Vol. 98, No. 4, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1981 Pages 1035-1043

IMMUNOREACTIVE Cu SOD AND Mn SOD IN THE CIRCULATING BLOOD CELLS FROM NORMAL AND TRISOMY 21 SUBJECTS

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Received November 4,1980

SUMMARY. - Copper and manganese superoxide dismutases (Cu SOD and Mn SOD) have been measured by radioimmunoassays in circulating blood cells from normal and trisomy 21 patients. Results show :

1- A 50% increase in the Cu SOD content of erythrocytes from trisomy 21 patients with no change in specific activity.

2- An excess of Mn SOD as compared to Cu SOD in the other circulating blood cells from normal patients, a result comparable to that obtained from human liver using enzymatic techniques.

3- An increase in Cu SOD levels in lymphocytes and granulocytes from trisomy 21 patients thus strenghtening the hypothesis of a gene dosage effect.

4- A decrease in platelet Mn SOD from trisomy 21 patients, with no change of Cu SOD, suggesting a relationship between the two enzymes, which is confirmed by the positive correlation between their levels in platelets from normal subjects.

Cu SOD, the gene of which is located on chromosome 21 (1), (2) has been shown to increase in erythrocytes (3) (4) (5) and platelets (5) (6) from trisomy 21 patients as compared to controls.

In order to study a possible role of this increase which has been interpretated as a gene dose effect, in the physiopathology of this disease, it is important to verify that an analogous variation also occurs in other cells. Particularly, the study of granulocyte Cu SOD has not been examined.

In contrast with enzymatic techniques (7), radioimmunoassays of SODs are easy, sensitive, and specific (8) (9), and so allow a correlative study of Cu SOD and Mn SOD in control subjects and trisomy 21 patients.

Moreover, the comparative estimation of specific enzymatic activity of erythrocyte Cu SOD from normal and trisomy 21 subjects, can be obtained by conjunction of both enzymatic and radioimmunological techniques (8).

0006-291X/81/041035-09$01.00/0

MATERIAL AND METHODS

Choice of subjects. Trisomy 21 subjects and control individuals of similar age were free of hepatic and inflammatory diseases. Their age ranged between 8 and 15 years inclusively.

Drawing of blood and isolation of blood cells. Blood (20 ml) was drawn by venous puncture, using 0.25 M Na_2 EDTA (0.4 ml) as anticoagulant. The red cells and platelet rich plasma (PRP) were separated by low speed centrifugation (1100 g, 4 min). Red cells were washed three times with two volumes of 0.15 M Na Cl, then lysed with one volume of distilled water, and frozen at - 20°C. The hemoglobin content was estimated according to the method of Drabkin, and Cu SOD was assayed by RIA in the crude hemolysate. Cu SOD was also assayed by both RIA and an enzymatic technique in ethanol chloroform extracts (ECE) of the hemolysates which were obtained using the method described in (5).

An aliquot part of PRP was treated by 0.5 vol. of 10% triton X100. After rotative agitation during 2 hours at 4°C, the samples were frozen at - 20°C.

A second aliquot part of PRP was centrifuged at 3000 g for 15 min and the platelet poor plasma (PPP) was also kept at - 20°C.

Granulocytes were isolated using the technique of Jemelin (10). 3.75 ml of 0.15 M NaCl containing 6% dextran were added to 5 ml of blood. After sedimentation for 45 mn, the supernatant was drawn and three volumes of 0.15 M NH_4 Cl were added to lyse contaminating erythrocytes. A centrifugation (10 min at 180 g) was carried out and the pellet was resuspended in 1 ml of normal serum. This washing was repeated two times. The purity of the preparation was assessed by light microscopy after May Grünwald Giemsa coloration and was always greater than 90%. The granulocytes were counted and were lysed by 0.5 vol. of 10% triton X100.

Lymphocytes were isolated according to the technique of Boyum (11), by Ficoll Triosil gradient centrifugation of 5 ml of blood. The lymphocytes were resuspended in 1 ml of normal serum and counted ; purity was assessed by light microscopy (greater than 90%). Lysis was carried out by 0.5 vol. of 10% triton X100.

SOD Assays. RIA of Cu SOD and Mn SOD were applied to erythrocyte granulocyte and lymphocyte lysates without further purification as earlier described (8) (9).

