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Cell damage by excess CuZnSOD and Down's Syndrome

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Summary – Down's Syndrome (DS), the phenotypic expression of human trisomy 21, is presumed to result from overexpression of certain genes residing on chromosome 21 at the segment 21q22-the Down locus. The "housekeeping" enzyme CuZn-superoxide dismutase (CuZnSOD) is encoded by a gene from that region and its activity is elevated in DS patients. Moreover, the recent discovery that familial ALS is associated with mutations in the gene encoding CuZnSOD, focused attention on the entanglement of oxygen-free radicals in cell death and neuronal disorders. To investigate the involvement of CuZnSOD gene dosage in the etiology of the syndrome we have developed both cellular and animal models which enabled us to investigate the physiological consequences resulting from overexpression of the CuZnSOD gene. Rat PC12 cells expressing elevated levels of transfected human CuZnSOD gene were generated. These transformants (designated PC12-hSOD) closely resembled the parental cells in their morphology, growth rate, and response to nerve growth factor, but showed impaired neurotransmitter uptake. The lesion was localized to the chromaffin granule transport mechanism. These results show that elevation of CuZnSOD activity interferes with the transport of biogenic amines into chromaffin granules. Since neurotransmitter uptake plays an important role in many processes of the central nervous system, CuZnSOD gene-dosage may contribute to the neurobiological abnormalities of Down's Syndrome. As an approach to the development of an animal model for Down's Syndrome, several strains of transgenic mice which carry the human CuZnSOD gene have been prepared. These animals express the transgene as an active enzyme with increased activity 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. To investigate the contribution of CuZnSOD gene dosage in the neuropathological symptoms of Down's Syndrome, we analyzed the tongue muscle of the transgenic-CuZnSOD mice. The tongue neuromuscular junctions (NMJ) in the transgenic animals exhibited significant pathological changes; withdrawal and destruction of some terminal axons and the development of multiple small terminals. The ratio of terminal axon area to postsynaptic membranes decreased, and secondary folds were often complex and hyperplastic. The morphological changes in the transgenic NMJ were similar to those previously seen in the transgenic NMJ and were similar to those previously seen in muscles of aging mice and rats as well as in tongue muscles of patients with Down's Syndrome. The findings suggest that CuZnSOD gene dosage is involved in the pathological abnormalities of tongue NMJ observed in Down's Syndrome patients. Reduced levels of the neurotransmitter serotonin in blood platelets is a clinical symptom characteristic of individuals with Down's Syndrome. To investigate the possible involvement of the CuZnSOD gene, in the etiology of that symptom, we examined blood platelets of the transgenic mice harboring the human CuZnSOD gene. It was found that platelets of transgenic CuZnSOD animals which overexpress the transgene contain lower levels of serotonin, due to a reduced rate of uptake of the neurotransmitter by the dense granules of the platelets. Furthermore, significantly lower than normal serotonin accumulation rate was also detected in dense granules isolated from blood platelets of DS individuals. These findings suggest that CuZnSOD gene dosage affects the dense granule transport system and is thereby involved in the depressed level of blood serotonin found in patients born with Down's Syndrome.

transfected cells / excess CuZnSOD / Down's Syndrome

Introduction

The human copper zinc superoxide dismutase (hCuZnSOD) is encoded by a gene residing on chromosome 21. This chromosome when triplicated causes the phenotypic expression of Down's Syndrome (DS) [7] and elevated production of CuZnSOD, due to gene dosage, is commonly found in DS patients [15, 32]. DS is the most common human genetic disorder occurring once in every 600-800 live births. The affected individuals suffer from a wide range of symptoms. Most obvious among these are morphological defects such as hypotonia in the neonate, short stature and the epicanthic eye folds which give rise

