COORDINATE EXPRESSION OF Mn-CONTAINING SUPEROXIDE DISMUTASE AND Cu,Zn-CONTAINING SUPEROXIDE DISMUTASE IN HUMAN FIBROBLASTS WITH TRISOMY 21

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SUMMARY
The amount of Mn superoxide dismutase (MnSOD) and the activity of Cu,Zn-superoxide dismutase (CuZnSOD) have been studied in human fibroblasts of five subjects with trisomy 21 and five subjects with normal karyotype, using nuclear magnetic relaxation and polarographic methods. In the trisomic fibroblasts we have found a mean molar amount of MnSOD 25.4% lower than in the control, and an amount of CuZnSOD 54.7% higher. A positive significant correlation between the activities of both enzymes has been observed indicating that the two enzymes dismutate the $\text{O}_2^-$ cooperatively. However, the increase of MnSOD per unit of CuZnSOD appears significantly lower in the trisomic fibroblasts, an effect that is not due to a diminished inducibility of MnSOD. These findings suggest that the MnSOD and CuZnSOD genes interact to preserve the normal level of total SOD activity.

INTRODUCTION
The regulation of protein synthesis is fundamental for the maintenance of the normal cell metabolism, the expression of differentiated functions, and the adaptation to changing environmental situations. Among the regulatory mechanisms discovered so far we can mention: the production of specific proteins or diffusible regulators that act upon regulatory sites on DNA or RNA (Conzelman & Sandhoff, 1978; Levin et al. 1978; Kielty, Povey & Hopkinson, 1981); the activity of regulatory sequences (Barta, Richards, Baxter & Shine, 1981; McGinnis, Shermoen, Heemskerk & Beckendorf, 1983; Davidson, Jacobs & Britten, 1983); RNA processing, splicing and degradation (Scandalios, 1979; Darnell, 1979, 1982; Amara, Jones & Rosenfeld, 1982; Safer et al. 1982; Rozeck & Davidson, 1983); postranscriptional and translational control (Revel & Groner, 1978); gene amplification (Brown & Dawid, 1968; Spradling & Mahowald, 1980). The emerging picture is very complex;

Key words: Mn superoxide dismutase, Cu,Zn-superoxide dismutase, gene regulation, trisomy 21.
however, and it is evident that many important steps or 'control points' (Brown, 1981) of eukaryotic gene regulation remain to be discovered. Relatively little is known, for instance, of gene interaction, a mechanism that must be present in high organisms to coordinate the expression of multiple genes. Such a mechanism could be suspected in the expression of two functionally identical enzymes, the manganese-containing superoxide dismutase (MnSOD) and the copper–zinc-containing superoxide dismutase (CuZnSOD), and tested in 21 trisomic cells where there is an increased production of CuZnSOD due to gene dosage effect (Sinet, Allard, Lejeune & Jerome, 1974; Crosti, Serra, Rigo & Viglino, 1976; Feaster, Kwok & Epstein, 1977; Baret et al., 1981). Both these enzymes dismute the superoxide anions O_2^- (McCord & Fridovich, 1969; Fridovich, 1975) and their structural genes are located on chromosome 6 and 21, respectively. One could expect either (1) that the overproduction of CuZnSOD might be balanced by a regulatory mechanism, which would maintain the total amount of SOD activity at the same level as in normal cells, or (2) that, in the absence of such a regulatory mechanism, the overproduction of CuZnSOD would upset the equilibrium between the two SODs with unfavourable consequences for the cell. We have studied the behaviour of the two enzymes in fibroblasts with trisomy 21 and have found that there exists a regulatory mechanism by which the increased level of CuZnSOD activity becomes rate-limiting for MnSOD production.

To our knowledge, this is the first example, in vertebrates, of genes interacting at the functional level to modulate their quantitative expression reciprocally.

