Expression of a Hybrid Cu/Zn-type Superoxide Dismutase Which Has High Affinity for Heparin-like Proteoglycans on Vascular Endothelial Cells*

(Received for publication, January 14, 1991)

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Since plasma levels of enzymes, such as superoxide dismutase (SOD), that scavenge reactive oxygen species are low, surface membranes of endothelial and parenchymal cells of various tissues are often exposed to oxidative stress. To dismutate superoxide radicals efficiently in and around vascular endothelial cells, we constructed a fusion gene encoding a hybrid SOD (HB-SOD) consisting of human Cu/Zn-SOD and a C-terminal basic peptide that binds to heparin-like proteoglycans. The fusion gene was expressed in yeast, and the resulting HB-SOD was highly purified. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, HB-SOD revealed a protein band with an apparent molecular weight of 20,000. HB-SOD bound to endothelial cells of aortic segments by a mechanism which was inhibited by heparin but not by antithrombin III. When injected intravenously to rats, 125I-labeled HB-SOD rapidly disappeared from the circulation; the rate of disappearance was decreased by heparin. Less than 1% of the injected HB-SOD was found in the urine 20 min after administration at which time more than 70% of SOD was excreted in its intact form. Immunohistochemical studies revealed that HB-SOD predominantly bound to heparin-like proteoglycans on endothelial cells of the artery and other tissues. HB-SOD might permit studies on pathophysiological roles of superoxide radicals in and around vascular endothelial cells in vivo.

Since most cells and tissues are highly enriched with enzymes, such as cytosolic Cu/Zn-SOD and catalase, that degrade reactive oxygen species (1–3), oxidative stress occurring intracellularly is efficiently inhibited by these enzymes. However, under pathological conditions, large amounts of superoxide and its metabolites are produced, and part of them comes out from cells (4, 5). For example, after ischemia, superoxide radicals are produced in reperfused tissues by several mechanisms including xanthine oxidase derived from vascular endothelial cells (6). It should be noted that a Cu/Zn-type SOD is also present in plasma and is called extracellular SOD. This enzyme is a glycoprotein and differs from the cytosolic Cu/Zn-SOD by the presence of a heparin-binding C-terminal polypeptide extension (7, 8). However, activity of enzymes, such as extracellular SOD, that degrade reactive oxygen species is significantly lower in plasma than that in intracellular compartments. Thus, when tissues are exposed to high levels of reactive oxygen species, membrane perturbation leading to cell death might be induced. Therefore, we propose that the plasma membranes of vascular endothelial cells and parenchymal cells should be protected from these reactive species particularly when animals were challenged with oxidative stress. Although cytotoxicity of reactive oxygen species is markedly inhibited by SOD and other enzymes in vitro (9, 10), the use of these scavenging enzymes in vivo is highly limited predominantly because of their unfavorable behavior, such as short half-life in the circulation. Because of the hazardous nature of reactive oxygen species, they should be scavenged efficiently at the site of generation and on plasma membranes of vascular endothelial cells. Endothelial cells are highly enriched with acidic proteoglycans, such as heparan sulfate. Proteins with high affinity for heparin-like proteoglycans have been shown to localize on the outer surface of vascular endothelial cells (7, 11, 12). The present work describes the construction of a cDNA encoding a hybrid SOD (HB-SOD) consisting of human Cu/Zn-type SOD and a basic peptide with high affinity for heparin. Physicochemical properties of HB-SOD and its selective binding to vascular endothelial cells are also described.

EXPERIMENTAL PROCEDURES

Materials—Human antithrombin III (AT-III) was obtained from Chemo-Sero-Research Institute (Kumamoto). Recombinant Cu/Zn-SOD was obtained from Suntory Co. (Osaka). All other enzymes were purchased from Toyobo Co. Ltd. (Osaka) and Takara Shuzo Co. Ltd. (Kyoto). cDNA synthesis kit was obtained from Amersham. Antiserum against human Cu/Zn-SOD was produced in New Zealand white rabbits as described previously (13). 125I-Labeled Bolton-Hunter reagent was from Du Pont-New England Nuclear. All other reagents used were of analytical grade.

