Free Rad. Res. Comms., Vols. 12-13, pp. 401-410
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RECOMBINANT HUMAN SUPEROXIDE DISMUTASES: PRODUCTION AND POTENTIAL THERAPEUTICAL USES

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In many pathological situations, tissue damage is caused by cellular generation of superoxide free radicals (O_2^-). These active species are generated during post-ischemic reperfusion of organs, in hyperoxic tissue, during acute and chronic inflammation and during exposure to ionizing radiation. Exogenous superoxide dismutase (SOD) was shown to significantly prevent such damage.

The genes for human cytosolic Cu/ZnSOD and mitochondrial MnSOD were cloned and introduced into an *E. coli* expression system. The proteins were expressed in high yields and purified to homogeneity, yielding pharmaceutical-grade materials. These enzymes were used in a variety of *in vivo* animal models for the demonstration of their protective effects against oxidative damage. Comparative pharmacokinetic studies in rats have revealed that the half-life of Cu/ZnSOD was 6-10 min., while that of MnSOD was 5-6 hours, thus indicating that MnSOD may be superior to Cu/ZnSOD for the treatment of chronic diseases. Indeed, MnSOD was found to be effective as an anti-inflammatory agent in the rat carrageenan induced paw edema acute inflammation model. Both enzymes were also effective in ameliorating post-irradiation damage in mice exposed to whole-body or localized chest X-ray radiation.

KEY WORDS: Cu/ZnSOD, MnSOD, superoxide dismutase, *E. coli* expression, inflammation, radioprotection.

INTRODUCTION

Superoxide radicals (O_2^-), produced as by-products of oxidative metabolism, mediate extensive damage to cellular macromolecules and organelles.[1,2] A family of metalloenzymes known as superoxide dismutases (SODs; superoxide: superoxide oxidoreductase, E.C. 1.15.1.1) have been proposed as O_2^- scavengers by McCord and Fridovich[3] thus providing the biological defense against oxygen toxicity. Three classes of SODs are known, distinguished by their catalytic metal, namely, Cu/Zn, Mn or Fe. They catalyze the dismutation of O_2^- to molecular oxygen and hydrogen peroxide via alternate reduction and oxidation of the active site metal, in a highly efficient manner.[1,3] They fall into several phylogenetic lineages: Cu/ZnSODs found primarily in eukaryotes, FeSODs in prokaryotes and MnSODs crossing the entire range from microorganisms to man.[4] Mn and FeSODs show a high degree of homology, suggesting common ancestry, whereas the Cu/ZnSODs are quite distinct.[4,5] Eukaryotic Cu/Zn and MnSODs are differentially compartmentalized to the cellular cytosol and mitochondria, respectively.[6-8]

The therapeutic potential of SODs for treatment of oxidative damage has provoked considerable interest. SODs have been proposed as clinically useful for a wide variety of applications including prevention of oncogenesis, tumor promotion, tumor invasiveness and UV-induced damage,[9-11] reduction of the cytotoxic and cardiotoxic effects of anticancer drugs[11] and as a measure against the aging process.[12] Major indications include protection of cardiac or renal tissues against post-ischemia or post-transplant reperfusion damage[13,14] reviewed in [15] and [16], and as anti-inflammatory agents[17] for treatment of rheumatoid and osteoarthritis (reviewed in [18]), brain trauma[19] and influenza induced lung pathogenesis.[20] In fact, bovine Cu/ZnSOD is being utilized for treatment of osteoarthritis in man and treatment of inflamed tendons in horses ([21] see also [18]).

Until recently, exploration of SODs' therapeutic potential has been hindered by their limited availability. This has been overcome by the biotechnology industry *via* cloning, expression and production of abundant amounts of fully active recombinant human SODs (r-hSODs). Human Cu/ZnSOD (hCu/ZnSOD) is a dimeric metalloprotein composed of identical noncovalently linked subunits, each of 16 kDa and containing one atom of copper and one atom of zinc.[22] Each subunit is composed of 153 amino acids of known sequence.[23,24] Human MnSOD (hMnSOD) is a homotetramer, composed of 22 kDa subunits, each containing one Mn atom.[25] Barra *et al.*[26] determined the amino acid sequence for the human liver enzyme. cDNA clones for both hSODs have been isolated and characterized.[27-30] Moreover, several groups have expressed r-hSODs in heterologous hosts.[31-34] Here we review the advances made by Bio-Technology General (BTG) in bacterial expression, production and formulation of r-hCu/ZnSOD and r-hMnSOD for treatment of free-radical-induced diseases. We report on their biochemical activity, pharmacokinetics and therapeutic effect in animal models of inflammation and irradiation.

