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An Arginine-213 to Glycine Mutation in Human Extracellular-Superoxide Dismutase Reduces Susceptibility to Trypsin-Like Proteinases

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Molecular genetic studies of extracellular-superoxide dismutase (EC-SOD) have shown that individuals with high serum EC-SOD content have a single base substitution generating the exchange of glycine for arginine-213 (R213G) in the heparin-binding domain of this enzyme [Sandström, J. *et al.* (1994) *J. Biol. Chem.* 269, 19163-19166], which causes the impairment of its binding ability to endothelial cell surface [Adachi, T. *et al.* (1996) *Biochem. J.* 313, 235-239]. Serum EC-SOD in healthy individuals without the above mutation is heterogeneous with regard to heparin affinity and consists of five fractions, forms (I) to (V), of which (IV) and (V) are the main fractions with high affinity for heparin [Adachi, T. *et al.* (1995) *J. Biochem.* 117, 586-590], whereas the major fraction in hemodialysis patients was serum EC-SOD form (I), which is thought to be the proteolytic truncated form. On the other hand, serum EC-SOD in both healthy individuals and hemodialysis patients with the R213G mutation consisted mainly of the high heparin-affinity type. This observation suggests that the susceptibility of EC-SOD to proteinases is reduced by the R213G mutation. The affinity of normal EC-SOD (n-EC-SOD) for heparin decreased by the treatment with trypsin, accompanied by a reduction in the molecular mass. The IC_{50} of trypsin for the heparin affinity of R213G mutant EC-SOD (m-EC-SOD) was 0.15 μg/ml, fivefold that for n-EC-SOD. Heparin affinity of n-EC-SOD was again more susceptible to neutrophils than that of m-EC-SOD. These results suggested that m-EC-SOD is more resistant to trypsin and neutrophil-release trypsin-like proteinases than n-EC-SOD, which causes the heparin affinity of serum EC-SOD to differ in individuals with and without the R213G mutation.

Key words: extracellular-superoxide dismutase, hemodialysis patient, heparin, neutrophil, trypsin.

There are three mammalian superoxide dismutase (SOD, EC 1.15.1.1) isozymes, copper- and zinc-containing SOD (Cu,Zn-SOD or SOD1), manganese-containing SOD (Mn-SOD or SOD2) and extracellular-SOD (EC-SOD or SOD3). EC-SOD is a secretory, tetrameric glycoprotein with a subunit molecular mass of about 32 kDa (*1, 2*) and the major SOD isozyme in plasma (*3, 4*). The content of EC-SOD in tissues is lower than those of Cu,Zn-SOD and Mn-SOD, which are intracellular enzymes (*5, 6*). A prominent feature of EC-SOD is its affinity for heparin, which differentiates it from the other SOD isozymes (*1*). The carboxyl-terminal portion of EC-SOD, especially the cluster of four arginine and two lysine residues at position 210-215, forms an essential part of the heparin-binding domain (*7-10*).

Serum EC-SOD levels from healthy persons are divided into two groups: Group I, a low-concentration group below 120 ng/ml; and Group II, a high-concentration group above 400 ng/ml (*4, 11, 12*). All healthy donors in Group II (frequency, 6.4%) have the same single base substitution, generating a change of arginine-213 to glycine (R213G) (*13, 14*), as determined from the known cDNA sequence (*15*). We purified mutant EC-SOD (m-EC-SOD) from sera of Group II donors (*16*). Mutant EC-SOD had slightly reduced heparin affinity compared with native EC-SOD purified from human umbilical cords (*10*) and recombinant EC-SOD (r-EC-SOD) (*2*), but its specific activity was similar (*16*).

Serum EC-SOD in Group I individuals is heterogeneous with regard to heparin affinity and can be divided into five fractions: Form (I), with very weak affinity; forms (II) and (III), with intermediate affinity; and forms (IV) and (V), with high affinity for heparin-HPLC (*17*). It has been suggested that EC-SOD form (V) is the primary form synthesized in the body (*17, 18*) and that EC-SOD forms

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Abbreviations: Cu,Zn-SOD, copper- and zinc-containing superoxide dismutase; DTPA, diethylenetriamine-*N,N,N',N",N"*-pentaacetic acid; EC-SOD, extracellular-superoxide dismutase; Mn-SOD, manganese-containing superoxide dismutase; m-EC-SOD, mutant EC-SOD; n-EC-SOD, normal EC-SOD; PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonyl fluoride; r-EC-SOD, recombinant EC-SOD; TPCK, L-1-*p*-tosylamino-2-phenylethyl-chloromethyl ketone; TLCK, *N*-α-tosyl-L-lysine-chloromethyl ketone.