Construction of a Fusion Gene Encoding HB-SOD—A full length cDNA encoding Cu/Zn-SOD was obtained from a human placental cDNA library (14) by screening with a synthetic nucleotide based on the published SOD gene sequence (15). An EcoRI restriction site was made prior to the initiation codon by site-directed mutagenesis (16). A 450-base pair EcoRI-Sau3A1 fragment of the SOD gene was sub-
cloned into EcoRI-BamHI sites of pBR322. The constructed plasmid was designated as pBRSD1. A nucleotide fragment, which encoded a heparin-binding peptide similar to that of extracellular SOD (8) with a stop codon and restriction sites of Sau3AI and SalI, was constructed by annealing four synthetic nucleotides, 5'-GATCCGGCCGGCGGGCTGAGGACGCGCCAGGCGGGCGGCACTA3' (A), 5'-TCTTGGCTCTGGAGTGCTCCCAGCGGGCGGGCA3' (B), 5'-CAAGGCAGGAAGGCGGGCGGGCGGAAGGTGGCGCTGGCAGCAATGCAAGC' (C), and 5'-TCGACTAGTACGGCCTTGTCATCAGC' (D), by the method of Ikehara et al. (17). This nucleotide fragment encoded the heparin-binding peptide of extracellular SOD (Cys310-Ala312) except that the Cys310 was replaced by arginine. The constructed fragments were ligated with a 4.5-kb BamHI-SalI fragment of pBRSOD1, and the constructed plasmid was designated as pBRHSOD1. pBRHSOD1 contained the HB-SOD gene encoding Cu/Zn-SOD with the heparin-binding domain on its C-terminal portion. A 0.549-kb EcoRI-SalI fragment of pBRHSOD1 was ligated with an 8.0-kb EcoRI-SalI fragment in a yeast expression vector pYHCC101 (14). The constructed plasmid was designated as pYHSI1. pYHSI1 contained TRP1 as a selectable marker for yeast, and the HB-SOD gene in the plasmid was controlled by the glyceraldehyde-3-phosphate dehydrogenase promoter in yeast. A yeast strain EH13-15 (Matα, trpl) (18) was transformed by pYHSI1 by the method of Ito et al. (19). The nucleotide sequence of both strands of the fusion gene was determined as described previously (20). Fig. 1 illustrates the construction of HB-SOD.

Expression and Purification of HB-SOD—Transformants were cultivated in Burkholder medium (21), and total cell proteins were obtained by the method of Yaffe et al. (22). Expression of HB-SOD was confirmed immunologically using anti-human SOD antibody. For purification of HB-SOD, the transformant was cultivated at 30 °C for 2 days in 30 liters of Burkholder medium containing 0.5% casamino acid (Difco), 0.1 mM CuSO4, 0.1 mM ZnSO4. After centrifugation, about 1 kg of cells (wet weight) was obtained. The harvested cell sample was rapidly cooled to 4 °C and centrifuged at 10,000 g for 20 min. To the supernatant fraction was added ammonium sulfate to give a final concentration of 50%. After centrifugation at 3,000 g for 20 min, the supernatant fraction was applied to a butyl-Toyopearl 650M column (10 cm in length, 1 cm in diameter) which was equilibrated with 20 mM phosphate buffer, pH 8.0, containing 35% ammonium sulfate. Fractions which were enriched with SOD activity were collected and dialyzed against 20 liters of 10 mM phosphate buffer, pH 8.0, containing 150 mM NaCl. The dialyzed sample was applied onto a heparin-Sepharose CL-6B column (10 × 20 cm) which was equilibrated with 10 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl. After washing the column with 4 liters of the dialysis buffer, proteins were eluted by 10 mM phosphate buffer containing 100 mM NaCl. Fractions enriched with SOD activity were combined and condensed to a small volume by an Amicon Diaflow. After dialysis against 20 liters of 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 2 mM dithiothreitol, the sample was stored at −20 °C until use.

Under such conditions, the enzyme could be stored at least 6 months without decreasing catalytic activity. When chromatographed on a Sephadex G-75 column (3 × 30 cm) in the absence of SDS, the purified enzyme was eluted as a single peak with an apparent molecular weight of 40,000. The specific activity of HB-SOD was 2,750 units/mg of protein (17-fold enrichment with 26% recovery from crude cell extract) as determined by the cytochrome c method (23). Protein concentration was determined by the method of Lowry et al. (24) using human SOD as the standard.