MATERIALS AND METHODS

Cell strains

Cultures were set up from skin explants of karyotypically normal individuals 8–25 years of age, 2 females and 3 males, and of three Down's syndrome patients, 14, 16 and 19 years old (1 female and 2 males). Two other trisomic strains were obtained from the human genetic Mutant Cell Repository at Camden, New Jersey (GM 2767, GM 2571) deriving from 2 females, 3 months and 14 years of age, respectively. Experimental and control cells were cultivated simultaneously in order to maintain culture conditions similar, as far as possible. The cells were grown at 37°C in air with 5% CO_2 in glass Roux flasks (170 cm²), with a medium combined of 45% TC 199 and 45% Eagle's MEM with Earle's salts, and supplemented with 10% foetal calf serum (FCS) and antibiotics (penicillin and streptomycin, 50 i.u. ml⁻¹ and 50 μg ml⁻¹, respectively. The medium was changed every 3–4 days. At confluence cells were trypsinized (0.25% trypsin, Difco) and collected. Cultures were continued for most strains up to passage 31.

Extract preparation

The cells (2×10⁶ to 5×10⁷) were washed three times with phosphate-buffered saline (PBS), without Ca²⁺ and Mg²⁺ at pH 7.2, and sonicated in 20 mM-Tris + 40 mM-KCl at pH 7.2 with Triton X-100 at a final concentration of 1%. The extracts were centrifuged at 100,000 g for 30 min. Protein determinations were done according to Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin (BSA) as standard.

Enzyme quantitative determination

Many attempts to determine MnSOD and CuZnSOD activity by estimating the inhibition of the oxidation, by O₂⁻, of different compounds (flavines, epinephrine, 6-hydroxydopamine) led us to
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the conclusion that they were lacking in precision and reliability. Good and reliable results had been achieved with the radioimmunoassay (Baret et al. 1979; Baret, Schiavi, Michelson & Puget, 1980); however, this method cannot distinguish between active and non-active molecules. Therefore we used two other methods, developed in one of our laboratories: the polarographic method and the \(^{19}\text{F}\) nuclear magnetic relaxation method, which allowed us to measure with greater accuracy both types of SOD (Rigo, Viglino & Rotilio, 1975; Rigo et al. 1979; Crosti et al. 1985).

RESULTS

Enzyme activity

The mean molar activities of CuZnSOD and MnSOD for the five trisomic cell strains and their controls are reported in Table 1. The values are given in moles rather than milligrams because the molecular weight of the human MnSOD is uncertain. The large variation observed between lines cannot be ascribed to sex or age of the donor or site of biopsy. Large variations of enzyme activities in cells \textit{in vitro} is not uncommon. Remarkable differences between strains were, for instance, noticed by Feaster \textit{et al.} (1977) and Marklund, Midander & Westnian (1984) in cultures initiated at different times. On the other hand, an identical trend of change is shown by simultaneously cultivated pairs of normal and 21 trisomic strains, thus supporting the hypothesis that culture conditions, among which, for instance, the variation in the constituents of FCS (Baret & Emerit, 1983) may be an important cause of the observed variability. The comparison of the SOD mean molar activities of the trisomic strains with their controls, is also reported in Table 1. This comparison shows: (1) that in the trisomic cells the activity of CuZnSOD is, on average, 54.7 ± 4.1% higher than in the control cells, as was expected due to the gene dosage effect; and (2) that in the trisomic cells the activity of MnSOD is, on average, 25.4 ± 9.5% lower than in control cells, a difference that is at the limit of significance \((t_4 = 2.378; P = 0.07)\), despite the positive increment in strain 5. We have no explanation for this apparent exception. However, we feel that it may be a

