The antioxidant enzyme extracellular superoxide dismutase (EC-SOD) is mainly found in the extracellular matrix of tissues. EC-SOD participates in the detoxification of reactive oxygen species by catalyzing the dismutation of superoxide radicals. The tissue distribution of the enzyme is particularly important because of the reactive nature of its substrate, and it is likely essential that EC-SOD is positioned at the site of superoxide production to prevent adventitious oxidation. EC-SOD contains a C-terminal heparin-binding region thought to be important for modulating its distribution in the extracellular matrix. This paper demonstrates that, in addition to binding heparin, EC-SOD specifically binds to type I collagen with a dissociation constant (K_D) of 200 nM. The heparin-binding region was found to mediate the interaction with collagen. Notably, the bound EC-SOD significantly protects type I collagen from oxidative fragmentation. This expands the known repertoire of EC-SOD binding partners and may play an important physiological role in preventing oxidative fragmentation of collagen during oxidative stress.

Reactive oxygen species (ROS),¹ such as superoxide (O_2^·), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^·), are important in pathophysiological events such as inflammation, cancer, atherosclerosis, and aging (1, 2). Because ROS damage DNA, proteins, lipids and the extracellular matrix (ECM), several defense mechanisms have evolved to protect against oxidant injury. Central to these defenses are the antioxidant enzymes. These include catalase, glutathione peroxidase, and superoxide dismutase (SOD).

Three SOD isoenzymes are known in humans, including CuZn-SOD found in the cytoplasm and nucleus (3), a mitochondrial Mn-SOD (4), and extracellular SOD (EC-SOD) (5). EC-SOD is a tetrameric protein found primarily in the ECM and, to a lesser extent, in the extracellular fluids (6). We have recently shown that the human EC-SOD subunit exists in two forms, each with a distinct disulfide bridge pattern. One form is enzymatically active (aEC-SOD), whereas the other displays no SOD-activity (iEC-SOD) (7). This novel property is likely to significantly impact the function of EC-SOD in vivo, although the physiological consequences of the observation remain to be described. An important feature of EC-SOD is its high avidity for heparin, which is thought to anchor EC-SOD to the cell surface and the extracellular matrix. The interaction is facilitated by a cluster of positively charged amino acid residues near the C-terminal of the protein (8). Purified EC-SOD is heterogeneous with respect to heparin affinity, and three populations of EC-SOD have been identified, including type A (no affinity), type B (intermediate affinity), and type C (high affinity) (8, 9). The heterogeneous affinity for heparin is caused by proteolytic removal of the heparin-binding regions and, thus, reflects the ratio of cleaved and intact subunits in the tetramer. The proteolytic event occurs intracellularly and is most likely carried out by a member of the furin family of proprotein convertases (10, 11). The affinity for heparin is believed to be important in regulating the distribution of EC-SOD in the ECM, and recent reports indicate that enhanced proteolysis of the heparin-binding region of EC-SOD significantly alters the tissue distribution during pathologic processes (12, 13).

EC-SOD catalyzes the dismutation of superoxide into hydrogen peroxide and oxygen with a forward rate constant that is almost diffusion limited (>10^9 M⁻¹ s⁻¹) (14). The hydrogen peroxide is subsequently metabolized by catalase and glutathione peroxidase. Superoxide and hydrogen peroxide are not considered to be the most damaging ROS. Hydroxyl radicals are several orders of magnitude more reactive than superoxide, are among the most reactive molecules known, and react rapidly with any molecule in its vicinity (15, 16). Significantly, hydroxyl radicals may be generated in vivo as a consequence of forming superoxide and hydrogen peroxide. Through Fenton chemistry, peroxide readily reacts with transition metals like ferrous iron to form hydroxyl radicals. Moreover, ferric iron will accept an electron from superoxide and cycle back to the ferrous state, thereby making it available for another peroxide molecule.

Experiments examining oxidative protein fragmentation using radiolysis have demonstrated that the number of peptides formed is directly proportional to the number of proline residues in the protein, suggesting that hydroxyl radicals may react with proline residues and lead to peptide bond cleavage.
The mechanism of peptide bond cleavage is not clear, but it seems to involve the formation of 2-pyrrolidone (18, 19). Notably, collagen contains 16% proline residues, whereas average human proteins contain only 5.6% (20). Collagen may thus be particularly sensitive to oxidative degradation (21, 22). In addition to the hydroxyl radical, superoxide may directly cleave collagen peptide bonds (21–23). Immunochemical studies have shown previously that EC-SOD strongly co-localizes with type I collagen in the ECM of airspaces and blood vessels (24). This location would be ideal for preventing oxidative fragmentation of type I collagen. In fact, mouse EC-SOD has recently been shown to inhibit oxidative fragmentation of proteins at proline residues in a model of bleomycin-induced pulmonary fibrosis, supporting the hypothesis that its association with collagen is important in protecting it from oxidative fragmentation (25).

