Substitution of glycine for arginine-213 in extracellular-superoxide dismutase impairs affinity for heparin and endothelial cell surface

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Extracellular-superoxide dismutase (EC-SOD) levels in sera divide into two discontinuous groups: a low-level group below 400 ng/ml and a high-level group above 400 ng/ml [Adachi, Nakamura, Yamada, Futenma, Kato and Hirano (1994) Clin. Chim. Acta **229**, 123–131]. Molecular genetic studies have shown that the donors in the high-level group have a single base substitution generating the exchange of glycine for arginine-213 (R213G) in the heparin-binding domain of EC-SOD [Sandström, Nilsson, Karlsson and Marklund (1994) J. Biol. Chem. **269**, 19163–19166; Yamada, Yamada, Adachi, Goto, Ogasawara, Futenma, Kitano, Hirano and Kato (1995) Jpn. J. Hum. Genet. **40**, 177–184]. The serum EC-SOD level in homozygote subjects was significantly higher than that in heterozygotes and in normal subjects. Serum EC-SOD from heterozygotes and homozygotes had equally decreased affinity for heparin, as judged by heparin-HPLC, as compared with that from normal donors. This result suggests that the serum EC-SOD in heterozygotes was mainly composed of the mutant form which has reduced heparin affinity. On the other hand, fibroblast cells derived from heterozygote subjects generated mRNA of both normal and mutant EC-SOD (m-EC-SOD), and expressed the corresponding proteins. EC-SOD is a tetrameric enzyme, and in heterozygote donors would be heterogeneous with regard to the constitution of normal and mutant subunits. The enzyme form consisting of only mutant subunits, the form with the weakest heparin affinity, can be preferentially driven out to the plasma phase, because EC-SOD in the vasculature exists in equilibrium between plasma and the endothelial cell surface. The binding of m-EC-SOD to bovine aortic endothelial cells was about 50-fold less than that of normal EC-SOD. This result suggests that the binding of m-EC-SOD to vascular endothelial cells is much decreased *in vivo*, which causes a high level of serum EC-SOD.

INTRODUCTION

Extracellular-superoxide dismutase (EC-SOD, EC 1.15.1.1.) is a secretory, tetrameric copper- and zinc-containing glycoprotein with a subunit molecular mass of about 30 kDa [1,2]. EC-SOD is the major SOD isoenzyme in plasma, lymph and synovial fluid [3,4], but occurs primarily in tissues, anchored to heparan sulphate proteoglycans in the glycocalyx of cell surfaces and in the connective tissue matrix; this form of the enzyme accounts for over 90% of the EC-SOD [5–7]. EC-SOD in plasma is heterogeneous with regard to heparin affinity [8,9] and can be divided into five fractions: form I, which lacks affinity; forms II and III, with weak affinity; and forms IV and V, with relatively strong affinity in heparin-HPLC [10]. Previously published data have suggested that EC-SOD form V, is the primary form synthesized in the body [11] and that EC-SOD forms I–IV with reduced heparin affinity seen in plasma are the result of endo- and exo-proteolytic truncations at the C-terminal end [12]. 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 [13,14]. In particular, the cluster of six basic amino acids, Arg-210–Arg-215, forms an essential part of the heparin-binding domain [12,15,16].

Serum EC-SOD levels from healthy persons are clearly divided into two groups: a low-concentration group (Group I, below 400 ng/ml) and a high-concentration group (Group II, above 400 ng/ml) [17,18]. A family study has suggested the genetic transmission of a high EC-SOD level in serum [19]. We found that $\approx 6.4\%$ of the Japanese population tested were high-plasma-level EC-SOD donors (Group II). In patients with various diseases, EC-SOD seemed to be divided into the above two groups. The frequency of Group II was significantly greater for haemodialysis patients than for healthy persons or other patients [18]. Sandström et al. also found persons having a high serum level of EC-SOD, and they represented about 2% of Swedish donors [20]. Furthermore, all of the donors in Group II were shown to have the same single-base substitution, generating the change of Arg-213 to glycine (R213G) [20], as determined from the known cDNA sequence [21]. In Japanese individuals having high serum EC-SOD, we also found the same gene mutation [22]. The 45 donors with a high serum level of EC-SOD were heterozygotes for the R213G mutation. Three homozygotes were found in haemodialysis patients.

