Superoxide Dismutases in Gastric and Esophageal Cancer and the Prognostic Impact in Gastric Cancer

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ABSTRACT

The oxidant-antioxidant balance is thought to be important in the initiation, promotion, and therapy resistance of cancer. In the present study, we assessed the expression of the antioxidants manganese superoxide dismutase (Mn-SOD) and copper/zinc superoxide dismutase in gastric and esophageal carcinomas and their relation with clinical outcome. Adenocarcinomas of the stomach (n = 81) as well as squamous cell carcinomas of the esophagus (n = 10) showed an enhanced immunohistochemical expression of Mn-SOD, which was accompanied by a significantly higher tissue level (P ≤ 0.007) compared with their corresponding normal mucosa. In contrast, copper/zinc superoxide dismutase was found to be marginally lower in these malignant tissues in comparison with the normal tissues. The superoxide dismutase levels were not found to be associated with major clinicopathological features of the gastric cancer patients. Univariate analysis revealed, however, that a high Mn-SOD level in gastric carcinomas, a low level in the normal gastric mucosa, and a high ratio of these two levels in gastric cancer patients are indicative of a poor overall survival. Multivariate analysis, including all clinicopathological parameters, revealed that the Mn-SOD ratio in particular is an independent prognostic parameter in gastric cancer patients.

INTRODUCTION

The antioxidant enzyme SOD, in combination with enzymes like myeloperoxidase, catalase, and glutathione peroxidase, forms the backbone of the primary cellular defense mechanism against ROMs (1–4). These ROMs, including true free radicals, such as superoxide anion (O2−) and hydroxyl radical (OH·), as well as nonradical compounds, such as hydrogen peroxide (H2O2), are continuously produced during aerobic metabolism (5). Under normal conditions, cells are protected against the toxic effects of high concentrations of ROMs by a balanced level of endogenous enzymatic and nonenzymatic antioxidants. However, oxidative damage can arise either from the overproduction of ROMs or from an imbalance of antioxidant defense mechanisms, and it has been implicated in the development and treatment of diseases such as cancer (3, 5–14).

The antioxidant enzyme SOD catalyzes the dismutation of O2− to H2O2, thereby preventing the accumulation of this free radical. Subsequently, H2O2 can be converted into H2O and O2 by catalase and/or glutathione peroxidase (1–4, 9). In human tissues, there are at least three different SOD enzymes. These include a cytoplasmic Cu/Zn-SOD, a mitochondrial Mn-SOD, and an extracellularly localized SOD (1, 2, 4, 9, 15). We recently showed that human colorectal cancer development is associated with a significant increase in Mn-SOD, whereas no major differences were found with regard to Cu/Zn-SOD (16).

In a larger study, the increased Mn-SOD antigen level of colorectal carcinomas was found to be an independent prognostic factor for the overall survival of the patients (17). Recently, Izutani et al. (18, 19) reported that cancers of the upper gastrointestinal tract are also characterized by an increased expression of Mn-SOD.

In the present study, we investigated SODs in gastrointestinal cancer further by evaluating 81 patients with gastric cancer and 10 patients with esophageal cancer. With respect to the gastric cancer patients, the relationship with several clinicopathological parameters and the prognostic value of the SOD content for the overall survival of the patients were determined as well.

MATERIALS AND METHODS

Patients and Study Design. Fresh tissue could be prospectively collected from 81 consecutive patients (21 females...
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and 60 males; mean age, 65.9 ± 1.4 years) who underwent resection for primary gastric cancer (adenocarcinoma) and from 10 patients (8 females and 2 males; mean age, 57.6 ± 3.5 years) who were operated on for primary squamous cell carcinoma of the esophagus at the Department of Oncological Surgery. Immediately after resection, the specimens were transported to the Department of Pathology, and samples of carcinomas and macroscopically normal mucosa, taken ~5–10 cm from the tumor, were snap-frozen and stored at −70°C until extraction, when available for research purposes. Immediately adjacent tissues were used for histological reconfirmation of disease and tissue type, and for immunohistochemistry. From the group of gastric cancer patients, several major clinical and pathological data were evaluated and registered or retrieved from their data files. Macroscopic pathological features of the gastric carcinomas, such as localization and diameter of the tumor, were assessed, and all tumors were histologically classified according to the TNM classification (20). Microscopic histological parameters, such as the WHO and Laurén (21) classification, the number of eosinophils, as well as the presence of intestinal metaplasia in the normal mucosa, were revised by one pathologist. With regard to the survival analyses of the gastric cancer patients, all patients entered the study at operation date, and the patients’ time experience ended in the event of death or, when still alive, at the common closing date, with a minimal follow-up of 33 months.