In this study, we show that human EC-SOD is not only associated with type I collagen but that it binds directly and reduces oxidative fragmentation of this structural protein. The interaction with collagen is mediated by the C-terminal region of EC-SOD, suggesting that this part of the protein functions as a ligand for both heparin and collagen. Although the affinity of EC-SOD for collagen is relatively low, the high concentration of collagen in the extracellular space makes it likely that this interaction affects the distribution of EC-SOD in the ECM.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Sequence-grade porcine trypsin was purchased from Promega. Bovine serum albumin, heparin-agarose (H-6508), o-phenylenediamine dihydrochloride substrate, hydrogen peroxide (H-1009), horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig, and proteinase inhibitors were from Sigma-Aldrich. HRP-conjugated rabbit...
EC-SOD Binds to Type I Collagen

**Purification of EC-SOD**—EC-SOD was purified as described previously (27). The membrane was subsequently blocked for 1 h in TBS containing 0.1% Tween 20 and incubated with the membrane for 1 h. The membrane was washed in TBS-T and bound a primary antibody subsequently detected by using an HRP-conjugated goat anti-rabbit antibody dilute in TBS-T and visualized using enhanced chemiluminescence (Amersham Biosciences). The dissociation constant ($K_d$) for the interaction between EC-SOD and type I collagen was determined by surface plasmon resonance analysis using a BIAcore 2000 instrument (Biacore, Uppsala, Sweden). The immobilization of native-type collagen fibrils in the flow cells of the sensor chips is not feasible. Hence, purified and soluble type I collagen was immobilized on a CM5 sensor chip as described previously using N-ethyl-N-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide (35). The resultant density of collagen was 71 fmol/mm$^2$. The binding analysis was performed in 10 mM Hepes, 150 mM NaCl, 1.5 mM CuCl$_2$, 1 mM EGTA, and 0.005% Tween 20, pH 7.4. Kinetic parameters were determined by the BIAevaluation 3.1 software (Biacore). The concentration of EC-SOD was determined by amino acid composition analysis and used to calculate $K_d$, fitting the data to a 1:1 binding model.

**ELISA**—Maxisorb microtiter wells (Nunc, Denmark) were coated with 0.1 mg of collagen in 100 ml of 30 mM Na$_2$HPO$_4$, 30 mM TES, and 135 mM NaCl, pH 7.4, overnight at 23 °C. This buffer is known to induce fibril formation of soluble collagen (35). The wells were emptied, and residual binding sites were blocked by the addition of 200 μl of 0.1% (w/v) bovine serum albumin in TBS for 1 h. A 2-fold dilution series of EC-SOD or trypsin-treated EC-SOD were made in 20 mM Na$_2$HPO$_4$, 50 mM NaCl, 0.05% (v/v) Tween 20, pH 7.4 (Buffer A). Limited digestion of EC-SOD with trypsin (100:1 w/w) removes the C-terminal dihedral amino acid compositions and used to calculate $K_d$, fitting the data to a 1:1 binding model.

**Protection of ROS-mediated Collagen Degradation by EC-SOD**—ROS were generated by the Cu(II)/H$_2$O$_2$ system. This system was chosen because it generates both superoxide anions and hydroxyl radicals. The reaction mechanism, shown below in Reactions 1–3,
has been proposed previously (36, 37). The first reaction proceeds faster at alkaline pH (preferably pH 11) but was carried out at pH 7.4 to lower the reaction rate. Reconstituted native-type collagen fibrils were made by incubating ~6 μg of purified collagen at 0.1 mg/ml 30 mM NaH₂PO₄, 30 mM TES, and 135 mM NaCl, pH 7.4 for 16 h at 26 °C (35). Several tubes were incubated for analysis. The visible fibrils were recovered by centrifugation, and the supernatant was removed. The supernatants were analyzed by SDS-PAGE and Coomassie Blue staining to verify that equal amounts of native-type collagen fibrils were generated in each tube. To the collagen fibrils were added 30 μl of 0.1 M NaH₂PO₄, pH 7.4 containing 50 μM CuSO₄ and EC-SOD at the indicated concentrations. Formation of ROS was initiated by the addition of 5 mM H₂O₂. The reaction was allowed to proceed for 30 min at 23 °C and stopped by the addition of 5 mM EDTA. The reaction mixtures were boiled in SDS-PAGE sample buffer and analyzed by non-reducing SDS-PAGE followed by Coomassie Blue staining.