During the studies on heparin affinity of serum EC-SOD, we observed that EC-SODs in sera obtained from heterozygotes and homozygotes had equally decreased heparin affinity. To determine whether both normal and mutant EC-SOD (m-EC-SOD) are expressed in heterozygote donors, EC-SOD mRNA in fibroblasts from heterozygotes was analysed. We then compared the heparin affinity of fibroblast-expressed EC-SOD obtained from normal, heterozygote and homozygote subjects.

It has been reported that in the vasculature, EC-SOD exists in equilibrium between the plasma phase and heparan sulphate proteoglycans in the glycocalyx of endothelial cell surfaces, and that the binding of EC-SOD to vascular endothelium correlates with its heparin affinity [8]. We have previously shown that EC-SOD could bind to cultured endothelial cells by binding to heparan sulphate proteoglycans on the cell surface [23]. To explain that the subtle decrease in the heparin affinity of m-EC-SOD detected by HPLC analysis causes a significant increase in

Abbreviations used: EC-SOD, extracellular-superoxide dismutase; r-EC-SOD, recombinant EC-SOD; m-EC-SOD, mutant EC-SOD; DTPA, diethylenetriamine-N,N,N′,N″,N″-penta-acetic acid; DMEM, Dulbecco's modified Eagle's medium; BAE cell, bovine aortic endothelial cell; FCS, fetal calf serum; TLCK, tosyl-lysylchloromethane; RT, reverse transcriptase.

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plasma EC-SOD levels in Group II individuals, the binding capacity of m-EC-SOD to cultured endothelial cells was also compared with that of normal EC-SOD in the present study.

EXPERIMENTAL

Materials

Human recombinant EC-SOD (r-EC-SOD), prepared as described previously [2], was kindly provided by SYMBICOM AB, Umeå, Sweden. *o*-Phenanthroline, PMSF and diethylenetriamine-N,N,N′,N″,N″-penta-acetic acid (DTPA) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Benzamidine was purchased from Tokyo Kasei, Tokyo, Japan.

SOD analysis

EC-SOD concentration was determined by ELISA as described previously [24].

cDNA analysis

Total cellular RNA was isolated from the fibroblasts (1×10^7 cells) using an RNA separation kit (ISOGEN, Nippon Gene, Inc., Tokyo, Japan). mRNA was separated by oligo(dT)-cellulose (Type 3, Collaborative Research, Inc., Bedford, MA, U.S.A.) chromatography, as described previously [25]. The first strand cDNA was generated using random hexanucleotide primers and reverse transcriptase (First-strand cDNA synthesis kit, Pharmacia LKB Biotechnology, Uppsala, Sweden). Then, the entire coding region of EC-SOD cDNA was PCR-amplified, similarly to the amplification from genomic DNA [22], and sequenced directly using specific primers. Amplification was carried out with 2 units of *Taq* DNA polymerase (Ampli-Taq, Perkin-Elmer obtained from Roche Molecular Systems, Branchburg, NJ, U.S.A.) and 250 ng of template DNA, in a final volume of 100 μl, using a program-controlled temperature system (PC-700 Astec, Fukuoka, Japan) of 40 cycles (94 °C, 1 min; 60 °C, 1 min; 70 °C, 2 min). The protocols for direct sequencing were slightly modified from that of the 7-de-aza-dGTP Reagent kit for Sequenase Version 2.0 (United State Biochemical Co., Cleveland, OH, U.S.A.), as described previously [25].

Fibroblast cell culture and preparation of EC-SOD in fibroblast-conditioned medium

Human skin fibroblast cell lines were initiated from skin punch biopsy specimens obtained from volunteers. Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10 % (v/v) fetal calf serum (FCS), 4 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin was used as medium, and the cells were kept in an atmosphere of 5 % CO_2/95 % air at 37 °C. Cultures were grown in 80-cm² culture flasks containing 10 ml of medium. When the cells had reached the subconfluent state, the medium was replaced with DMEM supplemented with glutamine, antibiotics and 0.5 % (v/v) FCS. Three days later, the conditioned medium was harvested. EC-SOD in the conditioned medium was precipitated by the addition of solid ammonium sulphate (80 % saturation). The precipitate was suspended in the minimum volume of distilled water and dialysed overnight against 25 mM sodium phosphate buffer, pH 6.5, containing 2 mM Na_2EDTA, 0.5 mM PMSF, 1 mM DTPA, 0.5 mM benzamidine and 0.5 mM *o*-phenanthroline at 4 °C.

