CLINICS

CLINICAL SCIENCE

Superoxide dismutase 2 as a marker to differentiate tuberculous pleural effusions from malignant pleural effusions

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OBJECTIVES: Our previous study demonstrated that superoxide dismutase levels were higher in tuberculous pleural effusions than in malignant pleural effusions, but that this difference could not be used to discriminate between the two. The objective of the present study was to investigate the levels of superoxide dismutase 2 in pleural effusions and to evaluate the diagnostic significance of pleural effusion superoxide dismutase 2.

METHODS: Superoxide dismutase 2 concentrations were determined in pleural effusions from 54 patients with tuberculous pleural effusion and 33 with malignant pleural effusion using an enzyme-linked immunosorbent assay (ELISA) kit. Pleural effusion interferon gamma and tumor necrosis factor alpha levels were also analyzed by ELISA. The Mann-Whitney U test was used to evaluate the significance of differences. Associations between superoxide dismutase 2 concentrations and sex, age and smoking habits were assessed using Spearman's or Pearson's correlation coefficient analysis. Receiver operator characteristic analysis was performed to evaluate the value of superoxide dismutase 2 levels in the discrimination of tuberculous pleural effusion from malignant pleural effusion.

RESULTS: Superoxide dismutase 2 levels were significantly higher in patients with tuberculous pleural effusion compared with those with malignant pleural effusion ($p<0.05$). When superoxide dismutase 2 was used to differentiate between tuberculous pleural effusions and malignant pleural effusions, the area under the receiver operator characteristic curve was 0.909 (95% confidence interval, 0.827-0.960; $p<0.01$). With a cut-off value of 54.2 ng/mL, the sensitivity, specificity, positive likelihood ratio and negative likelihood ratio were 75.8% (95%CI: 57.7-88.9%), 98.1% (95%CI: 90.1-99.7%), 40.91 and 0.25, respectively. Furthermore, significant correlations between pleural effusion superoxide dismutase 2 and interferon gamma ($r=0.579$, $p<0.01$) and between pleural effusion superoxide dismutase 2 and tumor necrosis factor alpha ($r=0.396$, $p<0.01$) were observed.

CONCLUSION: Pleural effusion superoxide dismutase 2 can serve as a biomarker for differentiating between tuberculous pleural effusions and malignant pleural effusions. Because of the high correlations of superoxide dismutase 2 with pleural effusion interferon gamma and tumor necrosis factor alpha levels, this marker may act as an inflammatory factor that plays an important role in the development of tuberculous pleural effusion.

KEYWORDS: Superoxide Dismutase 2; Tuberculous Pleural Effusion; Malignant Pleural Effusion; Biomarker.

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INTRODUCTION

Over the past few years, a large volume of research has been carried out on the redox state of *Mycobacterium tuberculosis* (*M.TB*) infection and cancer. *M.TB* is continually exposed to endogenous reactive oxygen species (ROS) during normal aerobic respiration, as well as exogenous ROS and reactive nitrogen species (RNS) generated by the host immune system in response to infection. To avoid oxidative damage, *M.TB* is equipped with various sophisticated redox sensors that detect oxidative stress to maintain redox homeostasis (1,2).

Oxidative stress is closely related to all aspects of cancer, from carcinogenesis to the cancer-bearing state. Important to carcinogenesis, the unregulated or prolonged production of cellular oxidants has been linked to gene mutation and the modification of gene expression and signal transduction

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pathways (3). The cancer-bearing state is also said to be under oxidative stress associated with active oxygen production by cancer cells and abnormal oxidation-reduction control, antioxidant enzyme over-expression and high levels of the non-enzymatic antioxidant scavengers are key components of cancer cell survival in an ROS-rich environment (4).

Superoxide dismutase (SOD) is an antioxidant enzyme involved in the defense system against ROS. SOD catalyzes the dismutation reaction of superoxide radical anion (O_2^-) to hydrogen peroxide, which is then catalyzed to innocuous O_2 and H_2O by glutathione peroxidase and catalase. An imbalance in this coordinated system leads to increased oxidative stress. When acclimating to increased levels of oxidative stress, SOD concentrations typically increase with increased in the magnitude in the stressful conditions. In our previous studies (5), a significant difference in SOD activity between tuberculous pleural effusions (TPEs) and malignant pleural effusions (MPEs) was observed. However, pleural effusion (PE) SOD levels were not an accurate biomarker for differentiating between TPE and MPE, exhibiting a sensitivity of 61.4% and specificity of 61.0%.

