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RESEARCH

CIRCULATING MANGANESE SUPEROXIDE DISMUTASE (Mn-SOD, $SOD_{2)}$ LEVELS AND ITS Ala16Val POLYMORPHISM IN PATIENTS WITH ALZHEIMER'S DISEASE

ABSTRACT

Introduction: Amyloid beta protein, DNA damage, oxygen free radicals and mitochondrial dysfunction are propounded mechanisms for pathogenesis of Alzheimer's disease (AD). In this study, we have focused on manganese superoxide dismutase (MnSOD, SOD-2), the most important scavenger enzyme in mitochondria. Ala16Val polymorphism, the most common variation in the SOD-2 gene, is considered to participate in the pathogenesis of neurodegenerative diseases. Therefore, in this study, we aimed to explain whether the MnSOD levels and its Ala16Val polymorphism are associated with Alzheimer's disease.

Materials and Method: We determined the protein SOD-2 levels and its Ala16Val polymorphism in patients with AD (n=55) and control samples (n=62) from age and sex matched healthy volunteers. Real time pcr and spectrophotometry were used for the analyses of Ala16Val polymorphism and SOD-2 levels respectively.

Results: We found significantly increased MnSOD levels in patients with Alzheimer's when compared to the healthy volunteers (144±67 U/gHb, 76±51 U/gHb respectively, p=0.001). But, there is no difference in Ala16Val polymorphism between the two groups.

Conclusion: We consider that MnSOD is a critical antioxidant enzyme for mitochondrial vitality in Alzheimer patients, but its polymorphic structure does not contribute to pathophysiology of Alzheimer's.

Key Words: Alzheimer Disease; Superoxide Dismutase; Polymorphism, Genetic.

ARAŞTIRMA

ALZHEİMER HASTALIĞI'NDA SERUM MANGAN SUPEROKSİD DİSMUTAZ (MnSOD, SOD_2) ENZİM DÜZEYLERİ VE Ala16Val GEN POLİMORFİZMİ

ÖZ

Giriş: Alzheimer hastalığının patogenezinde amiloid beta protein, DNA hasarı, serbest oksijen radikalleri ve mitokondrial fonsiyon bozukluğu gibi mekanizmaların sorumlu olduğu ileri sürülmektedir. Bu çalışma mitokondrideki en önemli radikal temizleyici olan mangan süperoksit dismutaz (MnSOD, SOD-2) üzerinde odaklanmıştır. Nörodejeneratif hastalıkların patogenezinde SOD-2 geninin en sık görülen varyasyonu olan Ala16Val gen polimorfizmi suçlanmaktadır. Bu nedenle SOD-2 enzim düzeylerinin ve Ala16Val gen polimorfizminin Alzheimer hastalığı ile ilişkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Çalışmaya Alzheimer tanısı almış 55 hasta ile yaş ve cinsiyet uyumlu 62 kontrol dahil edilmiştir. Örneklerdeki protein SOD-2 enzim düzeyleri ve Ala16Val polimorfizmleri spektrofotometrik yöntemle ve real time pcr ile tayin edilmiştir.

Bulgular: MnSOD düzeyleri Alzheimer hastalarında kontrol grubuna gore istatistiksel olarak anlamlı yüksek bulunmuştur (sırasıyla, 144±67 U/gHb, 76±51 U/gHb , p=0.001). Ancak her iki grup arasında Ala16Val polimorfizmi açısından anlamlı bir fark bulunmamıştır.

Sonuç: Alzheimer hastalarında MnSOD'un mitokondri fonksiyonları açısından kritik bir antioksidan enzim olduğu ancak polimorfik yapısının hastalığın patofizyolojisine katkıda bulunmadığı düşüncesindeyiz.

Anahtar Sözcükler: Alzheimer Hastalığı; Mn-Superoksit Dismutaz; Polimorfizm, Genetik.

