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EXPRESSION OF HUMAN CU-ZN SUPEROXIDE DISMUTASE GENE IN TRANSGENIC MICE: MODEL FOR GENE DOSAGE EFFECT IN DOWN SYNDROME

I. CEBALLOS,[1] A. NICOLE,[1] P. BRIAND,[2] G. GRIMBER,[1] A. DELACOURTE,[2] S. FLAMENT,[2] J.L. BLOUIN,[1] M. THEVENIN,[3] P. KAMOUN [1] and P.M. SINET[1]

[1] *URA CNRS 1335, Laboratoire de Biochimie génétique, Hôpital Necker 75743 Paris Cédex 15, France.*
[2] *INSERM U16, Laboratoire de Neurosciences, Faculté de Médecine, 59045 Lille, France*
[3] *Biochimie A, Hôpital Necker, 75743 Paris Cédex 15, France*

It was suggested that increased Cu-Zn superoxide dismutase (SOD-1) might be involved in the various biological abnormalities found in Down's syndrome (DS) such as premature aging and Alzheimer-type neurological lesions. As a model system for testing this hypothesis we have developed two strains of transgenic mice carrying only one copy of the human SOD-1 gene. In the first strain (TG1), no expression has been found by northern blot analysis. The second strain (TG2) exhibited human SOD-1 mRNA and increased SOD-1 activity in the brain (1.93 fold), in the heart (1.69 fold), thymus (1.49 fold) and to a lesser extent in muscle (1.25 fold), liver (1.19 fold), kidney (1.18 fold), spleen (1.35 fold), lung (1.26 fold) and erythrocytes (1.09 fold). In this strain, increased SOD-1 activity in the brain did not induce modifications in the seleno-dependent glutathione peroxidase, glutathione reductase and glutathione S-transferase activities. In brain homogenates, we have focused our studies on Tau proteins which are known to be the major antigenic components of paired helical filaments (PHF), both in DS and Alzheimer's disease. Our results suggested that, in our experimental conditions, the overexpression of SOD-1 did not induce the modifications of Tau proteins similar to those seen during neurofibrillary degeneration.

KEY WORDS: Cu-Zn superoxide dismutase, transgenic mice, Down syndrome, Tau proteins, paired helical filaments, free radicals.

INTRODUCTION

Human Cu-Zn superoxide dismutase (SOD-1; EC 1. 15. 1. 1.), a key enzyme in the metabolism of oxygen free radicals[1] is encoded by the SOD-1 gene located on chromosome 21 (band 21 q.22.1).[2]

It was postulated that the 1.5 fold increased SOD-1 activity in the cells of individuals with Down syndrome (DS), secondary to gene dosage effect, could be injurious to the brain[2-4] and might be responsible for or contributory to the premature aging and Alzheimer-type neuronal pathology,[2] which are part of the DS phenotype.[5,6] Because the function of SOD-1 is the conversion of the superoxide anion (O_2^-) to H_2O_2 which in turn is reduced by catalase and peroxidase, it was proposed that the increased SOD-1 activity could result in an increased generation of H_2O_2.[2,7]

Correspondence : I. Ceballos, URA CNRS 1335, Laboratoire de Biochimie Génétique, Hôpital Necker, 149 rue de sèvres, 75743 Paris Cédex 15, France.