to the eye shape characteristic of the syndrome. Patients are mentally retarded and those who survive past their mid-thirties usually develop Alzheimer's disease [10]. The risk of a child being born with trisomy 21 sharply increases as maternal age progresses into the fourth decade of life, and because presently many couples in Western societies postpone parenthood until these age groups, the incidence of DS is expected to increase. The presently available techniques for prenatal screening for DS (amniocentesis and chorion villi biopsy) are costly and not without risk; they are therefore applied routinely only to at-risk pregnancies, with the result being that most pregnancies are not screened at all for DS. Moreover, because of continuous improvements in all aspects of clinical treatment, the life expectancy of DS patients has tripled during the past two decades; middle-aged DS patients are no longer a rare occurrence. Thus, despite the medical and technological advances of recent years, the prevalence of DS individuals in society is not likely to be significantly decreased in the near future. Although DS was described more than a century ago and the relationship between trisomy 21 and the Down's phenotype has been known for over 30 years [20], very little is known about the way in which the additional chromosome 21 causes the disease. The syndrome is distinguished from most other genetic disorders; the latter are for the most part the result of a defect in a single gene causing a reduction in the activity of a gene product. Whereas in DS the wide range of symptoms is caused by an overexpression of several otherwise normal genes. The current concept is that the presence of extra copies of chromosome 21 genes results in the synthesis of increased amounts of gene products which creates an imbalance in various biochemical pathways; this in turn causes the physiological defects giving rise to the clinical picture of the syndrome. One of the main research efforts in the molecular genetics of DS is directed towards cloning of genes residing on chromosome 21 [25] and relating the consequences of their enhanced expression to the clinical symptoms of the syndrome through the use of model systems [7, 12, 16].

Model systems for gene dosage effects of CuZnSOD in DS

CuZnSOD is a key enzyme in the metabolism of oxygen-free radicals. Over-expression of this gene in DS may upset the steady-state equilibrium of active oxygen species within the cell resulting in oxidative damage to biologically important molecules. Indeed, Yim *et al* [37] have recently reported that CuZnSOD is able to catalyze the formation of hydroxy radicals from hydrogen peroxide and others have shown that elevated levels of SOD actually enhance the cytotoxicity of active oxygen radicals [13, 19, 28]. Excess oxidative activity produced by increased activity of CuZnSOD could in part be responsible for the phenotypic defects found in DS [17, 31]. The possible involvement of CuZnSOD overproduction in the etiology of the syndrome was investigated by us during recent years through the use of two types of model systems.

The reasons motivating our efforts to develop a suitable system for studying the molecular events underlying gene dosage effects in DS are two-fold. The first stems from difficulties attendant in research in man. Most of the pathological consequences of trisomy 21 are manifested during fetal development; research on human subjects, especially *in utero* is ethically complicated and practically impossible. A second reason is associated with the need to identify and sort-out the quintessence from the large number of genes residing on chromosome 21. It is not clear how many genes are involved in determining the characteristic DS phenotype and which one is doing what. Two types of model system were developed, a cellular system, consisting of cultured cells stably transfected and overexpressing the CuZnSOD gene and an animal model, employing transgenic mice, harboring the human CuZnSOD gene and producing increased levels of active enzyme.

Transfected cells with increased activity of CuZnSOD have altered properties

When the CuZnSOD gene is introduced by expression vector into cultured animal cells, the recipients resemble trisomy 21 cells except for one important difference. The imbalance is limited to one particular gene, rather than the whole chromosome. A cellular system of this type permits the study of the biochemical effects of the altered dosage of CuZnSOD in a defined background, irrespective of the overexpression of other chromosome 21 genes. The vector constructed, pg-SOD-SVneo (fig·1) contained a 12 Kb EcoRI-BamHI fragment encompassing the human CuZnSOD gene including its regulatory sequences [21]

