Heparin-affinity patterns and composition of extracellular superoxide dismutase in human plasma and tissues

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The tetrameric extracellular superoxide dismutase (EC-SOD) in human tissues and plasma has previously been found to be heterogeneous with regard to heparin affinity and could be divided into at least three classes: A, lacking heparin affinity; B, with weak affinity; and C, with strong affinity. Using rigorous extraction conditions and an extensive set of anti-proteolytic agents, tissue EC-SOD is now shown to be almost exclusively of native homotetrameric C-class. Plasma EC-SOD on the other hand is shown to be mainly composed of a complex mixture of heterotetramers with modifications probably residing in the C-terminal heparin-binding domain. Proteolytic truncations appear to be a major cause of this heterogeneity. The findings suggest that, since 99 % of the EC-SOD in the human body exists in the extravascular space of tissues, EC-SOD is primarily synthesized in tissues and secreted as homotetrameric native EC-SOD C. This tissue EC-SOD C should exist almost completely sequestered by

heparan sulphate proteoglycans. C-terminal modifications subsequently occurring in the EC-SOD C would weaken the binding to heparan sulphate proteoglycan, facilitate entrance to the vasculature through capillaries and lymph flow, and finally result in the heterogeneous plasma EC-SOD pattern. With the new extraction and analysis procedure, the tissue content of EC-SOD is found to be higher than previously reported. It is found, for example, when compared with Mn-SOD, to be higher in umbilical cord and uterus, about equal in placenta and testis and as high as that of CuZn-SOD in umbilical cord. The findings suggest that the protection level against superoxide radicals provided by EC-SOD in the tissue interstitial space, given the small distribution volume, is not much less prominent than that bestowed on the intracellular space by CuZn-SOD and Mn-SOD.

INTRODUCTION

Extracellular superoxide dismutase (EC-SOD, EC 1.15.1.1) is a secretory tetrameric copper- and zinc-containing glycoprotein [1,2]. It is the major SOD isoenzyme in extracellular fluids such as plasma, lymph and synovial fluid [3–6], but occurs primarily in tissues which account for 90–99 % of the EC-SOD of the mammalian body [7,8]. A prominent feature of EC-SOD is its heparin affinity. The physiological ligand of the enzyme is heparan sulphate proteoglycan [9,10] which exists in the glycocalyx of cell surfaces and in the connective-tissue matrix. EC-SOD of mammalian plasma [4,5] and also EC-SOD isolated from human lungs [1] has been found to be heterogeneous with regard to its heparin affinity and could be divided into at least three classes: A, which lacks affinity; B, which has weak affinity; and C, which has relatively high affinity. Intravenous injection of heparin results in release into the plasma of EC-SOD with high heparin affinity [4,5]. EC-SOD eluting from heparin–Sephacel columns above about 0.38 M NaCl shows this behaviour and is defined as belonging to class C [5]. Enzyme with lower affinity, but which still binds is then defined to be of B class. It appears that, in the vasculature, EC-SOD C exists bound to heparan sulphate on endothelial cell surfaces in equilibrium with the plasma phase and can be released into plasma by heparin. EC-SOD of classes A and B primarily exists in the plasma phase.

The binding to the negatively charged molecules heparin and heparan sulphate is of electrostatic nature [9]. The C-terminal end of the 222-amino-acid long EC-SOD C subunit, contains six arginines, three lysines and one histidine among the last 20 amino acids [11]. Based on studies on a series of EC-SOD

mutants with sequential truncations or amino-acid substitutions in the C-terminal end, it was recently concluded that this region forms the heparin-binding domain, with an unusual cluster of six positively charged amino acids in positions 210–215 constituting the essential part [12]. The truncation mutant studies, as well as co-transfection and co-incubation experiments [12], suggested further that the heterogeneity of EC-SOD heparin-affinity *in vivo* might be due to the occurrence of heterotetramers composed of subunits possessing and lacking heparin affinity. The novel findings prompted the present study in which we explored the heparin-affinity patterns and the molecular background to the heterogeneity of EC-SOD in human tissues and plasma.

