Differential Expression of Superoxide Dismutases in Lung Cancer

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Key Words: Tumor; Lung neoplasm; Radical; Redox; Antioxidant; Apoptosis; Cell proliferation; p53; Tissue array

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Abstract

Oxidant-antioxidant balance is known to regulate growth factors and invasion of tumor cells. Manganese superoxide dismutase (MnSOD), copper zinc SOD (CuZnSOD), and extracellular SOD (ECSOD), the first-line antioxidant defenses, were studied in lung carcinomas by immunohistochemical analysis (n = 139, 56, and 37, respectively) and in 8 lung tumor specimens by Western blot analysis and SOD activity measurement. Altogether, 49% of squamous cell carcinomas and 43% of the adenocarcinomas were positive for MnSOD by immunohistochemical analysis; corresponding values for CuZnSOD were 79% and 93%, respectively. MnSOD and CuZnSOD by Western blot analysis were 27% and 22% higher, and CuZnSOD activity was 93% higher (P = .06) in carcinomas than in nonmalignant lung tissue samples. ECSOD, a mainly extracellular enzyme, showed weak positivity only in 4 of 37 carcinomas, and by Western blot analysis showed 70% lower immunoreactivity (P < .0001) than in nonmalignant lung tissue samples. It is highly likely that low expression of ECSOD might have fundamental effects on the extracellular redox state of lung tumors with potential consequences on tumor behavior.

Lung carcinoma continues to be one of the major causes of cancer deaths in Western countries, and tobacco smoking is the primary cause of these cancers. Cigarette smoke contains reactive oxygen species (ROS) that are implicated in the pathology of neoplastic and nonneoplastic diseases. Oxidants also have multifactorial effects on cell proliferation and synthesis of growth factors and proteases that have fundamental effects on tumor angiogenesis and invasion.1,2

The first-line antioxidant protection against ROS includes 3 superoxide dismutases (SODs) that convert superoxide radicals to hydrogen peroxide and oxygen.3 All these isoforms, ie, cytosolic copper zinc SOD (CuZnSOD; SOD1), mitochondrial manganese SOD (MnSOD; SOD2), and extracellular SOD (ECSOD; SOD3), have been detected in human lung tissue.4-8 Studies on transgenic and knockout mice have suggested that MnSOD is the most important of the 3 SODs in vivo.9 However, the relative importance of these intracellular (MnSOD, CuZnSOD) and extracellular (ECSOD) enzymes in human lung and malignant lung diseases is poorly understood.

SODs have been studied widely in cultured cells, and they are known to be potential regulators of intracellular and extracellular redox states. The studies have concluded that overexpression of SOD by transfection abrogates growth and proliferation of cancer cells in vitro and in vivo.10-14 On the other hand, several human tumors have been reported to contain high MnSOD levels and/or activities, and, in many cases, a high MnSOD level or activity has correlated with a poor prognosis.15-22 In addition to providing significant protection against oxidative stress, enhanced expression of SODs has been found to increase the resistance of cancer cells to cytotoxic drugs and radiation.23
There are some studies on MnSOD or CuZnSOD in human lung carcinoma, but their results are controversial.\textsuperscript{4,24-26} ECSOD, which has not been studied in any tumors, is interesting, especially in the lung. Its activity in normal lung is high, and it is located mainly in the extracellular space.\textsuperscript{8,27} To better understand the importance of SODs in lung cancer, all SODs, MnSOD, CuZnSOD, and ECSOD, were assessed in nonmalignant lung tissue samples and various lung tumor samples by using immunohistochemical analysis, Western blot analysis, and specific activity measurement. The expressions of these enzymes were correlated with other markers of tumor genesis, p53, proliferation, apoptosis, and patient survival.

