RESEARCH COMMUNICATION

Low Activity of Manganese Superoxide Dismutase (MnSOD) in Blood of Lung Cancer Patients with Smoking History : Relationship to Oxidative Stress

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Abstract

Lung cancer is the primary cause of cancer death in the world. Although it is well established that tobacco smoke causes lung cancer, not all smokers develop lung cancer. Manganese superoxide dismutase (MnSOD), a major determinant of antioxidants in matrix mitochondria, plays a pivotal role in eliminating anion superoxide free radical generated from the tobacco smoke. The aim of this study was to analyze the enzyme activity of MnSOD in blood of lung cancer patients with a smoking history in relationship to oxidative stress. Samples were taken from leukocyte cells of 20 lung cancer patients in Persahabatan Hospital Jakarta. Control groups included 50 healthy smokers and 50 non smokers, all aged over 40 years. The MnSOD activity determined biochemically based on the inhibition of xanthin oxidase, of lung cancer patients was lower than the control group's ($p<0.001$). Plasma MDA levels, determined by reaction with thiobarbituric acid (TBA), were not significantly different ($p=0.479$), whereas plasma carbonyl levels were elevated ($p=0.003$). Free radical production in lung cancer patients thus appeared high. Smoker controls also tended to exhibit lower MnSOD and higher carbonyl radicals than their non-smoking counterparts. Continue cigarette smoke exposure may increase production of ROS and bring about a reduction of MnSOD, which could play a role in lunfg cancer development.

Key words: MnSOD activity - lung cancer - lipid peroxidation - carbonyl - smoking - Indonesia

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Introduction

Primary lung cancers are tumors of the bronchus and lung (Husain and Kumar, 2004). The Global cancer statistic indicated that lung cancer is responsible for over 1 million deaths each year (Sun et al., 2007). In Indonesia, lung cancer is the fourth most common cancer diagnose. Lung cancer is difficult to be detected and is often diagnosed at a late stage, only when symptoms are clinically proven (Sudoyo et al., 2006). There is a dose dependent relationship between the cumulative amount of smoking and the incidence of this disease (Perhimpunan Dokter Paru Indonesia, 2003; Ho et al., 2006; Sudoyo et al., 2006; Ho et al., 2007). Although most of lung cancer patients are smokers or ex-smokers (~90%), in fact many of them are also non-smokers (Abdi, 2006). Thus, the role of genetic factor for the cell defense mechanism against lung cancer, for example activity of enzyme manganese superoxide dismutase (MnSOD) should also be considered (Mollerup et al., 2002).

Reactive oxygen species (ROS) such as superoxide ($O_{2\cdot-}$) and hydrogen peroxide (H_2O_2) are constantly produced during metabolic processes in all living species. In a normal state, cellular ROS generation is counterbalanced by the action of enzymatic and non enzymatic antioxidant. When ROS levels exceed the antioxidant capacity of a cell, it is known as oxidative stress. It will cause oxidative damage on lipid, protein, carbohydrate, and nucleic acid molecules (Klaunig, 1998; Oberley, 2002; Reth, 2007). SOD (superoxide dismutase) is one of the key enzymes that detoxifies the anion superoxide ($O_{2\cdot-}$) and hydrogen peroxide (H_2O_2), which in turn detoxified by catalase and glutation peroxidase (Ho, 2006; Ho, 2007; Wang, 2001; Kinnula, 2003; Ho, 2001).

Among the three forms of SOD in humans, mitochondrial MnSOD is particularly important for antioxidant defense because mitochondria are the major site for cellular metabolism and the main site for the production of ROS (Ho, 2006). Oxidative stress was responsible in many diseases, including cancers.

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In this study, the parameter of oxidative stress that we used are specific activity of MnSOD, lipid peroxidation (malondialdehyde / MDA) (Saintot et al., 1996; Uzun et al., 2000; Liu et al., 2000; Güney et al., 2004; Pasupathi et al., 2008), and protein oxidation (carbonyl) (Saintot et al., 1996; Berlett and Stadtman, 1997; Liu et al., 2000; Rajesh et al., 2004; Winterbourn et al., 2008; Yagci et al., 2008).