Heparin-HPLC

Heparin-affinity chromatography was performed on a HPLC-column of TSKgel heparin-5PW (7.5 mm × 7.5 cm, Tosoh, Tokyo, Japan). The column chromatography was operated at a flow rate of 0.7 ml/min and 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) was used.

Purification of m-EC-SOD

Anti-(r-EC-SOD) monoclonal antibody was coupled to activated CH-Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden) at a concentration of 3.5 mg/ml swollen gel, essentially according to the manufacturer's suggestions. Pooled serum (126 ml) from heterozygote donors was supplemented with 2 mM Na_2EDTA, 0.5 mM PMSF, 1 mM DTPA, 0.5 mM benzamidine and 0.5 mM *o*-phenanthroline (final concentrations) and then dialysed against 0.5 M NaCl/0.1 M $NaHCO_3$ containing the above proteinase inhibitors. The dialysed serum was next passed through a paper filter and applied to anti-(r-EC-SOD), monoclonal antibody-conjugated Sepharose 4B (2.5 cm × 1 cm) equilibrated with 0.5 M NaCl/0.1 M $NaHCO_3$. After the column had been washed continuously with the same solution until the effluent showed no measurable absorbance at 280 nm, the adsorbed EC-SOD was eluted with 0.5 M NaCl/0.2 M Na_2CO_3. The pH of each fraction was neutralized with the minimum volume of 2 M acetic acid. The immunoreactive fractions (assayed by ELISA) were pooled and then dialysed against buffer A. The dialysed sample was applied to a heparin–Sepharose column (1 cm × 2.5 cm, Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with buffer A and then washed with the same buffer. The bound EC-SOD was subsequently eluted with a linear gradient of NaCl (0–1 M) in buffer A. EC-SOD was pooled and concentrated with a YM-10 membrane filter. This preparation was used as purified m-EC-SOD. For analysis of the C-terminal amino acid sequence, reverse-phase HPLC of m-EC-SOD was performed on a column of ULTRON 300 C_4 (4.6 mm × 15 cm, Shinwa Chemical Ind., Ltd., Kyoto, Japan) at flow rate of 0.7 ml/min. A programmed gradient system formed between solvent A (0.1 % trifluoroacetic acid in Milli Q water) and solvent B (0.1 % trifluoroacetic acid in acetonitrile) was used. The sample was eluted with a linear gradient up to 35 % of solvent B for 15 min, and continuously up to 55 % of solvent B for 40 min.

Amino acid sequence analysis of the C-terminal peptide

Purified m-EC-SOD or r-EC-SOD (70 μg) in a test tube was dissolved in 1 ml of 0.5 M Tris/HCl, pH 8.5, containing 7 M guanidinium chloride and 10 mM Na_2EDTA, and the tube was then flushed with nitrogen. The sample was thereafter reduced with 3 mg of dithiothreitol, flushed with nitrogen again, and incubated for 2 h at room temperature. After the addition of 8 mg of iodoacetic acid, the reaction mixture was incubated in the dark for 30 min, followed by extensive dialysis against 10 mM sodium acetate, pH 6.0, at 4 °C. After rotary evaporation, the residue was dissolved in 100 μl of 10 mM Tris/HCl, pH 8.6, and digested with 10 μl of 0.1 mg/ml tosyl-lysylchloromethane (TLCK)-chymotrypsin (Sigma, St. Louis, MO, U.S.A.) at 25 °C for 20 min.

The chymotryptic digests of the EC-SODs (m-EC-SOD and r-EC-SOD) were applied to a heparin-HPLC column equilibrated with buffer A. The column chromatograph was operated at a flow rate of 0.5 ml/min. The column was washed with the same buffer for 20 min and the buffer containing 0.15 M NaCl for 20 min. Then the bound peptides were eluted with buffer B.

These peptides were separated by C_{18} reverse-phase HPLC (column 3.9 mm × 15 cm, Nihon Waters Ltd., Tokyo, Japan) operated at a flow rate of 0.7 ml/min. A gradient system formed between solvent A and solvent B was used. Peptides were eluted with a programmed gradient (0 % B for 5 min, 0–60 % B for 60 min and 60–100 % B for 10 min). The peptides were detected on the basis of the A_{210}. The amino acid sequence was determined with an Applied Biosystems Protein Sequencer Model 473A (Foster City, CA, U.S.A.).