Briefly, RIA of human Cu SOD was carried out using a heterologous system (antibovine Cu SOD antiserum R S-5 iodinated rat Cu SOD), and purified human Cu SOD as standard. The assay was carried out with dilutions of the lysates (10^{-3} and 10^{-2} granulocyte and lymphocyte lysates ; pure and 10^{-1} platelet lysates).

RIA of human Mn SOD was carried out using a homologous system antihuman Mn SOD antiserum iodinated human SOD, and purified human Mn SOD as standard. The assays were carried out with the same dilutions as for Cu SOD assays. Results were expressed as µg/g Hb for erythrocyte Cu SOD, µg/10^6 cells for platelet, granulocyte and lymphocyte Mn SOD and Cu SOD.

The specific activity of erythrocyte Cu SOD was determined by conjunction of RIA and enzymatic assays, which were performed on ECE. The Riboflavin NBT technique of Beauchamp and Fridovich (12) was used and the results were expressed as units/mg Hb. Plasma Cu SOD and serum Mn SOD were also measured by RIA without prior treatment or dilution.

Table I : Erythrocyte Cu SOD, measured by RIA in crude hemolysates and Ethanol chloroform extracts, and riboflavin NBT assays in Ethanol chloroform extracts. Specific enzymatic activities are expressed in units/µg Cu SOD.

		Crude Hemolysates RIA ; ug/g Hb	ECE RIA ; ug/g Hb	ECE Enzymol.; U/g Hb	Specific enzymatic Activity ; U/ug SOD
	n	10	10	9	10
Controls	m	312.19	331.54	943.99	2.69
	sm	16.40	12.79	53.77	0.12
	n	15	15	15	15
Trisomy 21	m	481.32	468.29	1432.33	2.63
	sm	22.00	21.87	45.05	0.07
Percent Variation		+ 54.2	+ 41.25	+ 51.73	- 2
P		0.001	0.001	0.001	NS

RESULTS

Table I shows the results of erythrocyte Cu SOD assays performed on crude homogenates (RIA) and ECE (RIA and riboflavin NBT assay).

Since the RIA of Cu SOD gives similar results in crude hemolysate and ECE, the solvent extraction has no effect on Cu SOD. With the two techniques used and the two mediums assayed, an increase in Cu SOD ranging from 41% to 54% is shown in the erythrocytes of trisomy 21 patients. Moreover, the individual specific enzymatic activities which are obtained by computing for each subject the ratio

Table II : Mn SOD and Cu SOD levels in circulating blood cells, expressed as ng/10^6 cells

	Platelets		Granulocytes		Lymphocytes	
	Mn SOD	Cu SOD	Mn SOD	Cu SOD	Mn SOD	Cu SOD
n	14	14	17	17	11	11
Controls m	1.96	1.10	32.22	24.97	31.49	36.80
sm	0.17	0.10	2.37	2.36	2.63	4.06
n	13	13	14	14	11	11
Trisomy 21 m	0.99	1.22	36.24	41.77	31.44	63.41
sm	0.08	0.11	6.89	4.01	6.02	7.93
Percent Variation	- 49.5	+ 10.9	+ 12.5	+ 67.3	/	+ 72.3
P	< 0.001	NS	NS	< 0.001	NS	< 0.01

m, sm : ng/10^6 cells

between the results of ECE enzymatic activity and RIA appear to be identical in both control and trisomy 21 patients.

An increase in Cu SOD is also found by RIA in lymphocytes (+ 72.3%) and granulocytes (+ 67%) isolated from trisomy 21 patients, as shown in table II (and fig. 1). On the other hand, no changes in platelet Cu SOD were noted.

Mn SOD has been simultaneously measured in order to evaluate a possible contribution of mitochondrial SOD in trisomy 21. In normal subjects the results (Table II) show that the levels of platelet, granulocyte and lymphocyte Mn SOD from normal subjects are higher than or equal to those of Cu SOD. Indeed the ratio between the cell content of Mn SOD and Cu SOD ranges from 0.95 for lymphocytes to 1.85 for platelets (Table III).

There is no variation of granulocyte and lymphocyte Mn SOD from trisomy 21 patients as compared to control subjects (Table II) in contrast with Cu SOD. On the other hand, Mn SOD is significantly decreased in trisomy 21 platelets compared with normal controls as been shown earlier (6). The results of platelet SODs from trisomy 21 are hence distinguished by unchanged Cu SOD and decreased Mn SOD levels.