Protein Chemical Analysis—The enzyme samples and AT-III were radiolabeled by 1111-Bolton Hunter reagent as described previously (25). After extensive dialysis of the radioabeled samples against 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, they were used for experiments. The specific radioactivity of SOD, HB-SOD, and AT-III was 8.7 × 106, 8.6 × 106, and 2.6 × 106 cpm/mg of protein, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified HB-SOD was carried out using a 12.5% gel (26). After acid hydrolysis in 6 N HCl at 110 °C for 20 h, amino acid analysis of the enzyme was performed (27). The N-terminal amino acid of HB-SOD was analyzed by a gas-phase Sequencer (Applied Biosystem-470A) (28). Under nitrogen stream, HB-SOD was incubated for 10 min at 25 °C in 2 ml of 0.2 M phosphoric acid. Protein was precipitated with 10 ml of 100 mM EDTA, and dialyzed against 20 mM phosphate buffer, pH 7.2, 10 mM EDTA, and 10 mM dithiothreitol. The HB-SOD samples thus treated were isolated by Sephadex G-25 column chromatography (2 × 10 cm). Under nitrogen stream, the number of free SH groups of native and denatured HB-SOD samples was determined by 5,5'-dithiobis-(2-nitrobenzoic acid) as described previously (29). Free SH group of native and denatured HB-SOD was also determined without reduction by dithiothreitol.

Affinity Chromatography—The enzyme samples (1 mg) were applied onto a heparin-Sepharose column (1 × 5 cm) which was equilibrated with 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl. After washing the column with 15 ml of the same buffer solution, the bound fraction was eluted with the same buffer solution containing 0.5 M NaCl. Fractions of 1 ml were collected, and absorbance at 280 nm was determined.

Application of in Vito Behavior of HB-SOD—After overnight fast, male Wistar rats, 200 g, were used for experiments. Under pentobarbital anesthesia (50 mg/kg), animals were injected intravenously with radiolabeled enzyme samples (200,000 cpm/animal). At varying times after injection, 0.1 ml of blood samples were obtained from the left
femoral vein. Radioactivity in blood samples was determined. At the indicated times, animals were exsanguinated, and tissues were perfused through the abdominal aorta with 10 ml of ice-cold saline solution. The excised tissues were homogenized in 3 volumes of 5% trichloroacetic acid. After centrifugation at 3,000 x g for 10 min, radioactivity in acid-soluble and insoluble fractions was determined in a Tri carb scintillation spectrophotometer model-5130.

**Immunohistochemical Analysis**—Ten min after administration of either 0.2 ml of saline or 10 mg of SOD or HB-SOD to the rats, the abdominal aorta was excised and frozen in liquid nitrogen. Cryosections of the aorta were immunostained using specific antisera against human Cu/Zn-SOD and horse radish peroxidase-conjugated anti-rabbit IgG antibody as described previously (30).

**RESULTS**

**Physicochemical Properties of HB-SOD**—Physicochemical properties of the purified HB-SOD were studied. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue, HB-SOD gave a single protein band with an apparent molecular weight of 20,000; its mobility was slightly slower than that of human Cu/Zn-SOD (Fig. 2).

SOD is highly resistant to heat treatment (31). To know the heat stability of HB-SOD, SOD and HB-SOD were concurrently incubated at 70°C. After incubation for 10 min, more than 90% of the catalytic activity of both SOD and HB-SOD remained intact.

Human Cu/Zn-SOD is a homodimer, and each subunit has one disulfide bond (between Cys27 and Cys45) and two free SH groups (Cys8 and surface Cys111 residues) (32). HB-SOD has an additional cysteiny1 residue (Cys17') in the C-terminal domain. To know the third status of HB-SOD, the number of free SH groups of the enzyme samples was determined. Chemical analysis revealed that, in the presence or absence of denaturant, 5.5-5.7 and 3.4-3.8 mol of 5,5'-dithiobis-(2-nitrobenzoic acid) reacted with 1 mol of HB-SOD, respectively. After denaturation and reduction by dithiothreitol, 9.2-9.6 mol of the reagent reacted with 1 mol of HB-SOD.

Gas-phase Sequencer analysis of HB-SOD revealed that the N-terminal amino acid was blocked. It has been well documented that the N-terminal alanine of SOD is acetylated (33). Thus, the N-terminal amino acid of HB-SOD may also be acetylated. The amino acid composition of HB-SOD (Table I) was equal to the sum of those of human SOD and the C-terminal domain (PGWQRQ5ARESRKRRERECKAA) encoded by the synthetic DNA. Analysis of the amino acid composition and the N-terminal amino acid of the fusion gene produce is consistent with the anticipated structure of HB-SOD (Fig. 3). To confirm the structure of HB-SOD, the nucleotide sequence of the fusion gene was analyzed. The sequence analysis was also consistent with the structure of the HB-SOD gene. Because of the sequence of the synthetic DNA fragments, Ala125 and Gln-131 (C-terminal amino acid of SOD) are replaced by Arg and Gly, respectively.

