immunoassay provided one of the highest sensitivities compared with colorimetric enzyme immunoassays and chemiluminescence assays so far reported and is expected to be a better tool with which to quantitate limited SOD levels in tissues and body fluids. The comparison with results obtained from enzymatic assays such as the MCLA-dependent chemiluminescence assay is going to be further examined using crude SOD in various tissues.

Acknowledgement: We thank Professor K. Sato (College of Medical Care and Technology, Gunma University, Japan) for her helpful technical advice on fluorescence enzyme immunoassay and in use of the fluorescence microplate reader.

REFERENCES

[1] Oberley, L.W. (1982) in: Superoxide Dismutase (Oberley, L.W., Ed.) vol. 2, pp. 127–165, CRC Press, Boca Raton, FL.
[2] Oberly, L.W. and Buettner, G.R. (1979) Cancer Res. 39, 1141–1149.
[3] McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
[4] Oyanagui, Y. (1984) Anal. Biochem. 142, 290–296.
[5] Markland, S.L., Westman, N.G., Lundgren, E. and Roos, G. (1982) Cancer Res. 42, 1955–1961.
[6] Misra, H.P. and Fridovich, I. (1972) J. Biol. Chem. 247, 3170–3175.
[7] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
[8] Corbisier, P., Houbilon, A. and Remacle, J. (1987) Anal. Biochem. 164, 240–247.
[9] Bensinger, R.E. and Johnson, C.M. (1981) Anal. Biochem. 116, 142–145.
[10] Nakano, M., Kimura, H., Hara, M., Kuroiwa, M., Kato, M., Totsune, K. and Yoshikawa, T. (1990) Anal. Biochem. 187, 277–280.
[11] Goldstein, I.M., Kaplan, H.B., Edelson, H.S. and Weissmann, G. (1979) J. Biol. Chem. 254, 4040–4045.
[12] Nishikimi, M. (1975) Biochem. Biophys. Res. Commun. 63, 463–468.
[13] Kelly, K., Barefoot, C., Sehon, A. and Petkau, A. (1978) Arch. Biochem. Biophys. 190, 531–538.
[14] Gandy, S.E., Buse, M.G. and Crouch, R.K. (1982) J. Clin. Invest. 70, 650–658.
[15] Asayama, K. and Burr, I.M. (1985) J. Biol. Chem. 260, 2212–2217.
[16] Adachi, T. Usami, Y., Kishi, T., Hirano, K. and Hayashi, K. (1988) J. Immunol. Meth. 109, 93–101.
[17] Oka, S., Ogino, K., Matsura, S., Yoshimura, S., Yamamoto, K., Okazaki, Y., Takemoto, T., Kato, N. and Uda, T. (1989) Clin. Chim. Acta 182, 209–220.
[18] Portsmann, T., Wietshke, R., Jahn, S., Portsmann, B., Shmechta, H., Pergand, M. and Von Baehr, R. (1990) J. Immunol. Meth. 127, 1–10.
[19] Bartosz, G., Soszymski, M. and Petelwska, W. (1981) Mech. Ageing Dev. 17, 650–658.
[20] Oi, V.T. and Herzenberg, L.A. (1979) in: Selected Methods in Cellular Immunology (Freeman, W.H. ed.) p. 351.
[21] Jackson, S., Sogn, J.A. and Kindt, T.J. (1982) J. Immunol. Meth. 48 (3) 229–309.
[22] Sekoguchi, S., Nogata, K. and Sagai, H. (1988) in: Engineering Foundation's 4th Conference on 'Recovery of Bioproducts' in Hawaii.