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Genetics

Transgenic mice with increased Cu/Zn-superoxide dismutase activity: Animal model of dosage effects in Down syndrome

(Alzheimer disease/human chromosome 21/aneuploidy)

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ABSTRACT **Down syndrome, the phenotypic expression of human trisomy 21, is presumed to result from a 1.5-fold increase in the expression of the genes on human chromosome 21. As an approach to the development of an animal model for Down syndrome, several strains of transgenic mice that carry the human Cu/Zn-superoxide dismutase gene have been prepared. These animals express the transgene in a manner similar to that of humans, with 0.9- and 0.7-kilobase transcripts in a 1:4 ratio, and synthesize the human enzyme in an active form capable of forming human–mouse enzyme heterodimers. Cu/Zn-superoxide dismutase activity is increased from 1.6- to 6.0-fold in the brains of four transgenic strains and to an equal or lesser extent in several other tissues. These animals provide a unique system for studying the consequences of increased dosage of the Cu/Zn-superoxide dismutase gene in Down syndrome and the role of this enzyme in a variety of other pathological processes.**

Down syndrome (DS), the phenotypic expression of human trisomy 21 (Ts21), is manifested by morphogenetic abnormalities, mental retardation, immunological abnormalities, and the pathological manifestations of Alzheimer disease (AD) (1, 2). Although the mechanisms by which the presence of an extra chromosome 21 causes developmental and functional abnormalities are largely unknown (1), analyses of the activities or concentrations of the known gene products of this chromosome have revealed the existence of precise gene dosage effects. Trisomic cells contain close to 1.5 times as much as diploid cells of Cu/Zn-containing superoxide dismutase (CuZnSOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1), phosphoribosylglycinamide synthetase, cystathionine β-synthase, phosphofructokinase, and the α/β interferon receptor, and similar gene dosage effects have been observed in more than 40 additional loci examined in other human and mouse aneuploids (1). It has been proposed that gene dosage effects for specific loci affect development and function by perturbing a variety of intracellular processes and structures and intercellular interactions (1).

The study of the pathogenesis of a condition such as DS is greatly inhibited by the difficulties attendant on human research. Therefore, attention has turned to the development of animal models, and the trisomy 16 (Ts16) mouse and the Ts16↔2*n* chimeras have been identified as genetic models of human Ts21 and Ts21/2*n* mosaics, respectively (3–5). While useful for many studies, these models do not permit the effects of imbalance of individual loci to be studied directly in fully viable trisomic animals. Our attention has turned, therefore, to the development of animal models in which the dosage of specific human chromosome 21 genes is increased—an approach anticipated by Lederberg (6) more than 20 years ago.

Human CuZnSOD (h-CuZnSOD), a key enzyme in the metabolism of oxygen free radicals (for review, see ref. 7), is encoded by the gene *SOD1* in band 21q22.1 (8). This band is in the region to which the overall phenotype of DS has been attributed (9). The overexpression of the CuZnSOD gene could disturb the steady-state equilibrium of active oxygen species within the cell, resulting in oxidative damage to biologically important molecules (10, 11). It has been suggested that such a mechanism may be responsible for or contributory to either the mental retardation or Alzheimer disease, or both, which are part of the DS phenotype (12–15).

To permit the systematic investigation of the possible involvement of CuZnSOD overproduction in the etiology of DS, the gene and its cDNA were cloned (16–18) and then introduced into human HeLa cells and mouse L cells as part of recombinant plasmids containing the neomycin-resistance (Neo^R) selectable marker (19). Cell clones expressing elevated levels (up to 6-fold) of authentic enzymatically active h-CuZnSOD were isolated and found to have altered properties, with increased lipid peroxidation and higher resistance to the toxic effects of paraquat, a superoxide generator. These observations suggested that transgenic mice overexpressing CuZnSOD would provide an animal model for critically testing the hypothesis that the increased CuZnSOD activity present in DS has adverse phenotypic effects. To this end we have introduced the human gene for CuZnSOD into the germ line of mice and report that this gene is expressed as enzymatically active CuZnSOD in transgenic mice.

MATERIALS AND METHODS

Transgenic Mice. Transgenic mice carrying the h-CuZnSOD gene were produced essentially as described by Gordon and Ruddle (20). A linear 14.5-kilobase (kb) *Eco*RI–*Bam*HI fragment of human genomic DNA (Fig. 1) was excised from the recombinant plasmid pHGSOD-SVneo (19) and separated from plasmid sequences prior to microinjection. The *Eco*RI–*Bam*HI DNA fragment contained the entire hCuZnSOD gene, including the sequences required for expression in transfected cells (16, 19). Approximately 500 copies of the purified fragment were microinjected into the male pronuclei of CBYB/6 × B6D/2 zygotes in Rehovot and B6SJL zygotes in San Francisco. CBYB/6 is (BALB/c × C57BL/6J) F_1, B6D/2 is (C57BL/6J × DBA) F_1, and B6SJL is (C57BL/6J × SJL/J) F_1.