EXPERIMENTAL

SOD analysis

SOD enzymic activity was determined using the direct spectrophotometric method employing KO_2 [13] as modified in [14]. Cyanide (3 mM) was used to distinguish between the cyanide-sensitive isoenzymes CuZn-SOD and EC-SOD and the resistant Mn-SOD. One unit corresponds to 8.3 ng of human CuZn-SOD, 8.6 ng of human EC-SOD and 65 ng of bovine Mn-SOD. The ' KO_2 assay' is carried out at pH 9.5 and at relatively high superoxide concentrations. In comparison, the xanthine oxidase–cytochrome *c* SOD assay [15] is carried out under more physiological conditions, i.e. neutral pH and low superoxide concentrations. One unit in the ' KO_2 -assay' corresponds to about 0.024 units of CuZn-SOD and EC-SOD and 0.24 units of Mn-SOD respectively in the 'xanthine oxidase assay'. The ' KO_2 -

Abbreviation used: EC-SOD, extracellular superoxide dismutase.

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assay' is thus about 10 times more sensitive for CuZn-SOD and EC-SOD activity than Mn-SOD activity.

EC-SOD protein concentration was determined with an e.l.i.s.a. [16].

Addition of anti-proteolytic agents to all media

Because of the extreme susceptibility to proteolysis of the C-terminal heparin-binding domain of EC-SOD C [17], an extensive set of anti-proteolytic agents was added to all solutions used for homogenization and chromatography of plasma and tissues: 0.5 mM phenylmethanesulphonyl fluoride, 3 mM diethylenetriamine penta-acetic acid, 0.5 mM benzamidine, 0.5 mM *o*-phenanthroline, 90 mg/l aprotinin, 10 mg/l pepstatin, 10 mg/l chymostatin and 10 mg/l leupeptin.

Extraction of human tissues

Macroscopically normal human tissues were obtained as small pieces cut out during surgical procedures. The pieces were rapidly put on ice, brought to the laboratory, and immediately homogenized with an Ultraturrax homogenizer under cooling with ice in 10 vol. of 50 mM potassium phosphate, pH 7.4, with 0.3 M KBr (chaotropic salt that increases the degree of extraction of EC-SOD several fold), and the set of anti-proteolytic agents. The homogenates were then sonicated and finally extracted for 30 min at 4 °C. The extracts were then centrifuged (20000 *g* for 15 min). Unless immediately separated on heparin-Sephacrose, the supernatants were kept at -80 °C.

Human plasma obtained before and after heparin treatment

Heparin (200 i.u./kg body weight) was injected intravenously into two healthy persons fasted overnight. Blood samples were tapped into EDTA-containing vacuum tubes before and 10 min after the heparin injection. The tubes were immediately centrifuged (2500 *g*, 15 min) and the anti-proteolytic agents were thereafter added to the collected plasma. The plasma samples were kept at -80 °C.

Analytical separation of tissue extracts and plasma on a heparin-Sephacrose column

The chromatographies were carried out at room temperature in 1 ml heparin-Sephacrose columns (Pharmacia LKB Biotechnology, Bromma, Sweden) with 15 mM sodium cacodylate, pH 6.5, containing 50 mM NaCl as equilibration buffer and eluent. Plasma or tissue homogenates (0.5 ml) from about 0.3 g of tissue were applied, after exchange of medium to the cacodylate buffer using small Sephadex G-25 columns (PD-10, Pharmacia LKB). Bound components were eluted with a linear gradient of NaCl in the buffer (0–1 mol/l). In collected 0.65 ml fractions EC-SOD was determined with an e.l.i.s.a., and the chloride content measured with a chloride titrator (American Instrument Co., Silver Spring, MD, U.S.A.).

To allow immunoblotting studies (cf. Figure 2) on fractions from the plasma chromatogram larger amounts of EC-SOD and less contaminating protein had to be applied to the heparin-Sephacrose column. Pre-heparin plasma (4 ml), dialysed against 50 mM potassium phosphate (pH 7.6), was therefore applied to a 5 ml Q-Sephacrose column (Pharmacia LKB)

equilibrated against the same buffer. All EC-SOD was retained in the column and the enzyme was then desorbed with a gradient of potassium phosphate, pH 7.6, up to 300 mM. Fractions containing EC-SOD were pooled, concentrated and dialysed against 15 mM sodium cacodylate, pH 6.5, containing 50 mM NaCl and finally applied to the heparin-Sephacrose column. A purification factor of about 25 was achieved.

