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TARGETING SOD BY GENE AND PROTEIN ENGINEERING AND INHIBITION OF FREE RADICAL INJURY

MASAYASU INOUE, NOBUKAZU WATANABE, TOSHIHIKO UTSUMI and JUNZO SASAKI

Department of Biochemistry, Kumamoto University Medical School, 2-2-1 Honjo, Kumamoto 860, Japan

Although oxygen toxicity of tissues can be decreased by a variety of antioxidants and some enzymes, such as SOD and catalase, their protective effect on tissue injury in various diseases are fairly small predominantly because of their unfavorable *in vivo* behavior. To minimize oxidative stress in various diseases, such as ischemic myocardial injury, circulatory disturbance and corneal inflammation, we synthesized three types of SOD derivatives by gene and protein engineering technique. One type of SOD (SM-SOD covalently linked with hydrophobic anions) circulates bound to albumin with a half life of 6 h and accumulates in tissues whose local pH is decreased. The other type of SOD (AC-SOD covalently linked with long chain fatty acids via the ε-amino group of lysyl residues) anchors onto membrane/lipid bilayers of various cells. The last type of SOD (HB-SOD synthesized by constructing a fusion gene coding human CuZn-type SOD and a C-terminal heparin-binding domain) binds to heparin-like proteoglycans on vascular endothelial cell surface. Intravenous administration of either SM-SOD or HB-SOD markedly inhibited postischemic reflow arrhythmias in the rat. When the left anterior descending artery was occluded permanently, about 65 % of animals died within 30 min predominantly due to irreversible ventricular fibrillation; the motality of animals decreased to 15 % by administering SM-SOD either before or after occlusion. Topically administered AC-SOD bound to the corneal epithelial cell surface and polymorphonuclear leukocytes and efficiently dismutated superoxide radicals at their cell surface. Thus, endotoxin-induced keratitis was inhibited markedly by topical instillation of AC-SOD. Unmodified SOD itself failed to inhibit the pathologic events occurring in these disease models. Thus, these SOD derivatives permit *in vivo* studies on the mechanism and the site for oxygen toxicity in various diseases and provide a new strategy for targeting enzymes and bioactive peptides for medical use to appropriate site(s) of their action.

KEY WORDS: SOD, oxygen toxicity, protein engineering, targeting.

INTRODUCTION

A large number of human-type enzymes and bioactive peptides has been produced by gene engineering technique. Based on molecular mechanisms for various diseases, some patients with metabolic disorders have been successfully treated by such recombinant products without immunological complications. However, these recombinant products often failed to decrease the underlying tissue injury predominantly due to their unfavorable *in vivo* behavior and, hence, clinical application of these enzymes is highly limited. Thus, targeting protein peptide drugs to the appropriate sites of their action with effective concentrations is prerequisite to inhibit pathological metabolism occurring in these patients.

Reactive oxygen species are double-edged swords and, hence, play critical roles in both cellular defence mechanisms and oxidative tissue injury in various diseases.[1,2]

Send all proofs and all correspondence to: Dr. Masayasu Inoue, Department of Biochemistry, Kumamoto University Medical School, 2-2-1 Honjo, Kumamoto 860, Japan.

The hazardous effects of these reactive species can often be inhibited efficiently *in vitro* by adding SOD and/or catalase. Thus, reactive oxygens occurring in an extracellular space might predominantly by responsible for active oxygen mediated cell injury. Although both recombinant and naturally occurring CuZn-SOD often decreased cell injury *in vitro*, the intravenously injected enzyme was rapidly removed by the kidney[3] and, hence, dismutation of superoxide radicals in the circulation occurs only insufficiently by a single dose of SOD. Since hazardous oxygen metabolites rapidly react with a wide variety of biomolecules and impair their functions, they should be scavenged at or near the site of generation. Thus, in addition to extend the plasma half life of SOD, the enzyme should be targeted close to the site of cell injury. The present work describes a new strategy for targeting SOD by gene and protein engineering methods.