Analysis of Integrated Sequences. For Southern blot analysis, approximately 1 cm of mouse tail was placed in 1 ml of 50 mM Tris·HCl, pH 8.0/100 mM EDTA/0.5% sodium

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Abbreviations: AD, Alzheimer disease; DS, Down syndrome; CuZnSOD, Cu/Zn-containing superoxide dismutase; h-CuZnSOD, human CuZnSOD; Ts21 and Ts16, trisomy 21 and trisomy 16.

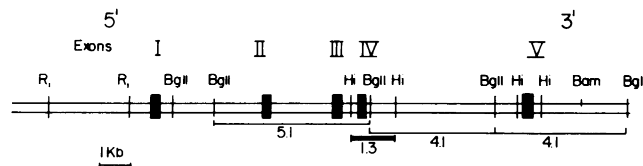

FIG. 1. Restriction enzyme map of the h-CuZnSOD gene sequence used in the construction of the transgene in transgenic mice. The 1.3-kb *Hin*dIII fragment used as a hybridization probe in Fig. 2 *Left* is represented as a solid bar below the map. The positions of the five exons are shown as solid boxes. The following restriction sites are shown: R_1, *Eco*RI; BglI, *Bgl* II; Hi, *Hin*dIII; Bam, *Bam*HI.

dodecyl sulfate/200 μg of proteinase K per ml and incubated at 50°C for 12–16 hr. DNA was extracted and analyzed as described (21). DNA (10 μg) was digested with either *Bgl* II or *Pst* I and blot-hybridized with either a ^{32}P-labeled 1.3-kb *Hin*dIII fragment of the h-CuZnSOD gene (Fig. 1), which contains only 140 base pairs (bp) of exon IV and shows low cross-reactivity with the mouse CuZnSOD gene or with a ^{32}P-labeled h-CuZnSOD cDNA clone.

RNA Blot Analysis. Total RNA was isolated from the brain and liver of transgenic and littermate control mice (22). Human RNA was isolated from simian virus 80 cells. RNA (10 μg) was blot-hybridized (18) to a ^{32}P-labeled h-CuZnSOD cDNA clone.

Analysis of h-CuZnSOD and Total CuZnSOD Activity. Two methods were used for assessment of CuZnSOD activity. For measurement of CuZnSOD specific activity, supernatant fluids from homogenized tissues were assayed as described (19, 23). For polyacrylamide gel analysis, tissues were perfused to remove blood and then homogenized in 50 mM Tris·HCl (pH 7.0). Homogenates were centrifuged at 16,000 × *g* for 15 min at 4°C. Each tissue extract (50 μg) was loaded onto either a 7.5% acrylamide/urea gel or a 10% acrylamide gel and electrophoresed for 3 hr at 150 V. Staining for SOD enzymatic activity was performed as described elsewhere (19) except that the gels were soaked in nitro blue tetrazolium, tetramethylethylene-diamine, and riboflavin in potassium phosphate for 50 min.

RESULTS

Transgenic Mice. Four founder females containing 1–5 copies of the h-CuZnSOD gene were obtained in Rehovot, and five founder animals (four females, one male) containing 1–12 gene copies were obtained in San Francisco (Table 1, Fig. 2). Each of the Rehovot founder females was mated to B6D/2 mice, and the San Francisco founders were mated to B6SJL mice. Three founders, TgHS-47, TgHS/SF-155, and TgHS/SF-218, transmitted the CuZnSOD gene to their offspring with frequencies ≥50%, consistent with Mendelian inheritance (Table 1). Although the other founders had transmission ratios of 18–38%, the numbers of animals tested were too small to establish whether any of these animals were germ-line mosaics. However, the difference in copy number between the founder and offspring in line TgHS-51 suggests that the former was a germ-line mosaic. Transmission in second-generation crosses was approximately 50% for all transgenic lines (offspring of TgHS/SF-229 are not yet tested). Both males and females, with male-to-male transmission, were found among the transgenic offspring, indicating that the h-CuZnSOD transgene(s) was on an autosome in each of the founders. Animals homozygous for the TgHS/SF-155 and -218 transgenes were bred and found to be viable. No gross physical or behavioral abnormalities were observed in any of the transgenic founders in their heterozygous or homozygous progeny.

