Changes in the Heparin Affinity of Extracellular-Superoxide Dismutase in Patients with Coronary Artery Atherosclerosis

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Extracellular-superoxide dismutase [EC 1.15.1.1] (EC-SOD) is a secretory glycoprotein with high affinity for heparin. This enzyme locates in blood vessel walls at a high enough level to suppress oxidative stress under normal conditions. EC-SOD is the major SOD isozyme in plasma, anchored to heparan sulfate proteoglycans in the glyocalyx of endothelial cell surfaces. Plasma EC-SOD is heterogeneous in heparin affinity and can be divided into five fractions, I to V, by heparin-HPLC. It has been suggested that EC-SOD form V is the primary form synthesized in the body and that EC-SOD forms with reduced heparin affinity are the result of proteolytic truncation of the C-terminal end of EC-SOD form V which is responsible for the binding with heparin. Recently, we reported that only plasma EC-SOD form V, with the highest heparin affinity, was increased by intravenous injection of heparin. The heparin affinity of plasma EC-SOD in patients with coronary atherosclerosis (CA+ patients) was compared in this study. The increase of plasma EC-SOD form V after heparin injection in CA+ patients was significantly less than that in subjects without evidence of stenosis in their major coronary arteries (CA− subjects). On the other hand, in CA+ patients, EC-SOD forms I to III, with low heparin affinity, were significantly increased compared to those in CA− subjects. EC-SOD in plasma apparently forms an equilibrium between the plasma phase and endothelial cell surface, and EC-SOD on the endothelial cell surface contributes to protecting the vessel wall against oxidative stress. These findings suggest that the quantitative and qualitative changes of EC-SOD, i.e., the decrease of bound EC-SOD on the endothelial cell surface, might suppress the defense systems against oxidative stress, which causes in part the development of coronary artery atherosclerosis.

Key words extracellular-superoxide dismutase; heparin; endothelial cell; coronary artery atherosclerosis

The occurrence of highly reactive oxygen species and their destruction by anti-oxidants are in equilibrium in healthy mammalian organisms, and disturbing this homeostasis causes numerous diseases. Active oxygen species can be produced extracellularly via the respiratory bursts of activated neutrophils and/or the enzymatic reaction of xanthine oxidase. It has been proposed that free radicals are involved in the initiation and progression of various cardiovascular diseases including atherosclerosis. Thus, the adequacy of the defense systems against active oxygen is critical for the susceptibility of blood vessel wall to oxidative damage. Moreover, tissue destruction may be accelerated by the uncontrolled release of proteinase from leukocytes and by the decrease and/or the inactivation of proteinase inhibitors in some pathological states of the cardiovascular system.

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). The blood vessel walls were found to contain large amounts of EC-SOD, whereas the levels of Cu,Zn-SOD and Mn-SOD were relatively low compared with other tissues. A prominent feature of EC-SOD is its affinity to heparan sulfate proteoglycans, which are located in the glyocalyx of endothelial cell surfaces. Nitric oxide (NO), one of the reactive oxygen species, is produced by a variety of vascular cells, including endothelium, macrophages, smooth muscle cells, platelets and fibroblasts. NO produced by the endothelium modulates vasomotor tone, inhibits platelet aggregation and leukocyte aggregation, properties that have been shown to be anti-atherogenic. Superoxide which is a substrate for SODs inhibits NO-mediated arterial relaxation. It was reported that EC-SOD protected NO against inactivation by superoxide more markedly than Cu,Zn-SOD. Compared with other heparin-nonbinding SOD isozymes, the EC-SOD property of heparin affinity enhances its protective action against superoxide formed under pathophysiological conditions.

The aim of this study was to investigate the changes of EC-SOD which might cause the progression of coronary artery atherosclerosis.

MATERIALS AND METHODS

Patients Subjects who had undergone coronary angiography were grouped as patients with coronary atherosclerosis (CA+ patients, 5 men and 5 women) defined as at least 75% stenosis in major epicardial arteries and subjects without evidence of stenosis in their major coronary arteries (CA− subjects, 7 men and 3 women). Patients who had been administrated heparin within one month for therapy against acute myocardial infarction or unstable angina were excluded. Patients who were under hemodialysis therapy with heparin were also excluded. The patients were receiving vasoactive agents, i.e., angiotensin converting enzyme inhibitor (imidapril hydrochloride, enalapril maleate, benazepril hydrochloride), β-blocker (atenolol, metoprolol tartrate), calcium antagonist (nifedipine, diltiazem hydrochloride), nitrate (isosorbide dinitrate, nitroglycerin) and anticoagulant (aspirin diauminate, ticlopidine hydrochloride). The administration of these agents was discontinued for a day to a week before the study whenever possible. Informed consent on the administration of heparin was obtained from all subjects.

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Blood samples for EC-SOD analysis at basal were collected into tubes containing EDTA-2Na after overnight fasting, followed by injection of heparin (100 international units per kilogram body weight). Twenty minutes later, blood samples at post-heparin were collected into EDTA-2Na containing tubes.