In favor of this hypothesis, it has been observed in trisomy 21 an increase in glutathione peroxidase activity in several tissues as well as an increased hexose monophosphate shunt activity in red cells.[2] H_2O_2 could increase lipid peroxidation, either directly or via the production of the highly reactive hydroxyl radical ($OH^{\cdot}$),[8] thereby leading to progressive tissue damage.[9] Several data further supported this hypothesis. (a) L and Hela cell lines transfected with a cloned human SOD-1 gene and containing 3 to 6-fold elevated SOD-1 activity showed an increase in lipid peroxidation.[3] (b) L and neuroblastoma cell lines transfected with a human SOD-1 gene and containing 2-fold increased SOD-1 activity showed an increased glutathione peroxidase activity.[7] (c) PC12 cell lines expressing the transfected human SOD-1 gene have shown impaired neurotransmitter uptake resulting from modifications of membrane properties of chromaffin granules, likely secondary to lipid peroxidation.[4] (d) the tongue neuromuscular junctions from SOD-1 transgenic mice exhibited pathological changes similar to those seen in muscles of aging mice and rats as well as in tongue muscle of patients with DS.[10] With regard to the possible role of SOD-1 and free radicals in neuronal pathology, it is also noteworthy that the large pyramidal neurones which are potentially susceptible to degenerative processes in Alzheimer's disease (AD), such as paired helical filaments (PHF) formation, have been shown to contain high amount of SOD-1 protein.[11] Moreover, the elevation of SOD-1 activity observed in familial Alzheimer's fibroblasts[12] could be involved in the formation of PHF (rich in hydroxy proline) by free radical hydroxylation of proline residues contained in PHF precursor protein.

To investigate the possible involvement of SOD-1 overexpression in the Alzheimer-type neuronal pathology in DS, strains of transgenic mice that carry the human SOD-1 gene and express elevated levels of SOD-1 were developed. Here, we studied (a) the possible effect of elevated SOD-1 activity in brain of transgenic mice on enzymes associated with oxygen-radicals metabolism and (b) the possible modifications of the electrophoretic pattern of Tau proteins which are known to be the major antigenic components of PHF.

MATERIALS AND METHODS

Transgenic mice

A linear 16-kilobase (kb) EcoR I DNA fragment containing the human SOD-1 gene[13] including sequences required for its expression in transfected cells[7] was purified and approximately 100 copies were microinjected into the male pronuclei of B6D2F1 hybrids.

Analysis of Integrated Sequences

For Southern-blot analysis, approximately 0.5 cm of mouse tail was placed in 5 ml of 50 mM TRIS-HCl, pH 8.0/100 mM EDTA/0.5% SDS (w/v) (sodium dodecyl sulfate) /proteinase K (50 μg per ml) and incubated at 37°C for 12–16 hours. DNA was extracted and digested with EcoR I, analyzed by electroporesis in 0.8% agarose gels and blots were hybridized with a ^{32}P-labeled human SOD-1 cDNA.[14]

Quantification of Copy Numbers Integrated in the Mouse Genome

The offspring of the two founders were analyzed using slot-blot analysis. This method

consisted in four steps: 1 – After denaturation in sodium hydroxide, varying amounts of tail DNA ranging from 0.5 to 1.5 μg were blotted on a Zetabind TM membrane using a slot-blot apparatus (Schleicher & Schuell, Minifold II). Membrane was loaded with DNA from three sources: a normal human DNA (containg 2 copies of SOD-1 gene), DNA of a free trisomy 21 patient (containing 3 copies of SOD-1 gene) and DNA tail of the offspring. 2 – Successive hybridizations with human SOD-1 cDNA probe and reference probe (mouse β-actin) were then carried out. 3 – Autoradiograms were quantified by densitometric scanning with a Schimadzu CS930 TLC scanner.

Northern-blot Analysis

Total RNA was prepared as described[15] and 10 μg were submitted to electrophoresis in a 1% agarose gel containing 6.6% formaldehyde. After transfer to nylon membrane (Zetabind), hybridizations were done with a random primed ^{32}P-labeled human SOD-1 cDNA [14] and 28 S probe as a reference probe.

Enzymatic Assays of SOD-1, Seleno Dependant-Glutathione Peroxidase (Se GSH-Px), Glutathione Reductase (GSSG-Reductase) and Glutathione S-Transferase (GST)

Supernatant fluids from homogenized tissues were used for enzymatic determination. SOD-1 and Se GSH-Px activities were assayed as previously described.[7] Total GST activity with CDNB (3,4-dichloronitrobenzene) as substrate was assayed as described.[16] GSSG reductase activity was assayed according to the method of Carlberg and Mannervik.[17] Statistical comparisons were made by using Student's t-test.