and a 2.7 Kb BamHI fragment containing the neo transcriptional unit. pg-SOD-SVneo was introduced into mouse L-cells and stable transformants expressing elevated levels of authentic enzymatically active h-CuZnSOD were isolated. The L-SOD clones exhibited altered properties, they were resistant to the toxic effect of paraquat and had increased lipid peroxidation, an indication for altered oxidative balance [8]. The pg-SOD-SVneo vector was also transfected into rat pheachromocytoman PC12 cells and clones expressing increased amounts of h-CuZnSOD were obtained. While outwardly maintaining their response to nerve growth factor and their typical appearance of cultured neurons, the cells expressing the extra gene had a greatly reduced capacity to take up certain types of neurotransmitters like dopamine or norepinephrine whereas the uptake of others like ascorbate or choline was normal (fig 2). Following detailed analysis of the phenomenon it was discovered that in the transformant – CuZnSOD cells, the chromaffin granules; the cellular organelles responsible for accumulating neurotransmitters, have a lesion in the transport mechanism. It was found that the pH gradient (ΔpH) across the membrane, which is the main driving force for neurotransmitter transport, was diminished in the PC12-SOD granules (fig 3A). This deficiency could have important consequences for neurons in the central nervous system that use a similar organelle-the synaptic vesicle – for accumulation of neurotransmitters. If a released transmitter substance persists for an abnormally extended period, new signals cannot get through at the proper rate. This observation demonstrated that even at the cellular level, an imbalance in the expression of the CuZnSOD gene has a deleterious effect which if it occurs in the central nervous system, would produce alterations in neuron function, which would impair the transduction of signals and mimic defects found in DS [9]. The PC12-SOD clones were also found to have impaired biosynthesis of prostaglandin E_2 (PGE_2). Prostaglandins (PGs) are un-