**Materials and Methods**

**Tissue Specimens for Immunohistochemical Analysis**

Samples of non–small cell lung carcinoma were retrieved from the files of the Department of Pathology, Oulu University Hospital, Oulu, Finland. The histopathologic and clinical data for the cases are given in Table 1. The diagnosis was made according to the World Health Organization classification of lung tumors.\textsuperscript{28} The conventional paraffin-embedded specimens included 73 cases, of which were evaluated for MnSOD immunoreactivity. In addition, approximately 1-mm diameter pieces from 207 specimens from 69 patients (3 specimens from each case) (62 men, 7 women) were drilled from the original paraffin blocks in the tissue array part of the study (Table 1). The samples were immunostained with MnSOD, CuZnSOD, and ECSOD. Owing to exhaustion of the samples, staining could not be performed in some cases. As control samples, nonmalignant lung tumor samples from 7 nonsmokers operated on for lung tumors were included. The mean ± SD follow-up period for the patients was 52.6 ± 47.5 months.

**Materials and Methods**

**Tissue Specimens for Immunohistochemical Analysis**

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**Table 1**

Clinical Data for Cases Analyzed Immunohistochemically*  
<table>
<thead>
<tr>
<th></th>
<th>Conventional Biopsy Specimens (n = 73)</th>
<th>Tissue Array Cases (n = 69)</th>
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<td></td>
</tr>
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<td>11</td>
<td>10</td>
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</table>

*Patient age ranged from 48 to 80 years at the time of surgery.
†At the end of the follow-up period.

**Immunohistochemical Staining**

We cut 4-µm-thick sections from representative tumor blocks and treated as described previously.\textsuperscript{5} Polyclonal rabbit antibodies for human MnSOD, CuZnSOD, and ECSOD\textsuperscript{6,27,29,30} were used with dilutions of 1:1,000 for MnSOD, 1:1,250 for CuZnSOD, and 1:200 for ECSOD. Immunostaining was performed using the Histostain-Plus Bulk Kit (Zymed Laboratories, San Francisco, CA), and the chromogen used was aminoethylcarbazole (Zymed Laboratories). For ECSOD, immunostaining was performed with the EnVision kit (DAKO, Hamburg, Germany), and the color was developed with aminoethylcarbazole. The sections were counterstained lightly with hematoxylin and mounted by using Immu-Mount (Thermo Shandon, Pittsburgh, PA). Rabbit isotype control (Zymed Laboratories) and phosphate-buffered saline, pH 7.2, were used for negative control samples.

Immunostains were evaluated separately for the intensity and quantity of staining and given scores as follows: intensity: 0, no reactivity; 1, weak intensity; 2, moderate intensity; and 3, strong intensity; quantity: 0, no immunoreactivity in tumor cells; 1, fewer than 10% of cells positive; 2, 10% to 40% of cells positive; and 3, more than 40% of cells positive. A combined score based on the sum of the intensity and quantity of immunostaining was obtained as follows: −, 0; +, 1 and 2; ++, 3 and 4; and ++++, 5 and 6.
For ECSOD only, the presence or absence of immunoreactivity in stroma or tumor cells was assessed. The evaluation of the immunostained samples was performed by 2 experienced pathologists (Y.S. and P.P.), and the association was calculated on the basis of the Cohen \(\kappa\) correlation on the SPSS program (SPSS, Chicago, IL).

**Western Blot Analysis**

For Western blot analysis, 50 µg of protein was run under denaturating and reducing conditions (BioRad, Hercules, CA), transferred to nitrocellulose membranes, and treated with MnSOD, CuZnSOD, and ECSOD antibodies (dilution 1:10,000, 1:20,000, and 1:2,500, respectively). \(\beta\)-actin was used as a marker for protein loading; for this purpose, mouse monoclonal anti--\(\beta\)-actin antibody (Sigma, St Louis, MO) at a 1:20,000 dilution was used. Secondary antibodies included donkey antirabbit and sheep antimouse antibodies (Amersham, Arlington Heights, IL). Proteins were detected by using an enhanced chemiluminescence system (ECL; Amersham), and luminol excitation was imaged on x-ray film. Expression was detected quantitatively by scanning densitometry (300A Computing Densitometry with Image Quant Software, v3.0 Fast Scan, Molecular Dynamics, Sunnyvale, CA).