RESULTS

Expression of rec-hCu/ZnSOD and hMnSOD in E. coli

Expression plasmids pMF-5520 and pMSE4, directing the production of hCu/ZnSOD and hMnSOD, respectively, are depicted in Figure 1. The plasmids were constructed by placing the authentic SOD coding sequence under control of bacterial regulatory signals: the *E. coli deo* operon promoters and ribosomal binding site (rbs) for constitutive expression of hCu/ZnSOD, and the inducible phage λ $P_L O_L$ promoter and cII rbs in the case of hMnSOD (Figure 1). *E. coli*, deficient in *deo* repressor and harboring pMF-5520, constitutively accumulate hCu/ZnSOD to levels about 13% of total cellular proteins (Figure 2). The overproduced r-hCu/ZnSOD comigrates with authentic enzyme on SDS-PAGE with an apparent molecular mass of 19 kDa (Figure 2) (an anomalous migration pattern as its calculated subunit size is approximately 16 kDa). Plasmid pMSE4 was propagated at 32°C in *E. coli* strain A4255, constitutively producing the thermolabile repressor CI857. Upon temperature shift to 42°C, repression is abolished permitting transcription and resulting in accumulation of hMnSOD to levels about 25% of total cellular proteins (Figure 2). The r-hMnSOD comigrates with native hMnSOD isolated from human liver with the expected monomer molecular mass of 22 kDa (Figure 2). The authenticity of the r-hSODs was confirmed by immunoreaction with corresponding rabbit antibodies elicited by the natural proteins.[32,34]

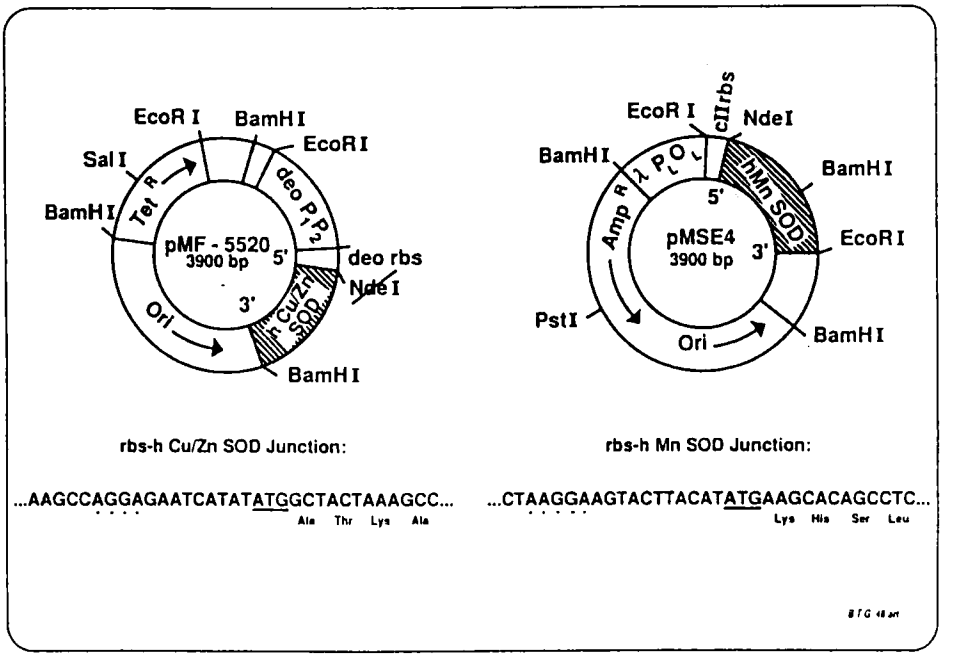

FIGURE 1 Schematic representation of r-hSOD expression plasmids. hSOD cDNA regions are shaded. The DNA sequences at the junction between the rbs and the 5′ end of the hSOD cDNAs are presented. The rbs is indicated with a dotted line, the ATG translation start codon is underlined and four codons, encoding the corresponding amino acids of the hSOD's HN_2-terminus, are shown.