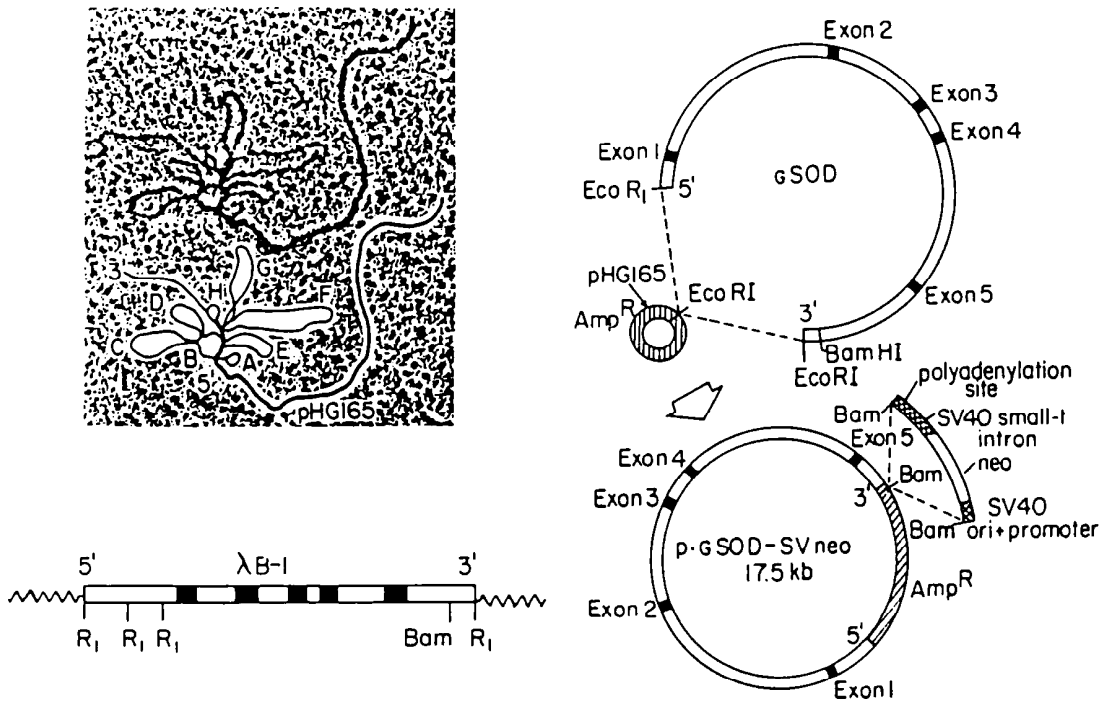

Fig 1. Structure of h-CuZnSOD gene and recombinant plasmid. (Right) Shuttle vector for expression of the CuZnSOD gene. Hatched segments represent the plasmid pG165 DNA that contains the pBR322 origin of DNA replication, the β-lactamose gene, and a polylinker. Open segment represents the neo gene, and the cross-hatched segment represents the SV40 transcription regulatory elements. Closed segments indicate the exons of the CuZnSOD gene. (Left) Electron micrograph and tracing of heteroduplex molecules between the CuZnSOD cDNA and the genomic fragment present in pg-SOD-SVneo which was also used for microinjecting mouse fertilized eggs. (Reprinted with permission from [14]).

saturated lipids derived from membrane-bound arachidonic acid, a known substrate for lipid peroxidation. The biosynthetic pathway of PGs involves free-radical mediated reactions and is greatly influenced by the oxidative balance within the cell. As shown in figure 3B, the synthesis of PGE_2 was significantly reduced in PC12-SOD clones. The rate limiting step in the biosynthesis of PGs is the cleavage of arachidonic acid from cellular phospholipids. This step is usually induced by calcium ionophores and cholinergic agonists. Addition of the calcium ionophore A23187 or the cholinergic agonist carbachol to PC-12 cells greatly enhanced the bio-