Immunoblot Detection of Tau Proteins

Brain tissue (1 gr) was homogenized in the Laemmli buffer (10 ml) and heat treated

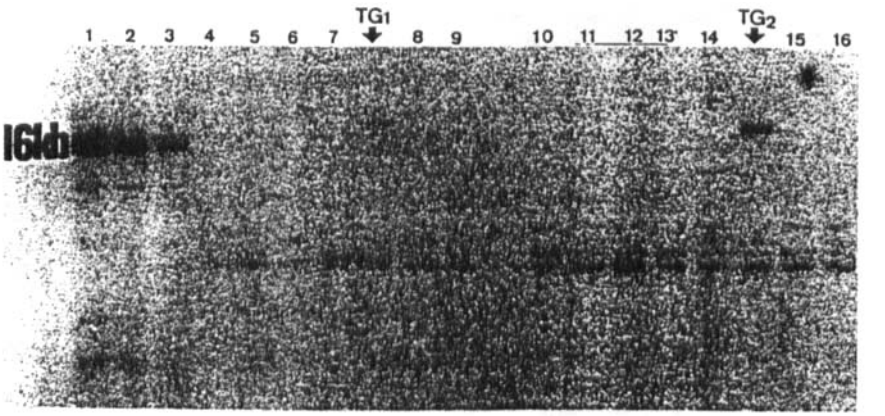

FIGURE 1 *Southern-blot analysis of transgenic founder mice.* 5 μg of mouse tail DNA were digested with ECoRI, electrophoresed through 0.8% agarose gel, transferred to Zetabind membrane and hybridized with a 32 P-labeled human SOD-1 cDNA. Lanes 1,2,3: human DNA digested with EcoR I, lane 1: 10 μg, lane 2: 7.5 μg, lane 3: 5 μg. Lanes 4–16: mouse tail DNA from negative transgenic mice. TG1: transgenic founder mice; TG2: transgenic founder mice.

(100°C, 10 minutes).[18] Brain proteins were separated by SDS-PAGE and transferred to nitrocellulose for Tau immunodetection as in.[18]

RESULTS

Transgenic Mice

Two founder mice containing the human SOD-1 gene were obtained (TG1 – TG2) (Figure 1) and have transmitted the human transgene to their offspring. Slot blot analysis was used to quantify the number of integrated copies of human SOD-1 gene. Figure 2 shows that, for equal quantities of DNA loaded on the membrane, the intensities of the hybridization bands with the SOD-1 probe were lower in transgenic mice than in human control or trisomy 21 DNA. In our experimental conditions, the human SOD-1 probe did not significantly cross-hybridized with mouse SOD-1 gene (see control mouse in Figure 2). These results indicate that only one copy of the SOD-1 gene was integrated in mouse genome.

Expression of the Human SOD-1 Transgene:

Northern blot analysis of total RNA from the two transgenic strains have revealed that TG1 strain (TG 37) did not express the human SOD-1 gene. Since no gene rearrangement was observed on Southern blot analysis, this situation likely results from the influence of the site of integration on expression of the transgene. By contrast, in the TG2 strain, two RNA transcripts of 0.9 kb and 0.7 kb (Figure 3) were detected with the human SOD-1 cDNA probe. This result indicates that the human SOD-1 gene is expressed in TG2 as in human tissues. By using a 28 S probe as a