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with reduced heparin affinity in plasma are the result of endo- and exo-proteolytic truncations at the C-terminal end (*8, 19, 20*). Sandström *et al.* showed that EC-SOD (I) fraction of human plasma contained mainly the low-molecular-mass band on SDS-PAGE analysis (*20*). On the other hand, serum EC-SOD in Group II individuals consists mainly of one form with the same heparin affinity as m-EC-SOD [eluted from heparin-HPLC between forms (III) and (IV) in Group I serum] (*16*).

During studies on heparin affinity of serum EC-SOD in Group I hemodialysis patients, it was observed that EC-SOD form (I) was the major fraction, whereas forms (IV) and (V) were predominant in sera from healthy individuals. As renal failure progress, leukocytes become activated (*21*). Phagocytic leukocytes secrete large amounts of various proteinases. EC-SOD in the vascular system may undergo proteolysis, particularly under pathological conditions (*22*). The hydrophilic C-terminal end of EC-SOD, which is rich in arginine and lysine and which contributes to heparin-binding, would be a target for proteinases. The finding of low levels of EC-SOD forms with weak heparin affinity in sera from hemodialysis patients with the R213G mutation indicated that the susceptibility to proteinases of m-EC-SOD and n-EC-SOD should be compared.

EXPERIMENTAL PROCEDURES

Materials—Human r-EC-SOD prepared as described (*2*) was kindly provided by SYMBICOM AB, Umeå, Sweden, and is described as n-EC-SOD in this report. Mutant EC-SOD was purified as described previously (*16*).

SOD Analysis—The EC-SOD concentration was determined by an enzyme-linked immunosorbent assay (ELISA) as described previously (*23*).

Preparation of Rat Neutrophil-Rich Fraction—Sixteen to 18 h after an intraperitoneal injection of 10 ml of 0.1% glycogen in saline to Wistar male rats (11 weeks, Japan SLC, Hamamatsu), an exudate rich in neutrophils was collected by washing the peritoneal cavity with 20 ml of HANKS solution (Nissui Pharmaceutical, Tokyo). The cells were collected in 50-ml centrifuge tubes and washed twice by centrifugation ($100 \times g$, 5 min) and re-suspension in HANKS solution.

Treatment of EC-SODs with Trypsin or Rat Neutrophil-Rich Fraction—Normal EC-SOD or m-EC-SOD (135 ng) was mixed with 0.5 to 50 ng of L-1-*p*-tosylamino-2-phenylethyl-chloromethyl ketone (TPCK)-trypsin (Sigma, St Louis, MO, USA) in 20 μl of 0.1 M Tris-HCl buffer, pH 8.6, containing 10 μg/ml BSA. After an incubation at 25°C for 12 h, 300 μl of 25 mM sodium phosphate buffer, pH 6.5, containing 2 mM EDTA-2Na, 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Wako Pure Chemical Industries, Osaka), 1 mM diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA, Wako Pure Chemical Industries), 0.5 mM benzamidine (Tokyo Kasei, Tokyo), 0.5 mM *o*-phenanthroline (Wako Pure Chemical Industries), and 10 μg/ml BSA was added, then heparin affinity was assayed. For the neutrophil-exposure experiment, the same amount of n-EC-SOD or m-EC-SOD was incubated with 6.5×10^7 cells/ml of rat neutrophil-rich fraction in the presence of 1 μM phorbol myristate acetate (PMA, Wako Pure Chemical Industries) in 30 μl of HANKS solution containing 10 μg/ml BSA at 37°C for the indicated periods. After adding 290 μl of 25 mM sodium phosphate buffer, pH 6.5, containing 10 μg/ml BSA and the above proteinase inhibitors, heparin affinity was analyzed.

Heparin-HPLC—Heparin-affinity chromatography was performed using a 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 buffer A (25 mM sodium phosphate, pH 6.5) and buffer B (25 mM sodium phosphate, pH 6.5, containing 1 M NaCl).