Synthesis of SOD Derivative that Circulate Bound to Albumin with Prolonged in vivo half life

Since renal glomerular filtration is the major mechanism for elimination of the circulating SOD, plasma half life of the enzyme could be prolonged by increasing its apparent molecular size. However, it should be noted that the rate of diffusion of a low molecular weight compound through interstitial tissues is higher than that of a high molecular weight compound. Thus, it would be of great benefit to extend the plasma half life of SOD without increasing its appraent molecular weight. It has been known that amphipathic organic anions, such as bilirubin. Thus, renal extraction of enzymes and bioactive peptides with smaller molecular size than the filtration limit could theoretically be inhibited by binding to plasma proteins, such as albumin. To test this possibility, we synthesized SOD derivatives (SM-SOD) by covalently linking poly-(styrene-co-maleic acid) derivatives (buthyl ester- or aliphatic amide-derivatives

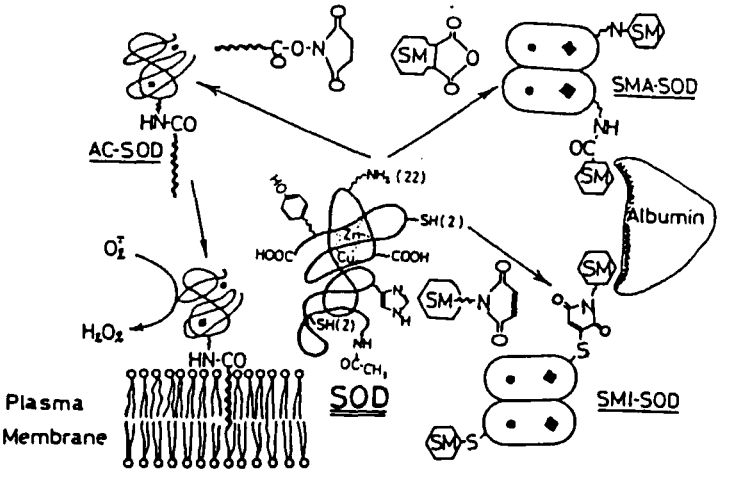

FIGURE 1 Synthesis of Site-Directed SOD Derivatives. To localize SOD at the site of tissue injury, -amino groups of lysyl- or Cys^{111}-residues of human CuZn-SOD were linked with 2 mol of poly(styren-co-maleic acid) derivatives. These SOD derivatives (SM-SOD) bind to the warfarin site on albumin. The lysyl residues of SOD were also linked with fatty acids with carbon chain length of C8-C14. The acylated SOD (AC-SOD) anchors onto plasma membrane/lipid bilayers via its acyl moiety.

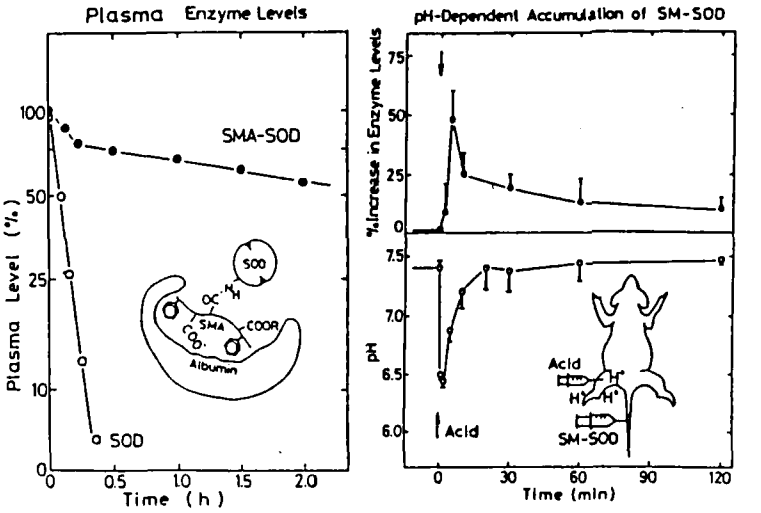

FIGURE 2 Fate of SM-SOD *in vivo*. When injected intravenously to the rat, SM-SOD circulated bound to albumin with prolonged *in vivo* half life (6–8 h) while unmodified SOD disappeared with a half life of 5 min predominantly due to its glomerular filtration (left). When animals were injected with a small amount of HEPES buffer, pH 6.0, to the femoral muscle, local pH 6.0, to the femoral muscle, local pH of the thigh rapidly decreased and then returned to normal levels. Predominantly due to amphipathic nature of SM-moiety of SM-SOD, the protonated enzyme became hydrophobic and bound to the plasma membrane surface of the acidified tissue (right).

