Heparin-Affinity of Human Extracellular-Superoxide Dismutase in the Brain

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Extracellular-superoxide dismutase (EC-SOD) is one of the three SOD isozymes in humans and has affinity for heparin-like sulfated glycosaminoglycans in the extracellular matrix. The C-terminal portion of EC-SOD is responsible for the heparin-affinity of this enzyme, but this portion should be a target of proteinases. Recently, Oury *et al.* reported that EC-SOD is localized within the cytoplasm of neuronal cells, while this enzyme is found in the extracellular matrix in most other tissues. It has been speculated that the heparin-binding domain of EC-SOD in the brain is sensitive to proteolysis, which may result in a loss of extracellular immunoreactivity. This study was performed to investigate the heparin-affinity of EC-SOD in the human brain. We found that human EC-SOD in brain tissue has high heparin-affinity similar to that in the umbilical cord. Moreover, heparin-affinity of EC-SOD in brain homogenate was not decreased by incubation at 37 °C for 72 h. The proteolytic activity in brain homogenate might be less than that in umbilical cord homogenates. The intracellular localization of EC-SOD could contribute to its slow clearance from the brain and maintenance of its physiological functions in the brain.

Key words extracellular-superoxide dismutase; brain; heparin-affinity; nitric oxide; reactive oxygen species; superoxide

Reactive oxygen metabolites have been implicated in a number of disease processes, and oxidative stress is efficiently inhibited by enzymatic defense mechanisms such as superoxide dismutase (SOD), catalase and glutathione peroxidase. There are three SOD isozymes, copper- and zinc-containing SOD (Cu, Zn-SOD), manganese-SOD (Mn-SOD) and extracellular-SOD (EC-SOD). EC-SOD is a secretory, tetrameric glycoprotein with a subunit molecular mass of about 30 kDa.¹⁾ A prominent feature of EC-SOD is its affinity for heparin, which differentiates this enzyme from other SOD isozymes.¹⁾ Its heparin-binding domain may facilitate localization of a relatively high concentration of EC-SOD in the extracellular matrix where scavenging of extracellular superoxide is of critical importance. The C-terminal portion of EC-SOD, which contains three lysines, six arginines and a histidine in the last 21 amino acids, is responsible for the heparin-affinity of the enzyme.²⁾ In particular, the cluster of six basic amino acids, Arg210—Arg215, forms an essential part of the heparin-binding domain.^{3,4)} However, the C-terminal end of EC-SOD may be a target for proteinases.⁵⁾ Proteinases released from activated neutrophils *etc.* reduce the heparin-affinity of EC-SOD and suppress defense against oxidative stress.^{6,7)} These observations suggest that the proteolytic processing pathway of EC-SOD might regulate its physiological functions.

EC-SOD is important for a variety of physiological neurobehavioral functions. Resistance against oxidative stress occurs in the brain. However, the content of this enzyme in the brain is very low compared to most other organs.⁸⁾ For example, EC-SOD transgenic mice were found to be protected against vasogenic edema⁹⁾ and ischemic brain injury.¹⁰⁾ Moreover, it has been reported that when EC-SOD levels were either higher (overexpressing mice) or lower (knockout mice) than those of controls learning was impaired.¹¹⁾ Optimal level of EC-SOD may be needed to maintain neurobehavioral homeostasis. It is possible that the heparin-affinity of EC-SOD is essential to maintain its optimal level in tissues, since the loss of heparin-affinity results in clearance of

this isozyme from the tissues to the extracellular fluid. Recently, Oury *et al.* reported that EC-SOD is localized to neurons in the adult mouse brain.¹²⁾ The finding that EC-SOD appeared to be localized in the cytoplasm of neuronal cells is in contrast to the localization of EC-SOD in most other tissues where it is found in the extracellular matrix.^{13,14)} It has been speculated that the heparin-binding domain of EC-SOD in the brain is susceptible to proteolysis and this would result in loss of extracellular immunoreactivity. We focused on the intracellular localization of brain EC-SOD and investigated the heparin-affinity of EC-SOD in the human brain and its sensitivity to proteolysis.

METHODS

Preparation of Samples Minimal volumes of brain tissue were obtained from two cadavers at autopsy (two men aged 41 years old and 44 years old), and each specimen was divided into two pieces. After excision, these pieces and umbilical cords were quickly frozen at –30 °C without any freeze-thaw cycling until use. Tissues were homogenized with a Polytron homogenizer in 5 volumes (brain) or 10 volumes (umbilical cord) of ice-cold 25 mM sodium phosphate, pH 6.5, containing 2 mM EDTA-2Na, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM diethylenetriamine-*N,N,N',N',N''*-pentaacetic acid, 0.5 mM benzamidine, 0.5 mM *o*-phenanthroline and 100 U/ml aprotinin. After centrifugation at 10000×*g* for 10 min, the supernatant was dialyzed overnight at 4 °C against the above buffer.