**RESULTS**

**Binding of EC-SOD to Type I Collagen**—The interaction between collagen and EC-SOD was investigated by affinity chromatography. An aorta homogenate was applied to a collagen-Sepharose column, and the bound proteins were subsequently eluted by NaCl (Fig. 1A). Analysis of the collected fractions by SDS-PAGE and Western blotting (Fig. 1B) revealed that EC-SOD bound to the column and was eluted with 100 mM NaCl. This suggests that the interaction is weak and electrostatic in nature. To examine the level of nonspecific binding, an undervatized Sepharose column was prepared. No binding of EC-SOD could be detected using the same conditions as were used for the collagen-Sepharose column (data not shown). We conclude that the interaction between EC-SOD and...
EC-SOD Protects Collagen against ROS-mediated Degradation—To examine the functional relevance of the EC-SOD/collagen interaction, we exposed a fixed amount of native-type I collagen fibrils to ROS in the presence of increasing amounts of EC-SOD. The degradation of the collagen was followed by non-reducing SDS-PAGE (Fig. 4). It is apparent that EC-SOD protects collagen from ROS-mediated modifications, because the intensity of the collagen bands decreases in the absence of EC-SOD. However, as the Cu(II)/H₂O₂ system also generates the hydroxyl radical, the complete inhibition of collagen degradation by EC-SOD could not be observed. Upon extended ROS exposure, collagen was completely degraded (not shown).

Discussion

It has been shown previously by electron microscopic immunocytochemistry studies that EC-SOD is localized in tissue areas containing high amounts of type I collagen (24). In the present study, the direct interaction between these two proteins was verified by affinity chromatography using collagen-Sepharose and by surface plasmon resonance analysis. The Kᵋ of the interaction was determined to be 200 nM. Taken together, these analyses imply that the interaction between EC-SOD and type I collagen is specific but of low affinity. This suggests that a dynamic relationship exists where transient interactions are favored. However, considering the repetitive nature of the collagen molecule, which may present several epitopes for EC-SOD binding, and the high concentration of collagen in the ECM, it is likely that EC-SOD is associated with collagen in the extracellular space. The Kᵋ for the interaction between recombinant EC-SOD and heparin was determined to be 120 nM (39). The affinities for these two EC-SOD binding partners are comparable, and it is thus likely that EC-SOD is distributed equally between collagen and heparan sulfate in the ECM. The dual binding capacity influences the distribution in the ECM and is likely important for the regulation of the local oxidant/antioxidant level.

In addition to facilitating the interaction with cell surface heparan sulfate and heparin, the C-terminal region of EC-SOD (Arg-210 to Ala-222) proved to be important for the collagen binding. The antiangiogenic protein pigment epithelium-derived factor has the capacity to bind both heparin and type I collagen. It has recently been shown that the amino acid residues involved in binding are located at distinct areas on the protein, suggesting that the binding-activities can be regulated independently (40). In contrast, the heparan sulfate and the collagen binding activities of EC-SOD both reside in the C-terminal region. Consequently, it is not possible to regulate the ligand binding activity independently by the intracellular proteolytic removal of this region (10). The high content of charged amino acid residues in the C terminus (seven Arg/Lys and two Glu) and the fact that the interaction between EC-SOD and collagen-Sepharose can be disrupted by 100 mM NaCl suggests that ionic interactions are at least partly responsible for the binding.

It has been shown recently that substitution of both Arg or Lys residues with Ala in the C-terminal region reduces the affinity for heparin (41). In addition, substitution of Glu residues with Ala increased the affinity. This suggests that the binding of EC-SOD to heparin is largely dependent on charge density and that the structural conformation of the C-terminal region may be of less importance. Whether the ionic interaction of EC-SOD with collagen relies on specific epitopes defined by secondary structural elements is not known. However, because the structure of collagen fibrils is repetitive in nature, EC-SOD might be able to bind several places along the collagen fibrils and thus provide global protection. Because the C-terminal region of EC-SOD supports the interaction with both heparin/heparan sulfate and collagen, we suggest the term “ECM-binding region” to indicate the multiple binding characteristics of this region.

The damage exerted by ROS, especially the hydroxyl radical, is confined to a small area because of their high reactivity toward other molecules. It is thus important that free radical scavengers are situated in the vicinity of the ROS production...
site. The binding of EC-SOD type B and C to heparan sulfate anchors EC-SOD to the cell surface, whereas the ability to bind collagen likely affects the distribution in a different way. In particular, the EC-SOD/collagen interaction is likely to protect collagen from ROS-mediated degradation. The ability of EC-SOD to protect proteins from oxidative fragmentation at proline residues was recently illustrated in vivo using EC-SOD knock-out mice (25). In this study, it was found that the lung tissue of ec-sod null mice subjected to oxidative stress contained elevated amounts of 2-pyrrolidone as compared with wild-type animals. As the presence of 2-pyrrolidone indicates oxidative cleavage of prolyl peptide bonds (18, 19), the effect was presumed to reflect an increased level of oxidative collagen fragmentation based on the fact that EC-SOD strongly co-localizes with type I collagen and on the high content of proline residues in this molecule. In this paper we have demonstrated that human EC-SOD does in fact reduce oxidative degradation of native-type collagen fibrils. Because the Cu(II)/H2O2 system generates both hydroxyl and superoxide radicals, the presence of EC-SOD does not result in protection of collagen during extended incubation in vitro (19). However, it is likely that it may slow down the reaction kinetics as observed previously (25, 36). Thus, in physiological conditions the EC-SOD/collagen interaction may slow down the reaction kinetics as observed previously (42–44).

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