Oxidative stress and motorneuron disease

Transgenic mice carrying mutated Cu/Zn superoxide dismutase genes provide insights into the pathogenesis of human motorneuron diseases and may be useful as models in the development and testing of therapies.

Amyotrophic lateral sclerosis (ALS) is a lethal degenerative disorder of motorneurons. Recently, the disease has been shown to be linked to mutations in a gene on chromosome 21 encoding Cu/Zn-dependent superoxide dismutase (SOD I); this linkage applies only to a small proportion of those ALS patients who have autosomal dominant inherited (familial) ALS [1]. The importance of this observation is two-fold: it provides an opportunity for comparing therapeutic approaches between this group of patients and the large majority of ALS patients for whom the disease is not inherited, and it also provides scope for the production of animal models of ALS on a rational basis.

SOD I has already been a focus of interest in studies of the neurodegenerative changes associated with Down's syndrome (trisomy 21), which resemble those occurring in Alzheimer's disease. Patients carrying only partial duplications of portions of chromosome 21 translocated to other chromosomes were analyzed in the search for the gene(s) responsible for the degenerative changes observed in Down's syndrome, and the 21q22 region was found to play a critical role [2]. Of the 100 or so genes within this region of chromosome 21, the SOD I gene was considered to be a good candidate for the gene affected in Down's syndrome: its duplication and consequent over-expression could be responsible for the degenerative changes observed. Indeed, over-expression of SOD I in PC12 phaeochromocytoma cells results in impaired uptake of the neurotransmitters catecholamine and serotonin [3]. In order to obtain more detailed information on the possible role of increased SOD I activity in neurodegenerative processes, transgenic mice were produced that over-express the human SOD I gene transcribed from its own promoter [4,5]. Surprisingly, over-expression of human SOD I in such mice [4] led to pathological changes in their motorneurons, such as the withdrawal and destruction of terminal axons at neuromuscular endplates and the development of multiple terminals smaller than those of wild-type age-matched mice [6].

The toxicity of excess SOD I activity in transgenic mice might be explained by the formation of hydroxyl ($HO^{\cdot}$) radicals from hydrogen peroxide (H_2O_2), the production of which from superoxide ($O_2^{\cdot -}$) is catalyzed by SOD I (Fig. 1). Whereas the superoxide radical itself is relatively unreactive [7], the hydroxyl radical can react with a great variety of molecules, in particular with the polyunsaturated fatty acids found in cell membranes. This reaction generates lipid peroxyl radicals, which themselves react with neighbouring membrane fatty acids, leading to a chain reaction that finally destroys membrane integrity. Recently, convincing evidence was presented by Schubert and co-workers [8] that the toxicity of the Alzheimer's-associated β amyloid protein for neuronal cells results from increased production of hydrogen peroxide and consequent lipid peroxidation. Accordingly, the enzyme