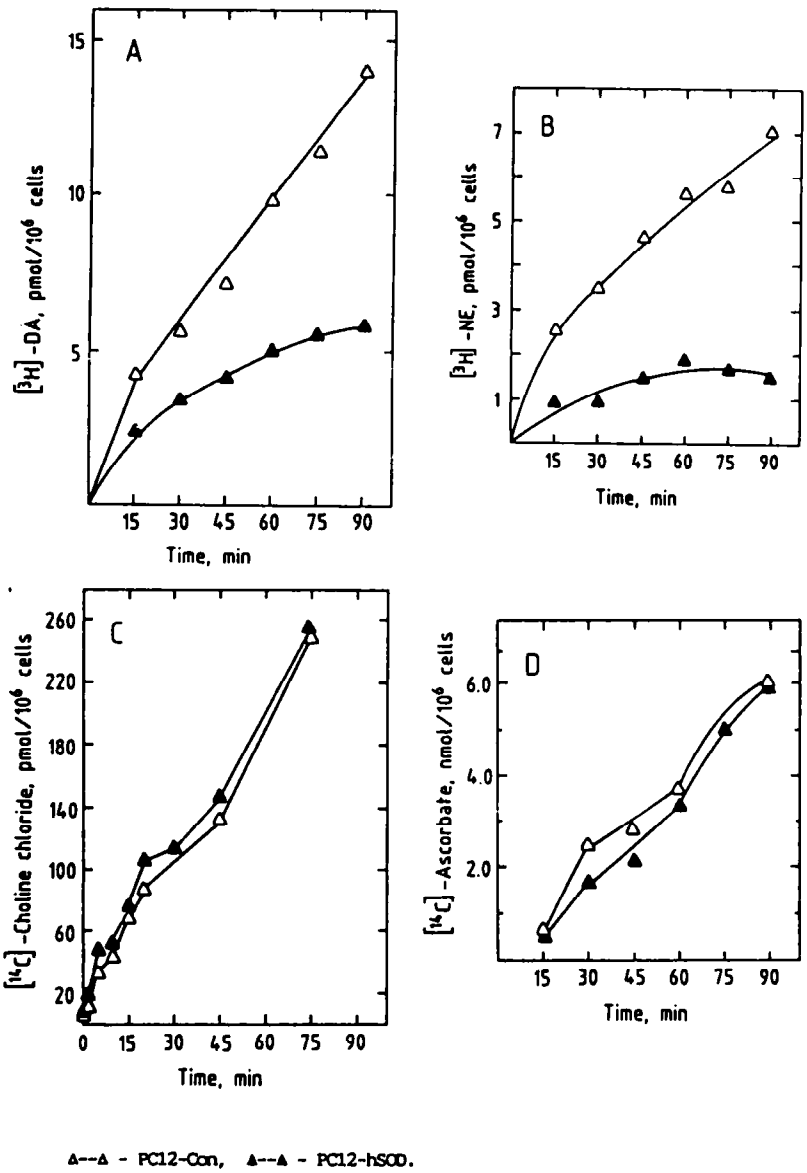

Fig 2. Accumulation rate of neurotransmitters by PC12-SOD transformants. Cultures were exposed for the lengths of time indicated to medium containing (**A**) 2.3 × 10^{-7} M ^{3}H-DA (**B**) 10^{-7} M ^{3}H-NE (**C**) 1 μM ^{14}C-cholin and (**D**) 50 μM ^{14}C-ascorbate. Each point represents the average of two determinations on duplicate cultures Δ---Δ PC12-Con. Δ---Δ PC12-SOD (Reprinted with permission from [9, 14]).

synthesis of PGE_2 (fig 3B), but even in the presence of these inducers the formation of PGE-2 by PC12-SOD amounted to only 50% of the non-induced PC12-control, indicating that it is not the process of generating free arachidonic acid which was impaired. Similar reduction of PGE_2 was measured in fibroblasts obtained from DS patients which bear among other anomalies elevated levels of CuZnSOD. In addition, primary cells isolated from three lines of transgenic CuZnSOD mice showed similar reduction of PGE_2 release. The findings strongly suggest that gene dosage of CuZnSOD causes a reduction in the formation of PGE_2 and link it to a similar reduction observed in trisomy 21 fibroblasts.

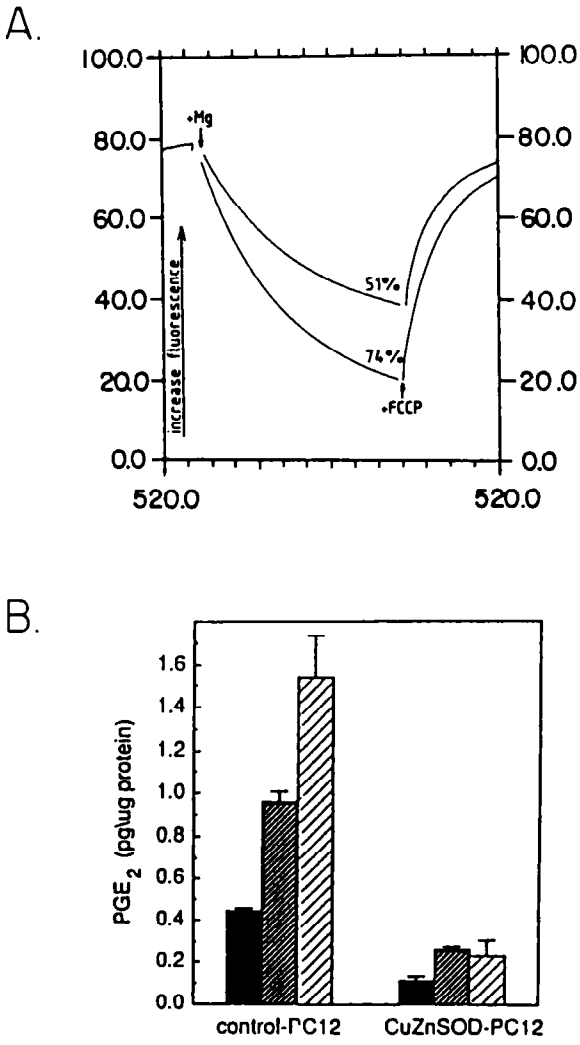

Fig 3. CuZnSOD gene dosage effects in PC12-SOD transformants. **A.** Proton uptake by chromaffin granules measured by acridine orange fluorescence quenching. Reactions were initiated by addition of $MgSO_4$ and terminated after 10 min by addition of FCCP. The quenching of fluorescence due to acidification amounted to 74% for PC12-Cont and 51% for PC12-SOD. **B.** Release of PGR_2 from control and PC12-SOD cells. A representative experiment, performed in triplicate. The vertical bars on top of the histograms represent the SEM. The amount of PGE_2 is expressed as pg PGE_2 released per mg cell protein. Untreated; treated with A23187 or with carbachol. (Reprinted with permission from [9]).