Metal co-factor activation of recombinant SODs

The overproduced SODs differ in their solubility pattern when produced in cultures grown in standard L-broth. Whereas r-hCu/ZnSOD concentrates in the soluble protein fraction of bacteria subsequent to sonication, r-hMnSOD is mostly insoluble (Figure 2). Both proteins are enzymtically inactive when produced in such conditions (Table I). Supplementation of the growth media with the appropriate metal cofactor, Cu^{++} for Cu/ZnSOD and Mn^{++} for MnSOD, results in activation of the recombinant proteins to full enzymatic potential (Table I). Moreover, the recombinant hMnSOD produced with Mn^{++} supplementation is mostly soluble as shown by partitioning to the sonicated cell supernatant (Figure 2; Table I).

Purification and characterization of r-hSODs

The r-hSODs were purified from fermented cultures supplemented with metal ions by a combination of heat-treatment and ion-exchange chromatography.[32,34] The final products (Figure 2) are >98% pure, with a specific activity of 3500 units/mg and overall yield about 30%. Molecular mass of the native recombinant enzymes, as determined by FPLC, was 32 kDa for the Cu/ZnSOD and 78 kDa for the MnSOD, reflecting the dimeric nature of the former and tetrameric nature of the latter, as observed for natural enzymes.[25] Analysis of metal content by atomic absorption revealed the presence of the appropriate metal ion(s) (one atom each of Cu^{++}/Zn^{++},