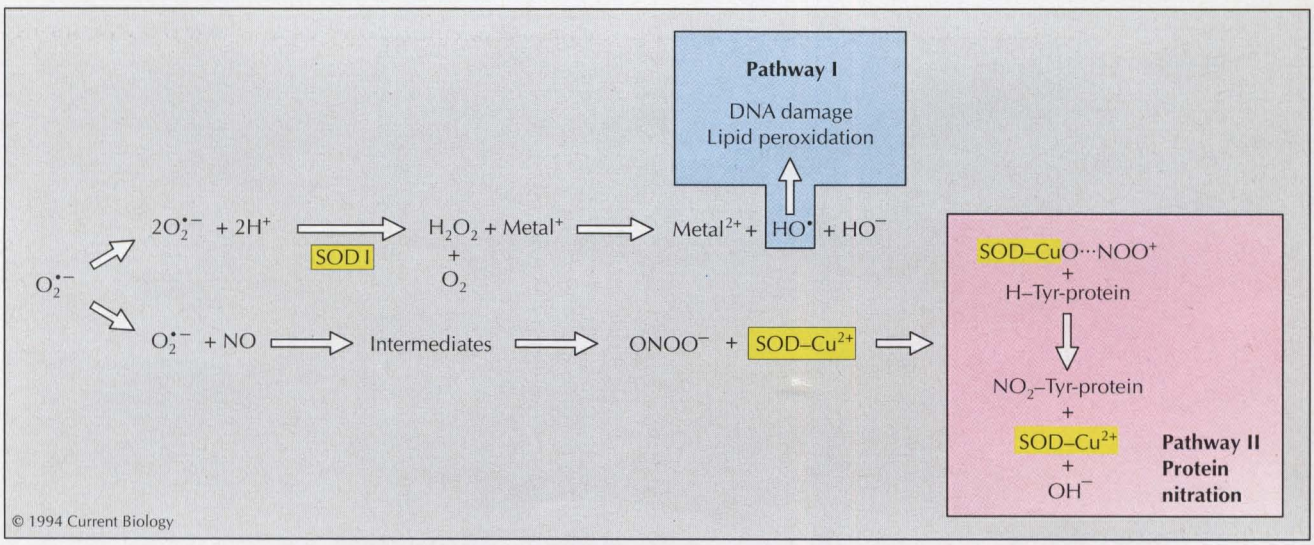

Fig. 1. Two possible neurotoxic pathways, involving SOD I, which could be responsible for motorneuron degeneration in familial ALS.

catalase, which catalyzes the conversion of hydrogen peroxide to water and oxygen, blocks the toxicity of β amyloid, providing further evidence that hydrogen peroxide mediates amyloid's toxicity in neuronal cells. These data clearly indicate that hydrogen peroxide and its derivatives such as hydroxyl radicals, can cause the lipid peroxidation that leads to neuronal cell death (Fig. 1).

On the basis of these findings, it seems puzzling that loss of SOD I enzyme activity has been proposed to cause motorneuron degeneration, given that an increase in the same enzyme's activity causes cell damage. Several point mutations have been identified within the coding sequence of the SOD I gene in patients with familial ALS [1,9–11], located in exons 1, 2, 4 and 5, but not in exon 3, which encodes most of the loop around the metal ions that forms the active site of the enzyme [12,13] (Fig. 2). Measurements of SOD I enzyme activity showed a reduction of about 50 % in most familial ALS patients [9,10,14], as expected for an autosomally inherited inactivating mutation that leaves one allele of the SOD I gene intact, but the way in which the mutation reduces enzyme activity remains unresolved. It has been suggested that decreased enzyme activity results from either an allosteric effect on the active center of the enzyme or interference with the formation of enzyme dimers [9].

Can reduced SOD I activity lead to cell damage? This question, which could be addressed by generating SOD I gene 'knockout' mice, has unfortunately not yet been conclusively answered. With SOD I knockout mice, it would be possible to address the question of whether other SOD enzymes, such as the extracellular SOD III or mitochondrial SOD II, can compensate for the lack of SOD I activity. If such compensation does not take place, accumulation of superoxide radicals and consequent cellular damage is to be expected.

Superoxide radicals are not as reactive toward lipids and proteins as are, for example, hydroxyl radicals; superoxide can, however, react with nitric oxide (NO) to yield peroxinitrite ($ONOO^-$) [15,16], and peroxinitrite has been shown to kill neurons in cell culture [15]. In addition, peroxinitrite itself can interact with SOD I, at low turnover rates, to generate nitronium-like intermediates that are prone to reaction with the tyrosine residues of proteins [16,17] (Fig. 1). This alternative reaction cascade has been suggested possibly to underlie the pathogenesis of familial ALS. But this reaction cascade requires the nitration of tyrosine residues with peroxinitrite to be catalysed by SOD within the cell and so would not be expected to occur in cells deficient in SOD. Altered forms of SOD I have, however, been characterized, which show reduced scavenging of superoxide without the enzyme's reactivity with peroxinitrite having been altered, endorsing this suggestion [17]. It will be very interesting to learn whether the mutations detected in the SOD I gene of familial ALS patients cause an increase in the enzyme's ability to catalyze tyrosine nitration but decreased catalysis of hydrogen peroxide formation.

