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LOCALIZATION AND QUANTITATION OF HUMAN SUPEROXIDE DISMUTASE USING COMPUTERIZED 2-D GEL ELECTROPHORESIS

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SUMMARY - The location of human superoxide dismutase has been identified on 2-D gels of cultured fibroblast extracts. Human superoxide dismutase was located on 2-D gels of a mouse-human somatic cell hybrid containing only human chromosome 21, and was absent in the mouse parental cell line. The native enzyme is a dimer composed of two identical monomers present on 2-D gels as a single spot of molecular weight approximately 16,000 and isoelectric point of pH 6.2. Comparison of the density of the SOD spot on gels of a trisomy 21 cell strain to that of a monosomy 21 cell strain showed a 3 fold increase indicating a gene dosage effect was present. This approach demonstrates the utility of quantitative 2-D analysis for gene dosage studies.

INTRODUCTION

Cytoplasmic superoxide dismutase (SOD, EC 1.15.1.1) performs an important protective role in cellular metabolism by the dismutation of the reactive species O_2 to H_2O_2 and O_2. Tan et al (1) showed that SOD co-segregates with human chromosome 21 in somatic cell hybrids. The gene for SOD has been further sublocalized to the region q21-q22 (2). This position is close to the subregion of the chromosome, q21-q22, to which the Down syndrome phenotype has been assigned (3). Gene dosage effects for SOD have been described in trisomy 21 cells. Feaster et al (4) reported 150% of normal enzymatic activity levels of SOD in trisomy 21 fibroblasts, while monosomy 21 fibroblasts had 50% of normal levels. These data suggest that the ability to measure elevated levels of SOD in cells may be useful for confirmation of suspected cases of Down syndrome. This may be particularly helpful in cases of chromosome 21 translocation or insertion, where the identification of the translocated or inserted chromosomal fragment may be in doubt. Pre-

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viously, the assay of SOD was based on enzymatic or immunological activity. We have now identified the location of SOD on 2-D gels and used computerized 2-D gel electrophoresis to directly quantitate the amount of SOD protein present.

MATERIALS AND METHODS

Cells were cultured in plastic 25 cm^2 flasks (Falcon) in a 5 % CO_2 atmosphere at 37°C with 90-100 % humidity. Eagles minimal essential medium with 10 % fetal calf serum was used for growth media. Four cell lines were selected for analysis. GM-2504 was a fibroblast strain derived from a trisomy 21 fetus and obtained from the Institute for Medical Research (IMR), Camden N.J. GM-230 was derived from a monosomy 21 fetus and was also obtained from IMR. WAVR4dF9-4a was derived from a hybridization of mouse fibroblastic cell line A9 and human diploid cell strain WI-38 (5) and was kindly supplied by Dr. Frank Ruddle. A9, a mouse fibroblastic cell strain, was obtained from the American Type Culture Collection (Rockville, Md).

Cells were labeled with ^{14}C leucine (12.5 uC/ml, New England Nuclear) in media without leucine but containing 10 % fetal calf serum. Cells were labeled for 24 hours beginning one day after confluent cultures were subcultivated at a 1:4 split ratio. Under these conditions the cells cultures were 75-90 % confluent at the beginning of the labeling period. The labeled cells were washed three times with phosphate buffered saline. The cells were lysed by the addition of 250 ul of lysis buffer consisting of 9.4 M urea, 2% NP-40, 5 % B-mercaptoethanol, and 2 % ampholines (4 parts pH 3.5-10 and 1 part pH 5-7). The cell extracts were centrifuged at 2000 g for 5 min to remove unlysed particles. Sample volumes with 0.5 x 10^6 cpm in 25-50 ul were applied to isoelectric focusing (IEF) gel electrophoresis. The two dimensional (2-D) gel electrophoresis technique of O'Farrell (6) was employed with minor modifications. 3.2 mm internal diameter pyrex glass tubes with lengths of 26 cm were used. Tubes were filled to 20 cm with IEF gel mixture (6) and allowed to polymerize. The samples were loaded and overlayed with 0.5 M urea in 1% agarose. Electrophoresis was carried out initially at 0.5 mA per tube until constant voltage of 800 was reached within 2-3 hrs and then continued for a total time of 15 hours, with a final hyperfocus of 1000 volts for one hour. The first dimention IEF disk gels were removed by gentle pressure and equilibrated for 2 hours in SDS sample buffer (6). They were layered on to a 20 cm long second dimension gel consisting of a 20 cm long running gel. The running gel consisted of 13 % polyacrylamide with a 1 cm stacking gel. An apparatus was constructed which allowed 4 gels of 20 cm length to be run simultaneously, modeled after that of Garrels (7). The 2nd dimension was run at 60 volts for 18 to 22 hours until a bromphenol blue marker reached within 1 cm from the bottom of the gel. The gels were fixed and stained with Coomassie Blue. They were dried on a high vacuum source to filter paper and exposed to Kodak X-Omat for 4 to 8 x 10^6 cpm-days until appropriate darkening was obtained.