MnSOD gene consists of 5 exons and 4 introns, is located in chromosome 6q25 (Bag, 2008; Clair, 2004; Li, 2005; Luo, 2001). MnSOD gene polymorphism has been reported in human population, i.e. Ala16Val, Ile58Thr, and Leu60Phe. In the Ala16Val polymorphism, Alanin (GCT) is substituted by Valin (GTT) at the 16th amino acid of mitochondrial targeting sequence (MTS) of human MnSOD. Shimoda et al predicted that Val allele would encode a β sheet conformation rather than a preferred α helical structure of MnSOD precursor protein that leads to an impaired transport of MnSOD from cytosol to mitochondria (Bag, 2008). Sutton et al. (2008) reported that spesific activity of MnSOD Val form is lower than Ala form and thus predisposes to greater risk of lung cancer (Ho et al., 2006; 2007). Experimental studies on rats shown MnSOD induction in lung after acute exposure to cigarette smoke , but this induction can be downregulated in severe oxidative stress condition (Harju et al., 2004). Antioxidant activity in tumor tissue at advance stage of lung cancer was higher than in normal lung tissue, but its activity was lower in blood (Ho, 2001). Previous study in 196 healthy subjects in Jakarta showed that the most frequent MnSOD genotype found in the population was Val/Val (95,9%), and the heterozygote genotype (Ala/Val) was only 4,1% (Wanandi et al., 2008). Low expression of MnSOD has often been accounted for different types of cancer formations, whereas overexpression of this enzyme has been linked with the inhibition of cancerous growth in humans, implicating it as a tumor supressor gene (Mariana et al., 2007). The role of MnSOD as tumor suppressor is still controversial. A study reported that overexpression of MnSOD in cancer cells has a correlation with poor prognosis Remmen et al., 2003). The aim of this study is to find whether lung cancer disease with smoking habits causes impairment of MnSOD spesific activity. Until now, there has not yet been found study about spesific activity of MnSOD in the blood of lung cancer patients with smoking history related to the oxidative stress.

Materials and Methods

This study was designed as a case-control study. The study was carried out in the Biochemistry and Molecular Biology Laboratory, Faculty of Medicine, University of Indonesia,m from June 2008 until December 2009. The ethical clearance of this study was approved by the Medical Ethics Committee FKUI No: 186/PT02.FK/ETIK/2008. Samples were taken from leukocyte cells of 20 lung cancer patients in Persahabatan Hospital who had no received chemo or radiotherapy. Controls were taken from leukocyte cells of 50 healthy active smokers and 50 non smokers subjects from factory X in Tangerang with age more than 40 years.

Blood collection

Venous blood (3 mL) in EDTA was taken from each subject. Red blood cells, buffy coat, and plasma were separated by immediate centrifugation at 2500 rpm for 15 minutes. Buffy coat and plasma were stored at -80°C until subsequent assay. Nuclei lysis solution 600 µL was added to the buffy coat sample and the solution was homogenized. The mixture was separated in two tubes, one tube for the DNA isolation, and the other one for the protein isolation.

DNA isolation

The mixture was incubated at 37°C overnight. After that, the mixture was added by a 2,5 µL RNase solution, was inverted, and was incubated again at 37°C for 15 min. Then it was added by a 100 µL protein precipitation solution, was centrifuged at 14,000 x g for 3 min in room temperature to obtain the brown deposit. The supernatant was added to 300 µL isopropanol in the new tube, was centrifuged at 14,000 g for 1 min in room temperature. Then the supernatant was discarded carefully by pipetting, add 300 µL etanol 70%, was centrifuged at 14,000 g for 1 min in room temperature. Next, the supernatant was discarded, air-dried, then was added by a 50 μL DNA rehydration solution. DNA were stored at -20°C until subsequent assay.

Protein isolation

The mixture was added by a 2 µL PMSF then make it homogen. Protein were stored at -80°C until subsequent assay.