SDS-PAGE and Western Blotting—SDS-PAGE was performed using 12.5% acrylamide gels (*24*). The SDS-PAGE gels were electroblotted at a constant current 100 mA for 1 h onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). EC-SOD was detected using rabbit anti-r-EC-SOD antibody (*10*) followed by peroxidase-conjugated goat anti-[rabbit IgG (H+L)] antibody (Organon Teknika, West Cheater, PA, USA), then visualized using 0.1 M Tris-HCl, pH 7.2, containing 0.05% 4-chloro-1-naphthol and 0.05% H_2O_2.

RESULTS AND DISCUSSION

Heparin Affinity of Serum EC-SOD—Serum EC-SOD samples obtained from healthy persons and hemodialysis patients with and without the R213G mutation were tested for affinity to heparin, and typical chromatograms are shown in Fig. 1. EC-SOD in sera obtained from healthy individuals in Group I (without mutation) was separated into forms (I) to (V) by heparin-HPLC. We found that $48.3 \pm 2.7\%$ ($n=6$) of EC-SOD was fractionated in high-heparin-affinity forms (IV) and (V) (shadowed fractions), as shown in Fig. 1A. On the other hand, EC-SOD forms (IV) and (V) were significantly decreased in sera from hemodialysis patients ($32.8 \pm 7.2\%$, $n=6$, $p<0.001$ *vs.* healthy control). Concomitantly, the level of form (I) was increased (Fig. 1B). It has been shown that the proteolytic truncation of the heparin-binding domain is a major cause of the presence of EC-SOD forms lacking heparin affinity (*20*). EC-SOD in sera from hemodialysis patients in Group II with the R213G mutation (heterozygotes) was eluted from the heparin-HPLC column as mainly one fraction at about 0.53 M NaCl (Fig. 1D), similar to that from healthy individuals with the R213G mutation (Fig. 1C). There was no significant difference in the percentages of high-heparin-affinity forms of serum EC-SOD between healthy individuals ($84.4 \pm 3.4\%$, $n=6$) and hemodialysis patients ($80.3 \pm 4.3\%$, $n=6$). To understand why there was an increase in the low-heparin-affinity forms of EC-SOD in the sera from patients without a mutation, but not in patients with the R213G mutation, we compared the susceptibility of EC-SODs to trypsin and neutrophil-secreted proteinases. EC-SOD form (V) in persons without the mutation (Fig. 1, A and B) and the major EC-SOD fraction in those with the mutation (Fig. 1, C and D) had the same heparin affinities as r-EC-SOD and m-EC-SOD, respectively. Therefore, we used r-EC-SOD as n-EC-SOD and m-EC-SOD in the *in vitro* experiments described below.

Treatment of EC-SODs with Trypsin—The C-terminal end of EC-SOD, which contains a cluster of six basic amino acids, Arg-Lys-Lys-Arg-Arg-Arg, in positions 210-215, is responsible for the heparin affinity of the enzyme and may

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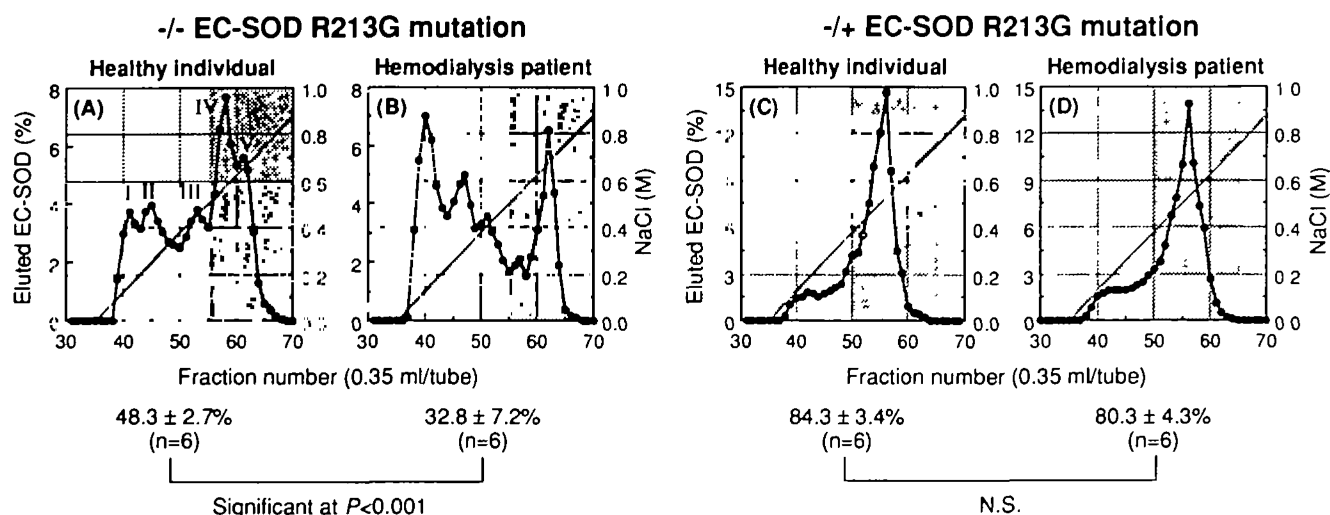