**Fig. 3. Structure of HB-SOD and its cDNA.** The area in the box shows the C-terminal heparin-binding domain of HB-SOD encoded by the synthetic DNA. Because of the sequence of the synthetic DNA fragments, Ala125 and Gln131 (C-terminal amino acid of SOD) are replaced by Arg and Gly, respectively.

| Amino acid | Theoretical | Found |
|------------|-------------|-------|
| Aspartic acid | 11 | 17.4 |
| Asparagine | 7 | 7.7 |
| Tryptophane | 8 | 7.7 |
| Serine | 12 | 11.4 |
| Glutamic acid | 15 | 18.8 |
| Glutamine | 3 | 9.5 |
| Proline | 6 | 5.8 |
| Glycine | 27 | 28.2 |
| Alanine | 12 | 11.3 |
| Cysteine | 5 | 4.7 |
| Valine | 14 | 13.1 |
| Methionine | 0 | 0 |
| Isoleucine | 10 | 7.4 |
| Leucine | 9 | 9.3 |
| Tyrosine | 0 | 0 |
| Phenylalanine | 4 | 3.8 |
| Histadine | 9 | 8.5 |
| Lysine | 14 | 13.2 |
| Arginine | 11 | 10.7 |
| Tryptophane | 2 | |

* Determined by 5,5'-dithiobis-(2-nitrobenzoic acid).

**Fig. 2. SDS-polyacrylamide gel electrophoresis of HB-SOD.** Polyacrylamide gel electrophoresis (12.5% gel) was performed in the presence of 0.1% SDS as described in the text. A, molecular weight markers; B, SOD; C, HB-SOD.
reversibly bind to heparin-like proteoglycans on endothelial cell surface. To test whether HB-SOD also binds to the endothelial cell surface, radiolabeled HB-SOD was incubated with aortic segments. HB-SOD rapidly bound to endothelial cells of aortic segments (Fig. 5). In the presence of 10 mg/ml heparin, binding of HB-SOD was inhibited by 70%. In contrast, chondroitin sulfate and AT-III failed to inhibit the binding of HB-SOD. AT-III also bound to aortic endothelial cells (Fig. 6). However, radioactivity associated with endothelial cells was significantly lower with AT-III than with HB-SOD. In the presence of 10 mg/ml heparin, binding of AT-III was also inhibited by 78%. Chondroitin sulfate and HB-SOD failed to inhibit the binding of AT-III. Under identical conditions, SOD did not bind to endothelial cells of aortic segments.

**Fate of HB-SOD in Vivo**—Fig. 7 shows the change in plasma radioactivity following intravenous administration of 125I-labeled HB-SOD and SOD. Plasma levels of radioactive HB-SOD decreased biexponentially; a faster phase with a half-life of 1 min followed by a slower phase with a half-life of 8 min. When HB-SOD was injected with heparin, the rate of disappearance of plasma radioactivity decreased significantly. The effect of heparin was more marked with the faster phase than with the slower phase. In contrast to the experiments with HB-SOD, SOD disappeared from the circulation with a half-life of 4 min; heparin had no effect on the rate of SOD disappearance. Twenty min after administration, significant radioactivity derived from HB-SOD accumulated in liver (11% of the dose), kidney (37% of the dose), and other tissues. More than 95% of the radioactivity associated with these tissues was recovered from trichloroacetic acid-precipitable fractions. Administration of HB-SOD with heparin (5,000 units/kg) decreased tissue-associated radioactivity by about 50% and increased plasma radioactivity by 55%. After 20 min of administration, less than 1% of the injected dose of radioactive HB-SOD was found in the urine. In contrast, intravenously administered SOD was rapidly filtered by the glomerulus and appeared in the urine in its intact form (29); about 70% of the injected dose was recovered in the urine within 20 min after administration. Heparin did not affect the urinary recovery of SOD.

**Immunohistochemical Localization of HB-SOD**—To know
the localization of HB-SOD, specimens were obtained from the aorta of animals 10 min after administration of either SOD or HB-SOD. Fig. 8 shows immunohistochemical localization of HB-SOD as demonstrated with specific antisera against human Cu/Zn-SOD. Consistent with our previous observation that the circulating SOD was rapidly excreted by the kidney (29), no significant binding of SOD was observed with the aorta. In contrast, significant amounts of HB-SOD bound to endothelial cells of the artery. When administered with heparin, HB-SOD failed to bind to vascular endothelial cells. Heparin-dependent binding of HB-SOD to endothelial cells was also seen with other tissues, such as liver and kidney (data not shown). Thus, HB-SOD might predominantly bind to heparin-like proteoglycans on vascular endothelial cells.