Lipid peroxidation determination in plasma

Lipid peroxidation products were measured as an index of MDA production by the method of Willis ED (Wills, 1987). In this reaction, MDA was reacted with thiobarbituric acid (TBA) reagent to generate a pink coloured product, which was read at 530 nm. The result was expressed as nmol / mL. tetraethoxypropane (TEP) was used as an external standard.

Determination of protein carbonyl levels

We followed the method described by Levine et al. (Rajesh et al., 2004) with a slight modification. Two tubes of 100 μl plasma sample were taken from protein isolate, one of the tubes was marked as "test" and the other as "control". 400 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 2,5 M HCl was added to the test sample and 400 μL of 2,5 M HCl alone was added to the control sample. The contents were mixed thoroughly and incubated in the dark (room temperature) for 1 hour. The tubes were shaken intermittently every 15 minutes. Then 500 μL of 20% TCA (w/v) was added to both tubes and

the mixture was left on ice for 5 minutes. The tubes were then centrifuged at 10,000xg for 10 minutes to obtain the protein pellet. The supernatant was carefully aspirated and discarded. This was followed by a second wash with 10% TCA as described above. Finally the precipitates were washed three times with 500 μL of ethanol:ethyl acetate (1:1,v/v) to remove the unreacted DNPH and lipid remnants. The final protein pellet was dissolved in 250 µL of 6 M guanidine HCl and incubated at 37°C for 10 min. The insoluble materials were removed by centrifugation. The carbonyl content was calculated from peak absorption (370 nm) using an absorption coefficient (e) of 22,000 M-1 Cm-1. The protein carbonyl content was expressed as nmol/mg protein.

Measurement of MnSOD spesific activity in leukocyte cells

The MnSOD enzyme specific activity was biochemically determined using RanSOD® kit. To inhibit the Cu/ZnSOD, at first natrium cyanide (5 mM) was added into each sample and the mixture was incubated for 5 minutes in room temperature. Xantin oxidase was then added into the mixture, followed by the measurement of light absorbance using spectrophotometer at 505 nm after 30 seconds and 3 minutes. The enzyme activity was calculated as a percentage inhibition of the samples plotted to the standard curve. The specific activity of the MnSOD enzyme was calculated as enzyme activity (in Unit) per mg protein. The protein concentration was measured using a spectrophotometer at 280 nm and plotted to the BSA (Bovine Serum Albumin) standard curve.

Statistic Analysis

The activity of MnSOD, MDA level, and carbonyl level data were expressed as mean ± SD. If the data distribution is normal, a one way ANOVA test were used to evaluate the spesific activity of MnSOD, MDA level, and carbonyl level data between cases and controls. A non parametric Kruskal Wallis test was used if the data distribution is not normal. The independent sample unpaired t-tests were used for analizing the effect of genotype to the spesific activity of MnSOD. Statistical analyses were performed using SPSS software for Windows version 17.0. The differences were considered significant at $p < 0,05$. To evaluate the correlation between plasma MDA and carbonyl level with MnSOD spesific activity we utilized the linier regression statistic analysis.

Results

Plasma MDA levels

In this study, mean of the plasma MDA level in the lung cancer was 0.78 ± 0.5 nmol/mL. Standard curve of the linear regression equation : y= 0.256x + 0.021 (y = absorbance at 530 nm; x = concentration). The plasma MDA level in the lung cancer was lower than the control's but it was not significantly different (p=0.479) (Figure 1). The MDA levels were higher in smokers (1.23 ± 1.03 nmol/mL) than in non smokers (1.19 ± 1.09 nmol/mL), but the difference was again not significant (Figure 1).

