EXTRACELLULAR SUPEROXIDE DISMUTASE CHANGES IN PATIENTS WITH DIFFERENT BRAIN TUMORS

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Abstract

The Specific activity of extracellular superoxide dismutase (EC-SOD) was measured in healthy persons and in patients with benign and malignant brain tumors. The results show decrease of the EC-SOD specific activity in sera of patients with benign and malignant brain tumors in comparison to that of control group.
This study concentrated on studying the changes that occur in sera EC-SOD activity of patients with benign and malignant brain tumors, in comparison to that of normal individuals. The result also revealed that this isoenzyme is present in many different molecular weights forms (as judged by polyacrylamide gel electrophoresis), some of these with no enzymatic activity. Conversion among these forms occurs in the malignant sera.

التغيرات في ال EXTRACELLULAR SUPEROXIDE DISMUTASE عند مرضى أورام الدماغ المختلفة

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الخلاصة

تم قياس فعالية انزيم Extracellular superoxide dismutase (EC-SOD) في مصول الاصحاء والمصابين باورام الدماغ الحميدة والخبيثة اذ اظهرت النتائج انخفاض في فعالية انزيم EC-SOD في مصول الاشخاص المصابين باورام الدماغ الحميدة والخبيثة مقارنة مع الاشخاص الاصحاء.
ركزت هذه الدراسة ايضا على دراسة التغيرات الحاصلة في فعالية المتناظر الانزيمي EC-SOD في مصول مرضى سرطان الدماغ الحميدة و الخبيثة مقارنة بفعاليته في مصول الأشخاص الأصحاء حيث لوحظ و باستخدام الرحلان الكهربائي، أن هذا المتناظر موجود بعدة أشكال و بأوزان جزيئية مختلفة ، البعض منها يظهر فعالية انزيمية و البعض الآخر بدون فعالية انزيمية و يتم تحول قسم من هذه الاشكال من الصيغة غير الفعالة في الحالة الطبيعية وعند المرضى المصابين بأورام الدماغ الحميدة إلى صيغة فعالة في المرضى المصابين بأورام الدماغ الخبيثة.

Introduction

The brain is exposed throughout life to oxidative stress, neurons are highly sensitive to oxidative stress [1], and certain disease of the brain and nervous system are thought to involve free radical process and oxidative damage, either as a primary cause or as a consequence of disease progression [2]. The ROS together with, unstable intermediates in the peroxidation of lipid, are well known inducers of cellular and tissue pathogenesis leading to numerous disease states, including cardiovascular disease[3] and age-related degenerative condition [4,5]. Neurodegenerative disease, such as Alzheimer's disease[6], and cancer[7], are also linked to damage from ROS as a result of an imbalance between the rate of radical generation and their scavenging[8].

The primary formation of most of the ROS is the reduction of molecular oxygen with the formation of superoxide [9, 10]. Although the reactivity of superoxide is quite low, it is capable of initiating free-radical chain reactions [11]. Superoxide (O_2 carrying an unpaired electron) undergoes a dismutation to form H_2O_2 spontaneously or enzymatically. The spontaneous distumation rate is low, whereas the reaction catalyzed by specific enzymes called superoxide dismutase (SOD) is 10^4 times as fast[9,12].

$$O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Extracellular superoxide dismutase (EC-SOD) is the major SOD isoenzyme in extracellular fluid like plasma, lymph and synovial fluid[13,14]. EC-SOD also occurs in tissue and in higher concentration (per g wet weight) than in plasma (per mL)[15]. It is localized predominantly in the extra-cellular matrix of tissue as well as in extracellular fluids depending on the presence of a carboxyl-terminal heparin- binding domain[13].

EC-SOD is a secretary tetramic CuZn-SOD containing glycoprotein, with a molecular weight of a round 135 KDa[16]. It is composed of four identical 30 KDa subunits, and has pI of 4.5[17]. EC-SOD in extracellular fluid is heterogeneous in its affinity for heparin[17,18], and three subtypes exist: type C with high affinity; type B with intermediate affinity; and type A without affinity for heparin. The heparin-binding domain is believed to be cluster of positively charged amino acids in the C-terminal region of the protein[19].

In a previous study carried out in our laboratory a decrease in sera total SOD specific activity was reported[20], in patients with different types of brain tumors. So in order to get a detailed information about the involvement of EC-SOD in the observed reduction the aim of this work was to determine the different levels of EC-SOD enzyme in patients with brain tumors in comparison with healthy persons. Moreover this work also involved the characterization of this enzyme and study the changes in its activity.

Materials and Methods

Serum samples were obtained from the same patients listed in [20].

Five milliliters of venous blood samples were collected in test tubes. The serum was separated immediately from the cells by centrifugation at 3000 x g for 5 min.