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INTRODUCTION

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder, characterized by a progressive decline in cognitive function. There are few mechanisms that play a crucial role in the development of AD. Most of these accused mechanisms focus on (a) amyloid b (Ab) deposition, the central constituent of senile plaques in brains of Alzheimer's patients (1,2), (b) DNA damage (3) and (c) mitochondrial dysfunction (4). Aerobic organisms require molecular oxygen (O_2) for vital cellular processes. As the consequence of respiration and enzymatic activities, cells can generate partially reduced forms of O_2 collectively referred as "reactive oxygen species" (ROS) which are highly toxic molecules and therefore must be eliminated from cells to maintain vital processes. The level of ROS and cellular redox homeostasis are regulated by different antioxidant systems, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), ascorbate and Vit E. There are three major superoxide dismutases: a cytosolic CuZn superoxide dismutase (SOD1), an intramitochondrial manganese superoxide dismutase (Mn-SOD, SOD2) and an extracellular CuZn superoxide dismutase (SOD3) (5) which catalyze the following reaction:

$$2H^+ + O_2^- + O_2^- = H_2O_2 + O_2$$

The SOD1 gene is localized to chromosome 21q22.1 and catalyzes dismutation of superoxide anions to hydrogen peroxide (H_2O_2) (6). Manganese-containing SOD, even though not coded in the mitochondrial DNA, is involved in controlling dioxygen toxicity in mitochondria, an organelle exposed to extreme oxidative load. Mitochondria include TCA cycle and electron transport system where highly energetic electrons are transferred to the oxygen and synthase ATP. Mitochondria also play critical roles in neuronal function and almost all aspects of these functions are altered in AD. There is increased oxidative damage to nucleic acids and mitochondrial dysfunction in AD brains and the cumulative evidence indicates that free radicals and their oxidative damage play a role in the pathogenesis of a number of diseases associated with neurodegeneration (7-10). Because the mitochondria are protected from $O_2^{\cdot-}$ by Mn-SOD enzyme, neurons may become susceptible to $O_2^{\cdot-}$ related damages when the activity of Mn-SOD in the mitochondria is reduced (11). The SOD2 null mice develop a severe neurological phenotype that includes behavioral defects, a severe spongiform encephalopathy, and a decrease in mitochondrial aconitase activity as a result of mitochondrial oxidative stress (12) while reduction of Sod2 in transgenic mice carrying amyloid precursor protein (APP) mutations triggers exacerbation of neuronal and vascular AD pathology (13) and authors consider that increasing Sod2 activity might be of therapeutic benefit. Mutations in genes involved in cellular mechanisms to repair oxidative damage may play a role in the pathogenesis of AD due to close relationship among mitochondria, ROS and neurodegenerative disorders (7). Of the SOD-2 polymorphic structures, the most common one is Ala16Val polymorphism located in exon 2. Therefore, we aimed to investigate Mn-SOD levels and SOD-2 Ala16Val (rs4880) polymorphism in these patients.

MATERIALS AND METHOD

The study was designed in Celal Bayar University, School of Medicine, Department of Clinical Biochemistry and Department of Neurology and was approved by the local ethical committee of the university hospital. In accordance with the Declaration of Helsinki, all subjects were informed about the procedure and asked to sign the written informed consent prior to participation in the study. We selected 55 Alzheimer's patients, and 62 control samples from age and sex matched healthy volunteers. All of the subjects were similar regarding life style, socio-economic status and none of them used any antioxidant or other kind of drugs except their routine AD treatment. Alzheimer's Disease was diagnosed according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association's (NINCDS- ADRDA) criteria (14).

Blood was drawn from antecubital vein into two tubes containing anticoagulant. While DNA extraction from the first tube was performed, the second one was kept at -70 °C to determine total SOD and SOD-2 enzyme levels until the day the measurements were conducted. The principle of the total SOD activity method is based, briefly, on the inhibition of nitroblue tetrazolium (NBT) reduction by O_2^- generated by the xanthine/xanthine oxidase system (15,16). One unit of SOD activity was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Activity was expressed as units per g hemoglobin (U/gHb). When NaCN was added into the medium, MnSOD was inhibited and CuZn-SOD values were measured, and finally Mn-SOD values were calculated by subtracting CuZn-SOD values from total SD values. Genomic DNA was extracted from anticoagulated peripheral blood using a high-pure template preparation kit (Cat. No: 11796828001, Roche Diagnostics) and stored at -80 °C for

Table 1— PCR Primer Sequences and Hybridization Probes for SOD-2.