**Activity Measurements**

SOD activity was measured by spectrophotometry by using cytochrome c (Sigma) and xanthine oxidase (Roche, Mannheim, Germany). The activity was assessed following the decrease in the rate of reduction (the rate of change in absorbancy at 550 nm) of cytochrome c. MnSOD activity was distinguished from CuZnSOD by its resistance to a 1-mmol/L concentration of potassium cyanide. The activity was expressed as units per milligram of protein.

**Determination of Apoptosis and Proliferation**

To detect apoptotic cells, in situ labeling of the 3’-ends of the DNA fragments generated by apoptosis-associated endonucleases was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD); the apoptotic index was assessed as described previously. For proliferating cell nuclear antigen (PCNA) immunostaining, the monoclonal antibody PC10 (dilution 1:50; DAKO, Glostrup, Denmark) was used; the secondary antibody was biotinylated antimouse antibody (dilution 1:300; Dakopatts, Copenhagen, Denmark). The avidin-biotin-peroxidase complex method was used, and the color was developed using 3,3’-diaminobenzidine. The percentage of PCNA-positive cells was counted in at least 6 high-power fields and was calculated in a manner similar to that used for apoptosis.

**Statistical Analysis**

SPSS for Windows (SPSS) was used for statistical analysis. The significance of the associations was determined by using a 2-tailed \(t\) test, the Fisher exact probability test, a 2-sample test of proportion, \(\kappa\) correlation analysis, and Pearson correlation. Probability values of .05 or less were considered statistically significant. The Cohen \(\kappa\) correlations for MnSOD immunoreactivity in conventional biopsy specimens and tissue array blocks were 0.32 and 0.54, respectively. The Cohen \(\kappa\) correlation for CuZnSOD was excellent (0.80).

**Results**

**Manganese SOD**

Nonmalignant lung tissue samples from the carcinoma samples showed variable MnSOD positivity in bronchial epithelium (weak in 41%, moderate in 17%, and strong in 17% of the cases) and alveolar macrophages. Of a total of 73 non–small cell carcinomas (conventional paraffin blocks), 41 (56%) showed positivity for MnSOD in the carcinoma cells. The staining was granular and usually weak; 9 samples (12%) had moderate staining. Altogether 61% of squamous

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<table>
<thead>
<tr>
<th>Sex/Age (y)</th>
<th>Tumor Type</th>
<th>Grade</th>
<th>Stage</th>
<th>Survival</th>
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<td>IA</td>
<td>Alive with metastasis</td>
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<td>IIIA</td>
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<td>Alive</td>
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</table>

NA, no information available.

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**Table 2**

### Clinical Information for Cases Analyzed for Enzyme Activity and by Western Blot Analysis

<table>
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<th>Tumor Type</th>
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<td>M/70</td>
<td>Squamous cell carcinoma</td>
<td>II</td>
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<td>M/57</td>
<td>Squamous cell carcinoma</td>
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<tr>
<td>M/71</td>
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<tr>
<td>M/62</td>
<td>Squamous cell carcinoma</td>
<td>III</td>
<td>IIA</td>
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cell carcinomas and 38% of adenocarcinomas were positive for MnSOD. In the tissue array blocks (66 cases could be evaluated) MnSOD positivity was seen in 42% of all carcinomas, 36% in squamous cell carcinomas and 47% in adenocarcinomas. The localization of MnSOD in lung tissue samples is shown in **Image 1**. A summary of the positive immunohistochemical findings for all carcinomas is shown in **Figure 1**. Western blot analysis for 9 lung specimens from separate patients (5 with adenocarcinoma and 4 with squamous cell carcinoma) revealed that the mean level of MnSOD protein was 12.5% higher in adenocarcinoma and 44% higher in squamous cell carcinoma than in the nonmalignant lung tissue samples **Figure 2A** and **Figure 2B**.