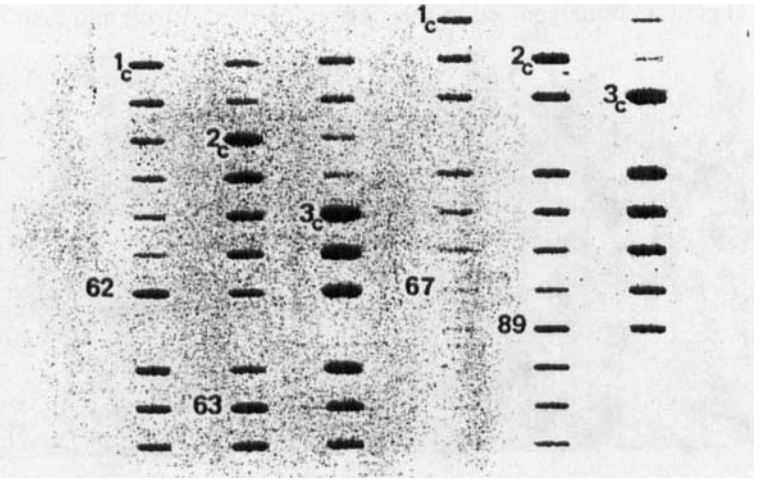

FIGURE 2 *Estimation of human SOD-1 copy number integrated in the mouse genome by slot blot method.* For each studied mouse (here n° 62,63,67 and 89), 6 different amounts of tail DNA ranging from 0.5 to 5 μg were blotted after denaturation on Zetabind membrane using a slot blot apparatus. A normal human DNA (2C) and DNA from a free trisomy 21 patient (3C) were used as controls for 2 and 3 copies of the human SOD-1 gene. Hybridization was performed with a ^{32}P-labeled human SOD-1 cDNA.

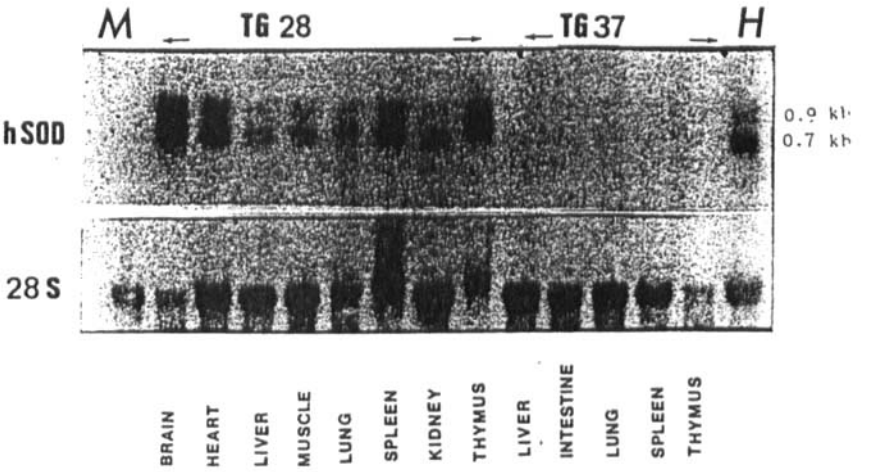

FIGURE 3 *Northern blot analysis of RNAs from various tissues of TG 28 (TG 2 strain) and TG 37 (TG1 strain) transgenic mice.* Each lane contained 10 μg of total RNA. Hybridization was done successively with a 32 P-labeled human SOD-1 cDNA and a 28 S probe as a reference. H: human RNA from fibroblasts with two SOD-transcripts of 0.9 kb and 0.7 kb. M: control mouse RNA.

reference (Figure 3), analysis of the same Northern blot suggested that the level of expression of the human transgene was tissue – specific with higher SOD-1 mRNA amounts in brain, thymus and heart than in liver, lung and kidney. Increased SOD-1 activity was detected in all tested tissues of the TG2 strain (Table I). Increased activity was more pronounced in brain (1.93 fold), heart (1.69 fold), and thymus (1.49 fold) than in muscle (1.25 fold), liver (1.19 fold), kidney (1.18 fold), spleen (1.35 fold), lung (1.26 fold) and erythrocytes (1.09 fold).

Consequence of Increased Brain SOD-1 Activity on Enzyme Activities Associated with Oxygen – Radicals metabolism

Despite the 1.93 fold increase in SOD-1 activity, the Se GSH-Px, GSSG reductase and

TABLE I

SOD – 1 activity (U/mg protein or hemoglobin) in various homogenized tissue supernatants from control and human SOD-1 transgenic mice. Results are expressed as mean ± S.D.; n: number of mice
Age of the mice was between 50 and 70 days. Comparison of the means were assessed by Student t-test.