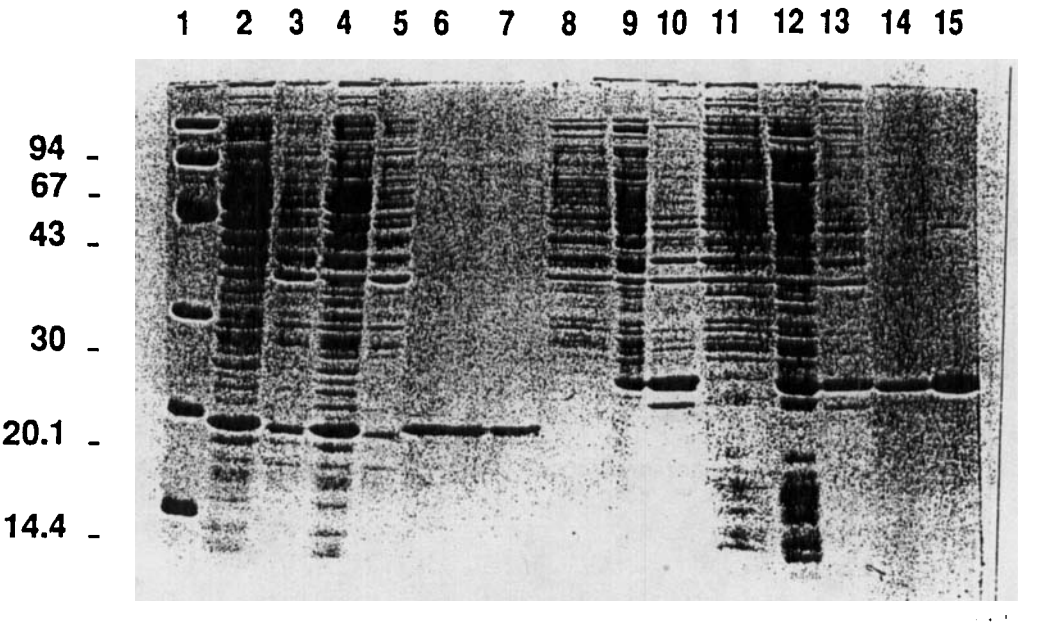

FIGURE 2 Expression of r-hCu/ZnSOD and r-hMnSOD in *E. coli*. Cultures of *E. coli* SØ930 harboring pMF-5520 (lanes 2–5) and *E. coli* A4255 containing pMSE4 (lanes 8–13) were grown in *L* broth supplemented with tetracycline (12.5 μg/ml) and ampicillin (100 μg/ml), respectively. In some experiments, growth media was supplemented with 75 ppm Cu^{++} (lanes 4 and 5) or 200 ppm Mn^{++} (lanes 11–13). Metal ions were supplied as the sulfate salts. Induction of cultures harboring PMSE4 was performed at 42°C for 2 hrs. Cells were harvested, sonicated and fractionated to soluble and insoluble proteins. Proteins were analyzed on 15% NaDodSO4/polyacrylamide gels. Each lane contained the equivalent of 100 μl of a culture at OD_{600} = 1. Proteins were stained with Coomassie brilliant blue. lane 1: protein size markers (Pharmacia). Lanes 2 and 3: soluble and insoluble proteins, respectivley, of cultures harboring PMF-5520 grown in *L* broth; lanes 4 and 5: same as 2 and 3, except that media was supplemented with 5 ppm Cu^{++}; lane 6: r-hCu/ZnSOD, purified to > 98% homogeneity; lane 7: authentic human liver Cu/ZnSOD, purified to > 98% homogeneity; lane 8: total proteins of cultures harboring PMSE4, grown in *L* broth at 32°C; lanes 9 and 10: Soluble and insoluble fractions, respectively, of cultures harboring pMSE4 grown in *L* broth and induced for 2 hrs at 42°C; lanes 11–13: same as 8–10 except that media was supplemented with 200 ppm Mn^{++}; lane 14: r-hMnSOD, purified to > 98% homogeneity; lane 15: authentic human liver MnSOD, purified to > 95% homogenity.

Mn^{++}) per enzyme subunit. NH_2-terminal AA sequencing, as determined by Edman degradation, showed that the r-hCu/ZnSOD had identical sequence to the authentic enzyme, suggesting *in vivo* processing of the initiating methionine residue. In contrast, r-hMnSOD retains the NH_2-terminal methionine preceding the native enzyme sequence. Absorption spectra of both r-hSODs were characteristic of the native enzymes, maximas shown at 279 nm for the Cu/ZnSOD and at 282 nm and 481 nm with a shoulder at 600 nm, for the MnSOD.[32,34] The pure r-hMnSOD has been crystallized and its three dimensional structure at 3 Å resolution has been analyzed.[35]

The purified recombinant enzymes were analyzed for pyrogenic contaminants in Limulus Amebocyte Lysate assay and for residual *E. coli* proteins by immunometric methods. The level of contaminants found were much below the limits of acceptance for use in human therapy.

TABLE I
Effect of metal supplementation on solubility and activity of r-hSOD in *E. coli*

Plasmid	Cu^{++} or Mn^{++} (ppm)	Percent soluble r-hSOD of soluble bacterial proteins	Specific Activity (units/mg-SOD)
pMF-5520	0	13	140
	50	13	1080
	100	13	1920
pMSE4	0	7	430
	50	15	1600
	100	17	2100

E. coli cultures harboring expression plasmids pMF-5520 and PMSE4 were grown in *L* broth supplemented with Cu^{++} or Mn^{++}, respectively, as indicated. Ions were supplied as the sulfate salts. Cultures harboring pMSE4 were induced for 2 hrs at 42°C. Extracts were sonicated and aliquots of soluble proteins were analyzed on NaDodSO4/polyacrylamide gels. Relative amounts of r-hSOD were determined by densitometric scanning of Coomassie blue-stained gels. Assays of SOD activity in crude extracts were performed as described,[3] human Cu/ZnSOD serving as standard.

Pharmacokinetic studies

Figure 3 shows the pharmacokinetic behaviour of r-hMnSOD administered to rats via intravenous and subcutaneous routes. For all time points, there was good agreement between the values of serum MnSOD levels obtained by RIA and those measured by