Three forms of SOD are present in humans, including SOD1 (Cu/Zn-SOD), SOD2 (Mn-SOD) and SOD3 (extracellular copper/zinc SOD) (6). Over the past few years, PE SOD1 levels have been evaluated in patients with PE, and the concentration of SOD1 in patients with TPE have been reported to be close to those found in patients with MPE (7). We also investigated PE SOD3 levels in patients with TPE and MPE. Although SOD3 levels tended to be higher in the TPE group, the difference was not significant (data not published).

Based on the problems highlighted above, we performed the present study to 1) determine the levels of SOD2 by ELISA and 2) examine the diagnostic accuracy of SOD2 in the differential diagnosis between TPE and MPE.

METHODS

Study design and patients

This study was retrospectively conducted by the Department of Lab Medicine, Shandong Provincial Chest Hospital, Jinan City, Shandong Province. The study protocol was approved by the Ethical Committee of the Shandong Provincial Chest Hospital.

Between March and June 2013, patients with pleural effusion were enrolled in this study and written informed consent was obtained from all enrolled individuals. Subsequently, the patients were included if the PE examinations and/or pleural biopsy specimens established a diagnosis of TPE or MPE. TPE was diagnosed by confirming one of the following: isolation of *M.TB* from PE or pleural tissue; pleural biopsy revealing granulomatous tissue without any evidence of other granulomatous diseases; detection of granulomas in the pleural tissue with a positive response (a progressive reduction of symptoms, effusion removed and radiologic improvement) to anti-TB treatment. MPE was diagnosed if the cytology or pleural biopsy specimen revealed an underlying malignancy.

Sample collection and analysis

PEs were collected within 24 h after hospitalization, before any treatment had been initiated. PEs were centrifuged at 1200 r/min and 4℃ for 15 min and the supernatants were immediately frozen in 1.5-mL Ep tubes and stored for three to seven months at -80℃. The MB/BacT Alert 3D (MB/BacT) system (Organon Teknika, Boxtel, The Netherlands) was used to isolate *M.TB*. PE SOD2 levels were determined using a commercially available enzyme-linked immunosorbent assay kit (Abnova Taiwan Corporation, Taipei, Taiwan) according to the manufacturers' protocols. PE IFN-γ and TNF-α levels were also analyzed using ELISA kits (Invitrogen, Camarillo, CA, USA).

Statistical analysis

Sample size estimation (power: 0.95, $\alpha = 0.05$) was performed using G*P 3.1.7 (8). Statistical analysis was carried out using SPSS 17.0 software and MedCalc Version 8.0.1.0. Distributions were evaluated using the Kolmogorov-Smirnov test for normality. Data were expressed as the mean ± standard deviation (SD) if they were normally distributed. Otherwise, median values were presented. Significant differences between the means were calculated using Student's t-test if the population distribution was normal; otherwise, the Mann-Whitney U test was used. Associations between SOD2 concentrations and sex, age, and smoking habits were assessed using Spearman's or Pearson's correlation coefficient analysis. Receiver operator characteristic (ROC) analysis was performed to evaluate the sensitivity and specificity of SOD2; the cut-off point was determined as the value of the parameter that maximized the sum of the specificity and sensitivity. Positive and negative likelihood ratios were also determined. A p-value <0.05 was considered statistically significant.

RESULTS

Patient characteristics

Of 421 patients with PE studied, 64 patients were excluded after being diagnosed with other diseases (not TPE or MPE) and 13 were excluded due to the presence of transudates according to previously defined criteria (9). Another 257 of the remaining 344 patients with PE were excluded because they did not satisfy the criteria for definitive diagnosis. Finally, 87 patients, 33 with malignant effusions and 54 with tuberculous effusions (48 patients with positive cultures, 4 patients with granulomas excluding other granulomatous diseases and 2 patients with granulomas and a positive response to anti-TB treatment), were included in our study. Table 1 presents the mean age, sex, smoking habits and other characteristics of the evaluated patients. The study population had a mean age of 43.07±20.10 years (range: 15 to 87 years) and 65.5% of the patients were male. The mean age of the 33 patients with MPE was 55.52 years (SD of 16.51 years) and 70.0% of these patients were men. The mean age of the patients with TPE was 35.46 years (SD of 18.32 years) and 63.0% were men. Significant differences were observed in the smoking habits (pack-years) and age between patients with TPE and those with MPE (all $p<0.01$). The severity of TPE relative to smoking habits (0.00, interquartile range: 0.00-2.500) was lower than that of MPE (2.00, interquartile ranges: 1.00-60.00). The levels of PE IFN-γ and TNF-α in patients with TPE (IFN-γ: 1128.44 (413.51-1350.51) pg/L; TNF-α: 180.88 (120.93-336.71) pg/L) were higher than those in patients with MPE (IFN-γ: 5.60 (4.97-6.95) pg/L; TNF-α: 28.15 (12.65-65.02) pg/L, all $p<0.01$). Spearman's correlation analysis revealed

Table 1 - Characteristics of the patients.