Tissue Extraction and Protein Concentration. Extractions were prepared from 50 to 100 mg of wet tissue samples, as described previously (22). The samples were weighed, and 1 ml of 0.1 M Tris-HCl (pH 7.5) with 0.1% (v/v) Tween 80/60 mg of sample was added. The tissue was homogenized for 2 min on ice in a Potter S (B Braun). The homogenates were centrifuged twice at 8000 × g for 2.5 min, 4°C, and the final supernatants were stored at −70°C. The protein concentration of the supernatants was determined using the method of Lowry et al. (23).

SOD Standards and Antibodies. The standards used were hr Mn-SOD and Cu/Zn-SOD, kindly provided by Dr. Z. Yavin from the Kryiat Weizmann Institute (Rehovot, Israel). The monospecific antibodies raised in rabbits showed no cross-reactivity between the two SOD isoforms (Mn versus Cu/Zn) and provided no signal with other proteins of tissue homogenates on Western blotting, as reported earlier (16, 24).

ELISA for Cu/Zn-SOD. The Cu/Zn-SOD antigen concentration of the tissue homogenates was determined by a modified ELISA, as described previously (16, 24). In brief, flat-bottomed polystyrene microtiter plates (Dynatech Laboratories; M129A) were coated with goat-α-human Cu/Zn-SOD [10 μg/ml in 0.05 M carbonate buffer (pH 9.6)] overnight at 4°C. Homogenates, diluted 1:600 in duplicate, were incubated for 2 h, and subsequently, rabbit-α-(hr) Cu/Zn-SOD polyclonal antiserum (dilution, 1:2500) was added to the wells and incubated for 1 h. Finally, a polyclonal goat-α-rabbit IgG conjugated to horseradish peroxidase (Dakopatts; P448; dilution, 1:5000) was added for 1 h, and the plates were colored with orthophenylendiamine for 30 min. The reaction was terminated with sulfuric acid, and the absorbance was read at 492 nm on a Titertek Multiscan (Flow Laboratories, Irvine, United Kingdom) plate reader. The Cu/Zn-SOD concentration of the samples was calculated from a calibration curve based on nine standards between 1.25 and 30 ng/ml (hr) Cu/Zn-SOD and expressed per mg of protein of the homogenate. The intra- and interassay coefficients of variation of this ELISA were 4 and 6%, respectively.

ELISA for Mn-SOD. This ELISA is similar to that for Cu/Zn-SOD, as described before (16, 24). The microtiter plates were coated overnight at 4°C with a rabbit-α-(hr) Mn-SOD polyclonal antibody, and the homogenates were diluted 1:150. The standard line of (hr) Mn-SOD ranged from 1.25 to 40 ng/ml. After duplicate incubation with the tissue homogenates, the plates were incubated for 90 min with rabbit-α-(hr) Mn-SOD coupled to horseradish peroxidase (dilution, 1:250). Bound antibodies were detected as described for Cu/Zn-SOD. The intra- and interassay coefficients of variation of this ELISA were 5 and 10%, respectively.

SOD Enzyme Activity Assay. The SOD activity was determined by the xanthine/xanthine-oxidase/cytochrome c method according to McCord and Fridovich (1), as described previously (16, 24). Xanthine/xanthine-oxidase-produced O_2^- reduces cytochrome c, which can be assessed spectrophotometrically at 550 nm. SOD competes with cytochrome c for the dismutation of O_2^•-, and total SOD activity was determined in the homogenates using a calibration curve from 1.25 to 12.5 μg/ml (hr) SOD. Mn-SOD activity was determined in the presence of 1 mM sodium cyanide, which inhibits Cu/Zn-SOD for ≥90%, and Cu/Zn-SOD was estimated by subtraction of the Mn-SOD from the total SOD activity. The activity is expressed in units/mg of protein, where one unit is equivalent to the SOD activity that causes 50% inhibition of the reaction rate in the absence of SOD. For Cu/Zn-SOD, one unit corresponds with 180 ng of active (hr) Cu/Zn-SOD, whereas one unit of Mn-SOD corresponds with 225 ng of active (hr) Mn-SOD.