When all 9 carcinomas were considered, the mean level of MnSOD was 27% higher in the malignant than in the nonmalignant lung tissue samples ($P = .05$; 2-tailed $t$ test).

There was no difference in MnSOD positivity between low- and high-grade tumors. No association was found between tumor extent and nodal metastasis and MnSOD expression. The mean ± SD apoptotic index in tumor material was 1.23% ± 1.17%, and the mean ± SD proliferation index was 27.1% ± 13.4%. Tumors showing MnSOD positivity had a significantly lower proliferation index determined by PCNA-staining ($P = .014$; 2-tailed $t$ test), whereas there was no association between the extent of apoptosis or patient survival and MnSOD positivity. When evaluated

**Image 1**

A. Manganese superoxide dismutase (MnSOD) immunoreactivity can be detected mainly in the bronchial epithelial cells of nonmalignant lung tissue sample (×200).

B. Copper zinc SOD (CuZnSOD) is located mainly in the bronchial epithelial cells (×200).

C. Peripheral nonmalignant lung tissue sample shows staining for MnSOD in alveolar macrophages, and alveolar epithelial cells are negative (×100).

D. Positive CuZnSOD immunostaining in the alveolar macrophages of a peripheral lung...
from array material, there tended to be more apoptotic cells in the MnSOD-positive cases \( (P = .06; \)  2-tailed \( t \) test). Previously, the expression of p53 was analyzed from this same material.\(^{32} \) The present study found no association between p53 and MnSOD positivity.

**Copper Zinc SOD**

Nonmalignant bronchial epithelium stained weakly positive for CuZnSOD; the size and location of the bronchi had no influence on the staining intensity. CuZnSOD could be analyzed in 56 tissue array samples. Altogether 46 (82\%) of the carcinoma samples were positive; 66\% weak and 16\% moderate. In adenocarcinomas, 93\% of the samples were positive; the corresponding value for squamous cell carcinoma was 79\% (Figure 1). The positive immunohistochemical findings for all carcinomas are summarized in Figure 1. By Western blot analysis, the mean CuZnSOD level was 25\% higher in adenocarcinoma and 17\% higher in squamous cell carcinoma than in the control nonmalignant tissue samples \( \) Figure 2C \( \) and \( \) Figure 2D \( \). If the 9 carcinoma cases were considered together, CuZnSOD was 22\% higher in the cancer samples than in the control samples \( (P = .11; \)  2-tailed \( t \) test). CuZnSOD activity was analyzed in the same 9 samples already described. The activity was 98\% higher in adenocarcinoma (mean from 5 samples) and 89\% higher in squamous cell carcinoma (mean from 4 samples) than in the

tissue sample \( (\times 100) \). \( \) E, Granular staining of MnSOD localizes to the cytosol of squamous cell carcinoma cells \( (\times 400) \). \( \) F, Diffuse staining for CuZnSOD in squamous cell carcinoma \( (\times 400) \). \( \) G, Granular staining for MnSOD in adenocarcinoma cells \( (\times 400) \). \( \) H, Negative control sample for a patient with squamous cell carcinoma \( (\times 400) \).
control samples. When all the carcinoma samples were considered, the activity was 93% higher in carcinoma than in the nonmalignant lung tissue samples \((P = .06; \text{2-tailed} \, t\, \text{test})\). CuZnSOD activity correlated significantly with CuZnSOD immunoreactivity \((r = 0.82; \, P = .007; \, \text{Pearson}\, \text{correlation})\).

No association between CuZnSOD positivity in grade I or II and grade III tumors was found. In addition, there was no significant association between tumor extent and CuZnSOD positivity. In patients with lymph node invasion, a trend toward lower CuZnSOD immunoreactivity was noted \((P = .05; \, \text{Fisher\, exact\, probability\, test})\). The expression of CuZnSOD also was analyzed in relation to proliferation index, apoptotic activity, and patient survival, but no association was found between these markers. p53 did not correlate with CuZnSOD reactivity.