PCR Primers	Hybridization Probes
SOD2NPCR-F 5'-CAGCCTGCGTAGACGGTCCC	*SOD2-Sensor 5'-CTCCGGCTTTGGGGTATCTG–FL*
SOD2NPCR-R 5'-CGTGGTGCTTGCTGTGGTGC	*SOD2-Anchor 5'-LC640-GCTCCAGGCAGAAGCACAGCCTCC–PH*

later use. Mn-SOD genotypes were determined by means of real time PCR on a LightCycler analyzer (Roche Diagnostics). The PCR primers and the hybridization probes were synthesized with Tib Molbiol (Berlin, Germany). The hybridization probes were used in combination with the LightCycler DNA Master Hybridization Probes kit (Roche Diagnostics). The sequences and the hybridization probes for SOD-2 Ala16Val are shown in Table 1. The PCR conditions were as follows: 3 mmol/l $MgCl_2$, 0.15 pmol/µL of each hybridization probe, 0,5 pmol/µL of each PCR primer, LightCycler faststart DNA master hybridization mix and 50 ng of genomic DNA in a final volume of 20 µl.

The analyzing program for SOD-2 Ala16Val starts with pre-incubation at 90 °C for 10 minutes and then 45 cycles of denaturation (10 sec. at 95 °C), annealing (20 sec. at 60 °C) and extension (20 sec. at 72 °C) are performed.

To assess the association between genotype and AD, odds ratio (OR) with 95% confidence interval (%CI) was calculated by using logistic regression model. Chi square test was used to compare between the control and study groups. Differences in SOD1 and SOD2 levels were assessed by parametric t-test. Statistical significance was defined by $p < 0.05$. All statistical analyses were performed with SPSS for Windows (version 11.0, SPSS, Chicago, IL).

Results

Demographic data of all participants are seen in Table 2. There was no difference in all parameters between the two groups. The distributions of SOD1 and SOD2 enzyme levels and SOD2 Ala16Val gene polymorphisms between the controls (group 1) and Alzheimer's patients (group 2) are shown in Table 3 and 4. We found significantly increased SOD2 levels in group 2 when compared to group 1 ($p=0.001$). But, there is no difference in Ala16Val polymorphism between the two groups.

Discussion

When antioxidant systems are not sufficient, free radical damage not only to macromolecules but also to DNA is inevitable. It is well known that mitochondrial DNA (mt DNA) is much more sensitive to oxidative damage than nuclear DNA. Richter et al. (17) have shown that 8 hydroxydeoxyguanosine (oh8dG), an oxidized base, is approximately 15 times higher in mt DNA than in nuclear DNA) (1/130000 vs 1/8000 bases respectively). This increase may be due to the lack of repair enzymes and protective histone proteins in mitochondria, and close proximity of mt DNA to ROS generated during oxidative phosphorylation. However, it must be kept in mind that mitochondria is the most important orga-

Table 2— The Demographic Data of Healthy Volunteers and Patients With Alzheimer Disease.

		Healthy Volunteers (n=62)	Patients With Alzheimer Disease (n=55)
Age*(%)	<65	16.6	13.2
	65-74	51.7	34.0
	>75	31.7	52.8
Total		100.0	100.0
Sex** (%)	Male	36.7	34.0
	Female	63.3	66.0
Total		100.0	100.0

*p=0.07, chi square.
**p=0.7, chi square.