Immunohistochemistry. Immunolocalization of Cu/Zn-SOD and Mn-SOD was performed by an indirect peroxidase-labeled antibody method with our polyclonal rabbit antibodies, as extensively described and validated previously (16), on 14 gastric and 10 esophageal carcinoma-normal mucosa pairs. Formalin-fixed, paraffin-embedded tissue samples were sectioned (4 μm), mounted on poly-L-lysine-coated glass slides, air-dried, deparaffinized, and rehydrated. Slides were subsequently submerged in 10 mM sodium citrate buffer (pH 6.0) and microwave-heated at 780 W for three 4-min intervals for antigen retrieval. The sections were incubated for 20 min in 5% normal goat serum to block nonspecific binding and then overnight at 4°C with rabbit-α-(hr) Cu/Zn-SOD (1:1000 dilution) or rabbit-α-(hr) Mn-SOD (1:250 dilution) polyclonal antiserum, diluted in TBS [50 mM Tris, 150 mM NaCl (pH 7.5)] containing 0.5% BSA. The sections were subsequently rinsed thoroughly, and biotinylated goat-α-rabbit immunoglobulin (Dakopatts; E0432; 1:200 dilution in TBS) was applied for 45 min as a bridging antibody, followed by a 45-min incubation with peroxidase-labeled streptavidin (Dakopatts; P0379; 1:100 dilution in TBS). Staining of the sections was performed by incubation in 0.1 M acetate buffer (pH 5.0) containing 0.03% 3-amino-9-ethylcarbazole and 0.03% H_2O_2 for 10 min, resulting in a red staining product. Finally, sections were counterstained in Mayer’s hematoxylin and mounted in Aquamount. As negative controls, TBS and preimmune serum were used instead of the primary antibody. The slides were independently evaluated by two readers, blinded to the other SOD assessments, to give a descriptive analysis.
RESULTS

Mn-SOD and Cu/Zn-SOD Concentrations. The mean Mn-SOD and Cu/Zn-SOD concentrations as determined by ELISA are shown in Table 1. The Mn-SOD content of both the gastric and esophageal carcinomas was significantly \( P < 0.0005 \) and \( P = 0.007 \), respectively, higher compared with that of corresponding normal mucosa. Paired evaluation revealed that a large majority of the carcinomas had higher Mn-SOD levels than the normal tissue, i.e., 78% (63 of 81) of the gastric and 90% (9 of 10) of the esophageal tumors. In contrast, both carcinoma types contained less Cu/Zn-SOD antigen compared with their corresponding normal mucosa \( (P = 0.001 \) and \( P = 0.08 \), respectively).

Table 1  Mn-SOD and Cu/Zn-SOD antigen levels (ng/mg of protein) in normal mucosa and carcinomas of patients with gastric or esophageal cancera

<table>
<thead>
<tr>
<th></th>
<th>Mn-SOD (n)</th>
<th>Cu/Zn-SOD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach ((n = 81))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>493 ± 30</td>
<td>718 ± 25</td>
</tr>
<tr>
<td>Carcinomas</td>
<td>911 ± 62a</td>
<td>606 ± 33b</td>
</tr>
<tr>
<td>Esophagus ((n = 10))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>530 ± 83</td>
<td>719 ± 63</td>
</tr>
<tr>
<td>Carcinomas</td>
<td>1151 ± 136b</td>
<td>518 ± 81</td>
</tr>
</tbody>
</table>

a Results shown are mean values ± SE.

b Versus normal mucosa \((P = 0.007)\).