**Extracellular SOD**

Immunohistochemical assessment of ECSOD revealed its expression mainly in the endothelial cells and matrix and usually weak immunoreactivity in bronchial epithelium and macrophages as previously published \((6,\, 27)\). ECSOD could be evaluated from 37 cases of the tissue array blocks. In carcinoma tissues, weak ECSOD immunoreactivity could be detected only in 2 of 37 cases, with faint stromal positivity in 4 cases. All cases showing tumor cell positivity also had stromal positivity. Of the positive cases, 2 were adenocarcinomas and 2 squamous cell carcinomas. There was no significant difference in ECSOD expression between adenocarcinomas and squamous cell carcinomas \((P = .91)\). A summary of the positive immunohistochemical findings for all carcinomas is given in Figure 1. Western blot analysis indicated that carcinoma tissue samples contained remarkably lower ECSOD than nonmalignant lung tissue samples. This finding was evident in adenocarcinoma \((76\% \, \text{less}; \, P = .003)\) and in squamous cell carcinoma \((65\% \, \text{lower}; \, P = .02)\); the mean decrease in all cancers was 70% \((P < .0001)\) \(\text{Figure 2E} and \text{Figure 2F}\).

**Discussion**

This report is the first describing the expression of all SODs in human lung tumors at the same time. We found a different profile in the expression of intracellular MnSOD and CuZnSOD on the one hand and extracellular ECSOD on the other in human malignant lung diseases. Both CuZnSOD and MnSOD could be detected in lung carcinomas by several methods. Both seemed to be higher in malignant than in nonmalignant lung tissue samples. On the other hand, ECSOD, which is mainly an extracellular enzyme, usually was nondetectable in the tumor cells. Stromal ECSOD immunoreactivity could be detected in only a few tumors. Low ECSOD expression, however, can have potential consequences on tumor invasion, which is known to be highly regulated by the redox state of the cells.

The findings of previous studies of SODs in lung cancer are controversial, suggesting increased\(^{24}\) or decreased\(^{4,25,33}\) expression. This variability might be due to multiple reasons, including methodological aspects such as differences in activity measurement protocols, standardization of the whole tissue homogenate to protein or DNA, or selection and preparation of tissue homogenates. Given the possible alterations in \(\beta\)-actin integrity and cohesion in neoplastic tissue,\(^{34,35}\) it was not included in the present study. Control tissue samples and their validity comprise one of the major reasons for variable results because SODs, especially MnSOD, are known to be induced, inactivated, or both by multiple agents in lung cells in vitro and in vivo.\(^{36}\) Most experimental studies have investigated MnSOD regulation after short exposures, which do not reflect the situation in vivo.\(^{3,36,37}\) In fact, previous immunohistochemical studies with the same antibody used in the present study and histopathologically healthy lung tissue samples could not detect MnSOD immunoreactivity in the bronchial epithelial cells or alveolar epithelial cells of nonsmokers, although MnSOD clearly could be found in nonmalignant lung epithelial cells of nonselected patients with cancer.\(^{5,38}\) Overall, our results suggest that MnSOD is elevated in lung carcinoma, and the increase seems to be more prominent in squamous cell than in other histologic types of lung carcinoma.