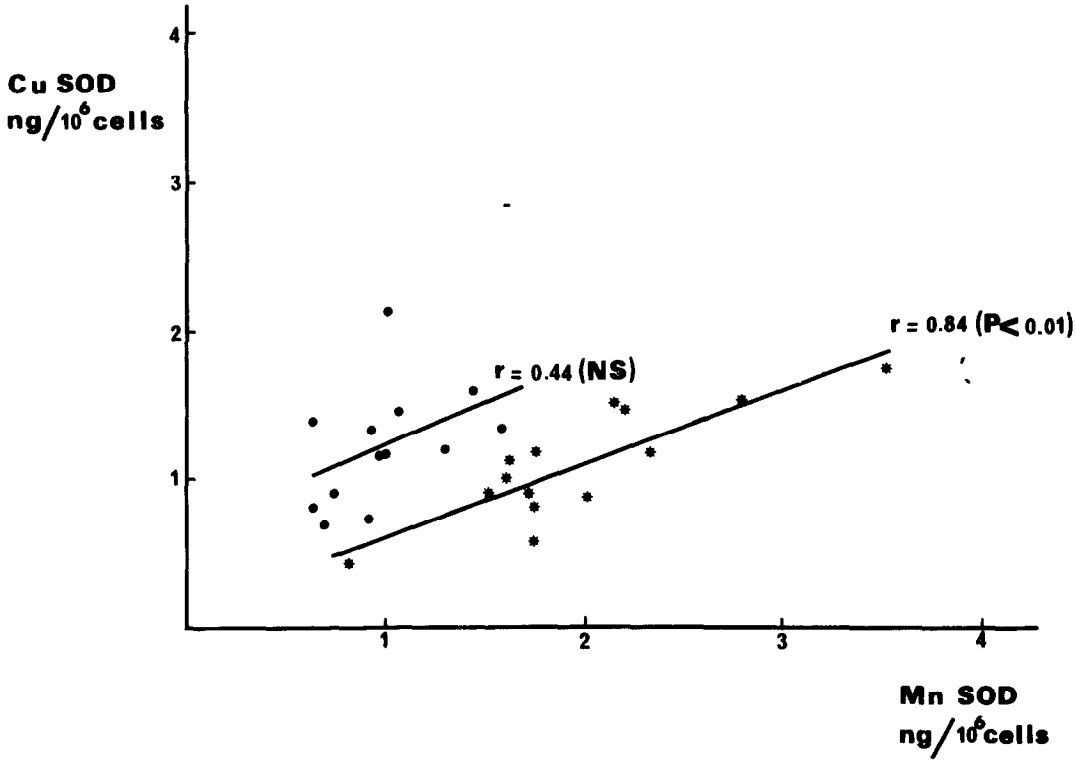

Figure 1 : Significant positive correlation (r = 0.84, P < 0.01) between Mn SOD and Cu SOD levels in platelets from normal subjects and unsignificant correlation (r = 0.44) in platelets from trisomy 21 patients. SOD levels expressed as ng/10^6 cells.

Table III shows a significant decrease of the ratios $\frac{\text{Mn SOD}}{\text{Cu SOD}}$ in the three cell preparations from 21 trisomy patients. This decrease appears to be related to an increase in Cu SOD in lymphocytes and granulocytes, and is related to a decrease in Mn SOD in platelets suggesting the possibility of a relationship between Mn SOD and Cu SOD platelets levels. Table III shows a significant positive correlation between the levels of the two proteins (n = 14, r = 0.84, p < 0,02) in the platelets from normal subjects. The specificity of this correlation is suggested by the lack of correlation between platelet Cu SOD level, expressed as ng/10^6 cells, and cell counts/mm^3 or total platelet proteins. Such a positive correlation is not significant in platelets from trisomy 21 patients, nor in lymphocytes and granulocytes from normal subjects.

Table IV shows the results of plasma Cu SOD and serum Mn SOD from controls and trisomy 21 patients. The increase of Cu SOD without

Table III : Ratios of Mn SOD to Cu SOD levels in platelets, granulocytes and lymphocytes.