Table 1. Characteristics of h-CuZnSOD-containing transgenic mice

Transgenic founder	Sex	Integrated copies (founder), no.	Transmission from founder to offspring, %	Integrated copies (offspring), no.
TgHS-41	F	1	18	1
TgHS-47	F	1	63	1
TgHS-48	F	3	0 (died)	—
TgHS-51	F	2	25	5
TgHS/SF-155	F	1	59	1
TgHS/SF-218	F	8	50	8
TgHS/SF-229	M	12	38	12
TgHS/SF-242	F	3	23	3
TgHS/SF-287	F	1	33	—

The copy number was determined by Southern blot analysis of tail DNA and comparison of the h-CuZnSOD signal in equal amounts of diploid human and transgenic mouse DNA preparations (three separate determinations) by scanning densitometry.

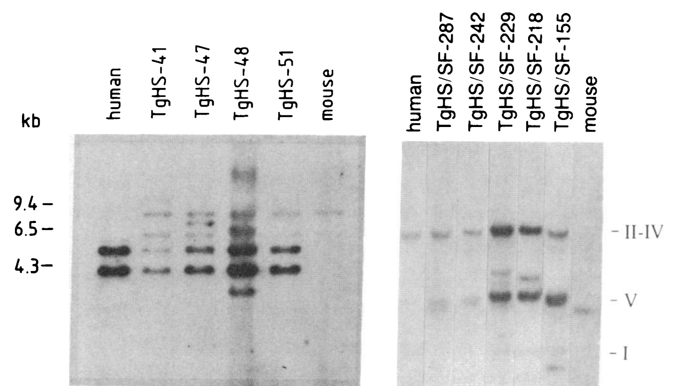

FIG. 2. (*Left*) Southern blot analysis of transgenic founder mice TgHS-41, -47, -48, and -51. (*Right*) Southern blot analysis of transgenic founder mice TgHS/SF-155, -218, -229, -242, and -287. Roman numerals indicate the fragments that contain the corresponding human sequences in Fig. 1. Ten micrograms of DNA was digested with *Pst* I and blot-hybridized with a ^{32}P-labeled human CuZnSOD cDNA clone (17).

Expression of Transgenomes. Blot-hybridization analysis of total RNA from four transgenic lines, TgHS-41, TgHS-51, TgHS/SF-155, and TgHS/SF-218, revealed that the h-CuZnSOD gene is expressed in a fashion similar to its expression in humans, with two RNA transcripts of 0.9 kb and 0.7 kb in a 1:4 ratio (Fig. 3) (18, 21). The level of h-CuZnSOD-

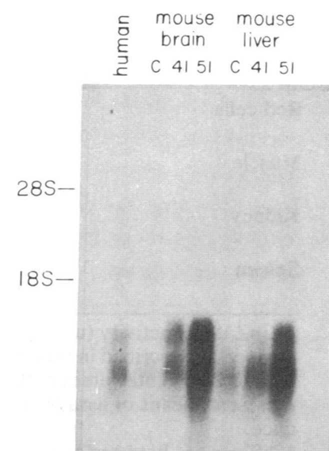

FIG. 3. Blot analysis of RNA from brain and liver of transgenic mice TgHS-41 and TgHS-51.

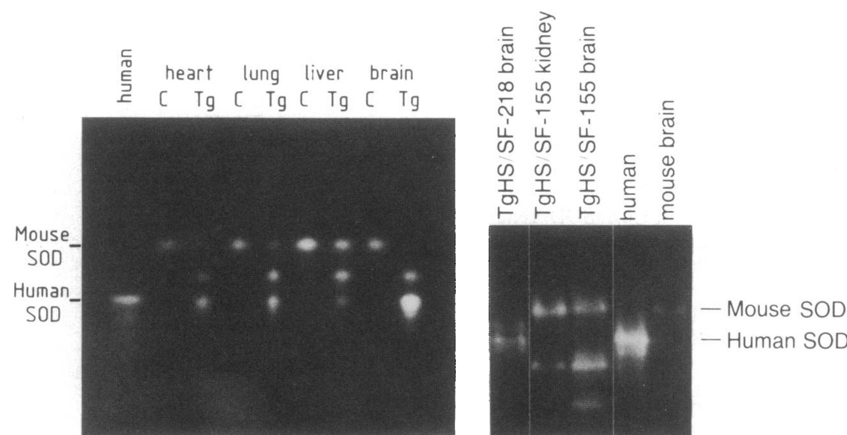

FIG. 4. Polyacrylamide gel analysis of CuZnSOD enzymatic activity in tissues of transgenic mice. (*Left*) Analysis of CuZnSOD activities in the heart, lung, liver, and brain of TgHS-51 (lanes Tg) and littermate (lanes C) tissues. (*Right*) Analysis of CuZnSOD activities in the brain and kidney of TgHS/SF-155 and the brain of TgHS/SF-218. The positions at which human and mouse CuZnSOD enzymatic activities migrate are shown.