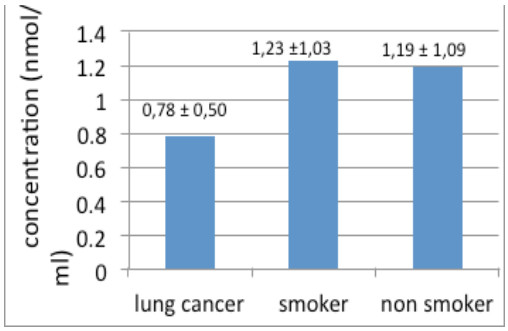

Figure 1. Plasma MDA Levels in Cases and Controls

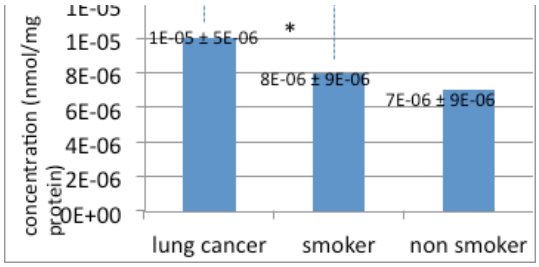

Figure 2. Plasma Carbonyl Levels in Cases and Control Groups. *significant difference between lung cancer and smoker control (p=0.003)

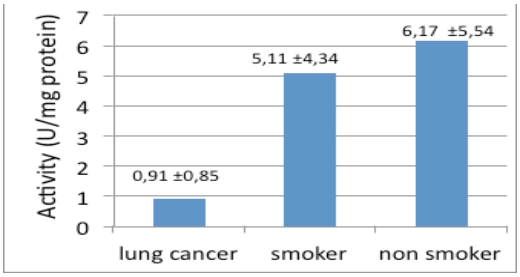

Figure 3. MnSOD Activity in Cases and Controls ** significant difference between lung cancer and smokers, *significant difference between lung cancer and non smokers

Protein carbonyl plasma levels

The mean plasma carbonyl level of lung cancer patients is 1E-05 ± 5E-06 nmol/mg protein. Plasma carbonyl level of lung cancer were higher than smoker's and non smoker's controls (p=0,003). Figure 2 showed the differences of the plasma carbonyl level between the case and control groups.

MnSOD specific activity

The mean of the MnSOD activity in the lung cancer was 0,91 ± 0,85 U/mg protein and lower significantly than control groups.(p=0,000). The standar curve of linier regression equation : y = 0,015x – 0,7. Figure 3 demonstrated that the MnSOD activity between case and control groups was different.

Discussion

High plasma carbonyl level can be caused by an increment in the production of ROS, reduction in the rate scavenging of ROS, and an increment susceptibility of the protein to oxidation (Rajesh et al., 2004). High plasma carbonyl level in lung cancer patients was possible as a consequence of the high production of ROS in lung cancer tissue. It also demonstrated that the oxidative damage implicated not only protein but also lipid, carbohydrate, and nucleic acid (Berlett et al., 1997).

The damage of the plasma protein by peroxidation have a long half-life. Therefore, the evaluation of the carbonyl group content in the plasma protein provides a significant clue to the magnitude of oxidative stress. Plasma carbonyl level in patients who are critically ill with severe sepsis also higher than healthy control (Winterbourn et al., 2008).

It was in accordance to Ho et al. study that the systemic MnSOD activity in the lung cancer is lower than healthy controls (2001). The low MnSOD spesific activity in the lung cancer suggested that as a consequence of high production of ROS in lung cancer tissue which escaped to the systemic circulation. The low spesific activity of MnSOD in blood may be caused, at least in part, by oxidant damage to the protein MnSOD. It is also caused by a defect in transport MnSOD from cytosol to mitochondria (Clerch et al., 1998). MnSOD spesific activity in smokers was lower than non smokers because MnSOD has been used for attacking high production of ROS from a continous cigarette smoking exposure.

Another study showed that SOD activity in a gastric carcinoma was lower significantly than in a non smoker, a healthy smoker, and a gastric carcinoma without smoking history (Pasupathi et al., 2008). The low levels of antioxidants in the circulation of cancer patients was possible due to their increased utilisation to the scavenge free radical as well as their sequestration by tumour cells (Sharma et al., 2007; Pasupathi et al., 2008). SOD activity in the blood circulation of cervical cancer patients before chemoradiation was also lower significantly than healthy controls (Sharma et al., 2007).

Study in rats with breast cancer showed a decreasing MnSOD spesific activity from 2 times until 10 times induction of DMBA than control groups. It suggested that continuing induction of DMBA will cause low MnSOD spesific activity like in this study (Nida, unpublished).

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