

MOLECULAR ANALYSIS OF EXTRACELLULAR-SUPEROXIDE DISMUTASE GENE ASSOCIATED WITH HIGH LEVEL IN SERUM

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Summary Extracellular-superoxide dismutase (EC-SOD) is one of the SOD isozymes mainly distributed in the extracellular fluid. In the vascular system, it is located on the endothelial cell surface according to studies on the heparin binding capacity. By measurement of serum EC-SOD levels of Japanese in healthy persons (n=103) and hemodialysis patients (n=150), 7 healthy subjects and 24 hemodialysis patients were classified into group II associated with high EC-SOD levels. By molecular analysis of the EC-SOD coding region from the group II individuals in Sweden, a single nucleotide substitution of G to C generating an amino acid change of arginine to glycine has been identified in the region associated with the heparin affinity of the enzyme. The same mutation was detected in the Japanese as a homozygote in both alleles of 2 hemodialysis patients and as a heterozygote in one allele of all the healthy group II individuals and 17 hemodialysis patients. The amino acid substitution may result in the decrease of the heparin affinity which is favorable for the existence of EC-SOD in the serum.

Key Words superoxide dismutase, endothelial cell, renal failure, hemodialysis

Received December 6, 1994; Revised version accepted April 3, 1995.

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INTRODUCTION

Reactive oxygen species have been implicated in many disease processes. Superoxide radicals are toxic radicals and play a major role as the source of other oxygen radicals. Superoxide dismutase (SOD) is the main protector against the superoxide anion in the living body. There are three isozymes; Cu,Zn-SOD in the intracellular space, Mn-SOD in the mitochondria and extracellular-superoxide dismutase (EC-SOD) in the extracellular space (Marklund, 1984a, b). A characteristic distinguishing EC-SOD from Cu,Zn-SOD or Mn-SOD is the heparin binding capacity (Karlson *et al.*, 1988; Adachi and Marklund, 1989). In the vascular system, EC-SOD binds on the surface of endothelial cells through the heparan sulfate proteoglycan, and eliminates the oxygen radicals from the NADPH dependent oxidative system from the neutrophils. Since the EC-SOD mainly exists on the surface of endothelial cells, a small amount of EC-SOD exists in the plasma and forms an equilibrium with the endothelial cell surface (Karlson *et al.*, 1993), reflecting the circumstances of endothelial surface.

Recently, we established an immunoassay system for EC-SOD and measured the EC-SOD level in the serum (Adachi *et al.*, 1992a). In healthy subjects, we found that 6% of the persons have a high EC-SOD level which was a 10- to 15-fold higher than the mean EC-SOD level in all subjects. The serum EC-SOD level in healthy persons defined by the heredity (Adachi *et al.*, 1993). Most recently, Sandström *et al.* (1994) also reported that about 2% of the plasma donors in Sweden had an 8- and 10-fold higher EC-SOD level and that a single base substitution of C to G at position 760 of the cDNA was responsible for the high level of EC-SOD in plasma. In this study, we performed molecular analysis of the EC-SOD gene from Japanese individuals having a high serum EC-SOD level and detected the same C to G mutation in healthy persons and hemodialysis patients.

MATERIALS AND METHODS

Subjects. The subjects were 103 healthy persons who had participated in an annual health check up and 150 renal failure patients receiving hemodialysis therapy. Healthy persons had no abnormalities in the physical examination and medical laboratory findings. Renal failure patients had been regularly receiving hemodialysis therapy for several years and did not have other severe diseases.

Measurement of serum EC-SOD levels. A two step enzyme-linked immunosorbent assay (ELISA) method was used for measurement of serum EC-SOD (Adachi *et al.*, 1992a). The lower limit of detection was 50 pg/ml and the working range was up to 50 ng/ml. This ELISA system showed no cross reactivities with other SOD isozymes.

Extraction of human genomic DNA. Human genomic DNA was isolated from blood leucocytes using DNA isolation kit (Micro-Turbogen, Invitrogen).

PCR amplification. The PCR mixture consisted of 50 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mg/ml gelatin, 0.15 mM of 7-deaza-2'-dGTP and 0.05 mM dGTP, 0.2 mM of each other dNTP, 1 μM of each primer, 2 units of *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer Cetus), and 500 ng of genomic DNA, in the final volume of 0.1 ml (Yamada *et al.*, 1993). Standard PCR amplification was carried out for 40 cycles (94°C, 1 min; 60°C, 1 min; 70°C, 2 min).

DNA sequencing. The protocol for direct sequencing was slightly modified from that of the 7-deaza-dGTP Reagent Kit for Sequenase Version 2.0 (United States Biochemical), as described previously (Yamada *et al.*, 1992). DNA sequences were entered into a personal computer and analyzed with a software of gene analysis, GENETYX version 9.0 (SDC, Japan).