Transgenic-CuZnSOD mice with elevated levels of the enzyme display phenotypic features of Down's Syndrome

Gene transfer into mice, leading to the creation of so-called transgenic mice, can be achieved by microinjecting a foreign DNA into fertilized eggs. The exogenously introduced gene becomes stably integrated into the mouse chromosome, and the resultant embyro develops into a mouse that carries an extra gene and transfers it to subsequent generations in a Mendelian fashion. Transgenic mice harboring the h-CuZnSOD gene have an advantage over transfected cultured cells in that they resemble more closely the natural situation; the transgene is present in every cell of the animal, and its influence is manifested throughout its entire developmental history. The results obtained with the transfected cells suggested that transgenic mice, overexpressing CuZnSOD would provide an animal model for investigating the possibility that increased CuZnSOD has an adverse phenotypic effect. Transgenic-CuZnSOD mice harboring the h-CuZnSOD gene were produced by microinjecting fertilized eggs with a linear 14.5 Kb EcoRI-BamHI fragment (fig 1) of human genomic DNA containing the entire gene, including its regulatory sequences [11]. Several strains of transgenic mice designated TgSH-41, 51, 69 and 70 carrying from one to several copies of the h-CuZnSOD gene were obtained. These animals expressed the transgene in a manner similar to that of humans, with two RNA transcripts of 0.9 and 0.7 Kb [29, 30]. The level of RNA in the transgenic animals correlated well with the activity of the human enzyme assessed after electrophoresis which separates the human and mouse enzymes (fig 4A). Increased CuZnSOD activity was recorded in nearly all tissues of the TgHS strains. While the ratio of transgenic h-CuZnSOD to control activity varied from tissue to tissue it remained constant in successive generations of heterozygous offspring. In all TgHS lines, expression was high in brain and

relatively low in liver (fig 4A). Comparable with other transgenic systems there was no correlation between the number of gene copies integrated and the observed level of transgene expression. This situation likely results from a combination of two factors: the influence of the site of integration on the regulation of expression and alterations in the DNA sequences of some of the integrated trans-