Isolation of EC-SOD[16,21]

SOD present in different isoenzymes among which EC-SOD is the only glycoprotein, therefore affinity chromatography using Con A-sepharose was used to isolate this isoenzyme from the other SOD isoenzymes and as summarized below[16,21]: Two milliliters of Con A-sepharose (Pharmacia) were placed in 5 mL column (5 mL syringe), then the column was washed with 2 x gel volume of 10mM phosphate buffer containing 1mM $MgCl_2.6H_2O$, 1mM $CaCl_2$, and 120 mM NaCl, pH6.5 (washing buffer) at a flow rate of 0.5 mL/min. One milliliter of (40 mg/mL) of samples was applied to Con A-sepharose column and remained in contact for 20 min with the gel to allow binding to lectin, after 20 min, 2 mL of the washing buffer were added and fractions of 1 mL was collected which contained CuZn-SOD and Mn-SOD of the sample. After that, the column was washed with 20 mL of washing buffer.

Partially purified EC-SOD was then eluted with 5 mL of 50 mM phosphate buffer containing of α-methyl-D-glucoside 150 mM, pH6.5 (elution buffer), added in 1 mL portions at 5 min intervals. After that the column was washed with 20 mL of washing buffer.

All fractions (1 mL each) were collected, and their absorbance were measured at λ=280 nm in order to detect the presence of the protein.

The protein concentration was determined using Lowry method[22] ,and bovine serum albumin as a standard. The activity of EC-SOD enzyme

in serum was assayed by riboflavin/NBT method[23].
Enzyme specific activity was expressed as unit of enzyme activity per mg of protein, where the unit of enzyme activity is defined as the amount of enzyme causing half the maximum inhibition of NBT reduction.
Fractions containing higher specific activity were used for subsequent analysis.

Gel electrophoresis

In order to detect the changes in the SOD isoenzyme that occur in the sera of the patient groups, analytical horizontal electrophoresis at pH 8.9 was performed as described by LKB application note 1977, for homogeneity determination using 7.5% acrylamide with a current of 40 mA and 15 V/cm voltages. The gels were stained for protein[24] and enzyme activity[25] detection.
EC-SOD subunits molecular weight were determined by poly acrylamide gel electrophoresis as described in LKB application note using catalase, aldolase, bovine serum albumin, and trypsin as standard.

Results

Isolation of EC-SOD

Pooled sera samples (40mg/mL) of normal, and patient with benign and malignant brain tumors were separated using Con A-Sepharose column[16] as described in table (1).

Table 1: Purification of human sera EC-SOD using affinity chromatography

Step	Volume (mL)	Protein (mg/mL)	SOD Activity (U/mL)	Specific activity (U/mg)
Normal				
a) Pooled Sera	3.5	20	51.73	2.587
b) Eluant	8	0.608	2.584	4.25
Benign				
a) Pooled Sera	1	40	45.04	1.126
b) Eluant	5	0.377	2.14	5.68
Malignant				
a) Pooled Sera	1	40	37.22	0.931
b) Eluant	9	0.239	1.47	6.15

EC-SOD activity

EC-SOD activities in sera samples were measured, after its separation from other SOD isoenzyme using Con-A-Sepharose. The affinity chromatography provides a useful method for distinguishing EC-SOD from other SODs and proves to be an effective procedure for enzyme purification[21,26].

Table 2: EC-SOD activity in sera of normal, patients with benign and malignant brain's tumors

Group	Serum EC-SOD activity (U/mL)	EC-SOD activity (%) in normal and patients with brain tumor
Control	2.58	100
Benign patients	2.14	82.95
Malignant patients	1.47	56.98

It is obvious from the result in table (2) that there is a decrease of 17.05% and 43.02% in EC-SOD activity in serum of benign and malignant groups respectively in comparison with that of control group.

Determination of approximate EC-SOD Molecular Weight

The approximate molecular weight of the isoenzyme was determined using gel electrophoresis.
In order to check the determination the approximate molecular weights of the different EC-SOD present in sera of normal, patients with benign and malignant brain tumors, standard protein kits, (catalase 232 KDa, aldolase 140 KDa, bovine serum albumin 67 KDa, trypsin 21 KDa), were electrophorized, to construct standard molecular weight curve.
This curve was obtained by plotting the logarithm of molecular weight of the standard proteins versus their relative mobilities as follows:

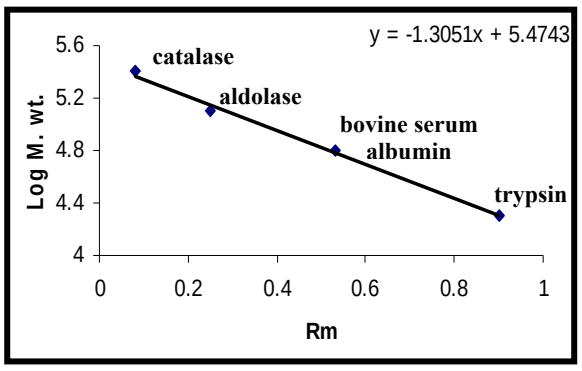

Figure 1: Standard molecular weights curve using (7.5%) PAGE

When the molecular weights of the bands stained from SOD activity in each sample were using the standard molecular weights curve, EC-SOD is present in different unit.

