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Dossier: Superoxide dismutases: recent advances and clinical applications

Anti-inflammatory properties of a chimeric recombinant superoxide dismutase: SOD2/3

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Abstract

While superoxide dismutase (SOD) may be useful in treating inflammation, the problems of getting it into the blood in the right concentration, for long enough periods, and to the intended organ, have limited its translation into human clinical medicine. None of the three naturally occurring forms of human SOD is well suited for use as a therapeutic agent. SOD1 and SOD2 are normally intracellular enzymes and are rapidly cleared by the kidney. SOD3 occurs outside cells, but binds so tightly to cell surfaces or to collagen fibrils in the intracellular matrix that it remains largely in the few organs that secrete it. The "stickiness" of SOD3 results from a positively charged region in the hydrophilic C-terminus of each subunit. We have genetically engineered a hybrid *chimeric* SOD called SOD2/3 with greatly improved pharmacological properties. It has the sequence encoding the mature human SOD2 fused to the C-terminus of human SOD3. This hybrid SOD2/3 is highly expressed and easily purified. The molecule binds to endothelial cells, but less tightly than SOD3, and circulates well enough to become widely attached to extracellular surfaces, presumably in many tissues. The loose binding appears to produce a buffering effect on enzyme concentration, effectively eliminating bell-shaped dose–response curves. Single IV injections of SOD2/3 have protected experimental animals against a variety of models involving inflammation or ischemia/reperfusion.

Keywords: Superoxide dismutase; SOD2/3; Anti-inflammatory; Ischemia

1. Introduction

In many laboratory models and in a few clinical trials, superoxide dismutase (SOD) has proven therapeutically useful in protecting injured tissues from the superoxide radical. Its degree of efficacy as a therapeutic agent depends on several factors such as its rate of plasma clearance [1], its ability to equilibrate between extracellular fluid compartments [2] and its ability to approach negatively charged cell surfaces [3].

Humans have three distinct SODs: the cytosolic Cu,Zn-SOD or SOD1, a 32 kDa dimer [4]; the mitochondrial Mn-SOD or SOD2, an 89 kDa tetramer [5]; and an extracellular Cu,Zn-SOD or SOD3, a 135 kDa tetrameric glycoprotein [6]. SOD1 and SOD2 are found in all tissues, but SOD3 is found appreciably in only a few tissues and at a much lower concentration than SOD1 or SOD2 [7,8]. It is, however, the major SOD in extracellular fluids. A hydrophilic positively charged carboxyl-terminal "tail" enables the enzyme to bind to heparan sulfate on endothelial surfaces [9,10] and partial proteolytic degradation of the tail gives rise to species (designated "A" and "B") with lower affinity for heparin than the intact "C" form [10].

Surface-bound SOD3 is thought to protect endothelial cell surfaces from superoxide generated by adherent activated neutrophils. However, we have shown that proteases released by inflammatory cells can cleave the SOD3 "tail" allowing the enzyme to become soluble and rendering the endothelium susceptible to superoxide attack [11]. Thus, SOD3, or an SOD with similar properties, might be the most attractive candidate for therapeutic use to enhance and/or replace normal levels of SOD3. Unfortunately, human SOD3 has resisted attempts at high-level recombinant expression in bacterial or

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yeast vectors; it has only been available from an expensive, low-yield mammalian expression system—Chinese hamster ovary cells [12]. Thus, relatively few studies have examined the use of SOD3 as an anti-inflammatory protein.

We recently described the construction of a chimeric fusion SOD that combines the desirable properties of SOD2 (smaller size and less negative charge) with the heparin-binding property of SOD3 [13]. This genetically engineered recombinant enzyme, called SOD2/3, contains the coding sequence from the mature (i.e. minus the mitochondrial targeting signal sequence) human SOD2 followed by the 26-residue carboxyl-terminal "tail" from human SOD3. We created a novel expression vector that enables high-level expression of this mutant enzyme [14]. Here, we describe some improvements in the expression system for SOD2/3, and review its performance in seven published studies of in vivo models involving inflammation and/or ischemia/reperfusion in mouse, rat and cat.