Tissue	Control mice (C)	Transgenic mice (T)	Comparison (t test)	Ratio T/C
Heart (n = 6)	17.21 ± 2.97	29.14 ± 3.59	$p < 0.001$	1.69
Lung (n = 6)	31.52 ± 3.35	39.83 ± 5.52	$p < 0.02$	1.26
Thymus (n = 6)	19.02 ± 2.68	28.34 ± 6.79	$p < 0.02$	1.49
Red Cells (n = 6)	10.83 ± 1.17	11.88 ± 0.94	NS	1.09
Liver (n = 6)	57.82 ± 1.08	68.86 ± 5.36	$p < 0.01$	1.19
Kidney (n = 6)	52.54 ± 4.92	61.93 ± 5.78	$p < 0.02$	1.18
Muscle (n = 3)	29.92 ± 2.33	37.44 ± 4.25	NS	1.25
Spleen (n = 5)	20.02 ± 1.94	26.98 ± 4.33	$p < 0.02$	1.35
Brain (n = 19)	29.55 ± 3.30	57.10 ± 6.84	$p < 0.001$	1.93

(NS: not significant)

TABLE II

Activities of enzymes of the oxygen free-radicals metabolism in the brain of human SOD-1 transgenic (T) and control (C) mice. Results are expressed as mean ± S.D.; n: number of mice
Age of the mice was between 50 and 70 days. Comparison of the means were assessed by Student t-test.

	Control mice (Whole brain)	Transgenic mice (Whole brain)	Comparison (t-test)	Ratio T/C
SOD-1 activity (Units/mg protein)	29.55 ± 3.30 (n = 19)	57.10 ± 6.84 (n = 19)	$p < 0.001$	1.93
Se-Glutathione peroxidase (nmol NADPH oxidized/ min/mg protein)	12.70 ± 1.77 (n = 13)	13.64 ± 2.27 (n = 12)	NS	1.07
Glutathione reductase (nmol NADPH oxidized/ min/mg prot)	40.47 ± 5.12 (n = 13)	39.91 ± 5.38 (n = 12)	NS	0.99
Glutathione S-transferase (nmol CDNB conjugated /min/mg prot)	175.61 ± 27.13 (n = 15)	171.16 ± 26.74 (n = 13)	NS	0.97

NS: not significant

GST activities were not found significantly modified in the brain of (1 – 3 months) transgenic mice (Table II).

Effect of Increased Brain SOD-1 Activity on Tau Proteins

In brain homogenates from transgenic mice. a Tau immunodetection profile observed after SDS-PAGE was the same as in control mice, showing mouse Tau proteins of 50 000 to 55 000 daltons (Figure 4).[19] Tau variants with a heavier molecular weight than normal Tau proteins which have been described in Alzheimer's brain patients[19] were not detected here neither with a anti-human Tau antibody nor with a anti-human PHF antiserum.

DISCUSSION

Our results show that the human SOD-1 gene[13] was successfully introduced into the germline of mice and stably expressed as an active enzyme in all tested tissues. In some tissues like brain, the increase in SOD-1 activity was within the range of that found in DS (1.93 fold increase in the transgenic brain). Therefore, these animals provide a unique system for testing possible links between biological and clinical abnormalities found in DS and increased gene dosage for SOD-1. The interest of this approach is supported by recent reports demonstrating abnormal neuromuscular junction in tongue[10] and diminished serotonin uptake in platelets of SOD-1 transgenic mice, [21] both pathological traits which are observed in DS.