Table 1. \textit{Mean activity of CuZnSOD and MnSOD (mol/mg protein} \(\times 10^{10}\text{)}\) in five fibroblast cell strains with trisomy 21 and five controls, and percentage increase in activity in trisomic cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>21 Trisomic cells</th>
<th>Control cells</th>
<th>Percentage increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of collections</td>
<td>Mean ± s.e.</td>
<td>No. of collections</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>1 3 2 8 3 8 4 13 5 14</td>
<td>0.45 ± 0.11 0.28 ± 0.01 0.21 ± 0.03 0.12 ± 0.01 0.42 ± 0.02</td>
<td>5 6 11 9 9</td>
</tr>
<tr>
<td>MnSOD</td>
<td>1 3 2 8 3 8 4 13 5 14</td>
<td>2.07 ± 0.78 0.69 ± 0.09 0.67 ± 0.10 0.41 ± 0.06 1.29 ± 0.15</td>
<td>5 6 11 9 9</td>
</tr>
</tbody>
</table>
consequence of the rather large variation observed between strains, leading to a chance overlapping of two extreme values. This assumption seems to be supported by other observations that will be reported and discussed later. If we take account of only strains 1 to 4, MnSOD average activity becomes 34.5 ± 3.3% lower than in control cells, a difference that is highly significant ($t_3 = 9.19; P < 0.01$). The decrease in MnSOD activity in the trisomic cells is also evident if the molar ratio of MnSOD to CuZnSOD is considered. The estimates reported in Table 2, while showing that the ratio in each strain with trisomy 21 is lower than in the respective euploid control, also indicate that the lower ratio in the former cannot be ascribed only to a 50% increase of CuZnSOD, but implies a real reduction in MnSOD. The average ratio of MnSOD to CuZnSOD in the control cells is approximately twice that in the trisomic cells.

Correlation between enzyme activities

We have fitted the least-squares regression line of the molar activity of MnSOD on the molar activity of CuZnSOD, using 46 independent pairs of measurements of the two enzymes from trisomic fibroblasts (Fig. 1). We have found a positive and highly significant correlation ($r = 0.77; P < 0.01$), as we had already observed in euploid fibroblasts (Crosti et al. 1985). The correlation could also be demonstrated within each strain, when the range of variability of the measurements was sufficiently wide (Fig. 2). The regression coefficients are 3.5 ± 0.4 for the total sample and 4.1 ± 1.4 and 5.2 ± 1.8 for two single strains. In two strains out of five we have also found a significant linear increase in MnSOD and CuZnSOD with the age of the culture. The regression lines of one strain are presented in Fig. 3.

DISCUSSION

Aneuploid fibroblasts with trisomy 21 produce less MnSOD than normal diploid fibroblasts. One could consider this reduction in MnSOD as a pathological effect due to the unbalanced genome. However, the analysis of our data suggests that the lower production of MnSOD in aneuploid fibroblasts is a functional compensation for the higher production of CuZnSOD. In favour of this hypothesis there is the fact that in trisomic fibroblasts the amount of MnSOD increases with the age of the culture as in normal diploid fibroblasts (Crosti et al. 1985), showing that, when necessary, trisomic cells are able to produce higher amounts of MnSOD. This increase with age is

<table>
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<tr>
<th>Strain</th>
<th>Trisomic 21</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>4.60</td>
<td>12.07</td>
</tr>
<tr>
<td>2</td>
<td>2.46</td>
<td>6.50</td>
</tr>
<tr>
<td>3</td>
<td>3.19</td>
<td>6.00</td>
</tr>
<tr>
<td>4</td>
<td>3.41</td>
<td>7.88</td>
</tr>
<tr>
<td>5</td>
<td>3.07</td>
<td>4.46</td>
</tr>
</tbody>
</table>
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not artificially induced, but is determined by endogenous stimuli, probably an overproduction of oxygen radicals from aged membranes. In support of the hypothesis of functional compensation there are also the findings of Vanella et al. (1982), which in ageing organisms have shown an increase in MnSOD accompanied by a decrease in CuZnSOD, and those of de Rosa, Keen, Leach & Hurley (1980), which have revealed higher amounts of CuZnSOD in rats fed with a manganese-deficient diet. Obviously, this hypothesis assumes that the two enzymes have the same specific activity at pH 7. This is precisely what Baret et al. (1981) found with purified human SODs, and our experiments have confirmed (unpublished data).