Binding of EC-SOD to bovine aortic endothelial (BAE) cells

BAE cells were kindly donated by Takeda Chemical Industries, Ltd., Osaka, Japan. The cells were cultured in DMEM supplemented with 10 % FCS, 4 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin as medium. They were kept in a 5 % CO_2 atmosphere at 37 °C. All experiments were performed with confluent cell monolayers in 35-mm tissue culture dishes. At confluence, the cells were maintained overnight in DMEM containing 10 mM Hepes without FCS and used the next day. Confluent BAE cell monolayers in 35-mm dishes were rinsed with DMEM containing 10 mM Hepes and 0.5 % BSA (DMEM-BSA), then 1 ml of DMEM-BSA containing various concentrations of m- or r-EC-SOD was added and the cells were incubated at 4 °C for 1 h. At the end of the incubation period, the unbound EC-SOD was removed and the cells were washed three times with PBS. The cells were then incubated at 4 °C for 30 min with 1 ml of DMEM-BSA containing 10 mg/ml heparin to remove the cell-surface-bound EC-SOD. The enzyme was then quantified by ELISA.

Binding of EC-SOD to immobilized heparin

A 200 μl portion of 10 mg/ml heparin in 0.1 M potassium phosphate, pH 7.0, containing 0.02 % Tween 20 was added to each well of a 24-well multi-dish and left to stand overnight at 4 °C. Each well was washed with 0.1 M potassium phosphate, pH 7.0, containing 0.3 M NaCl, 1 % BSA and 0.1 % NaN_3, and then 1 ml of the same buffer was added. The plate was left to stand at 4 °C before use (heparin-coated plate). After each well of the heparin-coated plate had been rinsed with DMEM containing 10 mM Hepes, 0.5 % BSA and 0.02 % Tween 20 (DMEM-BSA-Tween), 200 μl of DMEM-BSA-Tween containing various concentrations of m- or r-EC-SOD was added to each well and the plate was incubated at 4 °C for 1 h. At the end of the incubation period, the unbound EC-SOD was removed and the plate was washed three times with PBS. The plate was then incubated at 4 °C for 30 min with 200 μl of DMEM-BSA-Tween containing 1 M NaCl and 10 mg/ml heparin to remove the bound EC-SOD. The EC-SOD was then quantified by ELISA.

RESULTS AND DISCUSSION

EC-SOD levels in sera from subjects with and without the R213G mutation

We assayed the EC-SOD concentration in sera from subjects (53 healthy subjects and 66 haemodialysis patients) whose EC-SOD genes had been analysed by direct sequencing (Figure 1). The EC-SOD level in heterozygote subjects (1009 ± 512 ng/ml, 7 healthy subjects and 38 patients) was significantly higher than that in subjects without the mutation (186 ± 494 ng/ml, 45 healthy subjects and 25 patients). We found three homozygotes in patients. Their serum EC-SOD level (2531 ± 469 ng/ml) was significantly higher than that of the other groups. Four haemodialysis patients without the R213G mutation showed very high EC-SOD levels in their sera. When the entire coding region of their EC-SOD genes was re-analysed, however, no mutations which would participate in the increase in serum EC-SOD were found. The high levels of EC-SOD found in sera might result from the obstruction of excretion, hyper-production and inhibition of binding to endothelial cells of EC-SOD caused by renal failure.

Heparin affinity of serum EC-SOD

Serum EC-SOD samples obtained from 20 normal subjects, 20 heterozygotes and three homozygotes were tested for affinity for heparin, and typical chromatograms obtained are shown in Figure 2. Figure 2(A) shows that EC-SOD in serum obtained from a normal donor (N-1) was separated into five fractions I to V. An apparent peak of form V, eluted at 0.66 M NaCl, is a minor fraction in serum and may exist almost anchored to heparan sulphate proteoglycan in the glycocalyx of cell surfaces and in the connective-tissue matrix. The already published observation, that the intravenous injection of heparin led to a rise of only form V in serum, strongly supports the above suggestion [10]. The other forms of EC-SOD, I to IV, are secondary modified forms that arise by such processes as proteolysis of the C-terminal end [15,16].