SOD Analysis The EC-SOD concentration was determined by enzyme-linked immunosorbent assay (ELISA) as described previously.¹⁵⁾

Heparin-HPLC Heparin-affinity chromatography of the above samples was performed using an HPLC-column of TSKgel heparin-5PW (7.5 mm×7.5 cm, Tosoh, Tokyo). Chromatography proceeded at a flow rate of 0.7 ml/min, with a gradient system formed from 25 mM sodium phosphate, pH 6.5, and buffer containing 1 M NaCl.

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Fig. 1. Heparin-HPLC of EC-SOD in the Brain and Umbilical Cord

Brain homogenates (upper panels) and umbilical cord homogenates (lower panels) were incubated at 37 °C for 0 h (A and D), 24 h (B and E) and 72 h (C and F), followed by heparin-HPLC. Closed circles, EC-SOD concentration determined by ELISA; broken line, NaCl concentration in the mobile phase of HPLC. The shaded area shows fractions with high heparin-affinity.

RESULTS

As shown in Fig. 1 (A and D), EC-SOD in the brain had high heparin-affinity and was eluted at 0.70 M NaCl similarly to the enzyme in the umbilical cord and recombinant EC-SOD (kindly provided by Symbicom AB, Umeå, Sweden). We found that $78.0 \pm 5.4\%$ ($n=4$) of brain EC-SOD and $74.6 \pm 5.0\%$ ($n=4$) of cord EC-SOD were fractionated in high heparin-affinity forms. Next, we investigated the susceptibility of EC-SOD when the homogenates prepared without proteinase inhibitors were incubated at 37 °C. The immunoreactivity of EC-SOD in the brain and umbilical cord did not show significant decreases by incubation for 72 h, as shown in Fig. 2. However, the cord EC-SOD showed significant reduction of its high heparin-affinity form by incubation for 72 h ($59.9 \pm 6.5\%$, $n=4$, $p=0.013$ vs. 0 h) but not for 24 h ($80.1 \pm 7.6\%$, $n=4$) (Fig. 1, E and F). On the other hand, the heparin-affinity of EC-SOD in the brain did not significantly change (high heparin-affinity form = $74.6 \pm 4.8\%$ at 24 h, $n=4$ and $68.9 \pm 6.0\%$ at 72 h, $n=4$, $p=0.065$ vs. 0 h) (Fig. 1, B and C).

DISCUSSION

EC-SOD is the primary extracellular antioxidant enzyme and the characteristic property of this enzyme is its heparin-affinity. The binding ability of EC-SOD to heparin-like sulfated glycosaminoglycans is known to be essential for its function. On the other hand, the heparin-binding domain of EC-SOD is a target for proteinases. Regulation of this proteolytic event may be an important control point in modulating EC-SOD function in the tissues. EC-SOD in the umbilical cord has been shown to be least sensitive to proteolysis during freeze-thaw cycling of tissue samples compared to that of the kidney, placenta and prostate.⁵⁾ It is possible that proteolytic enzymes released by freezing-induced cellular and subcellular rupture cleave the heparin-binding domain of EC-

Fig. 2. Changes in Immunoreactivity of EC-SOD in Brain and Umbilical Cord Samples

Two brain homogenates (solid and open circles) and two umbilical cord homogenates (solid and open triangles) were incubated at 37 °C, and then their immunoreactivity was determined by ELISA.

SOD and decrease its heparin-affinity. The present results suggested that brain EC-SOD is not sensitive to proteolysis. There have been no previous reports of structural differences of EC-SOD among organs. The proteolytic activity in brain homogenate might be less compared to that in homogenate from the umbilical cord, although EC-SODs in the brain and umbilical cord have the same extent of heparin-affinity.

Nitric oxide (NO), one of the reactive oxygen species, is produced constitutively and functions as a neurotransmitter in the brain and is important for a variety of neurobehavioral functions.⁹⁾ NO reacts extremely rapidly with superoxide to produce peroxynitrite, a potent mediator of oxidant-induced cellular injury. SOD is a potent scavenger of superoxide and supports the functions of NO.¹⁶⁾ It is possible that the intracellular location of EC-SOD in the brain may be necessary for its function, such as the protection of NO produced by neuronal nitric oxide synthase (nNOS). The experimental results with EC-SOD transgenic mice^{9,10)} and knockout mice¹¹⁾

suggested that EC-SOD is essential for physiological neurobehavioral functions and resistance against oxidative stress in the brain. However, the intracellular content of EC-SOD is lower than those of Cu,Zn-SOD and Mn-SOD. EC-SOD in the brain was speculated to have a substantially higher ratio of enzyme activity to its mRNA level compared to that in other organs.¹⁷⁾ The intracellular localization of EC-SOD might contribute to its slow clearance from the brain and maintain its high level of activity in the brain.

In conclusion, our findings showed that brain EC-SOD has high heparin-affinity and is not sensitive to proteolytic cleavage of its heparin-binding domain. It is possible that the secretory process of EC-SOD within cells is well suppressed to maintain the superoxide scavenging activity in the intracellular space.

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