The significant positive correlation between the activities of the two enzymes indicates that the controlling genes are associated in their expression, that is that they cooperate in dismutating the superoxide ions. The simplest explanation of this correlation might be that both genes are induced by the same substrate, \( O_2^- \), so that an increased flux of \( O_2^- \) would induce the synthesis of both enzymes. Hassan & Fridovich (1977), investigating *Escherichia coli* grown in the presence or absence of oxygen and methyl viologen, and Crapo & McCord (1976), Kimball et al. (1976) and Crapo (1977), working on rat tolerance to hyperbaric oxygen, had already drawn attention to this point. It is interesting to note that the regression coefficient is constant within each group, euploid or aneuploid, suggesting that in each group the

\[ \text{CuZnSOD (mol/mg protein, } \times 10^10) \]

\[ \text{MnSOD (mol/mg protein, } \times 10^9) \]

Fig. 1. Regression line of MnSOD versus CuZnSOD activities in cells of five trisomic strains (\( n = 46; r = 0.77; P < 0.01; b = 3.5 \pm 0.43 \)).
relative amount of the two enzymes remains constant, whatever the cellular demand may be. However, the regression coefficient for the euploid cells \((b = 12.5)\) is larger than for the aneuploid \((b = 3.5)\). This seems so characteristic that even in the case of strain 5, in which the mean molar MnSOD activity does not appear to differ from that of the control strain, each strain can undoubtedly be recognized as belonging either to the Down's syndrome or control sample precisely by their regression coefficients. In this case the estimated regression coefficients were \(5.2 \pm 1.8\) for the trisomic cells and \(11.9 \pm 2.2\) for the control cells. The lower regression coefficient of fibroblasts with trisomy 21 suggests that the dismutation occurs as if the overproduction of CuZnSOD were sensed by the controlling unit of MnSOD, whose production is consequently reduced. In vivo, a decreased amount of MnSOD in 21 trisomic patients has been found by Sinet, Lavelle, Michelson & Jerome (1975) and by Baret et al. (1981) on platelets. However, Baetman et al. (1983) did not find such a decrease in lymphocytes and granulocytes. This discrepancy could be ascribed to different and specific roles for the two SODs in different cells. The absence of correlation between the two SODs in lymphocytes and granulocytes of normal subjects, according to the investigation of Baret et al. (1981), supports such an explanation. However, the difference in techniques must also be considered, since the radioimmunoassay used by Baret et al. measures the number of molecules, not the activity of the enzymes.

An objection to the hypothesis of the coordinate cooperation of the two genes in fibroblasts could be the restriction of the two enzymes to two separate and unrelated
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Fig. 3. Regression line of MnSOD and CuZnSOD activities for the number of culture passages in cells of a single aneuploid strain (MnSOD: $r = 0.92$, $P < 0.01$; CuZnSOD: $r = 0.88$, $P < 0.01$).

compartments, the cytosol or mitochondria, as proposed by Tyler (1975) and Rest & Spitznagel (1977). However, the objection is no longer tenable since our investigations on the intracellular localization of both SODs in normal and trisomic cells (Crosti et al. 1985) have demonstrated large amounts of CuZnSOD in all compartments, including mitochondria, and an appreciable amount of MnSOD in the lysosomal–microsomal fraction, that was also present, though in a smaller amount, in the cytosol. Similar observations have already been made by McCord et al. (1977) in the cytosol of primates.

In conclusion, the data presented and discussed here are very consistent with the hypothesis of an interaction between the two SOD genes, which maintains the total superoxide dismutase activity in the cells at a constant level. The fact that most living organisms have two different SODs, seems to imply that both SOD enzymes have some functional restriction or specificity that needs complementing for the optimal activity of superoxide dismutase in the cell. This optimal function seems to require, at least in some cell types, a coordinate cooperation of the two genes: a mechanism that, in the course of evolution, seems to have been privileged, by the selection of networks of coordinatively inducible structural genes. Our studies on 21 trisomic fibroblasts suggest that such a system is present also in man, to preserve the balance of all of the SOD activity. It follows that in fibroblasts, and possibly other cells, with trisomy 21 the $O_2^-$ dismutated, and possibly the $H_2O_2$ produced, should not be appreciably altered in respect of normal cells. Consequently, it appears doubtful that
overproduction of CuZnSOD per se is relevant to the pathogenesis of Down's syndrome.

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