On the other hand, EC-SODs in sera from a heterozygote (HE-1) and a homozygote (HO-1) consisted mainly of the high heparin affinity type, but with decreased affinity, as evidenced by their elution from the HPLC column at about 0.53 M NaCl (Figures 2B and 2C). This result suggests that serum EC-SOD also in the HE-1 individual was mainly composed of the mutant subunit. To confirm this possibility, the C-terminal peptide (Glu-201-C-terminus) of m-EC-SOD purified from heterozygote donors' sera was isolated by heparin-HPLC (Figure 3A) and C_{18} reverse-phase HPLC (Figure 3B), as described in the Experimental section, and the amino acid sequence of the peptide was analysed. An amino acid sequence of Glu-201–Ala-222 in r-EC-SOD, but with Arg-213 substituted by glycine, was detected. The reason for the presence of the multiple forms of EC-SOD in the normal population and not in the mutant population is unclear. However, it is possible that the susceptibility of EC-SOD to proteinases is decreased by the R213G mutation.

mRNA analysis

The serum results are consistent with the possibility that the heterozygotes generated only the mutant form, or that they generated normal as well as mutant forms. To answer this question, we used RT-PCR to amplify EC-SOD cDNA from the poly(A)$^+$ RNA isolated from the fibroblasts of donors N-1 and HE-1 and sequenced it directly. These sequencing patterns were the same as those in the genomic analysis [22]. The HE-1 donor expressed both normal and mutant EC-SOD mRNAs, since the direct sequencing of the reverse transcriptase (RT)-PCR product showed both C (normal) and G (mutant) bands.

Heparin affinity of EC-SOD in fibroblast-conditioned medium

Although heterozygotes generate the mRNA of both normal and mutant forms of EC-SOD, it is not clear that the corresponding proteins are expressed. To determine whether both forms of EC-SOD are expressed in heterozygote donors, the heparin affinity of fibroblast-expressed EC-SOD was compared. EC-SODs secreted by the fibroblast cells obtained from N-1, HE-1 and HO-1 donors were of the high heparin affinity type, the affinity of EC-SOD in HO-1 (eluted with 0.53 M NaCl; Figure 4C) was the weakest among the three. N-1 fibroblast-secreted EC-SOD

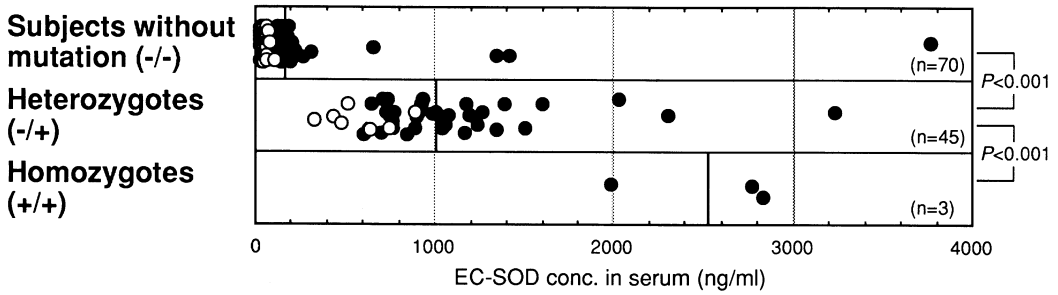

Figure 1 EC-SOD concentration in sera from subjects without mutation (−/−), heterozygotes (−/+) and homozygotes (+/+) for R213G

Serum EC-SOD levels for healthy subjects and haemodialysis patients are indicated by open circles and closed circles respectively. Vertical lines show mean values. The differences between the two groups were statistically significant at $P < 0.001$ by Student's t test.