with carbon chain length of C_4-C_{18}) (SM) via its Cys^{111} or lysyl residues (Figure 1).[3,4] When subjected to an albumin-Sepharose column chromatography, SM-SOD bound to the column and was eluted either by warfarin or sodium dodecylsulfate.[4] Under identical conditions, SOD did not bind to the column. These observations suggested that SM-SOD bound to the warfarin site on albumin with high affinity.

Fate of SOD and SM-SOD in vivo

Figure 2 shows the fate of SOD and SM-SOD in the circulation of intact rats. When injected intravenously, SOD disappeared from the circulation with a half life of 5 min while SM-SOD circulated bound to albumin with a half life of 6 h without being filtered by the kidney. Preliminary experiments revealed that, when the carboxyl group of SM was protonated, the lipophilic nature of the ligand increased significantly. Thus, when the local pH of a tissue is decreased, SM-SOD accumulated in an acidic tissue and bound onto plasma membrane surface (Figure 2).[5] Such a pH-dependent mobilization of SM-SOD also occurs in the heart whose left anterior descending artery was transiently occluded and reflowed *in vivo*.[6]

Effect of SM-SOD on Oxidative Tissue Injury

Since oxygen-free radicals are postulated to play critical roles in the pathogenesis of tissue injury, we tested the effect of SM-SOD on various types of diseases, such as ischemic myocardial injury and reperfusion arrhythmias of the rat. Post-ischemic reperfusion arrhythmias were prevented significantly by intravenous administration

of SM-SOD.[4,6] Furthermore, SM-SOD markedly decreased the motality of the rat whose descending artery was permanently occluded; motality of the control and SM-SOD-pretreated animals was 65 and 15%, respectively.[7] Administration of equimolar amounts of SM, SOD, or heat inactivated SM-SOD had no such inhibitory effect. To our surprise, SM-SOD equally decreased the motality of animals even if it was given 5 min after occlusion of the descending artery. These results suggest that superoxide radicals that trigger the lethal arrhythmias might occur at or near the area between ischemic and non-ischemic myocardium.[7]

Protective effect of SM-SOD was also tested on other types of tissue injury (Table I). So far as tested, SM-SOD markedly inhibited tissue injury in various types of diseases, such as cold-induced brain edema,[8] brain damage elicited by complete occlusion (for 18 min) followed by reflow of the carotid and vertebral arteries of the

TABLE I
Inhibition of oxidative tissue injury by SOD derivatives

Experimental Systems	Animals	Protection				References
		SOD	SM-SOD	AC-SOD	HB-SOD	
Cold-induced Brain edema	rat	no	yes	-	yes	8,16
Complete Brain ischemia (18 min)	dog	no	yes	-	-	9
Postischemic reflow arrhythmias	rat	no	yes	-	yes	6,16
Lethal arrhythmias caused by myocardial infarction	rat	no	yes	-	yes	7,16
Ischemic myocardial injury	dog	no	yes	-	-	22
Acute gastric mucosal injury	rat	no	yes	-	yes	10,16
Posthaemorrhagic transfusion-induced gastric injury	rat	no	yes	-	-	11
Postischemic reflow injury of the small intestine	rat	no	yes	-	-	13
Postischemia reflow injury of the liver	rat	no	yes	-	yes	12
Primary nonfunction of a graft after 30 min warm ischemia	rat	no	yes	-	-	14
Puromycin-induced renal injury	rat	no	yes	-	yes	23
Carrageenin-induced paw edema	rat	no	yes	-	yes	24
Endotoxin-induced keratitis	rabbit	no	-	yes	-	17,25

-, not tested.

dog,[9] stress-induced gastric injury,[10] posthaemorrhagic transfusion injury of the stomach,[11] postischemic reperfusion injury of the liver[12] and the small intestine,[13] and primary nonfunction of the transplanted porcine liver challenged with 30 min warm ischemia.[14] Thus, superoxide radical and/or its metabolites might play critical roles in the pathogenesis of such diseases with circulatory disturbance.