2. Materials and methods

E. coli Rosetta™ 2(DE3) (Novagen) was selected as the expression host strain. This strain is derived from BL21, which is protease-deficient (*lon* and *ompT*), and is further designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. The Rosetta™ 2(DE3) cells were transformed with plasmid pGB1-*sod2/3* as previously described [13]. After transformation, positive clones were identified and cultured at 37 °C for 16 h in Luria-Bertani LB medium supplemented with 200 μM $MnSO_4$, 30 μM paraquat and 50 μg/ml ampicillin. After centrifugation, the harvested cells were lysed by sonication in a 0.1 M sodium carbonate, 0.6 M NaCl, pH 10.5 buffer with 33 μg/ml Sigma P2714 protease inhibitor cocktail and 0.6 mg/ml lysozyme (Sigma). The cell debris was removed from the cell lysate by centrifugation for 20 min at 11,000*g*. The supernatant was subjected to heat treatment at 65 °C for 10 min, then cooled immediately in an ice bath. Deoxyribonuclease (100 U/ml) and ribonuclease (15 μg/ml) were added, with incubation for 1 h at room temperature, then centrifugation for 20 min at 11,000*g* to remove precipitated protein. Ultrafiltration with a Diaflo YM-30 membrane was used to concentrate the supernatant. The sample was then chromatographed through a 15 × 700-mm column of Sephadex G-75 in 10 mM Tris-HCl, 0.15 M NaCl at pH 7.4. Fractions were collected and assayed for SOD activity [4]. Higher-molecular-weight fractions exhibiting SOD activity were pooled, concentrated by ultrafiltration, and diluted with 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl buffer. To remove endotoxin, the pooled fractions were treated with 1% triton X114 (v/v), vortexed 20 s, placed on ice for 5 min, incubated at 37 °C for 5 min, and centrifuged for 20 s at 12,000 rpm. The upper phase containing the endotoxin-free SOD2/3 was recovered. (All subsequent steps used endotoxin-free triton absorbed water.) This sample was then applied to a heparin–agarose column. Fractions from this column were eluted by using a 0.15–1.0 M NaCl linear gradient in the buffer described above and subsequently assayed for SOD activity. Pooled fractions were then subjected to ultrafiltration, changing the buffer during this process to 10 mM potassium phosphate, 0.15 M NaCl, pH 7.4.

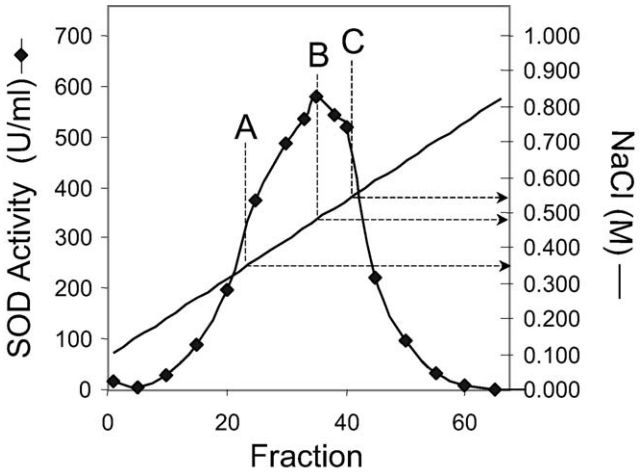

Fig. 1. Heparin–agarose chromatography of SOD2/3. Chromatography was performed as described in Materials and methods. Fractions were collected and SOD activity of each fraction was determined and expressed as units/ml of fraction volume. NaCl concentration was determined by conductivity of each fraction. SOD2/3 binds to the heparin affinity column, with the major component eluting at a NaCl concentration of approximately 0.48 M (position B). SOD2/3 purified by the previously described procedure elutes at 0.35 M (position A) [13], whereas authentic intact human SOD3 elutes at 0.55 M (position C) [10].

3. Results and discussion

The purification procedure for SOD2/3 described here differs in minor ways from the procedure originally described [13]. The yield of activity at each step, and the final specific activity of the SOD2/3 were not substantially different from the previous procedure, but the modifications result in a product that undergoes less proteolytic degradation and displays a significantly higher affinity for heparin as a result. The *E. coli* Rosetta™ 2(DE3) cells are deficient in the *lon* protease as well as the *ompT* protease, and the addition of the protease inhibitor cocktail at breakage further decreased the likelihood of proteolysis of SOD2/3 during purification. The incubation with deoxyribonuclease and ribonuclease was included to eliminate the binding of SOD2/3 to high molecular weight nucleic acids, ensuring more homogeneous behavior during subsequent steps. Fig. 1 shows the major SOD2/3 component eluting at a NaCl concentration of approximately 0.48 M. The previously described procedure [13] resulted in SOD2/3 that eluted at 0.35 M NaCl, probably reflecting partial proteolysis of one or more of the C-terminal hydrophilic tails. The heparin affinity of the current preparation of SOD2/3 is so close to that of intact SOD3 type C [10] (0.48 versus 0.55 M) that we believe that the small difference is more likely due to the differences in the geometric or spatial presentation of the tails than to the removal by proteolysis of even one lysine residue. The subunit structure of