Antisera to purified human SOD (Sigma) was obtained by subdermal injection in rabbits using Freund's complete adjuvant. Trisomy 21 fibroblasts were labeled, harvested by scrapping, frozen and thawed three times at -70°, and added to preimmune sera or the antisera to SOD. Goat anti-rabbit antisera was added to precipitate the immune complexes and the resulting immunoprecipitates were compared after 2-D gel electrophoresis.

A computer program was used to quantitate spot density. A program originally written by Lutin et al (8) was modified and employed on a PDP-11/70 computer at the Rockefeller University Computing Center. The program employed spot fitting to a two dimensional gaussian distribution. This allowed overlapping spots to be mathematically subtracted from each other and produced reproducible

spot volume determination. The developed autoradiograms were scanned using an Optronix microdensitometer and the output was entered by magnetic tape into the computer for analysis. The program first mathematically subtracted the background from the gels, and smoothed high frequency noise by a weighting filter. The densities were visualized as a topological contour map which gave a three dimentional perspective. Next spots were quantitated by fitting each peak maximun to a 2-D gaussian function, and overlaping spots were mathematically subtracted. The peaks were numbered and printed in order of size. For each peak, the coordinate, the volume density, and the relative volume as a percentage of total volume was determined. The peaks analyzed were then regenerated using their gaussian parameters and compared to the original gel contour plot. Spot volumes were determined to be linear with respect to spot density within the range examined. Ratios of the normalized spots were then calculated.

RESULTS

For identification and analysis of SOD, the hybrid cell strain WAVR4dF9-4a (abbreviated W-21) was used since this strain has been observed to have retained only the human 21st chromosome (5). This hybrid retains the 21st chromosome even though it is grown in nonselective media. To confirm that the 21st chromosome was present, we analysed W-21 by the alkaline giemsa G11 (9) and trypsin-Giemsa banding technique. We found the 21st chromosome had been retained in 84% of 50 cells examined.

The position of SOD on 2-D gels of fibroblast strains was determined in two ways, by co-electrophoresis and immunoprecipitation. Purified SOD was co-electrophoresed with labeled cell extracts. The stained gels were compared to similar gels of extracts to which purified SOD had not been added. As shown in figure 1, a spot was identified as SOD, which was present on the autoradiograms of W-21, GM-2504, and GM-230, but not A9. This spot had a molecular weight of about 16,000, a pH of 6.1, and overlayed the purified human SOD spot. Using both preimmune sera and antisera to SOD, a number of spots were found present in identical positions on autoradiograms of immunoprecipitates of trisomy 21 fibroblasts suggesting rabbit preimmune sera reacts with a number of human fibroblast proteins or that trapping was occuring. However, a set of 3 distinct spots were present on the autoradiogram of the immunoprecipitate using antisera to SOD which were absent on the preimmune sera precipitate. Two did not appear to overlay unique human proteins in comparing human and mouse cell 2-D gels, and may