**DISCUSSION**

The present work demonstrates that HB-SOD, a hybrid protein consisting of human Cu/Zn-SOD and C-terminal basic peptide, selectively binds to heparin-like proteoglycans on vascular endothelial cell surface. Analysis of the nucleotide sequence of the fusion gene, the amino acid composition, and the N-terminal amino acid of HB-SOD is consistent with the anticipated structure of the hybrid enzyme as described in Fig. 3.

When chromatographed on a Sephadex G-75 column in the absence of SDS, HB-SOD eluted as a single peak with an apparent molecular weight of 40,000. Thus, like Cu/Zn-SOD, HB-SOD may also be a dimer. HB-SOD and SOD have similar specific activity and heat stability, which suggests that the C-terminal heparin-binding domain does not affect the stability and catalytic function of the enzyme.

Human Cu/Zn-SOD has 2 surface Cys residues which are fully exposed to solvent and 2 Cys residues which react with thiol reagents only after denaturation of the enzyme (32). Titration of free SH groups in the presence or absence of denaturant suggested that Cys, Cys, and Cys residues of the enzyme might be of reduced condition. Thus, like naturally occurring Cu/Zn-SOD, the two subunits of HB-SOD might associate noncovalently and form a dimeric enzyme. The heparin-binding C-terminal domain (Arg-Ala) of HB-SOD has a structure identical with that of extracellular SOD (Cys-Ala) except that the cysteiny1 residue of the binding domain (Cys in the latter enzyme) is replaced by Arg. To elucidate whether Arg is important for the binding of the enzyme to negatively charged heparin-like proteoglycans, we also obtained the HB-SOD sample whose Arg was replaced by Cys. Kinetic analysis revealed that catalytic activity, affinity for heparin-Sepharose column, and in vivo behavior of Cys-HB-SOD were similar to those of Arg-HB-SOD (data not shown). Thus, the thiol status and Arg of the C-terminal domain of HB-SOD may not be critical for determining the biological properties of the enzyme. Since the C-terminal extension of HB-SOD is highly enriched with basic amino acids (Arg-A), these positively charged residues might interact with heparin-like proteoglycans. HB-SOD bound to the heparin-Sepharose column and was eluted with the buffer solution containing 0.5 mM NaCl. Since extracellular SOD was also eluted from a heparin-Sepharose column by a buffer solution containing 0.5 mM NaCl (34), the affinity of HB-SOD for heparin might be similar to that of extracellular SOD. In contrast, elution of AT-III from a heparin-Sepharose column requires fairly high concentrations of NaCl (>1 M) (35). Thus, the affinity of AT-III for heparin would be higher than those of HB-SOD and extracellular SOD.

Sephadex G-75 gel filtration chromatography revealed that, in the presence of 1 ml of rat serum, HB-SOD also eluted in a fraction with an apparent molecular weight of 40,000, suggesting that HB-SOD circulated without interacting with plasma proteins. Although the molecular weight of HB-SOD is smaller than the renal filtration limit (M, 50,000), less than 1% of radioactivity derived from HB-SOD was found in the urine 20 min after administration at which time more than 70% of the injected SOD was excreted intact in the urine (29). At this time, about 37% of radioactivity derived from I-labeled HB-SOD accumulated in the kidney; more than 95% of renal radioactivity was recovered from acid-insoluble fractions. Preliminary experiments revealed that HB-SOD localized on vascular endothelial cell surface and apical plasma membranes of renal proximal tubule cells as determined immunohistochemically. Since lumenal accumulation of HB-SOD increased with time both in control and heparin-treated animals (5,000 units/kg), these observations suggest that renal glomerular filtration might be responsible for the slower phase of disappearance of the circulating HB-SOD. Since less than 3% of the injected HB-SOD appeared in the urine even after 60 min of administration, the C-terminal heparin-binding domain of HB-SOD might inhibit urinary excretion of the lumenal enzyme presumably through binding to apical plasma membranes of tubule cells. Renal proximal tubule cells have been known to reabsorb some plasma proteins in glomerular filtrate by a basic amino acid-inhibitable mechanism (36). Biochemical properties of the site(s) for HB-SOD binding on apical membrane surface of proximal tubules should be studied further.

Preliminary experiments in vivo revealed that carrageenan-induced paw edema, cold-induced brain edema, and postischemic reperfusion injury of the liver and heart were inhibited significantly by a single intravenous dose of HB-SOD (6 mg/kg) (12, 37). These observations suggest that superoxide radical and/or its reactive metabolites on vascular endothelial cells may play critical roles in the pathogenesis of tissue injury. Thus, HB-SOD might permit in vivo studies on the mechanism for oxidative injury of vascular endothelial cells and of renal proximal tubules.

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