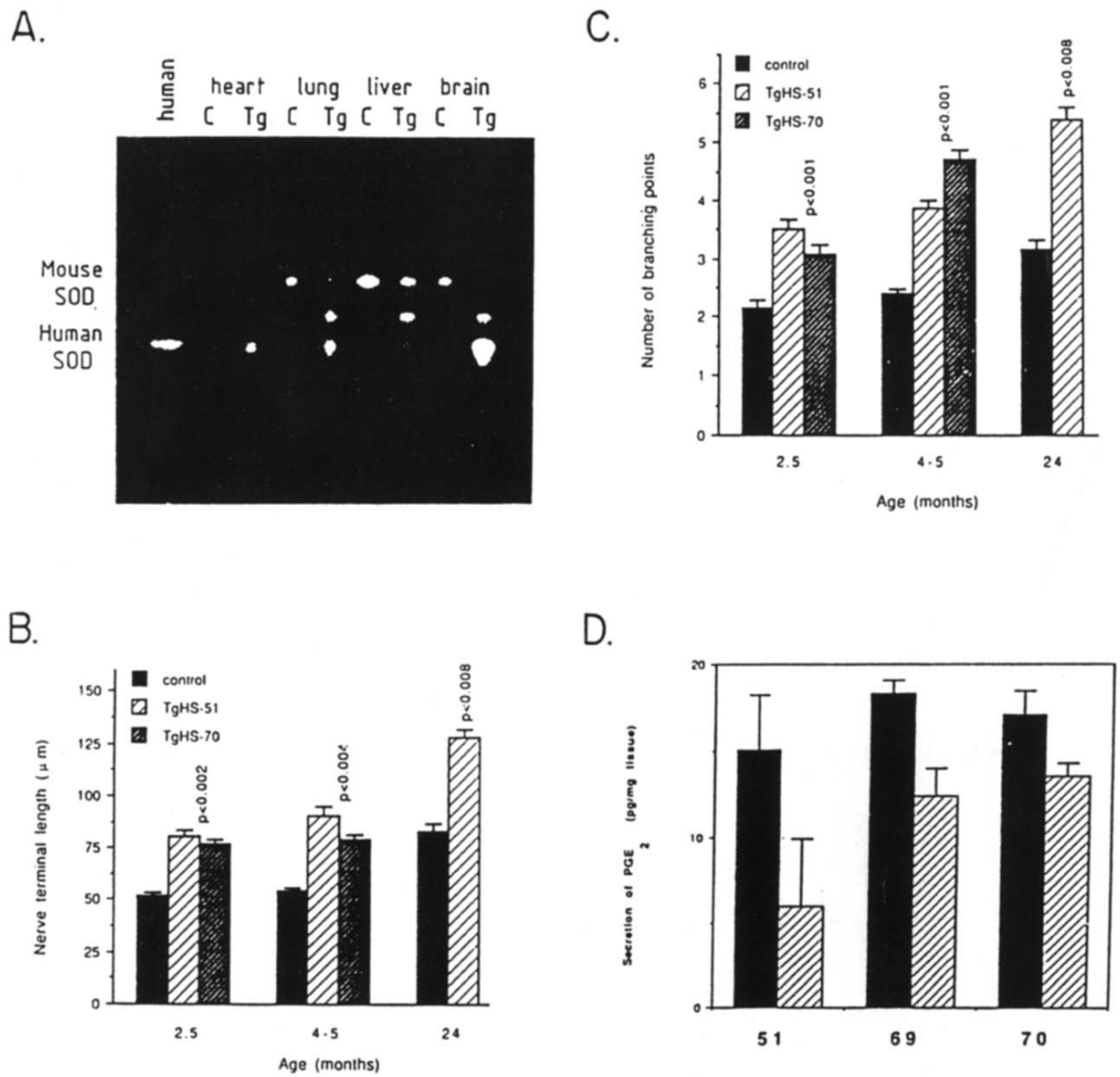

Fig 4. Expression of the h-CuZnSOD gene in transgenic mice and the consequent phenotypic effects. **A.** Polyacrylamide gel analysis of CuZnSOD enzymatic activity in tissues of the transgenic mice – TgHS 51 (Tg-lanes) and littermate (c-lanes). **B, C.** NMJ morphometry in soleus muscle (**B**) Nerve terminal length and (**C**) number of nerve terminal branching points. At each age, 4-6 mice were analyzed. Results are expressed as mean ± SE. The changes between controls and transgenics are indicated. Control mice (2.5 and 4-5 month-old) differed significantly from 24-month-old controls ($P < 0.001$). Transgenic mice (2.5 and 4-5 months-old) differed significantly from 24-month-old transgenics ($P < 0.001$). **D.** Release of PGE_2 from kidneys of transgenic-CuZnSOD and non-transgenic control mice. Experiments were performed in triplicate with each of the three strains 51.69 and 70 of TgHS mice. The vertical bars on the top of the histograms represent the SEM. The amount of PGE_2 released is expressed as pg PGE_2 per mg of tissue non-transgenic, transgenic-CuZnSOD. (Reprinted with permission from [3, 16]).