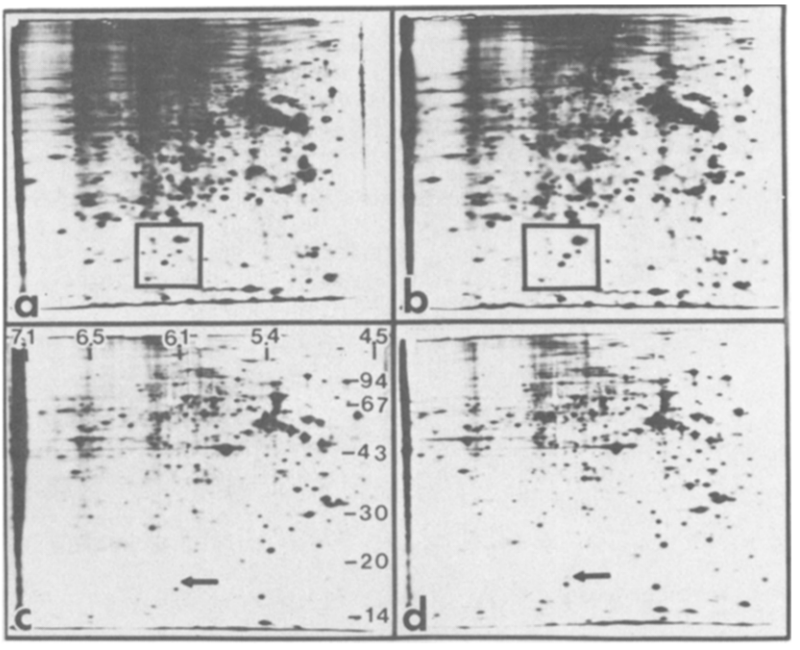

Fig. 1 gel electrophoresis of cellular extracts showing the location of SOD. (a) Monosomy 21 (GM-230), (b) Trisomy 21 (GM-2504), (c) Mouse strain (A9), and (d) Mouse-human hybrid which has retained only human chromosome 21 (WAVR4dF9-4a). Boxed areas in a and b enclose the region containing SOD which is enlarged in Fig 2 for analysis. The arrow points to the location of SOD which is absent in c and present in d. Molecular weight markers (Pharmacia) are indicated in (c) and included Phosphorylase b (94,000), Albumin (67,000), Ovalbumin (43,000), Carbonic anlydrase (30,000), Trypsin Inhibitor (20,100) and Lactalbumin (14,400). The pH measurements, made by direct surface electrode, are indicated in (c).

represent cross-reacting human and mouse antigens. The third spot was the most intense and had the same position as the spot identified as SOD on fibroblast extracts.

The quantity of SOD present on the gel of the trisomy 21 fibroblast strain (GM-2504), was compared to the quantity present on the monosomy 21 cell strain (GM-230), along with the quantities of 15 other protein spots in the vicinity of the SOD spot as shown in Fig 2. The data for the quantities expressed as a percent of the total volume is give for each of the spots in Table 1. The ratio of the quantity of SOD in Trisomy 21 to Monosomy 21 was found to be 3.01. This indicated the amount of SOD present in the trisomy cells was equivalent to the number of copies of chromosome 21 present and was consistent with a gene dosage effect for SOD.