Fig. 1. **Heparin-HPLC of sera from healthy individuals and hemodialysis patients with and without the EC-SOD R213G mutation.** Chromatography proceeded as described in "EXPERIMENTAL PROCEDURES." Closed circles, EC-SOD concentration assayed by ELISA; dotted line, NaCl concentration in the buffer. Shadowed area shows fractions with high heparin affinity. The numbers indicate the percentage of EC-SOD with high heparin affinity. The differences between the two groups, (A) and (B), were statistically significant at $p<0.001$ by Student's t test. There were no significant (N.S.) differences between (C) and (D).

be a target for trypsin. Normal EC-SOD and m-EC-SOD were incubated with various concentrations of TPCK-trypsin at 25°C for 12 h, then heparin affinities were examined. m-EC-SOD had slightly decreased heparin affinity compared with n-EC-SOD, as evidenced by their elution from the heparin-HPLC column at about 0.53 M NaCl and about 0.7 M NaCl, respectively (Fig. 2, A and B, and Ref. *16*). Roughly half of the n-EC-SOD lost its high affinity for heparin (fractions in shadowed area in figures) after treatment with 0.025 μg/ml trypsin, whereas only about 20% of the m-EC-SOD lost high heparin affinity (Fig. 2, C and D). At 0.075 μg/ml trypsin, no high-heparin-affinity form of the n-EC-SOD remained, whereas 67% of m-EC-SOD retained high affinity (Fig. 2, E and F). At trypsin concentrations above 0.25 μg/ml, all m-EC-SOD also lost much of its high heparin affinity. The high heparin affinity of n-EC-SOD decreased at lower concentrations of trypsin (IC_{50} of about 0.03 μg/ml) than that of m-EC-SOD (IC_{50} of about 0.15 μg/ml). Figure 3 shows immunoblots of n-EC-SOD and m-EC-SOD exposed to trypsin (samples used for the above heparin-HPLC). The initial major band with a molecular mass of about 32 kDa was accompanied by a few faint bands (lane 1). This may be due to a heterogeneous structure of the carbohydrate chain, because non-glycosylated EC-SOD variant showed no heterogeneity on SDS-PAGE (*25*). The major band of n-EC-SOD was converted into another major band with an apparent molecular mass of about 30 kDa, whereas the major band of m-EC-SOD was not affected by 0.075 μg/ml trypsin (lane 3). At trypsin concentrations of 0.25 μg/ml and above, n-EC-SOD gave some degraded bands of lower molecular mass, while m-EC-SOD showed a major converted band with a molecular mass that was decreased by about 2 kDa (lanes 4 and 5). The behavior of EC-SODs on SDS-PAGE agreed with the results of the heparin-HPLC as shown in Fig. 2, and suggests that proteolysis is a major contributor to the decrease of heparin affinity of EC-SODs.

Mutant EC-SOD with a substitution of R213G located in the center of the heparin-binding domain had slightly reduced heparin affinity. It is predicted from secondary structure algorithms that the C-terminal region of EC-SOD assumes an α-helix, and that the R213G mutation results in a turn in the region (*26*). The decrease in positive charge and the change of secondary structure may impair the affinity of this enzyme for linear heparin. The heparin-binding ability of n-EC-SOD proved highly susceptible to trypsin ($IC_{50}=0.03$ μg/ml), whereas no major change in its immunoreactivity was evident. The highly hydrophilic C-terminal end including the heparin-binding domain should extend into the solvent and be easily accessible to trypsin. Moreover, a cluster of basic amino acids contributing to heparin-binding is a good substrate for trypsin. The results suggested that the R213G mutation reduced the susceptibility of EC-SOD to limited proteolysis by trypsin. Glycine-213 in m-EC-SOD may interfere with the progress of proteolysis at the heparin-binding domain.