Statistical Analyses. The significance of the differences in the SOD antigen and activity levels, expressed in mean ± SE, between pairs of normal mucosa and carcinoma were evaluated using the paired Student’s \( t \) test. For comparison of data from different patient and sample groups, ANOVA and the unpaired Student’s \( t \) test were used; estimates for equality of variances showed no significant differences according to Levene’s \( F \)-test. For the statistical survival analyses of the group of gastric cancer patients, the clinicopathological parameters were dichotomized as follows: gender into males versus females; age in years into \(<66.2\) versus \(\geq 66.2\); TNM stage in stage III/IV versus stage III/IV; Laurén classification in diffuse/mixed versus intestinal; WHO classification in differentiated (papillary, tubular, mucinous, and adenosquamous) versus undifferentiated (signet cell and undifferentiated); tumor localization in antrum versus corpus, fundus, and cardia; diameter of the tumor into \(<5\) cm versus \(\geq 5\) cm; number of eosinophils in many versus few/ moderate; and intestinal metaplasia in normal mucosa in present versus absent. The optimal cutoff points of the age, diameter, and the SOD parameters in carcinoma and normal mucosa were assessed by slowly increasing the level until the point of best discrimination, i.e., optimal dichotomization. For age and diameter, no such point was found, and therefore, the median and 5 cm were used, respectively, to obtain comparable groups.

Univariate survival analysis was performed with the Cox proportional hazard model \((25)\), using the SPSS 6.0 statistical software package (SPSS, Inc., Chicago IL), resulting in the identification of covariates that significantly correlated with the overall survival of the patients. Multivariate survival analyses were performed using the Cox proportional hazards method by separately adding the significant SOD variables to the nine dichotomized clinicopathological parameters \( (i.e., \text{gender and age of the patients, TNM stage, Laurén classification, WHO classification, localization, diameter, number of eosinophils of the tumors, and intestinal metaplasia of the normal mucosa}) \). Overall survival curves were constructed using the method of Kaplan and Meier \((26)\). The statistical significance of the difference in survival of the groups was calculated using the log-rank test. Differences were considered significant when \( P \leq 0.05 \).

### Table 2  Mn-SOD and Cu/Zn-SOD antigen level (ng/mg of protein) in normal gastric mucosa and gastric carcinoma \((n = 81)\), dichotomized according to various clinicopathological parameterb

<table>
<thead>
<tr>
<th>Parameter dichotomized (n)</th>
<th>Normal mucosa</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>Mn-SOD</td>
<td>Cu/Zn-SOD</td>
</tr>
<tr>
<td>Alive (15)</td>
<td>560 ± 56</td>
<td>830 ± 49</td>
</tr>
<tr>
<td>Deceased (66)</td>
<td>477 ± 34</td>
<td>692 ± 27</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (60)</td>
<td>458 ± 32</td>
<td>733 ± 30</td>
</tr>
<tr>
<td>Female (21)</td>
<td>592 ± 69b</td>
<td>674 ± 40</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt;66.2) (40)</td>
<td>477 ± 41</td>
<td>740 ± 32</td>
</tr>
<tr>
<td>(\geq 66.2) (41)</td>
<td>508 ± 44</td>
<td>696 ± 38</td>
</tr>
<tr>
<td>Laurén</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse/mixed (30)</td>
<td>516 ± 50</td>
<td>703 ± 42</td>
</tr>
<tr>
<td>Intestinal (50)</td>
<td>478 ± 38</td>
<td>724 ± 31</td>
</tr>
<tr>
<td>WHO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiated (54)</td>
<td>464 ± 37</td>
<td>711 ± 30</td>
</tr>
<tr>
<td>Undifferentiated (26)</td>
<td>551 ± 53</td>
<td>726 ± 47</td>
</tr>
<tr>
<td>TNM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stages I + II (49)</td>
<td>485 ± 43</td>
<td>694 ± 33</td>
</tr>
<tr>
<td>Stages III + IV (32)</td>
<td>504 ± 39</td>
<td>754 ± 37</td>
</tr>
<tr>
<td>Localization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antrum (34)</td>
<td>556 ± 46</td>
<td>715 ± 34</td>
</tr>
<tr>
<td>Other (47)</td>
<td>447 ± 38</td>
<td>719 ± 35</td>
</tr>
<tr>
<td>Diameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt;5) cm (47)</td>
<td>522 ± 40</td>
<td>753 ± 33</td>
</tr>
<tr>
<td>(&gt;5) cm (34)</td>
<td>452 ± 44</td>
<td>669 ± 36</td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Many (9)</td>
<td>342 ± 103</td>
<td>651 ± 110</td>
</tr>
<tr>
<td>Few/moderate (71)</td>
<td>514 ± 31</td>
<td>725 ± 25</td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent (39)</td>
<td>488 ± 38</td>
<td>725 ± 33</td>
</tr>
<tr>
<td>Present (42)</td>
<td>497 ± 46</td>
<td>711 ± 37</td>
</tr>
</tbody>
</table>