**SOD Analysis** Human recombinant EC-SOD, prepared as described, was kindly provided by Symbion AB, Umeå, Sweden. The EC-SOD concentration was determined by an ELISA as described previously.

**Heparin-HPLC** Heparin-affinity chromatography was performed using a HPLC column of TSKgel heparin-5PW (7.5 mm × 7.5 cm, Tosoh, Tokyo, Japan). 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).

**Statistical Analysis** The student's t-test was used to compare the data between each group. Results were considered significant at a p-value of <0.05.

**RESULTS AND DISCUSSION**

**Effect of Heparin Injection on Plasma EC-SOD** We have reported that EC-SOD in plasma is homogeneous with regard to heparin-affinity, 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. An intravenous injection of heparin into healthy volunteers led to an immediate rise of the plasma EC-SOD level, caused by an increase of form V only. This result suggested that form V exists anchored to heparan sulfate proteoglycan on endothelial cell surfaces, in equilibrium with the plasma phase, and can be released into plasma by heparin. On the other hand, EC-SOD forms I to IV primarily exist in the plasma phase.

Table 1 summarizes the characteristics of the subjects in the CA+ and CA- groups. There were no significant differences between the two groups in all factors. In this study, the increase of plasma EC-SOD form V by intravenous heparin injection to CA+ patients was compared to that in CA- subjects, and typical chromatograms are shown in Fig. 1. An intravenous injection of heparin to CA- subjects led to an increase of EC-SOD V by 8.21 ± 1.37-fold (n = 10), which was significantly greater than that in CA+ patients (5.13 ± 1.85-fold, n = 10, p = 0.016). The heparin-releasable EC-SOD level in the CA+ patients (150 ± 30.7 ng/ml) was significantly lower than that in CA- subjects (206 ± 73.5 ng/ml, p = 0.034), whereas the plasma EC-SOD levels before and after heparin injection were not significantly different between the two groups, as shown in Fig. 2. This finding raised two possibilities: first, EC-SOD V was released less by the intravenous heparin injection in CA+ patients because the heparin affinity of the enzyme form V in the patients was higher than that in CA- subjects; second, EC-SOD forms with weaker-heparin-affinity are increased and the basal amount of the enzyme bound on the endothelial cell surface decreased in the patients. Therefore, we compared the heparin affinity of EC-SOD in CA- and CA+ subjects.

**Heparin Affinity of Plasma EC-SOD** Plasma EC-SOD samples obtained from CA- subjects and CA+ patients were tested for affinity to heparin, and typical chromatograms are shown in Fig. 3. EC-SOD in plasma obtained from CA- in-

| Table 1. Clinical Characteristics of CA- and CA+ Subjects |
|---|---|---|
| Age | 61.5 ± 10.2 | 67.1 ± 8.5 | 0.200a |
| Sex (male/female) | 5/5 | 7/3 | 0.361 |
| Systolic blood pressure (mmHg) | 126.8 ± 19.8 | 129.3 ± 33.2 | 0.844a |
| Diastolic blood pressure (mmHg) | 70.2 ± 8.3 | 70.7 ± 13.5 | 0.927a |
| Fasting blood sugar (mg/dl) | 100.7 ± 17.0 | 101.9 ± 45.8 | 0.938a |
| Uric acid (mg/dl) | 6.0 ± 2.0 | 5.7 ± 1.1 | 0.111 |
| Total cholesterol (mg/dl) | 188.1 ± 33.1 | 190.7 ± 34.7 | 0.868a |
| VLDL cholesterol (mg/dl) | 47.4 ± 16.3 | 43.1 ± 11.1 | 0.617a |
| LDL cholesterol (mg/dl) | 109.3 ± 18.5 | 112.4 ± 29.1 | 0.802a |
| HDL cholesterol (mg/dl) | 38.5 ± 13.5 | 33.3 ± 9.7 | 0.313a |
| Total triglycerides (mg/dl) | 139.5 ± 61.3 | 132.9 ± 22.8 | 0.742a |
| Lp(a) (mg/dl) | 18.7 ± 12.7 | 33.1 ± 29.6 | 0.249a |
| History of smoking (+/-) | 4/6 | 3/7 | 0.639a |
| History of diabetes mellitus (+/-) | 1/9 | 3/7 | 0.264 |
| History of hypertension (+/-) | 3/9 | 1/9 | 0.264 |
| History of hyperuricemia (+/-) | 4/6 | 2/8 | 0.329 |
| History of hyperlipoproteinemia (+/-) | 2/8 | 1/9 | 0.531 |

Data are presented as the mean ± S.D. a) The figures indicate the number of subjects. (+/-) indicates with and without history of smoking or of the diseases.