Immunoblotting

Samples separated by SDS/PAGE in 12.5% (w/v) polyacrylamide gels were electroblotted at a constant current (2 mA/cm²) for 30 min on to poly(vinylidene fluoride) membranes (Bio-Rad, CA, U.S.A.). Blots were developed using highly purified rabbit anti-(human-EC-SOD) antibodies followed by a biotinylated secondary antibody and finally a biotin/avidin-peroxidase complex (Dakopatts, Copenhagen, Denmark). For staining of blots of tissue samples 1 mg/ml diaminobenzidine and 0.01% H₂O₂ were used. The immunoblot of plasma samples were stained with the Enhanced Chemiluminescence (ECL) kit (Amersham International, Amersham, Bucks., U.K.).

Other analyses

Protein was analysed with Coomassie Brilliant Blue G-250 [18]. Human serum albumin was used for standardization. DNA was determined by fluorimetry as a complex with {2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)benzimidazole, 3 HCl (Hoechst 33258)} [19] using calf thymus DNA as standard.

RESULTS

Plasma

Figure 1 shows the result of heparin-Sephacrose separation of human pre- and post-heparin plasma. Both samples contained nearly equal amounts of A- and B-class EC-SOD, whereas there was much more C-class EC-SOD in the post-heparin sample. The post-heparin C-class EC-SOD was also displaced to the right in the chromatogram and was apparently mainly composed of a major peak eluting at about 0.54 M NaCl. Samples from the second person analysed displayed very similar patterns (results

Figure 1 Separation of pre- and post-heparin plasma on heparin-Sephacrose

Human plasma (0.5 ml) collected before and 10 min after intravenous injection of 200 i.u./kg body weight heparin was separated on a heparin-Sephacrose column as described in the Experimental section. I, II, III, IV and V denote the position in the NaCl gradient of fractions from pre-heparin plasma analysed by immunoblotting, cf. Figure 2.

Figure 2 Immunoblot of fractions from heparin-Sepharose separation of pre-heparin human plasma

The immunoblot was performed as described in the Experimental section. The pre-heparin plasma sample was separated on Q-Sepharose before application on the heparin-Sepharose column, cf. the Experimental section. I, II, III, IV and V in Figure 1 mark the position in the NaCl gradient of the fractions analysed by immunoblotting. T209, a 209-amino-acid-long EC-SOD C-terminal truncation mutant [12]; C, recombinant EC-SOD C. Left, position of molecular-mass markers in kDa.

not shown). To further characterize the pre-heparin plasma chromatogram, the central A-class fraction (I) and two B- (II, III) and C-class (IV, V) fractions were analysed by immunoblotting, Figure 2. The analysed fractions displayed two main bands, one large band with a mobility indistinguishable from that of 222-amino-acid long recombinant EC-SOD C and one smaller band with a mobility similar to that of a recombinant C-terminal 209-amino-acid long truncation variant, T209 [12]. The A fraction contained mainly the low-molecular-mass band, whereas the B and C fractions contained more of the high-molecular-mass band as the heparin affinity increased.

Figure 3 Separation of human tissue homogenates on heparin-Sepharose

Human tissues were homogenized, extracted and separated on heparin-Sepharose as described in the Experimental section.

Tissues

Homogenates of the human tissues for which data are presented in Table 1, were all separated on a heparin-Sepharose column, and some of the results are shown as examples in Figure 3. The chromatograms contained uniformly a minimal A-class peak and a large narrow C-class peak, which eluted at the same position in the NaCl gradient as isolated recombinant and native EC-SOD C. Whether EC-SOD A really exists in the tissues to any significant extent, or whether it is the result of proteolysis during handling, cannot be judged with certainty; see below.

Table 1 Content of SOD isoenzymes, protein and DNA in human tissues

Human tissues were homogenized and the extracts were assayed for their contents of CuZn-SOD and Mn-SOD activity, EC-SOD protein, total protein and DNA. The results for EC-SOD protein converted into activity units are also presented. The conversion factor 8.6 ng per unit was used. To obtain the CuZn-SOD activities, the cyanide-sensitive SOD activities were subtracted with these calculated EC-SOD units. Note that the SOD activity assay used [13,14] is 10 times less sensitive to Mn-SOD activity than to CuZn-SOD and EC-SOD activity.