a Results shown are mean values ± SE.
b Versus male \((P = 0.05)\).
other relevant associations between SOD levels and patient- or tumor-related features were observed.

**SOD Activity.** The SOD activity levels were determined in 15 gastric normal mucosa-carcinoma pairs. There were no significant differences in the Mn-SOD and Cu/Zn-SOD activity levels, expressed as units/mg of protein, between normal gastric mucosa (4.5 ± 0.4 and 3.9 ± 0.5, respectively) and carcinomas (4.7 ± 0.6 and 3.8 ± 0.4, respectively). The Pearson’s correlation coefficient between the antigen and activity levels of Mn-SOD in these pairs was highly significant (overall $r = 0.65, P < 0.0005$; normal mucosa $r = 0.84$ and carcinoma $r = 0.66$), whereas for Cu/Zn-SOD, this linear correlation was only marginally significant (overall $r = 0.36, P = 0.05$; normal mucosa $r = 0.33$ and carcinoma $r = 0.44$). The corresponding protein levels, however, did show an increase of the Mn-SOD isoform from $509 \pm 64$ to $861 \pm 133 (P = 0.01)$ and a nonsignificant ($P = 0.08$) decrease of the Cu/Zn-SOD isoform from $612 \pm 38$ to $479 \pm 56$ ng/mg of protein, respectively.

**Immunohistochemistry.** In normal gastric mucosa, Mn-SOD was predominantly localized in the cytoplasm of the parietal cells, showing a granular staining pattern. Also, the mucous cells at the outer top of the foveolar epithelium contained Mn-SOD in their cytoplasm, whereas the other foveolar and glandular cells were negative or weakly positive (Fig. 1A). The tumor cells within the gastric adenocarcinoma showed a relatively strong Mn-SOD immunoreactivity as compared with their normal epithelial counterparts (Fig. 1B). Both in the normal mucosa and carcinoma tissue, most stromal cells (myofibroblasts and macrophages) only showed a weak to moderate Mn-SOD immunoreactivity.

The Mn-SOD expression of the esophagus was mainly confined to the luminal site of the normal squamous epithelium, whereas in the carcinoma tissue, most tumor and stromal cells were moderately to strongly positive (Fig. 2, A and B).

Cu/Zn-SOD was found to be localized diffusely in the cytoplasm of the glandular cells of the normal gastric epithelium. Parietal cells showed cytoplasmic and/or nuclear Cu/Zn-SOD immunoreactivity (Fig. 1C). Although Cu/Zn-SOD was found in the cytoplasm and/or nucleus of some tumor cells, most gastric carcinoma showed a less intense Cu/Zn-SOD immunoreactivity as compared with normal mucosa. Stromal cells in the normal mucosa and carcinoma tissue were negative for Cu/Zn-SOD (Fig. 1D). Concerning normal esophageal epithelium, the majority showed a strong cytoplasmic and/or nuclear staining of the deepest part of the basal layer, whereas the other cells only showed a weak to moderately cytoplasmic Cu/Zn-SOD immunoreactivity (Fig. 2C). The tumor cells of the esophageal carcinoma were either negative or positive showing a very weak staining intensity of the cytoplasm and/or the nucleus (Fig. 2D). Foci of negative and positive tumor cells were present within the same section. Stromal cells both in normal and carcinoma tissue were mainly negative.

Intestinal metaplasia, characterized by the presence of goblet cells, was sometimes found in the normal gastric mucosa. These metaplastic parts were strongly positive for both Mn-SOD and Cu/Zn-SOD, with negative goblet vacuoles (data not shown).