specific mRNA was significantly higher (×3) in TgHS-51 than in TgHS-41 (Fig. 3). TgHS-47 did not express the transferred gene.

The level of RNA in the transgenic animals correlated well with the activity of h-CuZnSOD in the transgenic mice, assessed either after gel electrophoresis, which separated the human and mouse enzymes (Fig. 4), or by spectrophotometric assay (Table 2). Increased CuZnSOD activity was detected in nearly all tissues of the TgHS-41, TgHS-51, TgHS/SF-155, and TgHS/SF-218 lines. The ratios of transgenic to control CuZnSOD activity varied considerably from tissue to tissue within a given line but remained constant in successive generations of heterozygous offspring of TgHS-41, TgHS-51, and TgHS/SF-218. In all lines, expression in liver was quite low. These findings suggest that the transgenes have tissue-specific patterns of expression that are not identical with that of the endogenous mouse CuZnSOD. In addition, as has been observed in other transgenic systems, there was no correlation between the number of gene copies integrated (Table 1) and the level of gene expression observed (Table 2). This situation likely results from a combination of two factors: the influence of the site of integration on the regulation and expression of the transgene(s), and alterations in the DNA sequences of some or all of the integrated transgenes when multiple copies are present.

CuZnSOD is a dimer composed of two identical subunits. Therefore, in transgenic animals the enzyme forms a heterodimer (mouse plus human), which is seen in Fig. 4 as the middle band in the tissues of TgHS-51 and TgHS/SF-155. When the level of the human enzyme was high, as in the brain of TgHS-51 (Fig. 4 *Left*) and TgHS/SF-218 (Fig. 4 *Right*), it bound most of the mouse subunits and the upper mouse homodimer band almost disappeared. The h-CuZnSOD homodimer of TgHS/SF-155 had a faster mobility than native

Table 2. Specific activity of CuZnSOD in tissues of transgenic and control mice

Tissue	TgHS-41		TgHS-51		TgHS/SF-155		TgHS/SF-218	
	Tg	Cont	Tg	Cont	Tg	Cont	Tg	Cont
	(ratio)		(ratio)		(ratio)		(ratio)	
Brain	32.2*	15.6	82.3*	13.7	53.0†	33.8	55.4†	24.4
	(2.1)		(6.0)		(1.6)		(2.3)	
Heart	6.6*	5.5	24.7*	5.0	54.6	43.0	61.4†	27.6
	(1.2)		(4.7)		(1.3)		(2.6)	
Lung	18.6*	12.2	29.3*	9.0	51.8	42.8	61.2†	43.4
	(1.5)		(3.3)		(1.2)		(1.4)	
Liver	43.4*	29.3	51.3*	22.4	67.6	69.8	57.6	49.8
	(1.5)		(2.3)		(1.0)		(1.2)	
Red cells			8.8*	1.84	9.02†	5.68	12.3†	7.70
			(4.8)		(1.7)		(1.6)	
Muscle					49.8	36.6		
					(1.4)			
Kidney					58.8	55.8		
					(1.1)			
Spleen					54.0†	46.6		
					(1.2)			

CuZnSOD activity (units per mg of protein or hemoglobin) in homogenized tissue supernatants was assayed as described in refs. 19 (Rehovot) and 23 (San Francisco). Because of differences in the assays used, the absolute values of the activities obtained differ between the two laboratories. Activities listed represent means of assays on ≥5 independent samples per tissue. Cont, control mice; Tg, transgenic mice.

*Differences between transgenics and controls were significant at $P \leq 0.01$ by the Mann–Whitney U test.

†Differences between transgenics and controls were significant at $P \leq 0.05$ by the Wilcoxon test for pair differences (1-sided).

h-CuZnSOD (even though their mRNAs were apparently of the same size), and the mobility of the heterodimer was accordingly displaced (Fig. 4 *Right*). The actual cause of the altered mobility of the h-CuZnSOD subunit has not been determined, but presumably it is a result of a mutation in the structural gene associated with its integration into the mouse chromosome. In this regard, it should be noted that the restriction pattern of the TgHS-155 transgene is somewhat altered from the normal pattern (Fig. 2 *Right* and data not shown).