Targeting SOD by Gene Engineering

Reactive oxygens are 'double-edged swords' and are involved in both physiological and pathological processes. Thus, to minimize hazardous oxygen toxicity, they should be scavenged specifically at an appropriate site(s) in injured tissues. Although SM-SOD could efficiently be mobilized to the acidic area of an injured tissue, no such decrease in local pH would occur in other types of diseases than those with acute circulatory disturbance. To target the enzyme exactly to the site of tissue injury independent from changes in local pH, other strategy for recognizing injured cell surface should be considered. Since most parenchymal cells are surrounded by vascular endothelial cells, the latter might play important roles in protecting the former from being attacked by extracellular reactive oxygens. Since endothelial cells are highly differentiated from one tissue to another, they might have specific molecules on their cell surface. Among various membrane proteins, proteoglycans, such as heparan

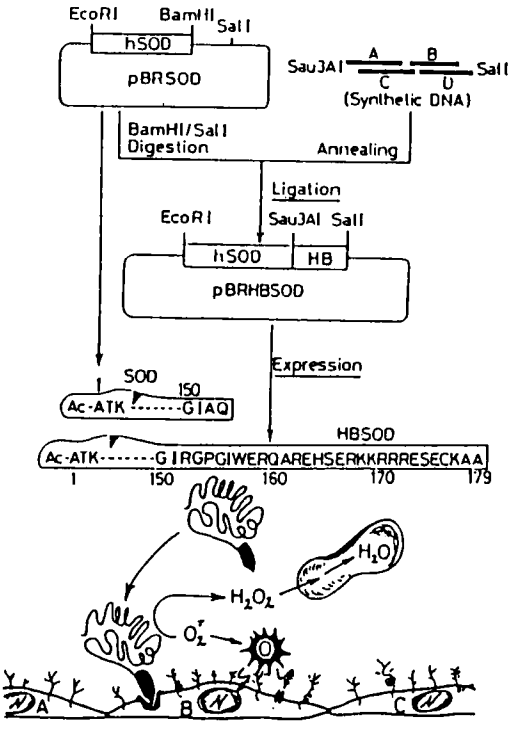

FIGURE 3 Construction of a fusion gene for targeting SOD to vascular endothelial cell surface. Fr. A–D, synthetic DNA fragments encoding heparin binding peptide; HB-SOD antithrombin(AT-III)-type SOD.

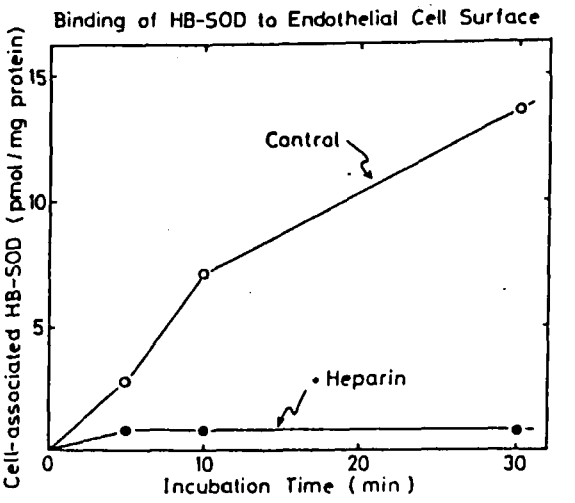

FIGURE 4 Binding of HB-SOD to heparan sulfate on endothelial cell surface. When radiolabeled HB-SOD was incubated with cultured endothelial cells, it bound to cell surface in a time dependent manner. This binding was inhibited by the presence of heparan sulfate but not by chrondroitin sulfate and dermatan sulfate.