**Survival Analyses of the Gastric Carcinoma Patients.**
Sixty-six patients with a gastric carcinoma (81.5%; 19 females and 47 males) had died during follow-up, with a median survival time of 11.5 months (range, 0.5–74 months). Only 15 (18.5%; 2 females and 13 males) were still alive at the end of the study, with a median follow-up time of 50 months (range, 33–118 months). The patients who had died were slightly older (66.3 ± 1.4 years) compared with those still alive (63.9 ± 4.1). Furthermore, there was a decreased survival of the patients with TNM stage III or IV (15.6%) compared with TNM stage I or II patients (20.4%).

Univariate Cox survival analysis of the dichotomized clinicopathological parameters showed that several had some association with a poor survival; for example, female sex (survival, 9.5% versus 21.7% for males) and a large tumor diameter (survival, 14.7% versus 21.3% for small tumors), but only a high number of eosinophils within the carcinoma (survival, 0% versus 19.7% with low eosinophil numbers) and the absence of intestinal metaplasia of the normal mucosa (survival, 10.3% versus 26.2% of those with metaplasia) were significant (0.02 ≤ $P \leq 0.05$) predictors of a poor overall survival. In the multivariate analysis, only the number of eosinophils of the tumor remained significantly associated with the overall survival (hazard ratio, 0.4; 95% confidence interval, 0.2–0.9; $P = 0.03$). Gender and age of the patients, TNM stage, Lauren classification, WHO classification, localization, and diameter of the tumor were found not to be significantly associated with the overall survival of the patients.

Optimal dichotomization of the antigen concentration in the carcinomas revealed that a high Mn-SOD content (>450 ng/mg of protein) was associated with a relatively poor overall survival of the patients (Table 3; Fig. 3A). In contrast, a cutoff point could also be identified in the normal mucosa, at 335 ng/mg of protein Mn-SOD, which indicated that a low SOD level of the normal mucosa was associated with a relatively poor survival of the patients (Table 3; Fig. 3B). Because of this diametrical observation, we additionally assessed whether the carcinoma:normal mucosa ratio of the Mn-SOD concentration within the patients was related to the survival as well. At a ratio >2.03, the survival was found to be significantly lower than below this ratio (Table 3; Fig. 3C).

Multivariate analysis, in which the dichotomized SOD variables were adjusted to the assessed nine clinicopathological parameters, revealed that the carcinoma:normal mucosa Mn-SOD ratio remained significantly associated ($P = 0.03$) with a relatively poor overall survival of the patients, indicating its independent prognostic value. In these multivariate analyses, a high eosinophil content of the carcinomas and the absence of intestinal metaplasia in the normal gastric mucosa were consistently found to be significantly associated with the overall survival ($0.4 <$ hazard ratio $< 0.6$; 95% confidence interval, 0.2–1.0; 0.03 $< P \leq 0.05$).

**DISCUSSION**
In the present study, we showed that gastric adenocarcinomas and esophageal squamous cell carcinomas are characterized by a significantly increased expression of Mn-SOD. These results are in agreement with studies by Izutani et al. (18, 19) who...
previously reported a greatly enhanced expression of Mn-SOD, at both the mRNA and protein level, in the same type of cancers, and with our report on SOD in the colorectal cancer sequence (16). Moreover, the present study indicates that also in gastric cancer, the Mn-SOD level is of prognostic significance for the overall survival of the patients, similar to that as reported for colorectal cancer (17).

In most cancer cells, there is an imbalance of antioxidant enzymes, as recently reviewed by Oberley and Oberley (27). Although originally, SOD levels were thought to be lower in malignant cells and (experimental animal) tumors, when compared with normal nonmalignant cells and tissues (11, 27, 28), several investigators challenged the concept that low (Mn-)SOD levels are a general feature of human neoplasia. For example, Beno et al. (29) showed that the antioxidant activity levels, including SOD, were increased in the gastric mucosa of patients at increased risk of gastric cancer. Also, colorectal carcinomas (30) and renal adenocarcinomas (31) were reported to have increased (Mn-)SOD activity levels. In addition, human tumors of the skin, ovary, brain, lung, and kidney have been reported to contain elevated Mn-SOD antigen levels, assessed either immunohistochemically or by ELISA of serum samples or tissue.