More than one form of EC-SOD was separated, which their approximate molecular weight was tetramer (269–275 KDa), trimer (202-214 KDa), dimer (129-145 KDa), and monomer (68 KDa). The presence of more than EC-SOD activity bands in the gel may be caused by partial degradation or denaturation of the enzyme during the isolation procedure. The existence of the genetic variants is another possibility[16], since pooled samples were used for analysis. Besides, also it may be due to carbohydrate microheterogenity[27]. Such a possibility requires further study to be confirmed.

This agrees with what was reported previously by others such as Oury, Crapo, et al, who have referred to the presence of different forms of EC-SOD in human aorta [28].

Electrophoresis Methods for the Analysis *of EC-SOD*

Conventional electrophoresis was carried out using polyacrylamide gel (7.5%) to analyze crude samples and the EC-SOD, which was isolated from other SOD isoenzymes using con A-sepharose.

Figures (2a), (2b) show the electrogram of protein staining profile using CBB-G 250 as a stain and SOD activity staining profile of the native gel respectively.

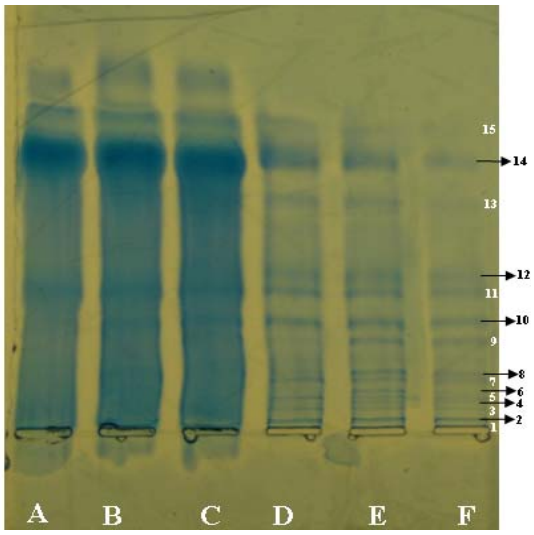

Figure 2a:Conventional polyacrylamide gel electrophoresis (7.5%). The gel was stained for protein[24] and the samples used were:

A- Crude serum (control). D- P.P.EC-SOD (control).
B- Crude serum (benign). E- P.P.EC-SOD (benign).
C-Crude serum (malignant). F- P.P.EC-SOD (malignant).

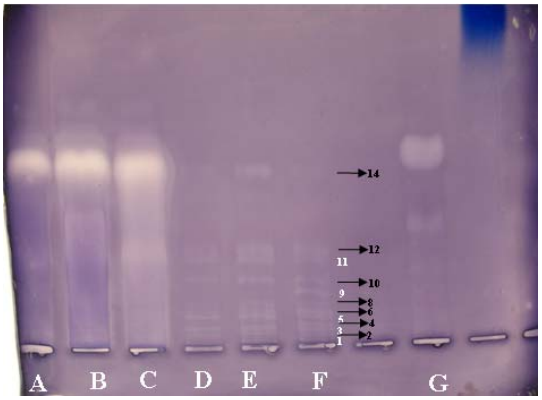

Figure 2b: Conventional polyacrylamide gel electrophoresis (7.5%). The gel was stained for SOD activity[25] and the samples used were:

A- Crude serum (control). D- P.P.EC-SOD (control).
B- Crude serum (benign). E- P.P.EC-SOD (benign).
C-Crude serum (malignant). F- P.P.EC-SOD (malignant).

Upon comparison of the crude samples lanes (A, B, and C) with that of isolated eluant SOD lanes (D, E, and F), figure (3-5b), it is clear that many of the enzyme bands disappeared, which represent the cytosolic SOD, and Mn-SOD.