Tissue		EC-SOD		CuZn-SOD (unit/g wet weight)	Mn-SOD (unit/g wet weight)	Protein (mg/g wet weight)	DNA (mg/g wet weight)
		(ng/g wet weight)	(unit/g wet weight)				
Kidney	A*	9080	1060	21 100	1540	66	2.2
	B	7970	927	23 600	1530	61	2.9
	C	4750	552	28 900	1810	68	2.5
	D	6080	707	27 050	1500	70	3.6
Lung	A	9630	1120	4 380	295	43	3.1
	B	3540	412	4 710	523	52	4.1
Placenta	A*	2330	271	14 700	10	61	2.0
	B	3530	411	3 890	23	32	1.7
	C	2520	293	8 870	71	49	2.0
Prostate gland	A*	7990	930	4 620	929	16	1.3
	B	11 800	1370	6 840	495	47	2.5
	C	11 300	1320	6 470	209	40	1.5
Testis		5880	684	10 500	93	49	2.6
Umbilical cord	A*	7300	848	1 840	19	20	0.48
	B	9150	1060	1 730	51	22	0.19
	C	6070	706	689	60	18	0.16
Uterus	A	20 300	2360	6 810	219	57	4.4
	B	21 700	2523	6 310	122	45	1.8

* The tissues for which heparin-Sepharose chromatograms are presented in Figure 3.

Figure 4 Immunoblot of human tissues

Immunoblot of the peak-C fractions from heparin–Sephacel separations of human tissue extracts, cf. Figure 3. Lane 1, the EC-SOD truncation mutant T209 [12]; lane 2, umbilical cord; lane 3, kidney; lane 4, placenta; lane 5, prostate gland; and lane 6, recombinant EC-SOD C. Left, position of molecular-mass markers in kDa.

Figure 4 shows the result of immunoblotting of the central fractions of the C-class peaks of the chromatograms presented in Figure 3. All four tissues display dominating high-molecular-mass bands with mobilities indistinguishable from that of recombinant EC-SOD C. There were only traces of bands at the position of the truncation variant T209.

Table 1 presents the SOD isoenzymes, protein and DNA contents of the investigated tissues. Compared with the previous analysis of these components in human tissues [7], the tissues were, in the present case, immediately extracted in buffer with the anti-proteolytic agents, and EC-SOD was determined directly with e.l.i.s.a. instead of as enzymic activity after concanavalin A–Sephacel chromatography. In the other tissues previously studied [7], kidney, lung and uterus, the CuZn-SOD and Mn-SOD activities were very similar to the present findings. The present results for EC-SOD are, however, distinctly higher. EC-SOD emerges as a much more prominent SOD isoenzyme than previously perceived. Compared with Mn-SOD, the activity is higher in umbilical cord and uterus and about equal in placenta and testis. In umbilical cord the EC-SOD and CuZn-SOD activities are roughly equal. This is apparently related to the low cellularity in the umbilical cord, cf. the low DNA content, and is in accord with the notion that EC-SOD exists in the tissue interstitial space.

Analysis of EC-SOD C in tissues caused problems. Initially we rapidly froze small samples and kept them at -80°C . They were then thawed to temperatures just above freezing and were homogenized in the buffer containing anti-proteolytic agents as described in the Experimental section. When the homogenates were then separated on heparin–Sephacel, the resulting patterns were variable with differing amounts of A-, B- and low-affinity C-class EC-SOD peaks in addition to the ‘native’ EC-SOD C seen in Figure 3. Of the tissues tested, the problem appeared greatest with kidney and least with umbilical cord. We interpreted the observed effects to be due to proteolytic enzymes released by freezing-induced cellular and subcellular rupture cleaving the heparin-binding domain of EC-SOD C. We then adopted the procedure described in the Experimental section, which apparently essentially preserved the heparin-affinity pattern of tissue EC-SOD.