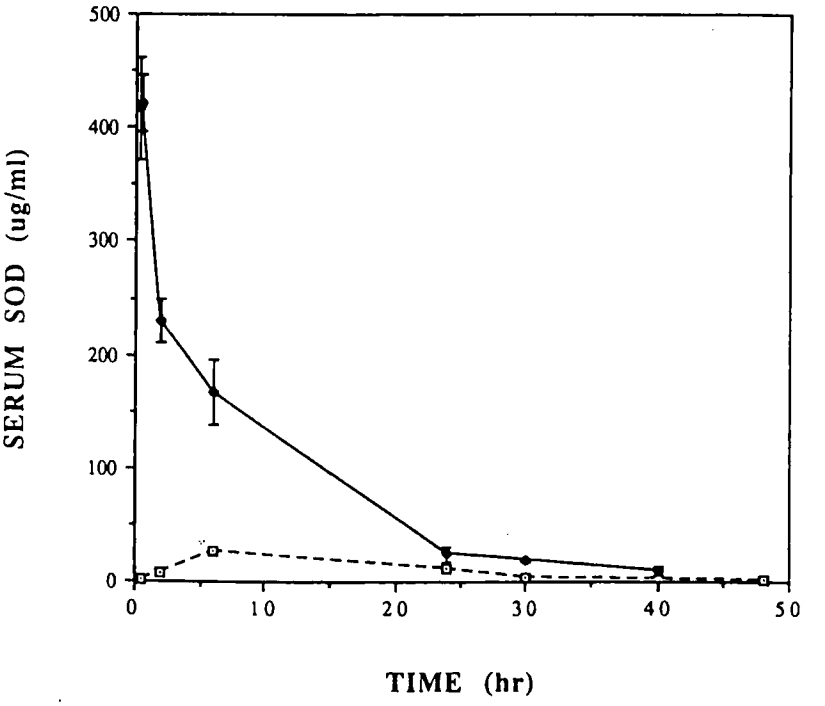

FIGURE 3 Pharmacokinetics of r-hMnSOD in rats. Rats were injected intravenously (full line) or subcutaneously (broken line) with 25 mg/Kg of r-hMnSOD and serum levels were determined by both radio immunoassay and enzymatic measurements. The vertical brackets represent the S.E.M. (N = 3 rats/point).

TABLE II
Anti-inflammatory Properties of r-hCu/ZnSOD and r-hMnSOD

Route of* injection	Time before carrageenan	Dose (mg/kg B.W.)	Inhibition of Paw Swelling r-hMnSOD	r-hCuZnSOD
Subcutaneous	2 h	50	50%	50%
Subcutaneous	24 h	50	40%	0
Intravenous	10 min	8	50%	0
Intravenous	10 min	20	80%	10%

*A — The recombinant enzymes were given s.c. (50 mg/kg) 2 h or 24 h before the carrageenan challenge (N = 8 rats/group). Control animals received carrageenan only.

B — The recombinant enzymes were given i.v. 10 min before carrageenan (N = 6 rats per group).

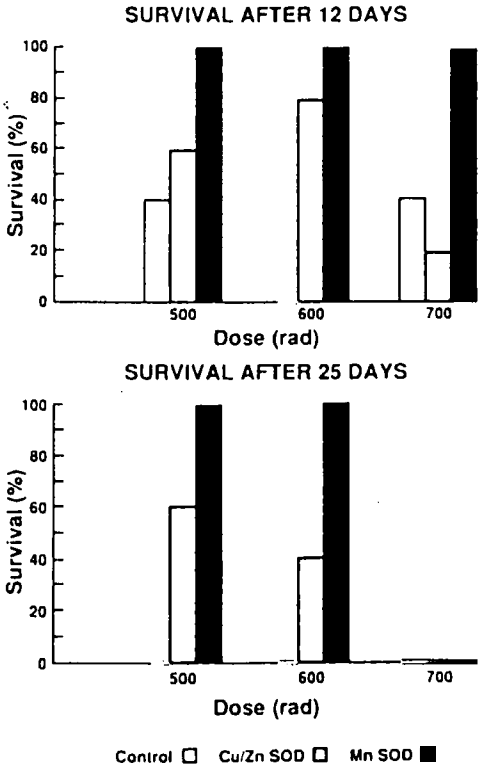

FIGURE 4 Radioprotective effect of SODs after whole body irradiation in mice. Balb-C mice were given r-hCu/ZnSOD (100 mg/Kg S.C. at −2 h +50 mg/Kg i.v. at 0-time + 100 mg/Kg 2 h after irradiation; shaded bars) or r-hMnSOD (100 mg/Kg at 0.5 h before irradiation; dark bars). Another group of mice were not injected and served as control. Groups of animals from each treatment (N = 5 rats) were exposed to whole body x-ray irradiation at doses of 500, 600 or 700 rad.

the enzymatic assay. High initial serum levels (430 μg/ml) were obtained by i.v. administration. This level dropped sharply during the first 2h, then the rate of clearance of MnSOD followed first-order kinetics with a half life ($t_{1/2}$) of 6.2 h. In contrast, Cu/ZnSOD adminsitered intravenously is rapidly cleared from the blood with a half-life time of 6 minutes (data not shown).

Subcutaneously-administered r-hMnSOD (Figure 3) resulted in a low rate of entry into the serum, reaching low levels of only 27 μg/ml after 4 h, followed by a first-order decline with a $t_{1/2}$ of 7.3 h. A sustained release effect could also be obtained after subcutaneous administration of Cu/ZnSOD but the time scale is accordingly shorter; peak levels are attained after two hours, followed by a gradual decline to an unmeasurable level after 4 hours (data not shown).