Discussion

EC-SOD was reported to play critical role in a great variety of physiological functions via its control over the levels of extracellular superoxide $O_2^{-\cdot}$[29]. The observed decrease in EC-SOD activity in the present study may occur with an increased production of superoxide. This ion interacts directly either with the circulating EC-SOD, or with EC-SOD in the vascular wall, that is, in equilibrium with this isoenzyme serum level[4]. Another suggestion for such decrease in sera EC-SOD activity among patients with brain tumors may be due to reduced production of this enzyme by tumor cells, thereby rendering individual more susceptible to oxidative damage[4].

Important EC-SOD effects in brain tissue are likely to be expressed through its control over nitric oxide (NO) metabolism and alterations in (NO) systems. EC-SOD was reported to have an important role in controlling NO bioactivity, since extracellular superoxide is primary and highly efficient scavenger of NO[30-32]. NO functions as neurotransmitter in the brain and thus it is important for variety of neurobehaviour [33]. The ability of NO to diffuse rapidly across

$O_2^{-\cdot}$

extracellular spaces, which is highly dependent on there being very low levels of with NO, is extraordinarily rapid.

$$O_2^{\cdot -} + NO \rightarrow OONO^-$$
$$K \cong 6x10^9 mole/sec$$

Thus even tiny amounts of superoxide secreted by membrane oxidase on neurons could, if not scavenged by EC-SOD, regulate or disrupt NO mediated neurotransmission[33].

EC-SOD is generally unlike other SODs, is glycoprotein. Plant lectins due to their unique ability of binding to sugar moieties of glycoprotein, have become a valuable tool in the separation and identification of many glycoproteins among them EC-SOD[34]. Concanavalin A (Con A), the lectin from the jack bean, can react with branched terminal non-reducing α-D-glucopyranosyl, α-D-mannopyranosyl, and B-D-fructopyranosyl residues[35], and also with other binding sites[36]. Therefore Con A-Sepharose was used in this study as a tool for EC-SOD isolation from other isoenzymes.

During the isolation of EC-SOD, the presence of calcium chloride in the washing buffer is very important, since without it, the enzyme is not retained. It is necessary also that the column must be washed thoroughly to ensure that the sample does not become contaminated with dissociable concanavalin[26].

SODs are soluble enzymes and can be easily separated by gel electrophoresis under non-denaturing conditions[37-39]. The used SOD activity staining method, that was used here, is relatively simple method which is widely used, and which is considered to be the most reliable and reproducible method[37,40].

The result of electrophoresis in polyacrylamide gel, shown in figure (2a), indicate the presence of eight narrow and sharp bands from (1 to 8) stained for protein in lanes (D, E, and F) except band (6) which disappeared in lane (D) upon comparison of the protein staining profile, figure (2a), with that of enzyme activity profile, figure (2b).

It is obvious that bands (7, 13, and 15), in figure (2a), lanes (D, E, and F) were stained for protein, but cannot be visualized in the corresponding lanes using the enzyme's activity staining, figure (2b).

The bands which cannot be visualized in the SOD activity stains may represent an impurity[16]. The other possibility is that human EC-SOD was reported to exist in two forms: one form (active EC-SOD = aEC-SOD), which is enzymatically active, contains a disulphide bridge pattern similar to that of CuZn-SOD, therefore stained for protein and enzymatic activity. The other form (inactive EC-SOD = iEC-SOD) has a different disulphide bridge pattern and is enzymatically inactive. So, such form appears when the gel is stained for protein while it is absent when the gel was stained for enzymatic activity [41].

Also, it is clear that band (9), which is present in the protein stained profile, figure (2a) lanes (D, E, and F), disappeares when the gel was stained for the enzyme activity in lanes (D and E). This means that iEC-SOD form in normal, and benign samples were converted to aEC-SOD in malignant samples.

It was reported that one gene may produce proteins with different disulphide bridge arrangement. The rearrangement of the disulphide bonds of iEC-SOD to aEC-SOD, or visa versa requires the breakage and reformation of two disulphide[41]. If the assembly of the EC-SOD tetramer during the biosynthesis is a random event, six possible combinations of tetramers exist: aa-aa, aa-ai, aa-ii, ai-ai, ai-ii, and ii-ii EC-SOD. These tetramers will express decreasing levels of SOD activity depending on how many iEC-SOD monomers the EC-SOD molecule contains. The number of products from the human genome may be higher than anticipated[42]. Thus the different bands intensity among normal, benign and malignant in SOD activity staining gel may be due to conversion of aEC-SOD monomers to iEC-SOD monomers or vise versa. The form of iEC-SOD has unknown biological role at present.

Conclusion

In previous work[20] a decrease in total SOD activity was measured in patients with different types of brain tumors. The result of the current study showed that the EC-SOD isoenzyme seems to contribute in the occurrence of the oxidative stress reported in our laboratory[43]. To role out the participetation of other SOD isoenzyme in this oxidative stress further work is being carried out.

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