Table 3— The Total SOD and MnSOD Enzyme Activities Among Healthy Volunteers and Patients With Alzheimer Disease

	Healthy Volunteers mean±SD (Median)	Patients With Alzheimer Disease mean±SD (Median)
Total SOD* (U/gHb)	198.2±93.7 (203.5)	224.2±73.9 (239.1)
MnSOD** (U/gHb)	76.1±51.2 (75.0)	144.1±66.5 (141.4)

*p=0.1, Student's t test.
*p=0.001, Student's t test.

nelle of free radical production in mammalian cells due to electron transfer system of the inner membrane. In addition to electron transport chain reactions of the inner membrane, the outer membrane enzyme monoamine oxidase catalyzes the oxidative deamination of biogenic amines and is a quantitatively large source of H_2O_2. This source contributes to an increase in the steady state concentrations of ROS within the mitochondrial matrix and the cytosol. For example, the steady state concentration of $O_2^{\cdot-}$ in the mitochondrial matrix is about 5 to 10 times higher than other subcellular sites (18). In order to overcome increased free radical load, mitochondria has several antioxidant mechanisms. SOD2 is located in the mitochondria and is the only isoform that is induced and regulated by reactive oxygen species (19).

Free oxygen radicals and defective antioxidant defense mechanisms may contribute to some degradative processes such as aging (20,21), oxidative stress induced apoptosis (22,23), impaired fluidity of cell membranes (24), and oxidative modifications of cellular proteins, RNA and DNA (especially mt DNA) (25).

In this preliminary study which will be improved with future studies including larger number of cases we found increased total SOD and significantly increased MnSOD levels in Alzheimer's patients, which supports overloaded free radical metabolism in the pathogenesis of AD. We consider that this increase in Mn-SOD levels may be a response to overwhelming ROS to protect mitochondria, which has crucial function in central nervous system. Many authors have proved that deposited beta-Amyloid peptide (A beta) in extracellular matrix produces free radicals which may attack cell membranes, initiate lipid peroxidation, damage membrane proteins, and alter membrane permeability resulting in neurodegeneration (26-28). Similarly, Kanski J (29) and Butterfield DA (30), have reported that Met 35 in Ab (1-42) is the key molecule of free radical generation processes in Ab peptide and contributes to the peptide's toxicity in brains with Alzheimer's disease.

We consider that higher Cu-Zn SOD enzyme, a radical intracellular scavenger, and found to be high in our study, works as a protective mechanism against lipid peroxidation sources which form radicals like a beta protein. However, concomitant increase in SOD2 reveals that pathophysiology of AD is not only dependent on the transmembrane or extracellular matrix but also on the intramitochondrial region. Whatever the cause is, when ROS exceeds dismutational capacity of SOD2 in AD, oxidative damage will commence and damage mitochondrial components especially mt-DNA and may lead to mitochondrial dysfunction. The more diffuse and the longer the oxidative damage is the more rapid will be the dysfunctional process. On the other hand, Gulesserian T. et al. (6) have found significantly increased SOD-1 levels in Down Syndrome brain cortex, whereas decreased SOD-1 levels in the AD temporal cortex and SOD-2 was comparable. They have suggested that decrease of SOD1 may reflect an antiapoptotic mechanism or simply cell loss in the brain.

In conclusion, MnSOD has the utmost importance in protecting neuronal mitochondria and maintains neuronal function against free radical damage in especially neurodegenerative disorders. While MnSOD and mitochondrial dysfunction

Table 4— The Distribution of SOD2 Ala16Val Alleles Among Healthy Volunteers and Patients With Alzheimer Disease*

	Healthy Volunteers (n:62)	Patients With Alzheimer Disease (n:55)	OR (95% CI)	
Ala/Ala (%)	10.3	12.5	1	
Ala/Val (%)	50.0	50.0	0.82 (0.23-2.90)	p=0.7
Val/Val (%)	39.7	37.5	0.78 (0.21-2.84)	p=0.7
Total	100,0	100,0		

*p=0.9, chi square.

are vital, polymorphic structure of MnSOD does not participate in pathophysiology of AD. As higher MnSOD levels do not depend upon genetic polymorphism like Ala16Val, we consider that this high level may be due to other possible intramitochondrial ROS sources such as unknown substance deposition. AD is a multifactorial pathological procedure which consists of Ab peptide, ROS generation, anti-oxidant defense mechanism, mitochondrial dysfunction and neurodegeneration and increasing SOD2 activity might be useful in managing of AD.

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