It has been suggested that increased SOD-1 activity could produce oxidative damages within cells and might be involved in some major neurobiological abnormalities found in DS such as Alzheimer-type neuronal pathology.[2,11,12,22] Looking for biological modifications which could support these hypotheses, we have studied in the

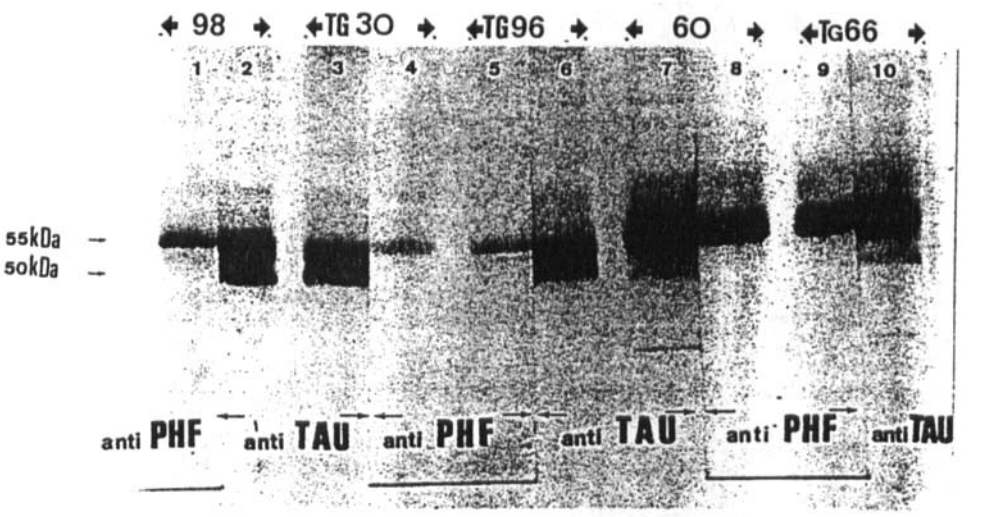

FIGURE 4 *Immunodetection of Tau proteins in the whole brain from transgenic and control mice.* 50 µl of mouse brain homogenates were loaded in large wells at the top of 10–20% polyacrylamide gel gradients. Once separated, proteins were electroblotted to nitrocellulose membrane. Migrating tracks (revealed with Ponceau red) were divided in two parts, the first was treated with the anti serum Tau (1/10.00)[2,3,6,7,10] and the other the anti-human PHG antiserum (1/200)[1,4,5,8,9]. 98 : one month old control mouse. TG 30 and TG 96: one month old transgenic mice. 60: seven months old control mouse. TG 66: seven months old transgenic mouse.

brain of SOD-1 transgenic mice the activities of enzymes involved in oxygen radical detoxification and the electrophoretic pattern of Tau proteins.

Although SOD-1 activity was increased 1.9 fold in the brain of the transgenic mice, Se GSH-Px, GSSG reductase and GST activities were not modified. Then, it appears that, at least in young mice and in a normal oxygen environment, an increase in SOD-1 activity similar to that found in DS do not by itself produce alterations of the enzymes associated with oxygen radicals metabolism. Thus, unlike what has been observed in DS erythrocytes and other tissues[2,23,23] as well as in transfected-cells expressing the human SOD-1 gene[7] but similarly to what was found in DS fetal brain[25] and mouse trisomy 16 fetal brain,[26] SeGSH-Px was not increased in the brain of SOD-1 transgenic mice.

In whole brain homogenates from SOD-1 transgenic mice, the Tau immunodetection profile after SDS-PAGE was the same as in control brain. Thus, Tau variants with heavier molecular weights than normal Tau proteins, which are antigenic components of PHF[20,27,28] were not detected in transgenic animals. These results suggest that in our experimental conditions, brain overexpression of SOD-1 "per se", does not induce modifications of Tau proteins similar to those seen during neurofibrillary degeneration.[18,20,27,29] Since all these experiments were performed on whole brain homogenates and in young animals, other studies, focused on specific brain regions, like hippocampus or cortex, which are brain areas where the human SOD-1 transgene was well expressed (unpublished data), and in animals of various ages are required for accurately assessing the possible consequences of a SOD-1 excess in brain cells.

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