RESULTS AND DISCUSSION

We examined the serum EC-SOD content and its distribution in healthy persons and hemodialysis patients. The distribution of the serum EC-SOD contents was discontinuous in healthy persons (n=103), and the contents were clearly separated into two groups; a lower levels (group I) below 400 ng/ml and a higher levels (group II) above 600 ng/ml, as described previously (Adachi *et al.*, 1992a). Ninety six healthy persons were classified into group I with a mean level of 52.7 ± 18.4 ng/ml and 7 into group II with that of 573.2 ± 176.6 ng/ml. The serum EC-SOD content in hemodialysis patients (n=150) was also classified into group I (126 subjects, 191.6 ± 65.8 ng/ml) and group II (24 subjects, $1,695.2 \pm 947.1$ ng/ml). The frequency of persons belonging to group II and the mean of the serum EC-SOD values were higher in the hemodialysis patients than those in the healthy persons.

To study the qualitative heterogeneity of serum EC-SOD, we performed molecular analysis of the EC-SOD gene. The nucleotide sequence of human EC-SOD cDNA (J02947 in EMBL database) has been reported by Hjalmarsson *et al.* (1987). Based on the sequence, 10 specific oligonucleotide primers were designed for polymerase chain reaction (PCR) and direct sequencing (Fig. 1). The entire coding region of EC-SOD was amplified as two separate DNA fragments; PC104 (480 bp), using primers, SE1A and SE4B; PC201 (614 bp), using primers, SE2A and SE1B, and directly sequenced using all the primers. First, we analyzed the cDNA synthesized from mRNA in the fibroblasts, in which EC-SOD was expressed relatively high (Marklund, 1990). Although both fragments could be amplified by RT-PCR adding 7-deaza-2'-dGTP, the reproducibility was not good. The reaction by reverse transcriptase might fail because of high GC contents of mRNA (Hjalmarsson *et al.*, 1987). Therefore we tried to analyze the genomic DNA since the entire coding region of EC-SOD seems to be contained in one exon (personal discussion by Dr. Marklund, of the Umea University Hospital, Sweden). Amplifications from the genomic DNA resulted in the same products as those produced from the cDNA. As Fig. 2 shows, a single nucleotide substitution of G to C at nucleotide position (nt.) 760 was identified by the analysis of genomic DNA. A substitution of Arg (codon

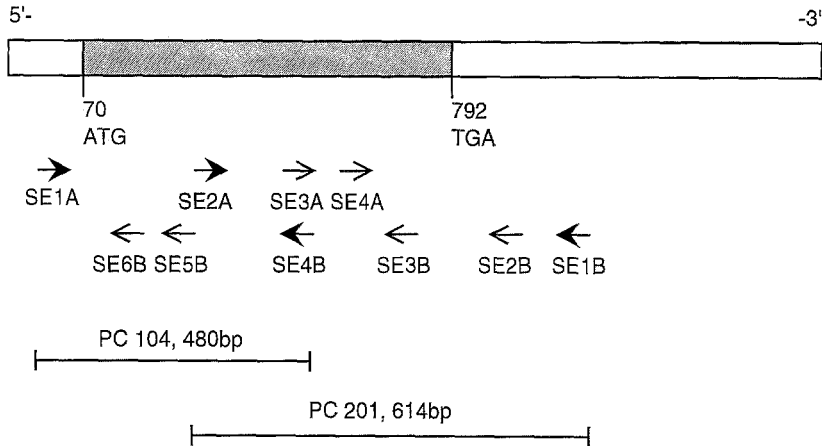


Fig. 1. Molecular analysis of the entire coding region of EC-SOD. 10 oligonucleotide primers (indicated by arrows) were provided: 4 sense primers; SE1A (5'-AGGA-GCTGGAAAGGTGCCCGACTC-3'), SE2A (5'-TTCCGACCGAGCCGAA-CA-3'), SE3A (5'-GACTTCGGCAACTTCGCGGTCC-3'), SE4A (5'-GCCAG-CGTGGAGAACGGGAA-3') and 6 antisense primers; SE1B (5'-GGATGGT-GGGTCTCGGTATAGGGA-3'), SE2B (5'-GAGTGGAAAGGTGTCTGTTGG-AG-3'), SE3B (5'-ACACGCCACCACGCAGCAG-3'), SE4B (5'-GGACCGC-GAAGTTGCCGAAGTC-3'), SE5B (5'-AAGCTGCCGGAAGAGGACGA-3'), SE6B (5'-CTCCGTGACCTTGGCGTACAT-3'). Two separated DNA fragments, PC104 and PC201, covering the entire coding region of EC-SOD were amplified using primer pairs SE1A-SE4B and SE2A-SE1B, respectively, and sequenced directly using all the primers. Open boxes show the noncoding region and a screened box indicates the coding region of EC-SOD cDNA. The position numbers refer to those from the database (J02947 in EMBL DNA database).