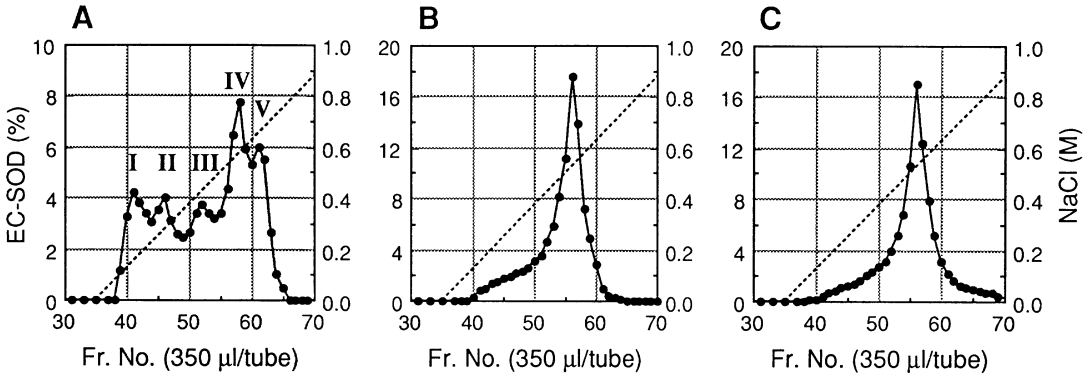

Figure 2 Heparin-HPLC of serum from an individual without mutation, N-1 (A), a heterozygote, HE-1 (B) and a homozygote, HO-1 (C)

Chromatography was conducted as described in the Experimental section. ●, EC-SOD concentration assayed by ELISA; - - -, NaCl concentration in buffer.

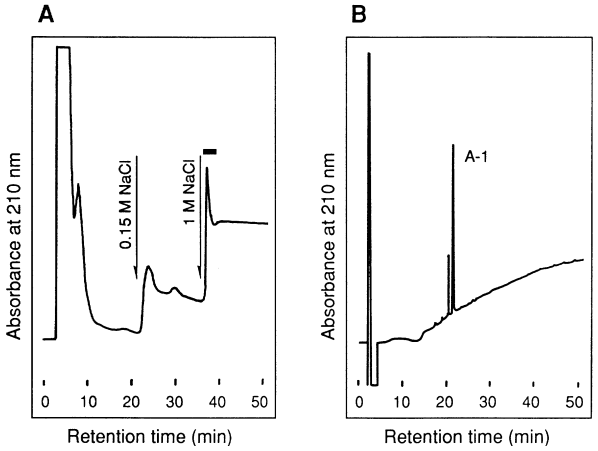

Figure 3 C-Terminal amino acid sequence of mutant EC-SOD purified from the sera of heterozygotes

Purified EC-SOD was digested with TLCK-chymotrypsin as described in the Experimental section. TLCK-chymotrypsin-digested peptides were applied to a heparin-HPLC column (**A**). The fraction indicated by the horizontal bar was subjected to C_{18} reverse-phase HPLC (**B**). The amino acid sequence of the main peptide isolated (A-1) was determined.

(Figure 4A) and r-EC-SOD (results not shown) had highest affinities and were eluted at 0.70 M NaCl. As shown in Figure 4(B), HE-1 fibroblast-expressed EC-SOD was eluted as ternary peaks, with the main peak at 0.63 M and the two minor ones at 0.53 and 0.70 M NaCl. Fibroblast-conditioned media from other subjects (nine normal subjects, a heterozygote and a homozygote) showed similar chromatograms to those described above.

The multiple peaks may consist of EC-SODs with heterogeneous heparin affinities, due to the co-operative effect of normal (high affinity) and mutant (low affinity) subunits. It is known that EC-SOD occurs mostly anchored to the connective-tissue matrix and to heparan sulphate proteoglycan in the glycocalyx of cell surfaces [7]. Among the various EC-SOD forms with different heparin affinities, the EC-SOD tetramer consisting of only mutant monomers may be preferentially released from the tissue interstitium heparan sulphate into the plasma phase and appear as the main form in the plasma of heterozygote donors.

Binding of EC-SOD to BAE cells

We have posed the question as to why the subtle difference in heparin affinity detected by HPLC analyses between normal and mutant EC-SOD (shown in Figure 4) causes the significant differences in serum EC-SOD levels between subjects with and without the R213G mutation (shown in Figure 1). As described in the Introduction, EC-SOD in the vasculature forms an equilibrium between plasma and endothelial cell surface. Therefore, the binding capacity of m-EC-SOD to BAE cells was compared with that of r-EC-SOD. The estimated maximum binding of m-EC-SOD to BAE cells, 14.2 ng/dish (78.0 ng/mg of cell protein), was 50 times lower than that of r-EC-SOD, 820 ng/dish (4505 ng/mg of cell protein), as shown in Figure 5. On the other hand, the maximum binding of m-EC-SOD to heparin-coated multi-dishes, 2.69 ng/well, was not significantly different from that of r-EC-SOD, 2.77 ng/well.