sulfate, are one of the major component on endothelial cell surface. Thus, heparan sulfate could be a good marker for targeting the circulating enzymes close onto vascular endothelial cell surface. To test whether reactive oxygens on the outer surface of vascular endothelial cells play critical roles in the pathogenesis of various diseases, we constructed a fusion gene encoding human CuZn-type SOD with additional C-terminal domain (HB-SOD) that specifically bound to heparan sulfate on endothelial cell surface (Figure 3). When subjected to heparin-Sepharose column chromatography, the recombinant HB-SOD bound to the column and was eluted by increasing NaCl concentration in the elution buffer.[16] HB-SOD also bound to cultured endothelial cell surface by a heparin-inhibitable mechanism (Figure 4). Furthermore, immunocytochemical examination revealed that the intravenously injected HB-SOD specifically bound to the outer surface of vascular endothelial cells. To test whether dismutation of superoxide radicals on vascular endothelial cell surface inhibits oxygen toxicity *in situ*, HB-SOD was injected to animals that were challenged with various types of oxidative stress. SM-SOD-inhibitable tissue injury, such as cold-induced brain edema, carrageenin-induced paw edema, stress-induced gastric mucasal injury and postischemic reperfusion arrhythmias, was markedly inhibited by HB-SOD. Thus, superoxide radical and/or its metabolites occurring on the outer surface of vascular endothelial cells might be of critical importance for eliciting tissue injury in such diseases.

Inhibition of Oxygen Toxicity in Avascular Tissues

Although SM-SOD and HB-SOD accumulates in an injured tissue with decreased pH and at the two-dimensional space on vascular endothelial cells, respectively, they cannot be mobilized efficiently to avascular tissues, such as the cornea. Hence, oxygen toxicity occurring at the corneal surface could not effectively be decreased by systemic

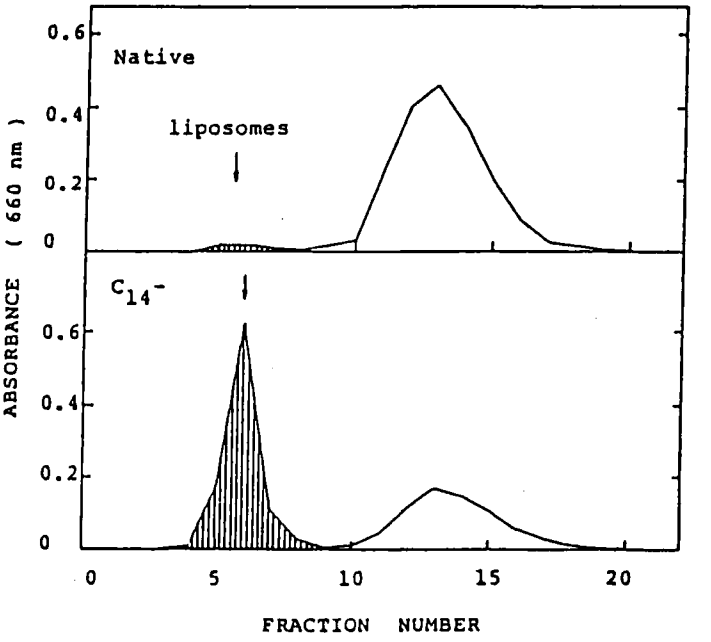

FIGURE 5 Binding of AC-SOD to liposomal membranes. SOD and AC-SOD which linked with 1 mole myristic acid (0.2 mg enzyme) was mixed with DPPC-liposomes (2 mg) and subjected to Sephadex G-200 column chromatography (1 × 25 cm). Chromatography was carried out in phosphate buffer saline, pH 7.4, and fractions of 1.5 ml were collected.

administration of SM-SOD. To increase the local concentration of an agent in surface tissues, topical instillation would be more efficient than systemic administration. Unfortunately, the topically administered SOD rapidly washed away from the corneal surface by tears and, hence, its local concentration decreased rapidly. To stabilize SOD close to the outer surface of corneal cell surface, we also synthesized acylated SOD derivatives (AC-SOD) (see Figure 1). Because of hydrophobic nature of the acyl moiety of AC-SOD, the enzyme anchored onto liposomal membranes (Figure 5). Binding of AC-SOD to liposomal surface increased with increased carbon chain length of the acyl groups. AC-SOD also bound to biomembranes of various cells, such as corneal epithelial cells and polymorphonuclear leukocytes.[17] Thus, superoxide radicals generated on the outer surface of activated neutrophiles were efficiently dismutated by pretreating cells with AC-SOD (Figure 6). To dismutate superoxide radicals efficiently *in situ*, AC-SOD was topically instilled to the cornea. Endotoxin-induced corneal inflammation was decreased efficiently by topical instillation of AC-SOD.[18]