Our study is by far the largest on the immunohistochemical cell-specific expression of MnSOD in lung tumors. Altogether, 139 specimens were analyzed. MnSOD was
Figure 2 | Representative Western blot analysis of manganese superoxide dismutase (MnSOD) (A and B), copper zinc SOD (CuZnSOD) (C and D), and extracellular SOD (ECSOD) (E and F) in control lung tissue samples (h), adenocarcinoma (ac), and squamous cell carcinoma (sc). Five control and 5 adenocarcinoma cases were included in the series of adenocarcinomas; 4 cases were included in the squamous cell carcinoma group. When all 9 cases were combined, MnSOD and CuZnSOD were 27% and 21% higher, respectively, and ECSOD was 71% lower in carcinomas than in the nonmalignant lung tissue sample.
positive in adenocarcinoma and squamous cell carcinoma, showing that cancer cells are capable of synthesizing MnSOD. MnSOD was localized to cancer cells; the staining typically was granular, referring to its mitochondrial origin. Even though part of the carcinoma specimens were negative, this result does not rule out enzyme expression in these cells. In contrast with healthy lung tissue samples from nonsmokers, bronchial epithelium and alveolar macrophages in the nonneoplastic lung tissue samples from patients with carcinoma showed variable, often weak to moderate, MnSOD immunoreactivity. It is likely that MnSOD is induced by cytokines and possibly cigarette smoke in the nonmalignant and malignant cells of patients with carcinoma.

One study investigated CuZnSOD immunoreactivity by Western blot analysis in lung carcinoma, and in that study, no significant difference between malignant and nonmalignant tissue samples could be detected. In our study, CuZnSOD immunoreactivity by Western blot analysis showed a nonsignificant tendency to be elevated in lung carcinoma. Consistently, the only previous histochemical study on nonmalignant respiratory epithelium and lung tumors detected CuZnSOD, especially in adenocarcinoma. Overall, elevated CuZnSOD activities (93% higher) and immunoreactivities by Western blot analysis and immunohistochemical analysis suggest that CuZnSOD might be elevated in lung carcinoma.

The relationship of p53 and MnSOD from in vitro studies prompted us to study markers of tumorigenesis in lung carcinoma. There are no studies on the tumor suppressor protein p53 or markers of apoptosis and proliferation measured by PCNA immunoreactivity in association with SODs and human lung carcinoma tissue samples. ROS are known to be inducers of several proteins, including p53, and they are known to have a role in the execution of p53-dependent apoptosis. MnSOD has been suggested to be a potential target for p53, because of a finding of excess MnSOD expression in the context of p53 deficiency in animal models and cell lines. The lack of p53, along with elevated MnSOD levels, is associated with some cell types showing enhanced resistance to a number of cytotoxic drugs. In previous studies on our lung cancer material, p53 staining was found to be positive in dysplasias and carcinomas, whereas adjacent nonneoplastic tissue samples were negative. In the present study, there was no association between MnSOD or CuZnSOD and p53 positivity in lung carcinoma. No differences were found between adenocarcinomas and squamous cell carcinomas. This might suggest that p53 does not influence the induction of the SODs in lung carcinoma in vivo.

Tumor cell proliferation was assessed in relation to MnSOD immunoreactivity, because previous findings have shown an inverse correlation between MnSOD overexpression and cell proliferation in vitro and also in experimental models in vivo. In line with these earlier studies, cell proliferation was lowered significantly \((P = .014)\) in the MnSOD-positive lung carcinomas. One might expect to see high SOD levels correlating with increased cell proliferation, if it is assumed that SODs enhance lung carcinogenesis. These results suggest that MnSOD-positive tumors might grow more slowly than MnSOD-negative tumors.

ECSOD is the major extracellular antioxidant enzyme in human lung. It also is the major determinant in decreasing extracellular oxidant stress and availability of superoxide to the reaction with nitric oxide extracellularly. Furthermore, ECSOD being expressed in the vascular endothelium modulates the redox milieu in these cells, which might have multiple effects on cell growth. Low ECSOD expression in lung tumors can have potential effects on extracellular regulation of multiple factors that regulate angiogenesis and invasion.

We found significant levels of the major intracellular superoxide dismutases, MnSOD and CuZnSOD, in lung adenocarcinoma and squamous cell carcinoma, whereas the extracellular SOD, ECSOD, seemed to be remarkably lower in lung carcinoma than in the nonmalignant lung. Because cellular redox states have multiple cellular effects, including proliferation, drug resistance, and angiogenesis, intracellular and extracellular SODs might have significant but different roles in the resistance and growth of lung tumors.