Anti-inflammatory properties of r-hMnSOD and r-hCu/ZnSOD

A rat model of acute inflammation based on carrageenan-induced edema was utilized. The two enzymes were equi-potent in the inhibition of paw swelling when administered subcutaneously (50 mg/kg) 2 h prior to carrageenan challenge (Table II). The advantage of the longer half-life of MnSOD over Cu/ZnSOD was evident when the two SODs were subcutaneously injected 24 h prior to the carrageenan challenge. In this case, r-hMnSOD was inhibitory to the inflammatory response, whereas r-hCu/ZnSOD has lost its effectiveness.[39] The antiinflammatory potential of the two enzymes was also examined using the i.v. route of adminsitration (Table II). As seen, r-hMnSOD was markedly active in inhibiting paw swelling at 8 and 20 mg/kg, while r-hCu/ZnSOD was only marginally active at the high dose and ineffective at the lower dose.[39]

Protection against radiation-induced damage

1. Whole body irradiation The protective effect of r-SODs on mortality rate after whole body irradiation in mice is shown in Figure 4. At a high dose of radiation (700 rad) both SODs were ineffective in preventing death (no survival after 25 days). However, the r-hMnSOD had an effect on prolonging survival beyond 12 days. At lower radiation doses MnSOD provided a full protection, whereas Cu/ZnSOD was only partially effective (100% vs. 40–60% survival at Day 25, respectively). The results obtained with Cu/ZnSOD are consistent with the studies reported by Petkau *et al.*[40] which has similarly shown protection by Cu/ZnSOD in whole-body irradiated mice.

2. Localized chest irradiation The radioprotective effect of the r-hSODs was further evaluated in a system involving localized chest irradiation culminating in extensive lung damage in mice. In this model, radiation-induced death, which is the result of a fibrotic lung disease, occurs 2–5 months after exposure. Significant radioprotective effects (dose reduction factors of 1.2–1.3; Figure 5) were observed using both SODs. Superior effectiveness of r-hMnSOD is evident at the higher radiation dosage (Figure 5).

DISCUSSION

Expression of human erythrocyte Cu/ZnSOD and liver mitochondrial MnSOD has

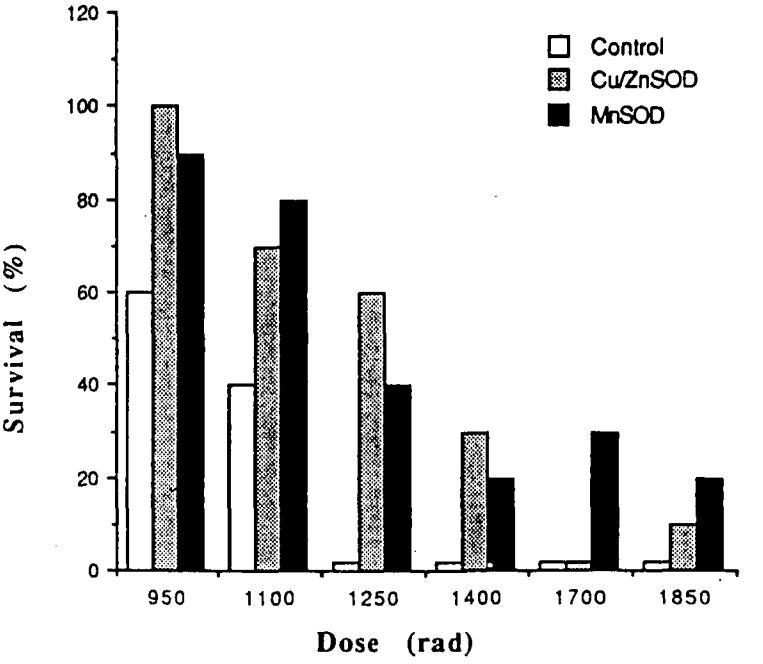

FIGURE 5 Radioprotective effects of SODs after chest irradiation in mice. Balb-C mice were treated wtih r-hCu/ZnSOD (shaded bars) or r-hMnSOD (dark bars) as described in the legend to Fig. 4. Control animals (open bars) were not injected. Chest x-ray irradiation was ensured by a carefully-patterned lead shield. The bars represent survival rates observed up to 8 months after irradiation.