Fig. 1. Heparin-HPLC of Pre- and Post-heparin Plasma from CA- Subjects and CA+ Patients

Chromatography proceeded as described in Materials and Methods. Closed circles, chromatogram of EC-SOD in plasma obtained after heparin injection (120 min after intravenous injection of 100 i.u. heparin/kg body weight); open circles, chromatogram of EC-SOD in plasma obtained before heparin injection; dotted line, NaCl concentration in buffer. Shadowed areas show fraction with high heparin affinity.
individuals was separated into forms I to V by heparin-HPLC similar to the already published observations.\textsuperscript{15} We found that 47.2±5.74% (n=10) of EC-SOD was fractionated in high-heparin-affinity forms IV and V (shadowed fractions) in CA− subjects. On the other hand, EC-SOD forms IV and V were significantly lower in plasma from CA+ patients (39.6±6.74%, n=10, p=0.015 vs. CA− subjects). Concomitantly, the level of forms I to III were increased in patients.

It has been shown that proteolytic truncation of the heparin-binding domain, a cluster of basic amino acids in the C-terminal portion, is a major cause of the presence of EC-SOD forms lacking heparin affinity.\textsuperscript{16,17} 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.\textsuperscript{6,18} In particular, the cluster of six basic amino acids, Arg-210—Arg-215, forms an essential part of the heparin-binding domain.\textsuperscript{16,17,18} In the previous study, we showed that the heparin affinity of EC-SOD was reduced by treatment with trypsin accompanied by a reduction in the molecular mass.\textsuperscript{20} McCord et al.\textsuperscript{21} noted that trypsin-like proteinase released by neutrophils may cleave the C-terminus of EC-SOD. It is known that proteinase release of neutrophils and suppression of anti-proteinase activity are stimulated under arteriosclerosis conditions.\textsuperscript{21} These pathophysiological changes may cause the decrease of heparin affinity of EC-SOD shown in Fig. 3.

As arteriosclerosis progresses, leukocytes and macrophages activated, and/or present in the vessel wall, release cytokines, such as tumor necrosis factor, interleukin-1, interleukin-6, fibroblast growth factor, platelet-derived growth factor, and transforming growth factor-β. It was reported that cytokines suppressed the synthesis of heparan sulfate on the endothelial cell surface.\textsuperscript{22} In arteriosclerosis patients, a decrease of glycosaminoglycans was observed with a reduction in the proportion of heparan sulfate.\textsuperscript{23} In addition, in diet-induced atherosclerosis models with rabbits and monkeys, changes in the proteoglycan of a relatively greater proportion of chondroitin sulfates and dermatan sulfate and a smaller proportion of heparan sulfate in aorta were observed.\textsuperscript{24,25} The binding of EC-SOD to heparan sulfate was 10 and 150 times more efficient than that to dermatan sulfate and chondroitin sulfate, respectively.\textsuperscript{5} EC-SOD bound on the endothelial cell surface should be reduced in CA+ patients because the abilities of both endothelial cells and EC-SOD to bind each other are reduced in those patients. Therefore, we may have observed the reduced rise of plasma EC-SOD after the heparin injection in patients.

It is apparent that dysfunction of the vessel wall in arteriosclerosis is due in part to stimulated production of active oxygen species and proteinases and to the loss of the physiological action of NO. The microenvironment created by the neutrophil-endothelial cell interaction plays an important role in the propagation of injury, because the superoxide concentration at the site is sufficient to inactivate NO and some anti-proteinases.\textsuperscript{26} NO reacts extremely rapidly with superoxide to be inactivated and produce peroxynitrite, a potential mediator of oxidant-induced cellular injury, at or near the diffusion-limited rate (4.3 to 6.7×10⁷ M⁻¹ s⁻¹). Peroxynitrite is a potent oxidant, capable of oxidizing thiols\textsuperscript{27} and DNA bases,\textsuperscript{28} and of causing tyrosine nitration\textsuperscript{29} and initiating lipid peroxidation.\textsuperscript{30} EC-SOD is a potent scavenger of superoxide at the rate of 2×10⁷ M⁻¹ s⁻¹ and prevents the inactivation of NO. EC-SOD in the vascular system apparently forms an equilibrium between the plasma phase and the endothelial cell surface, and EC-SOD on the endothelial cell surface should contribute in efficient protection of the vessel wall against oxidative stress. The decrease of heparin affinity of EC-SOD shown in this study may cause the suppression of the defense system against oxidative stress occurring in the

![Fig. 2. Changes in Plasma EC-SOD Level in CA− Subjects and CA+ Patients by the Intravenous Injection of Heparin](image)

The increment was significantly different (p=0.034) between CA− subjects (n=10) and CA+ patients (n=10). EC-SOD levels of pre- and post-heparin plasma were not significantly different (N.S.) between the two groups.

![Fig. 3. Heparin-HPLC of Plasma from CA− Subjects and CA+ Patients](image)

Chromatography proceeded as described in Materials and Methods. Closed circles. EC-SOD concentration assayed by ELISA; dotted line, NaCl concentration in buffer. Shadowed areas show fractions with high heparin affinity.
vascular system, and result in the progress of coronary artery atherosclerosis.

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