	TPE (54 patients)	MPE (33 patients)
Age	35.46 ± 18.32	55.52 ± 16.51
Sex, % male	63.00%	70.00%
Smoking habit (pack-years)	0.00 (0.00-2.500)	2.00 (1.00-60.00)
SOD2 (ng/mL)	73.5 (64.7-82.9)	42.1 (34.4-56.9)
IFN-γ (pg/L)	1128.44 (413.51-1350.51)	5.60 (4.97-6.95)
TNF-α (pg/L)	180.88 (120.93-336.71)	28.15 (12.65-65.02)

Data are presented as the medians (interquartile ranges); IFN-γ: interferon gamma; TNF-α: tumor necrosis factor alpha.

significant correlations between SOD2 and IFN-γ ($r = 0.579$, $p<0.01$) and between SOD2 and TNF-α ($r = 0.396$, $p<0.01$).

SOD2 in TPEs and MPEs

The levels of SOD2 in the PEs of patients with TPE or MPE are presented in Table 1. The median PE SOD2 concentration in patients with TPE was 73.5 ng/mL (interquartile range: 64.7-82.9 ng/mL), compared with 42.1 ng/mL (interquartile range: 34.4-56.9 ng/mL) in patients with MPE. PE SOD2 levels were significantly higher in patients with TPE compared with those with MPE ($p<0.05$) (Figure 1). SOD2 levels in the TPE or MPE patients were not correlated with age (all $p>0.05$) and were independent of the sex and smoking habits (all $p>0.05$).

Diagnostic performance of SOD2 in the differentiation between TPE and MPE

The ability of SOD2 to differentiate between TPEs and MPEs was evaluated using receiver operating characteristic curve analysis (Figure 2). When SOD2 was used to differentiate TPEs from MPEs, the area under the curve value was 0.909 (95% confidence interval [CI]: 0.827-0.960; $p<0.001$). With a cut-off value of 54.2 ng/mL, the sensitivity, specificity, positive likelihood ratio and negative likelihood ratio were 75.8% (95%CI: 57.7-88.9%), 98.1% (95%CI: 90.1-99.7%), 40.91 and 0.25, respectively.

DISCUSSION

In this study, we investigated the presence of SOD2 in pleural effusions (PEs) and evaluated the diagnostic significance of PE SOD2 in the differentiation between TPEs and MPEs. PE SOD2 levels were significantly higher in patients with TPE compared with those with MPE ($p<0.05$). When SOD2 was used to differentiate TPEs from MPEs, the area under the ROC curve was 0.909 (95%CI: 0.827-0.960). With a cut-off value of 54.2 ng/mL, the sensitivity, specificity, positive likelihood ratio and negative likelihood ratio were 75.8% (95%CI: 57.7-88.9%), 98.1% (95%CI: 90.1-99.7%), 40.91 and 0.25, respectively. Meanwhile, significant correlations between PE SOD2 and IFN-γ ($r = 0.579$) and between PE SOD2 and TNF-α ($r = 0.396$) levels were observed. These data demonstrate that PE SOD2 could serve as a biomarker for differentiating TPEs from MPEs. Because of the high correlations between SOD2 and PE IFN-γ and TNF-α levels, the inflammatory factor SOD2 may play an important role in the development of TPE.

Pleural effusion is a diagnostic challenge for clinicians, as it can arise from various causes. In developing countries, TPE and MPE are the two most common types of pleural effusion (PE) (10,11). However, differentiating between TPE and MPE represents a critical problem. In our previous studies (5), we have demonstrated a significant difference in SOD activity between TPEs and MPEs, with significantly higher SOD levels observed in TPEs compared with MPEs. However, SOD activity in PE was not an accurate biomarker for differentiating TPEs from MPEs. Identifying the SOD isoform that contributes to the difference in SOD levels between TPEs and MPEs is crucial to understand the mechanism and improve the differential diagnostic value of this marker. The present study represents the first demonstration that PE SOD2 levels were significantly higher in patients with TPE compared with those with MPE. Our data show that SOD2 levels in the pleural fluid could serve as a

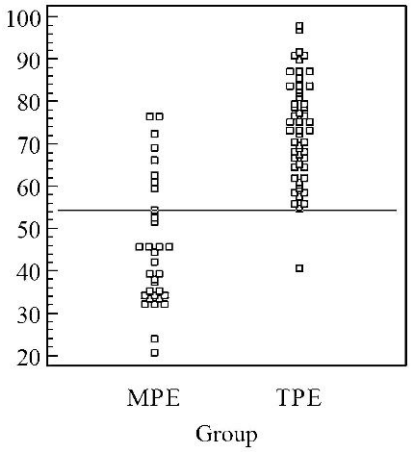

Figure 1 - Comparison of pleural effusion SOD2 concentrations between patients with TPE (n = 54) and those with MPE (n = 33). The horizontal bar indicates the cut-off point (54.2 ng/mL).