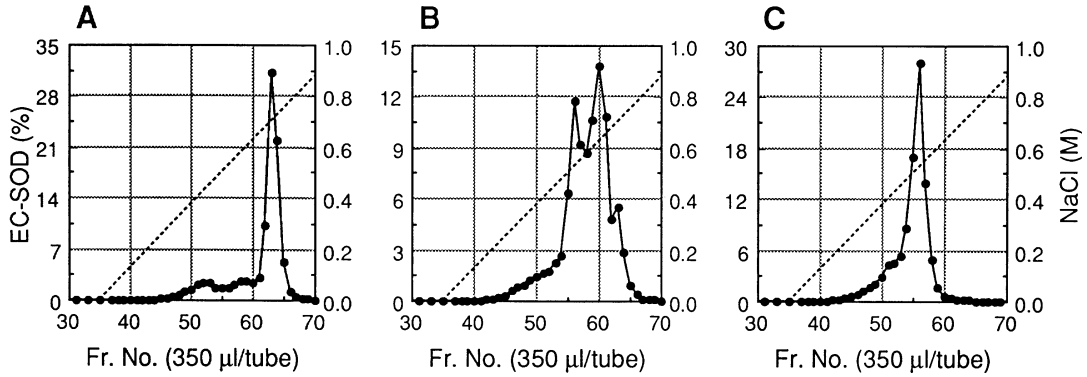

Figure 4 Heparin-HPLC of media conditioned by fibroblast cells from an individual without mutation, N-1 (A), a heterozygote, HE-1 (B) and a homozygote, HO-1 (C)

●, EC-SOD concentration assayed by ELISA; - - -, NaCl concentration in buffer.

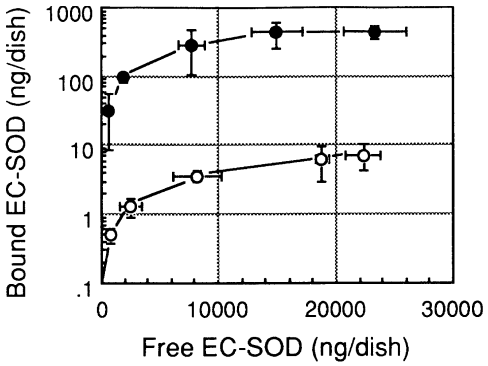

Figure 5 Binding of EC-SODs to BAE cells

Dishes containing confluent BAE cells were used. A 1 ml volume of DMEM-BSA containing the indicated concentrations of m-EC-SOD (○) or r-EC-SOD (●) were added and the cells were incubated at 4 °C for 1 h. After the incubation, the medium was removed and the cells were rinsed with PBS. Bound EC-SOD was estimated by the treatment of cells with 10 mg/ml heparin in DMEM-BSA, followed by ELISA. Results represent the means ± S.D. of triplicate experiments.

The maximal amounts of enzyme bound to BAE cells were much different between m-EC-SOD and r-EC-SOD, whereas the amounts of both EC-SODs bound to immobilized heparin were not significantly different from each other. Heparin is known to be the most potent (at least 10-fold stronger) ligand of EC-SOD compared with other sulphated glycosaminoglycans such as heparan sulphate, dermatan sulphate and condroitin sulphate [5]. Sulphated glycosaminoglycans on the cell surface are heterogeneous, and components with low affinity for EC-SOD may not be able to trap the m-EC-SOD. This result suggests that m-EC-SOD binds weakly to the endothelial cell surface *in vivo*, and causes the high serum levels of EC-SOD found in individuals with the R213G mutation. It is possible that the R213G mutation affects the stability of EC-SOD *in vivo*, because the heparin-binding ability of EC-SOD may contribute to the internalization and metabolism of EC-SOD *in vivo* [23].

We have reported previously that serum EC-SOD levels were distinctly higher in patients with renal diseases and that the increase in this enzyme was accompanied by a lack of renal function [18]. Recently, it was found that the mutation frequency of EC-SOD in haemodialysis patients was about twice that in healthy persons [22]. There are data to suggest that the binding of EC-SOD to cellular surfaces is an especially efficient way of protecting cells against external superoxide anion. Further studies will be undertaken to determine whether or not this mutation is a prognostic factor in diseases, particularly chronic renal disease.

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