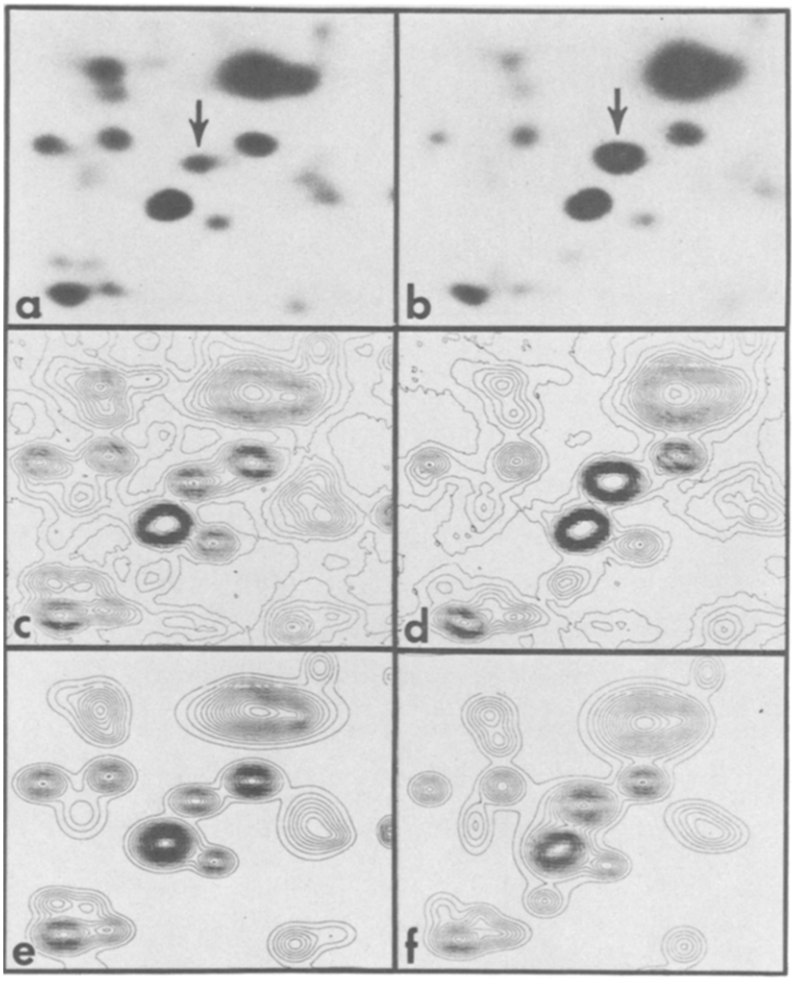

Fig. 2. Computer Quantitation of SOD. Arrows show the position of SOD from monosomy 21 (a) and trisomy 21 (b). Sections enlarged from Fig 1. (c) and (d) show the corresponding contour plots derived from data scans of (a) and (b). (e) and (f) show reconstructed 2-D gaussian contours fitted to the spots in (c) and (d).

DISCUSSION

We have identified the position for human SOD on 2-D gels and have demonstrated a gene dosage effect which is proportional to the number of 21st chromosomes present. The method described here made use of direct quantitation using computerized analysis. SOD is known to be a dimer of molecular weight 32,000 composed of two identical subunits of 16,000 (10). This is consistent with our findings of a single polypeptide with molecular weight 16,000.

TABLE 1
QUANTITATION OF 2-D GEL SPOTS INCLUDING SOD

Spot No.	Position X	Y	Volume % M-21 (a)	Volume % T-21 (b)	Ratio a/b
1	4.1	1.0	28.92	31.75	1.10
2	2.6	3.3	12.31	10.99	0.89
3	5.2	3.0	8.40	6.39	0.76
4	1.5	0.9	8.32	5.97	0.72
5	0.8	4.6	7.32	6.25	0.85
6	4.1	2.2	6.43	5.39	0.84
7	0.6	2.2	4.10	2.50	0.61
8	1.7	2.1	3.80	4.05	1.07
9	3.2	2.5	3.68	11.09	3.01**
10	4.7	4.6	3.16	2.55	0.81
11	1.2	2.7	2.87	2.51	0.87
12	1.6	4.2	2.57	3.06	1.19
13	0.9	4.1	2.35	1.58	0.67
14	3.4	3.4	2.34	2.49	1.06
15	5.2	0.2	1.78	1.82	1.02
16	1.4	4.2	1.63	1.60	0.98

** - SOD
X and Y position in cm from upper left corner of (a) and (b) in Fig 2.

Using 2-D gel electrophoresis, McConkey defined a set of polypeptides which were related to the presence of human chromosme 11 in somatic cell hybrids (11). Cox et al (12) have presented similar results for the X chromosome. These results and our data have indicated that more than 50 % of human proteins may be present in positions that do not appear to overly mouse proteins. By combining the analysis of somatic cell hybrids retaining and lacking a specific chromosome, with the quantitative analysis of cells aneuploid for a specific chromosome, it should now be possible to identify other human gene products which map to specific chromosomes. Proteins which are expressed in somatic cell hybrids may be demonstrated to show gene dosage effects by this method.

Down syndrome (trisomy 21) is a common cause of mental retardation, and shows many features suggesting accelerated senescence. Martin (13) noted that Down syndrome had a higher number of specific features associated with normal aging than any other human genetic disease. These features included premature greying of hair, amyloid deposition, premature brain changes of senile dementia (Alzheimer disease), hypogonadism, autoimmunity, DNA repair defects, chromosomal abberations, increased stem cell defects, slow virus susceptibility, lipofuscin

deposition, diabetes mellitus, degenerative vascular disease, cataracts, and redisposition of adipose tissue. The cause of accelerated senescence in Down syndrome is unknown. It is likely to be related to the expression of the tripled dosage of genes present due to three copies of chomosome 21. Disturbed gene dosage could lead to regulatory, metabolic, or developmental abnormalities which may initiate or accelerate certain aspects of the normal aging process. To understand these mechanisms we will need to know what genes are involved. The approach described here, of direct quantitation of gene dosage effects in trisomy 21 compared to monosomy 21 cells, may allow for the identification and definition of other gene products of the 21st chromosome. Some of these may be involved in the specific pathogenesis of Down syndrome and in accelerating aging.

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