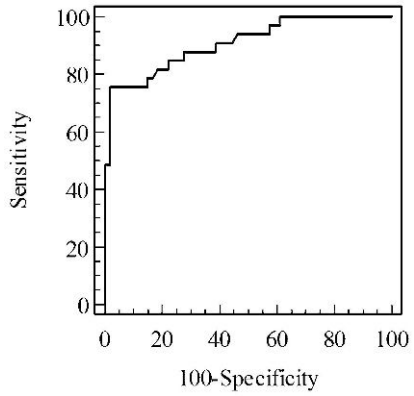

Figure 2 - Receiver operating characteristic curve of SOD2 for the differential diagnosis of TPEs and MPEs. The area under the curve was 0.909 (95%CI: 0.827-0.960).

biomarker for differentiating TPEs from MPEs. Compared with other pleural effusion biomarkers, such as IFN-γ and adenosine deaminase (data not shown), SOD2 could not be used to accurately diagnose tuberculous pleural effusion and had limited diagnostic value.

SOD catalyzes the dismutation of superoxide anion (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2). An imbalance in this coordinated system will lead to increased oxidative stress. Many studies have examined oxidative stress during tuberculosis and lung cancer (the major cause of MPE) (12-14). There are three distinct SOD isoforms (SOD1, SOD2 and SOD3) found in humans (15). SOD1 is the predominant SOD isoform in most cells and tissues, accounting for 70-80% of the total cellular SOD activity. SOD2 is a key mitochondrial antioxidant enzyme. More than 90% of SOD3 is found in the interstitial space within tissues and extracellular fluids; SOD3 accounts for the majority of plasma SOD activity (16-18). We evaluated SOD3 in PEs and found no significant difference in SOD3 levels in the pleural fluid of patients with TPE compared with those with MPE (data not published). Another report indicated that the concentration of SOD1 in patients with TPE was similar to that in patients with MPE (7).

The results of this work demonstrated that SOD2 levels were significantly increased in TPE patients. Due to their role in cellular metabolism, mitochondria are the major sites of ROS production. SOD2 is the only known superoxide scavenger in the mitochondria and is therefore likely to be important in the regulation of overall cellular ROS levels. By controlling ROS, SOD2 plays an important protective role against oxidative stress in lung tissue (19-22). Although ROS represent the first line of defense against microbial invasion and replication, the over-expression of ROS can cause collateral tissue damage. ROS levels were significantly higher in *M.TB*-infected macrophages compared with normal macrophages. Host cells may increase SOD2 expression to decrease ROS production and avoid damage (23-26). Thus, ROS levels are an important cause of increased SOD2 in TPEs compared with MPEs. Furthermore, SOD2 down-regulation has been reported in a variety of cancer cells with diverse tissue origins and forced overexpression of this enzyme in carcinoma cells decreases their tumorigenicity. Mitochondria play an important role in apoptosis and cellular proliferation and SOD2 may also be directly involved in carcinogenesis by protecting against mitochondrial damage. Significant correlations between PE IFN-γ and TNF-α levels and SOD2 levels were observed in our study, implying that SOD2 might serve as an inflammatory factor and play an important role in the development of TPE. IFN-γ and TNF-α are crucial Th-1-type cytokines that control TB infection (27,28). IFN-γ stimulates the killing capacity of macrophages via the induction of ROS production (29-32) and increased TNF-α levels (33,34). Thus, SOD2 levels may increase to counteract the overexpression of ROS induced by IFN-γ and TB infection.

The limitation of this study is its retrospective nature, which might lead to selection bias and thus the inability to interpret or generalize the results. Hence, a future approach will be to analyze PE SOD2 levels in a prospective, larger cohort of patients to validate the utility of SOD2 as a candidate biomarker in differentiating TPEs from MPEs.

In conclusion, we have demonstrated that SOD2 levels were increased in patients with TPEs compared with those with MPEs. We further described, for the first time, that PE SOD2 levels could be used to differentiate TPEs from MPEs. The precise pathophysiological roles of SOD2 in TPE and MPE require further investigation. Because of the high correlations of SOD2 levels with IFN-γ and TNF-α levels, SOD2 may act as an inflammatory factor that plays an important role in the development of TPE.

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AUTHOR CONTRIBUTIONS

Wang MS and Wang XF conceived the study. Zhang ZQ and Wang MS conducted the research. Wang MS wrote the manuscript.

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