		Platelets	Granulocytes	Lymphocytes
Controls	n	14	14	13
	m	1.85	1.53	0.95
	sm	0.11	0.20	0.091
Trisomy 21	n	14	14	16
	m	0.84	0.91	0.54
	sm	0.065	0.083	0.057
P		< 0.001	< 0.01	< 0.001

change of Mn SOD can be related to a higher degree of hemolysis in trisomy 21 patients.

DISCUSSION

The increase in erythrocyte Cu SOD from trisomy 21 patients which has already been reported using enzymatic techniques (3) (4) (5), is confirmed by radioimmunoassay. The value of this increase shown by these two different techniques is quite similar. Hence it can be concluded that the increase in enzyme activity in erythrocytes from trisomy 21 subjects is accounted for by an increase in the synthesis of Cu SOD and that the structure of the enzyme is not altered since the specific enzymatic activity is identical with that of control values. The consequence in vivo of this increase could be as already mentioned (20) a slight hemolysis of the erythrocytes, which could explain the increase of plasma immunoreactive Cu SOD without concomitant change of serum Mn SOD.

The lack of sensitivity and specificity of enzymatic techniques could explain why the levels of Cu SOD and Mn SOD have been poorly studied. The RIA of SODs in granulocytes, lymphocytes and platelets from normal subjects show that the levels of Mn SOD are similar to or higher than that of Cu SOD. On the other hand, enzymatic techniques, performed on lymphocytes (18) and platelets (15) (19), and using differential sensitivity of Cu SOD and Mn SOD to cyanide, gave opposite results, characterized by a great excess of

Table IV : Serum Mn SOD and plasma Cu SOD levels from control subjects and trisomy 21 patients. Results expressed as ng/ml.

		Plasma Cu SOD	Sérum Mn SOD
Controls	n	18	21
	m	40.14	33.7
	sm	5.24	2.57
Trisomy 21	n	18	24
	m	103.69	31.59
	sm	12.56	1.52
P		< 0.001	NS

m, sm : ng/ml

Cu SOD. These discrepancies cannot be explained by a difference between the specific enzymatic activities of Cu SOD and Mn SOD, since the purified enzymes we have used as standard in radioimmunoassays exhibit quite similar specific activities. Moreover, an excess of Mn SOD over Cu SOD has been shown to occur in human liver by enzymatic techniques (21), and also by radioimmunoassay. In fact, the in vitro (and perhaps the in vivo) lability of Mn SOD already suggested by some authors (5) and which is strongly reduced by resuspending the cells in normal serums could explain these discrepancies.

The increase in lymphocyte and granulocyte immunoreactive Cu SOD, with no change of Mn SOD strenghtens the hypothesis of a gene dosage effect in trisomy 21 subjects, and its role in some aspects of the physiopathology of this disease can be considered. A decrease in bactericidal potency of granulocytes has been described (13), but the NBT test has given contradictory results (1) (4) (15). Hence, the increased sensivity of trisomy 21 to infections might not be due to a lack of O_2^- in granulocyte and the high intracellular level of Cu SOD could be counteracted by an increase in the production of O_2^-. Another possibility is an alteration of lymphocyte

function as suggested by the modifications of the lymphocyte reactivity in trisomy 21 (16) and by the action of copper penicillamin complex, showing a dismutating activity towards $O_2^{\cdot-}$, on the function of helper cells (17).

The absence of significant variations of platelet Cu SOD from trisomy 21 patients compared to granulocytes and lymphocytes as measured by radioimmunoassay does not correspond to the results already obtained by a luminol enzymatic technique (6) ; this discrepancy could be explained by the different techniques of platelet isolation. Indeed, the radioimmunological technique allows the direct assay of SODs in PRP, whereas the enzymatic techniques need a further isolation of platelets. In contrast with the lack of variation of granulocyte and lymphocyte Mn SOD, there is a decrease of platelet Mn SOD from trisomy 21 patients so that the variations of the $\frac{\text{Mn SOD}}{\text{Cu SOD}}$ ratios in the three populations of circulating cells are quite similar. This fact, together with the positive correlation between platelet levels of Cu SOD and Mn SOD from normal subjects, suggests a possible relation between these two enzymes in platelets.

Acknowledgements

This work was supported by grants from DRET (n° 78/1018) and INSERM (ATP n° 79 110).

We are grateful to A.M. MICHELSON for his encouragements and critical examination of this work and to Dr. Paul REBUFFEL for his kind help in collecting blood samples.

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