DISCUSSION

Apart from being secretory, the most distinguishing and interesting property of EC-SOD is the heparin affinity of the

enzyme. Heparin does not normally exist extracellularly, and the likely ligands of EC-SOD *in vivo* are heparan sulphate proteoglycans [9,10] which exist in the glycocalyx of cell surfaces and in the connective-tissue matrix. Tissue EC-SOD is now shown to be almost exclusively of homotetrameric C-class, and the heparin affinity of that fraction appears identical to that of native and recombinant EC-SOD C. EC-SOD C forms an equilibrium between the fluid phase and heparan sulphate proteoglycan bound to the endothelium in the vasculature [4,5,16], to the exterior of cells in culture [10] and immobilized on Sepharose [9]. Since the interstitial fluid volume is small in tissues, the total cell-surface areas are large and since heparan sulphate is also present in the connective-tissue matrix, virtually all tissue EC-SOD (C) should exist in sequestered form. EC-SOD bound in that fashion retains most of its enzymic activity [20]. EC-SOD is the least abundant SOD isoenzyme of tissues, but the content is apparently not much less than that of Mn-SOD, (Table 1, [7]). The distribution volume is, however, small, which is why the protection of the cell surfaces and the tissue interstitium given by EC-SOD should be considerable and probably not much less prominent than that bestowed on the interior of the cells by the intracellular isoenzymes CuZn-SOD and Mn-SOD.

Unlike the case of CuZn-SOD and Mn-SOD, EC-SOD is synthesized by only a few cell types. A comprehensive analysis of EC-SOD expression by human cell lines indicated that fibroblasts and glia cells may be important sources of EC-SOD in the body [21]. In all cases investigated, the synthesized EC-SOD was of C class [21]. The present findings of uniform EC-SOD C presence in human tissues, that account for about 99 % of the EC-SOD in the body, suggest that EC-SOD is primarily synthesized and secreted as the homotetrameric C-class form. The low-affinity C-class EC-SOD and B- and A-class EC-SODs (cf. Figure 1) are consequently likely to be the result of secondary modifications. The heparin-binding domain of EC-SOD in the C-terminal end is highly hydrophilic, and should extend into the solvent and be easily accessible for proteolytic cleavage and other modifications. A high susceptibility to both proteolysis [17] and non-enzymic glycation [22,23] has indeed been found.

The heparin injection resulted in a large increase in the heterogeneous C-class peak in the plasma chromatogram, Figure 1. The relative increase was greater the stronger the binding to the heparin. It is apparent that the C-class EC-SOD forms an equilibrium between plasma and the endothelial cell surfaces, more displaced towards the surfaces the stronger the heparin affinity is. EC-SOD preparations containing A-, B- and C-class enzymes have previously been produced both by co-transfection of Chinese hamster ovary cells with plasmids defining wild-type EC-SOD C and an A-class truncation variant T213 [12], and by limited proteolysis of EC-SOD C with trypsin [17]. Analysis with immunoblotting in both those studies suggested that the different heparin affinities were dependent on the proportion of subunits in the tetramer with and without heparin-binding domains. The present study with immunoblotting suggests that proteolytic truncation of the heparin-binding domain is also a major cause of the EC-SOD heterogeneity seen in plasma. However, the differences in proportion of large and small subunits are not as distinct between fractions II, III, IV and V as those that were seen in the other studies. The difference may be due to the fact that non-enzymic glycation of lysine residues in the heparin-binding domain also contributes to the loss of heparin affinity in plasma EC-SOD [22,23]. The two-band EC-SOD pattern found in plasma (Figure 2) is clearly more distinct than that obtained with trypsin [17] and is reminiscent of that seen in the co-transfection experiment [12]. We suggest that the distinct low-molecular-mass band is the result of carboxypeptidases removing

basic C-terminal amino acids, which occur abundantly *in vivo* [24–26]. Any endoproteolytic event *in vivo* resulting in exposure of basic amino acids in the cluster in positions 210–215, would according to this analysis subsequently be followed by degradation down to amino acid 209 (cf. data in [12]).

To conclude, we suggest that EC-SOD, primarily synthesized as the homotetrameric C-class form in the tissues, is slowly continuously modified in the C-terminal end by proteolysis and glycation, resulting in enzyme less strongly bound to heparan sulphate proteoglycans. The modified enzyme should more easily enter the vasculature through capillary walls and via the lymph flow, resulting in the complex heparin-affinity pattern of plasma EC-SOD. According to this analysis, EC-SOD should be regarded as a tissue interstitial enzyme rather than as a plasma enzyme.

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