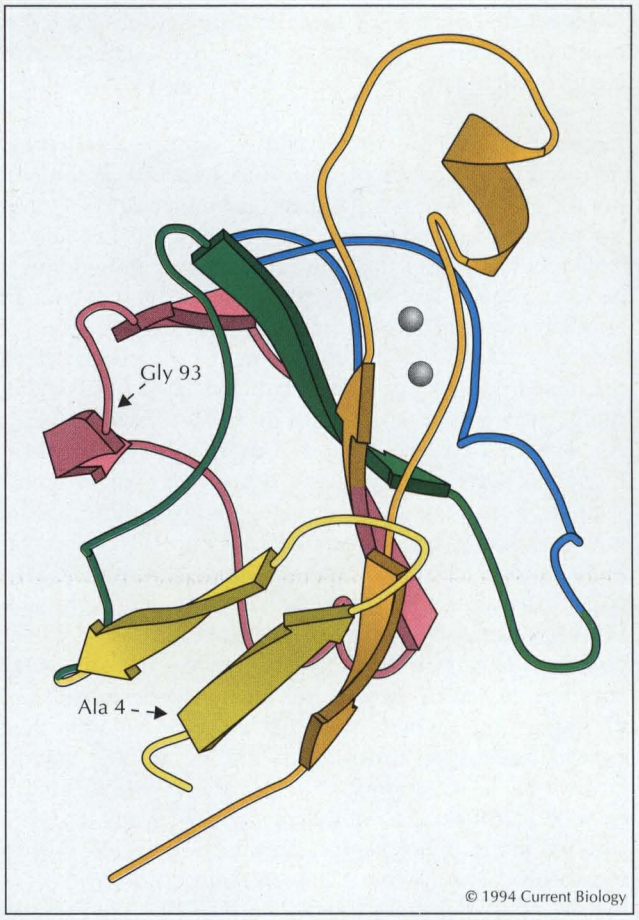

Fig. 2. Three-dimensional structure of the SOD I enzyme. The Cu^{2+} and Zn^{2+} are shown in grey, and the protein domains encoded by the exons 1–5 in yellow, green, blue, pink and orange, respectively; β-sheet structures are indicated by arrows. The positions of the two point mutations, Ala 4 and Gly 93 are indicated by broken arrows.

Recently, Gurney and co-workers [18] have established transgenic mice that over-express two mutated forms of human SOD, bearing an Ala 4→Val mutation and a Gly 93→Ala mutation. The Ala 4→Val mutation was found to be the most frequent mutation in SOD I in initial analyses of familial ALS [9]; it is localized within the β-strand region of the enzyme, but it does not affect the major loop region that forms the channel around the active site Cu^{2+} and Zn^{2+} (Fig. 2). This mutation is expected to destabilize dimers, which are the normal biologically active form of the enzyme (as demonstrated by X-ray crystallography) [13]. Indeed, the SOD I enzyme activity in red blood cells from patients heterozygous for the Ala 4→Val mutation is less than 50 % of control levels, indicating that the mutation reduces SOD activity [9]. In the case of the other mutation analyzed, the Gly 93→Ala substitution, the mutated residue is again not localised within the loop region around the active center; instead it lies in the backbone of the enzyme (Fig. 2). SOD activity in red blood cells from

patients heterozygous for this Gly 93→Ala substitution is reduced to about one third of the activity detectable in control patients [9].