Treatment of EC-SODs with Neutrophil-Rich Fraction—Proteinase released from neutrophils might attack EC-SOD *in vivo* (*27*). Therefore, we compared the heparin affinity of m-EC-SOD and n-EC-SOD incubated with rat neutrophils. Because rat and human neutrophil proteinases display considerable homology (*28*), we used rat neutrophils to assess the susceptibility of EC-SODs to neutrophil-derived proteolytic enzymes. By exposure to 6.5×10^7 neutrophils/ml for 12 h, about 55% of the n-EC-SOD lost its high heparin affinity and it eluted from the heparin-HPLC column as multiple peaks with heterogeneous heparin affinity, like the EC-SOD in serum obtained from Group I individuals, whereas about 70% of the m-EC-SOD retained its high heparin affinity (Fig. 4). The reduction of heparin affinity by neutrophils was completely inhibited by 1 mM N-α-tosyl-L-lysine-chloromethyl ketone (TLCK, Sigma). Exposure to 2.5 μg/ml elastase from human leukocytes (Sigma) and 2.5 μg/ml cathepsin G from human leukocytes (Sigma), which are major neutrophil-released proteinases, did not impair the heparin affinity of either EC-SOD (data not shown).

Neutrophil-derived proteinases have been implicated in

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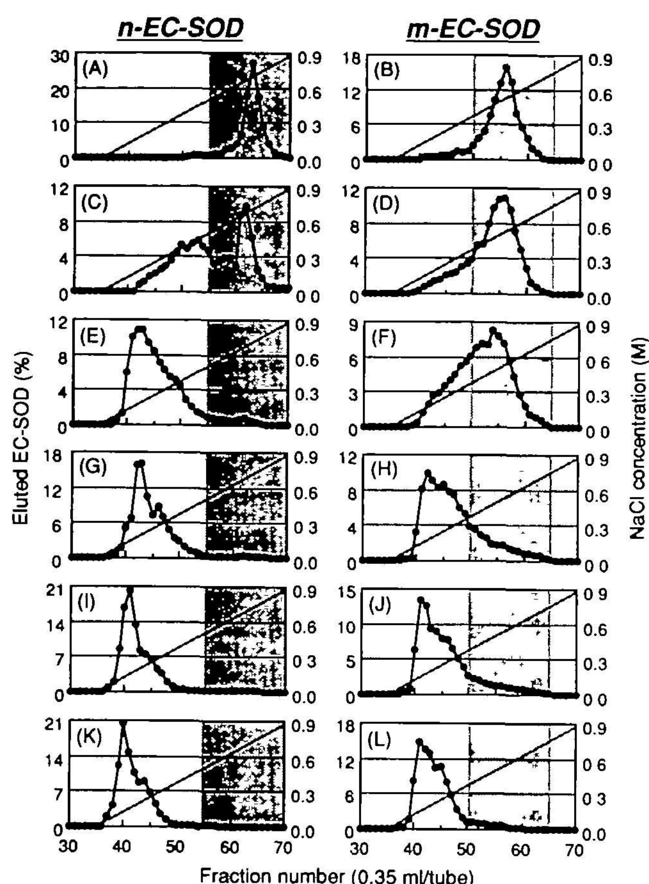

Fig. 2. **Heparin-HPLC of EC-SODs treated with trypsin.** Normal EC-SOD (left panels) and m-EC-SOD (right panels) were treated with 0 (A and B), 0.025 (C and D), 0.075 (E and F), 0.25 (G and H), 0.75 (I and J), and 2.5 (K and L) μg/ml of TPCK-trypsin at 25°C for 12 h, followed by heparin-HPLC as described in "EXPERIMENTAL PROCEDURES." Closed circles, EC-SOD concentration assayed by ELISA; dotted line, NaCl concentration in the buffer. Shadowed area shows the high-heparin-affinity fractions.

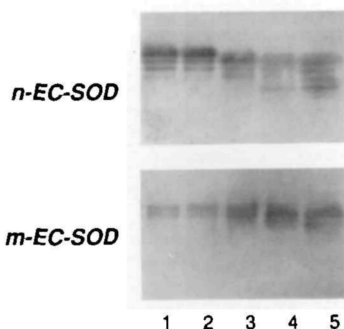

Fig. 3. **Western blots of EC-SODs treated with trypsin.** Normal EC-SOD (upper panel) and m-EC-SOD (lower panel) were treated with 0 (lane 1), 0.025 (lane 2), 0.075 (lane 3), 0.25 (lane 4), and 0.75 (lane 5) μg/ml of TPCK-trypsin at 25°C for 12 h, followed by SDS-PAGE and Western blotting.