CGG) at amino acid position 213 by Gly (codon GGG) is deduced from this mutation. This mutation in Japanese subjects is the same single base substitution of C to G at position 760 in 2% of plasma donors in Sweden by Sandström *et al.* (1994). This mutation was detected in both alleles of 2 hemodialysis patients in group II, in one allele of all healthy persons in group II and 17 hemodialysis patients in group II (Table 1). The frequency of the identified mutant allele in hemodialysis patients (21/300) was about twice of that in healthy persons (7/206). In 5 hemodialysis patients in group II, any changes which would participate the increase of serum EC-SOD were not found in the coding region of EC-SOD.

The 21 amino acids at the carboxyl terminal (C-terminal) of the EC-SOD protein include 9 basic amino acids (6 Arg and 3 Lys) and play an important role for the binding capacity with heparan sulfate (Karlsson *et al.*, 1988; Adachi and Marklund, 1989). Furthermore, recent reports (Adachi *et al.*, 1992b; Sandström *et al.*, 1992; Karlsson *et al.*, 1993) support that the cluster of six basic amino acids, Arg-Lys-Lys-Arg-Arg-Arg, at aa.210-215, forms the essential part of the heparin binding

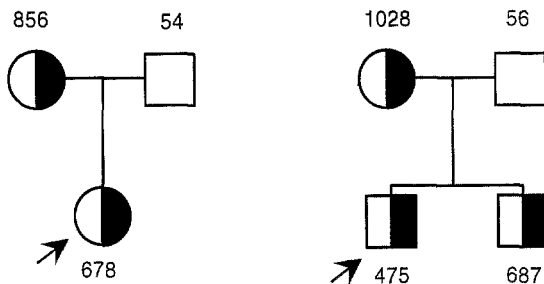


Fig. 3. Family studies of two healthy individuals on the relationship between the mutation and EC-SOD level in serum. Individuals of male (square) and female (circle), are diagnosed as normal (open) and heterozygotes of the mutation (half-filled) by direct sequencing. Circles and squares indicate female and male, respectively. Arrows indicate probands. The values show EC-SOD level in serum (ng/ml).

in heparin affinity between the EC-SOD in group I and that in group II were demonstrated by heparin-Sepharose column chromatography. The serum EC-SOD in group I consisted of three approximately equal fractions; fraction A without affinity, fraction B with weak affinity, and fraction C with relatively strong heparin-affinity, whereas that in group II consisted mainly of fraction C. Since the absolute level of EC-SOD A and B in group II were almost equal to those in group I, the difference in EC-SOD level resulted from the content of EC-SOD C (Adachi *et al.*, 1992a). Furthermore, by preliminary studies using heparin HPLC chromatography (data not shown), we could observe differences in the elution of fraction C among individuals with and without the mutation. Fraction C from persons in group I is mainly eluted with about 0.65 M NaCl, but that with the mutation is eluted with about 0.52 M NaCl. The elution pattern of the fractions in 5 hemodialysis patients in group II without mutation, was similar to that in group I. The detailed studies by the heparin HPLC including the differences between the homozygote and heterozygote of the mutation, are now in progress. We hope to publish the results as soon as possible.

The serum EC-SOD level in chronic renal failure became high as a result of the obstruction of the excretion from the blood to urine through the kidney by the renal dysfunction (Adachi *et al.*, 1994). Five hemodialysis patients had a high EC-SOD level, nevertheless no substitution of basic amino acids was detected. In the hemodialysis patients, the high EC-SOD level in the serum might result from the obstruction of excretion, hyper production, and inhibition of binding to endothelial cells of EC-SOD caused by the renal failure.

Oxygen radicals play a major role in the pathogenesis in experimental kidney diseases (Shah, 1989) and other diseases such as carrageenan-induced paw edema and postischemic cardiac arrhythmias (Oyanagui *et al.*, 1991; Inoue *et al.*, 1991a). Inoue *et al.* synthesized the modified SOD consisting of human Cu,Zn-SOD and

a basic peptide attached to the carboxyl terminal that binds to heparin-like proteoglycans, and studied its pathophysiological roles (Inoue *et al.*, 1991b). The modified SOD with high affinity for endothelial cells suppressed carrageenan-induced paw edema in mice (Oyanagui *et al.*, 1991) and the postischemic cardiac arrhythmias (Inoue *et al.*, 1991a), but native Cu,Zn-SOD did not. The heparin binding capacity in this case was the most important factor to express the pharmacological activities for the modified SOD. The low heparin affinity was strongly suggested to be a disadvantage for the protective activity of the EC-SOD. In this study, we identified the mutation which seems to decrease the heparin affinity of EC-SOD. Although the data may be insufficient, two homozygotes for the detected mutation alleles were found only in hemodialysis patients and the frequency of the mutant allele in the patients was calculated to be twice that in the healthy persons. The decrease of the protective capacity on the endothelial surface by EC-SOD might accelerate the renal dysfunction. Further studies are underway to determine, whether the existence of this mutation is an important prognostic factor in chronic renal disease or not.

Acknowledgments We thank Dr. Marklund, Umea University Hospital, Sweden, for valuable suggestions about the genomic structure of EC-SOD.

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