Interestingly, no reduction of SOD I enzyme activity was observed in the brains of mice into which these mutant human SOD I proteins had been introduced [18] — but the mice do still carry two intact mouse SOD I alleles. This result indicates that the formation of heterodimers between mutant and wild-type SOD I, which in human familial ALS patients is thought to be responsible for reduced enzyme activity, either might not occur at all in the mice or might not lead to reduced SOD I activity. In mice over-expressing human SOD I with either Ala 4→Val or Gly 93→Ala mutations, significantly higher SOD I activities were detected in their brains than in those of wild-type mice, corresponding to the increased gene copy number and presence of the human SOD I protein. As a comparison, mice of the G12 line, which have an average of 2.2 additional copies of human SOD I with the Gly 93→Ala mutation, have very similar concentrations of human SOD protein and SOD enzyme activity in their brains to the levels in mice of the N1026 line, which have an average of 3.3 additional copies of wild-type human SOD I. This indicates that, in contrast to the situation in human patients [9], the mutation does not lead to any reduction of SOD enzyme activity in transgenic mice. In addition, mice with a transgene carrying the Ala 4→Val mutation also have increased SOD enzyme activity, with the level depending on transgene copy number.

Degenerative changes in the motorneurons were observed only in mice over-expressing human SOD I with the Gly 93→Ala mutation. Mice of this transgenic line had the highest levels of SOD I enzyme activity in their brain extracts when compared to transgenics over-expressing either wild-type human SOD I or Ala 4→Val mutated SOD I, as a result of the integration of 18 copies of the transgene (the highest copy number of any of the lines). How should these data be interpreted? Could it be that degeneration of motorneurons in these transgenic mice is not due to the mutation, but results simply from increased SOD I enzyme activity? This would be consistent with the earlier studies by Avraham *et al.* [4,6], who observed that over-expression of wild-type SOD I in transgenic mice leads to degenerative changes in their motorneurons.

Why then in the experiments of Gurney and co-workers [18] did control mice, over-expressing wild-type SOD I, not develop motorneuron degeneration? The answer may lie in the amount of transgenically expressed SOD I. The mice analyzed by Avraham *et al.* [4] had up to ten times higher SOD I enzyme activity in brain extracts than did controls, compared to a maximum of three to four times higher levels in the transgenic mice over-expressing wild-type SOD I that were studied by Gurney and co-workers [18], and two-fold higher levels in the SOD I transgenic animals reported by Ceballos-Picot *et al.* [5]. In the latter two cases, motorneuron disease did not develop. The question of whether animals with the genetic background used by Gurney *et al.* [18] are capable of developing motorneuron disease should be easily resolved by cross-breeding different founder strains of transgenic mice to increase the copy numbers of wild-type SOD I or the Ala 4→Val mutant form. The results of Gurney and co-workers [18] could alternatively be explained by the possibility that the Gly 93→Ala mutation changes the activity of SOD I for specific substrates. For example, if the mutation increases the affinity specifically for peroxinitrite, then a pathogenic mechanism involving peroxinitrite could come into play, as proposed by Beckman *et al.* [16,17].

In conclusion, these experiments suggest that not only loss of SOD I function, but also excess function or a change of substrate specificity, could be responsible for motorneuron degeneration in familial ALS patients who have mutations in the SOD I gene. It will be very interesting to learn whether over-expression of catalase, or other mechanisms leading to the detoxification of hydrogen peroxide, can reduce the pathological defects observed in transgenic mice over-expressing human SOD I. Moreover, the use of drugs such as vitamin E, acetylcysteine, L-ascorbate and others, which are known to protect cells from hydrogen peroxide and its derivatives [8], could be evaluated in these mice. Although some of these compounds have already been tested in patients suffering from ALS, no therapeutic effects have been demonstrated. It would, however, be of particular interest to re-evaluate this approach in patients who are known to carry SOD mutations.

The possibility of treating ALS patients with systemic or intrathecally administered recombinant SOD protein, or with gene therapeutic strategies aimed at over-expression of the human SOD I gene in motorneurons, have to be considered with care. It is questionable whether administration of SOD could be expected to correct the pathogenic free radical production in motorneurons. Similarly, viral gene therapy would only be a sensible choice if the expression of transduced SOD I could be precisely regulated, and if the possibility can be excluded that the disease normally develops as a result of the loss of SOD I enzyme activity, and not because of a gain in function of alternative enzymatic pathways. The experimental results described here hold promise for the treatment of human motorneuron disease, but translating them into therapies will require caution.

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