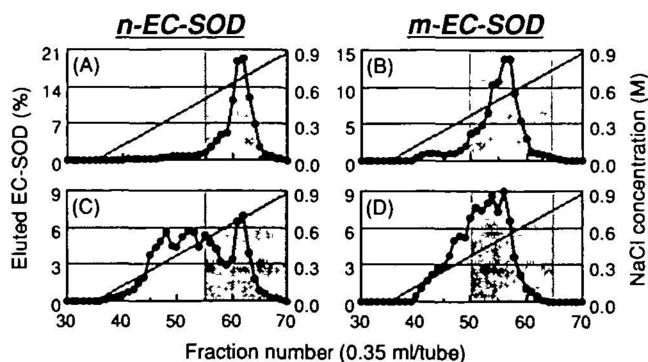

Fig. 4. **Heparin-HPLC of EC-SODs treated with the neutrophil-rich fraction.** Normal EC-SOD (left panels) and m-EC-SOD (right panels) were treated with rat neutrophil-rich fraction at 37°C for 0 h (A and B), 12 h (C and D), followed by heparin-HPLC analysis as described in "EXPERIMENTAL PROCEDURES." Shadowed area shows the high-heparin-affinity fractions.

the development of tissue injury in an increasing number of diseases including renal failure (*22, 27, 29*). The environment created by neutrophil-endothelial cell interaction plays an important role in the propagation of injury. The proteinases degrade proteoglycan molecules as well as many plasma proteins, and therefore play a key physiological role in modulation of inflammatory reactions. Although the proteinases are rapidly complexed and inactivated by proteinase inhibitors under normal conditions, the activities of inhibitors are often degraded under pathological conditions. Neutrophil superoxide production is primed by the proteinases (*30*), and superoxide at this site is sufficient to suppress the potentiality of proteinase inhibitors (*22*).

Neutrophil-released trypsin-like proteinases might reduce heparin affinity, because that of n-EC-SOD was easily reduced compared with m-EC-SOD, and the reduction was inhibited by TLCK. McCord *et al.* (*31*) have noted that trypsin-like proteinase released by neutrophils may cleave the C-terminal of EC-SOD coated endothelium and release EC-SOD A, which has no heparin affinity. The heparin affinity of n-EC-SOD was more sensitive to proteinases released from neutrophils than that of m-EC-SOD. This result is consistent with the fact that m-EC-SOD has the same primary structure as n-EC-SOD, except for the substitution of glycine for arginine-213, which would be a target of trypsin-like proteinases. The difference in susceptibility between them may cause serum EC-SOD in individuals without the R213G mutation, but not in those with it, to be heterogeneous in its heparin affinity, as shown in Fig. 1. In hemodialysis patients, neutrophils are activated and release large amounts of proteinases. This may increase the level of EC-SOD form (I), a proteolytic truncated form of EC-SOD (V), making it the major fraction in sera from these patients. On the other hand, EC-SOD in sera from individuals with the R213G mutation is not heterogeneous with regard to heparin affinity. This is consistent with the finding that m-EC-SOD is more resistant to trypsin and neutrophil-released trypsin-like proteinases than n-EC-SOD. It is known that adherence of neutrophils to endothelial cells is an important step for development of inflammation (*32*), and proteinases released from adhered neutrophils could injure the surrounding tissues without the interference of proteinase inhibitors in the microenvironment created by the neutrophil-endothelial cell interaction (*22*). It is possible that m-EC-SOD has little opportunity to be exposed to adhered neutrophil-released proteinases,

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because m-EC-SOD has lower affinity for aortic endothelial cells (*16*).

The R213G mutation of EC-SOD results in a significantly higher level of serum EC-SOD (*14, 16*). Among individuals without the mutation, the EC-SOD level in hemodialysis patients was significantly higher than that in healthy volunteers (data shown in Ref. *16*). An increase of form (I) in patients would also lead to a higher level of serum EC-SOD.

In conclusion, the R213G mutation of EC-SOD reduces not only its affinity for heparin, but also its susceptibility to trypsin-like proteinases, which causes quantitative and qualitative changes of this enzyme in the vascular system.

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