Pathophysiological Significance of Scavenging Oxygen Radicals

The present work demonstrates that site-directed SOD derivatives apparently decreased tissue injury in various types of diseases. However, there are many *in vitro* studies showing that hydrogen peroxide is more toxic than superoxide radical. If this also hold true *in vivo*, dismutation of superoxide radical to hydrogen peroxide would

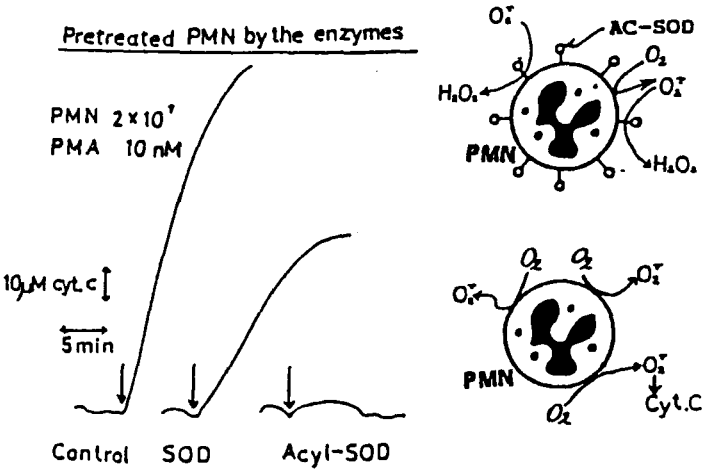

FIGURE 6 Efficient dismutation of superoxide radicals on leucocyte cell surface. Polymorphonuclear leucocytes (2×10^7 PMN) were preincubated with either saline, SOD or AC-SOD at 37 °C for 20 min. Then, cells were washed with ice-cold saline to remove unbound enzymes. PMN thus pretreated were stimulated by 10 nM phorbol myrystate acetate. The rate of superoxide radical formation in the medium was determined by the cytochrom C method.

have increased tissue injury. Two explanations for the discrepancy between *in vitro* and *in vivo* experiments would be possible; the one is that superoxide radical might directly inactivate some critical membrane molecule(s) for aerobic life. The other possibility is that hydrogen peroxide generated extracellularly could efficiently be degraded *in vivo*. In this context, Watanabe *et al.*[19] revealed that a fairly small number of erythrocytes as well as catalase significantly inhibited oxidative injury of cultured myocardial cells which were challenged with O_2^--generating xanthine/xanthine oxidase system. Topological analysis revealed that intracellular enzymes, such as catalase and glutathione peroxidase, efficiently degraded extracellular hydrogen peroxide. Since hydrogen peroxide, but not superoxide anion, is an amphipathic neutral molecule, it is not surprising that extracellularly generated hydrogen peroxide is rapidly translocated across plasma membrane lipid bilayers and degraded to water by intracellular enzymes. Since most of parenchymal cells also possess high levels of catalase and/or peroxidases, such a metabolic sink mechanism for scavenging extracellular hydrogen peroxide might also operate in other cell types than erythrocytes.

The present work demonstrates that efficient degradation of reactive oxygens in various diseases apparently decreased cellular injury. However, the classical concept that 'inflammatory reaction is a sequence of events essentially required for defence mechanism' has been widely accepted from a long time ago. Recent studies on CuZn-SOD gene dosage revealed that trangenic mice that express elevated intracellular levels of human SOD showed similar abnormality as that found in aging rodents and patients with Down's syndrome.[20] Preliminary experiments in this laboratory also revealed that some drug-induced hepatotoxicity could be enhanced by administering long acting SOD derivatives.[21] Thus, inhibition of inflammatory reactions by various scavengers for reactive oxygens should be performed carefully.

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