DISCUSSION

To understand at the molecular level how overexpression of normal genes produces DS, the genes involved in the pathology associated with the syndrome must first be identified by demonstrating a connection between overproduction of their gene products and the phenotypic manifestations of DS (1). It has been shown (20) that elevated levels of CuZnSOD in L cells transfected with h-CuZnSOD cause an increase in lipid peroxidation, and it has been argued that this may alter the structure and function of cell membranes and thus be involved in some of the clinical symptoms of the syndrome. The results reported here indicate that the h-CuZnSOD gene can be transferred into mice and be stably expressed as an active enzyme at levels exceeding those of the endogenous mouse gene and in the range to be expected in a trisomic state. Because the gene is expressed in most if not all cells of the animal throughout its entire developmental history, these animals provide a unique system for correlating the consequences of increased gene dosage of CuZnSOD to clinical symptoms found in DS.

The importance of the approach described here is heightened by recent reports that there may be duplications (resulting in three gene copies) of small regions of chromosome 21 either in individuals with DS with a normal karyotype (24) or in patients with Alzheimer disease (AD) (24, 25). In the former, duplication of the *ETS2* protooncogene and of the loci for CuZnSOD (*SOD1*) and the amyloid β protein has been described (24). The situation is still unresolved in AD, with extra copies of the *ETS2* protooncogene and amyloid β protein gene being described by one group (24) and of the region of the CuZnSOD locus by another (25).

Current thinking about the role of chromosome 21 in the pathogenesis of AD has been significantly influenced by two recent findings. The first is that a locus for familial AD has been identified on chromosome 21 (26). The second is that the gene for the putative precursor of the amyloid β protein, which is deposited in the neurons and cerebral vessels of individuals with AD, is also on chromosome 21 (27–30), in the same vicinity as the familial AD locus (26, 27). While some genetic derangement of the amyloid β protein locus may be etiologically implicated in AD, a role for CuZnSOD has certainly not been ruled out. Therefore, the transgenic lines described here provide an important system for studying the effects of elevated CuZnSOD activity on the brain.

That transgenic mice carrying the h-CuZnSOD gene(s) are not grossly phenotypically abnormal is not surprising, since we certainly did not expect that elevation of CuZnSOD activity would be involved in the pathogenesis of the major dysmorphic features of DS. If CuZnSOD is indeed involved in the genesis of the DS phenotype, we would expect its effects to be on more subtle aspects of tissue function and integrity, particularly in tissues that might be affected over long periods of time by altered metabolism of superoxide radicals. The nervous system is, of course, a prime candidate for investigation, and studies are now under way to determine whether biochemical or morphological alterations are detectable. The important point to consider is that the availability of the transgenic animals makes it possible to investigate in a prospective manner whether increased activity of CuZnSOD does lead to structural, biochemical, or functional abnormalities and, thereby, to assess the possible role of this enzyme in the pathogenesis of the DS phenotype. Although earlier studies on trisomy 16 mouse fetuses did not show increased lipid peroxidation in fetal brains, these investigations were limited by the short time the animals could be observed and by the many other alterations in brain structure and biochemistry resulting from the imbalance of the many loci on chromosome 16. Both problems are obviated by the approach described here. Furthermore, the successful preparation of the CuZnSOD transgenic lines also serves as a precedent for the construction of lines of transgenic mice carrying other human chromosome 21 genes, such as *ETS2* or those for the amyloid β protein or phosphoribosylglycinamide synthetase, either individually or in sets of two or more.

In addition to their relevance to the study of DS and AD, the h-CuZnSOD transgenics will also be of importance in the investigation of the role of CuZnSOD in protection against the adverse effects of a variety of exogenous agents. Thus, exogenous CuZnSOD has been reported to protect against the *in vivo* and *in vitro* lethal effects of radiation (31), drugs (32–34), and oxygen toxicity (35–36); against the oncogenic actions of radiation and chemical agents (37); and against posttraumatic and postischemic cell and tissue injury (38–41). However, such protection experiments are not always successful or easily replicated, possibly because of the difficulty of introducing CuZnSOD in a reproducible manner into intact tissues and cells *in vivo* (42). These difficulties should be obviated by the availability of lines of transgenic mice with different levels of CuZnSOD activity. Such lines also will facilitate investigation of the potential roles of free radicals and CuZnSOD in phenomena such as aging and other degenerative processes (43).

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