been achieved at BTG utilizing *E. coli* as host organisms. The proprietary BTG expression elements, consisting of the inducible P_L promoter, CII rbs and CI857 thermolabile repressor[32,34] or the *E. coli deo* promoters and rbs, in a deo repressor deficient host, were highly efficient in producing abundant quantities of the r-hSODs, up to 25% of total cellular proteins. It must be emphasized that production of fully active r-hSODs required supplementation of growth media with the appropriate metal cofactor, although no effect on overall level of expression was noted. Moreover, the overproduced recombinant enzymes were soluble within the bacterial host reflecting appropriate folding in the heterologous milieu. This is unlike many other *E. coli*-expressed recombinant proteins which compartmentalize in dense inclusion bodies. Hallewell *et al.*,[31] at Chiron Corp, has similarly reported expression of active hCu/ZnSOD in *E. coli* under control of the *tac* promoter, yet make no mention of cofactor supplementation.

Purified r-hSODs were identical to the authentic enzymes by the following criteria: monomer molecular mass, metal content, absorption spectra, specific activity, and structural combinations. Moreover, the Cu/ZnSOD was deficient of the terminal methionine, so commonly residual in recombinant proteins, indicating its access to *E. coli* processing enzymes.[32] In fact, the only obvious structural difference between the recombinant and authentic Cu/ZnSOD is the lack of NH_2-terminal acetylation,[4,22-24] which appears to have no effect on enzymatic activity. As to the r-MnSOD, the one structural difference indicated is residual methionine, preceding the NH_2-terminal lysine.[34] This is in accordance with the inability of *E. coli* methionyl aminopeptidase

to cleave the met-lys substrate.[36] Although the sequence of human Cu/ZnSOD implies two asn-linked glycosylation signals, the presence of sugar residues in the enzyme isolated from human erythrocytes was never corroborated. In fact, the addition of glucose residues by glycation in plasma causes irreversible inactivation of SOD and is mostly found in pathological situations such as diabetes mellitus and aging.

The longer half-life shown for r-hMnSOD as compared with its Cu/Zn counterpart, in the rat model, corresponds to that of the authentic enzymes[37] and indicates its advantage for long-term clinical administration. It may also explain its superior efficacy as an anti-inflammatory agent in the rat model of carrageenan-induced edema when administered 24 h prior to challenge, as well as its higher protective potential in whole body and localized irradiation. These results correlate with previous observations that MnSOD had improved efficacy in preventing O_2-induced "suicide" of polymorphonuclear leukocytes during phagocytosis.[38] The demonstrated superiority of MnSOD in preventing experimentally induced inflammation suggests its therapeutical potential in clinical manifestations of inflammation.

In addition MnSOD was more effective than Cu/ZnSOD as a protective agent against radiation induced damage in models involving whole body and localized exposure. Radiation-induced damage is a two-step process involving (i) direct effects of newly formed reactive oxygen radicals and (ii) a later stage of inflammation involving neutrophil infiltration. From the localized irradiation model it appears that both SODs may be equipotent in the scavenging of induced superoxides, at low radiation levels; the superior effect of MnSOD is revealed only at higher doses. This notion is supported by studies of whole body irradiation which additionally indicates that MnSOD may be more suitable in providing protection during the prolonged stage of secondary inflammation.

The successful production of human enzymes which catalyze the dismutation of superoxide radicals, by recombinant DNA techniques, has opened new avenues for enzyme therapy. In fact, the potential use of r-hCu/ZnSOD is currently being evaluated in clinical studies for ischemia-reperfusion related disorders. Among these situations are damages inflicted by reperfusion therapy for acute myocardial infarction, acute tubular necrosis subsequent to renal transplantation and bronchio-pulmonary dysfunction in neonates treated with high oxygen respiration. The use of MnSOD in clinical applications remains to be studied.