genes when multiple copies are present. CuZnSOD is a dimer comprised of two identical subunits. Therefore, in the transgenic animals the enzyme formed a heterodimer (mouse plus human) which is seen in figure 4A as the middle band in extracts from tissues of TgHS-51 mice. When the level of the human enzyme was high, as in TgHS-51 brain, it bound most of the mouse subunits causing the disappearance of the upper band representing the mouse homodimer. To rule out the possibility that the phenotypic effects were due to insertional mutagenesis, several strains of TgHS were prepared and analyzed. The different hybridization pattern in Southern blot analysis of the transgenic lines indicated different integration sites in each of them. In DS patients, the additional 21 chromosome may cause alterations in the expression of various genes resulting in abnormal levels of gene products in specific organs or even certain regions within the organ. It was therefore interesting to examine the expression pattern of the h-CuZnSOD transgene in the brain of the transgenic animals. The pattern of the human enzyme expression was determined by immunohistochemistry using anti h-CuZnSOD antibodies which do not cross-react with the mouse enzyme. Forebrain regions such as cortex, hippocampus and basal ganglia, as well as brain stem nuclei (substantia nigra, central gray, locus coeruleus) exhibited higher expression of the transgene as compared to other regions. This expression pattern was similar to that of the mouse enzyme visualized in control littermate mice. These results indicated that overexpression of the h-CuZnSOD was confined to brain regions in which the resident mouse gene was expressed. Outwardly, the transgenic-CuZnSOD mice appeared normal, without any obvious deformities. This was not surprising, because there was no reason to expect that elevation of CuZnSOD activity alone causes the major dysmorphic features of DS. Rather, it was anticipated that overexpression of CuZnSOD will effect more subtle aspects of tissue function and integrity, particularly in those tissues that might be affected by altered metabolism of oxygen-free radicals. Bearing in mind the effect observed in the cellular system (PC12-SOD), the neurotransmitter serotonin was quantitated in the blood of the TgHS mice and was found to be significantly lower than the value in non-transgenic littermate mice [26]. This observation generated much interest, because reduced concentration of blood serotonin has, for some time, been observed as a well known clinical symptom among DS patients. When this deficiency was first noticed in the 1960s, it aroused considerable attention because of the possible relevance of serotonin uptake by blood platelets to neurotransmitter function in the central nervous system, hence, its involvement in the hypotonia and mental retardation of DS [4, 5]. At that time, attempts were made to raise the levels of blood serotonin in DS infants by administration of its precursor, 5-hydroxytryptophan; muscular tone, motor activity, and sleep abnormalities were reported to improve concomitantly with its administration. However, the development of infantile spasms, a severe seizure syndrome, in 17% of the patients receiving the drug brought these studies to a halt [6]. Serotonin is an important neurotransmitter in the central nervous system, both in the embryonic state and in infants. It usually does not appear free in the blood circulation because of its efficient uptake by platelets, where it is accumulated and stored in the dense granules. Detailed analysis of platelets isolated from the transgenic-CuZnSOD mice revealed that the uptake process in these granules was impaired, and this constituted the cause for the reduced concentrations of blood serotonin in these animals. The dense granules of the platelets are in many respects similar to the chromaffin granules of PC12 cells, which, as described above, were affected by the increased activity of CuZnSOD. It is intriguing that this same lesion appears both in the PC12-SOD cellular system and in the transgenic-CuZnSOD mice and that the consequent defect is a well-known deficiency diagnosed in DS.

The transgenic-CuZnSOD mice also had abnormalities in the connections between nerve terminals and the muscles, the so-called neuromuscular junctions (NMJ). Such a defect was first noticed in the tongue muscle of DS patients. In many of them, the large protruding tongue is a striking clinical feature. The reasons for the protrusion have been considered as being related to hypotonia of the lower lip and localized tongue enlargement in the region of the lingual tonsil. Studies conducted by Yarom *et al* [34, 35] on the tongue muscle of patients with DS revealed pathological changes in the NMJ. The pathology consisted of terminal axon degeneration and changes in the end plates and myofibers. In addition, significant increases in the concentrations of copper and calcium were recorded in the DS tongues. It was suggested that the latter may be due to the abnormally high levels of CuZnSOD

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