References

1. I. Fridovich (1986) *Archives in Biochemistry and Biophysics*, **247**, 1–11.
2. B.A. Freeman and J.D. Crapo (1982) *Laboratory Investigations*, **47**, 412–426.
3. J.M. McCord and I. Fridovich (1969) *Journal of Biological Chemistry*, **244**, 6049–6055.
4. H.M. Steinman (1982) *In Superoxide Dismutase* Oberley, L.W. ed. CRC, Boca Raton, Fl Vol. I, pp. 11–68.
5. J.I. Harris, *et al.*, (1980) *European Journal of Biochemistry*, **106**, 297–303.
6. R.A. Weisiger and I. Fridovich (1973) *Journal of Biological Chemistry*, **248**, 4793–4796.
7. B.L. Geller and D.R. Winge (1984) *Methods in Enzymology*, **105**, 105–114.
8. J.W. Slot, *et al.* (1986) *Laboratory Investigation*, **55**, 363–371.
9. K. Shinkai, *et al.* (1986) *Cancer Letters*, **32**, 7–13.
10. K. Toda, *et al.* (1986) *Journal of Investigations in Dermatology*, **86**, 519–522.
11. L.W. Oberley and G.R. Buettner (1979) *Cancer Research*, **39**, 1141–1149.
12. J.M. Talmasoff, *et al.* (1980) *Proceedings of the National Academy of Sciences, USA*, **77**, 2777–2781.
13. J.M. McCord, (1985) *New England Journal of Medicine*, **312**, 159–163.
14. G. Ambrosio, *et al.* (1986) *Circulation*, **74**, 1424–1433.
15. G.B. Bulkley, (1987) *British Journal of Cancer*, **55**, Suppl. VII, 66–73.

16. B.R. Lucchesi, (1987) *In: Thrombosis and Platelets in Myocardial Ischemia*. J.L. Mehta, ed. pp. 35–48.
17. W. Huber (1981) *European Journal of Rheumatology and Inflammation*, **4**, 173–182.
18. L. Flohe, (1988) *Molecular and Cellular Biochemistry*, **84**, 123–131.
19. A.M. Michelson, *et al.* (1988) *Free Radical Research Communications*, **4**, 209–224.
20. T. Oda, *et al.*, (1989) *Science*, **244**, 974–976.
21. W. Puhl, *et al.* (1984) *In: Oxygen Radicals in Chemistry and Biology*. Bors, W., Saran, M. and Tait, D. eds. Walter de Gruyter & Co. Berlin. pp. 813–820.
22. J. Hartz and H.F. Deutsch (1972) *Journal of Biological Chemistry*, **247**, 7043–7050.
23. J.R. Jabusch, *et al.* (1980) *Biochemistry*, **19**, 2310–2316.
24. D. Barra, *et al.* (1980) *FEBS Letters*, **120**, 53–56.
25. J.M. McCord, *et al.* (1977) *In: Superoxide and Superoxide Dismutase*. Michelson, A.M., McCord, J.M. and Fridovich, I. eds. Academic Press, London. pp. 129–138.
26. D. Barra, *et al.* (1984) *Journal of Biological Chemistry*, **259**, 12595–12601.
27. J. Lieman-Hurwitz, *et al.* (1982) *Proceedings of the National Academy of Sciences USA*, **79**, 2808–2811.
28. L. Sherman, *et al* (1983) *Proceedings of the National Academy of Sciences USA*, **80**, 5465–5469.
29. Y. Beck, *et al.* (1987) *Nucleic Acids Research*, **15**, 9076.
30. Y. Beck, *et al.* (1988) *In: Oxy-Radicals in Molecular Biology and Pathology*. Alan R. Liss, Inc. pp. 257–269.
31. R.A. Hallewell, *et al.* (1985) *Nucleic Acids Research*, **13**, 2017–2034.
32. J.R. Hartman, *et al.* (1986) *Proceedings at the National Academy of Sciences USA*, **83**, 7142–7146.
33. R.A. Hallewell *et al* (1987) *Bio/Technology*, **5**, 363–366.
34. Y. Beck, *et al.* (1988) *Bio/Technology*, **6**, 930–935.
35. U.G. Wagner *et al.* (1989) *Journal of Molecular Biology*, **206**, 787–788.
36. A. Ben-Bassat, *et al.* (1987) *Nature*, **326**, 315.
37. A. Baret, *et al.*, (1984) *Biochemical Pharmacology*, **33**, 2755–2760.
38. J.M. McCord *et al.* (1977) *In: Movement, Metabolism and Bactericidal Mechanisms of Phagocytes*. Ross A., Patriarca, P.L. and Romeo, D. eds. Piccin Medical Books Pub. Padoa, Italy. pp. 257–264.
39. A. Nimrod *et al.* (1989) *In: Medical, Biochemical and Chemical Aspects of Free Radicals*, Hayaishi, O., Niki, E., Kondo, M. and Yoshikawa, T. eds. Elsevier Science Publishers, B.V. Amsterdam, pp. 743–746.
40. A. Petkau (19XX) *Photochemical Photobiology*, **28**, 765–774.

Accepted by Prof. G. Czapski