Fig. 1 Immunohistochemical staining of gastric tissue for Mn-SOD (A and B) and Cu/Zn-SOD (C and D). The granular cytoplasmic staining of Mn-SOD is predominantly present in the parietal cells (black arrow) of the normal mucosa (A) and more intensely expressed in the gastric carcinoma cells (B). Cu/Zn-SOD was found to be diffusely expressed in the cytoplasm and nucleus of parietal cells (black arrow) within the normal mucosa (C) and only at a low level in the carcinomas (D). ×200.
homogenates (32–37). The exact cause of the higher levels in tumor cells is not clear yet, but because Mn-SOD is an inducible enzyme (38, 39), it is possible that elevated levels of ROMs (40, 41) or cytokines (42–45) in the neoplastic tissue are responsible for its up-regulation. Moreover, in vitro studies have indicated that transfection of the Mn-SOD gene inhibits cell proliferation (46, 47), which even led to the assumption that this enzyme is antioncogenic. In this context, the increased Mn-SOD level might also be an adaptive response of the body to try to control tumor cell proliferation and the oncogenic process.

Whereas the Mn-SOD content was increased, the Cu/Zn-SOD content of gastric and esophageal carcinomas was decreased compared with the normal mucosa. In contrast to differences in the antigen level, carcinomas and normal mucosa showed comparable SOD activity levels, an observation similar to that in colorectal cancer (16). This discrepancy between the SOD antigen and activity levels is thought to be attributable to endogenous inhibitors of the SOD enzymes (32), to interferences in the enzymatic activity assay (48, 49), and to the contribution of the third isoform of SOD, i.e., extracellularly localized SOD, in the activity assessment.

Immunohistochemical analysis of normal gastric mucosa revealed that the SOD expression was relatively low, except for the metabolically active acid-producing parietal cells. Normal

Fig. 2 Immunohistochemical staining of esophageal tissue for Mn-SOD (A and B) and Cu/Zn-SOD (C and D). Mn-SOD was particularly expressed in the luminal part of normal squamous epithelium of the esophagus (A) and was prominently present within the squamous cell carcinomas (B). In contrast, Cu/Zn-SOD was most strongly expressed in the cells of the deepest part of the basal layer in normal esophageal mucosa (C) and only marginally expressed in the carcinomas (D). ×200.
Table 3  Univariate and multivariate analysis of the dichotomized Mn-SOD parameters of carcinomas and normal mucosa in relation to the overall survival of patients with gastric cancer

<table>
<thead>
<tr>
<th>Parameter dichotomized</th>
<th>Survivors/total n/n (%)</th>
<th>Median survival (mo)</th>
<th>Cox hazard ratio (95% CI, P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Univariate</td>
</tr>
<tr>
<td>CA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn-SOD level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤450</td>
<td>6/17 (35.3)</td>
<td>36</td>
<td>2.1 (1.1–4.1, 0.03)</td>
</tr>
<tr>
<td>&gt;450</td>
<td>9/64 (14.1)</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>NM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn-SOD level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤335</td>
<td>2/30 (6.7)</td>
<td>10</td>
<td>0.6 (0.3–0.9, 0.02)</td>
</tr>
<tr>
<td>&gt;335</td>
<td>13/51 (25.5)</td>
<td>22</td>
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</tr>
<tr>
<td>Mn-SOD ratio CA:NM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2.03</td>
<td>11/44 (25.0)</td>
<td>27</td>
<td>1.7 (1.0–2.7, 0.04)</td>
</tr>
<tr>
<td>&gt;2.03</td>
<td>4/37 (10.8)</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

*a* Multivariate analysis was performed by adjusting each SOD parameter to the clinicopathological parameters gender and age of the patients, TNM stage, Laureánchez classification, WHO classification, localization, diameter, number of eosinophils of the tumors, and intestinal metaplasia of the normal mucosa.

*b* CI, confidence interval; CA, carcinoma; NM, normal mucosa.

*c* ng/mg of protein.

esophageal epithelium was characterized by the distinct localization of Mn-SOD in the luminal part and that of Cu/Zn-SOD in the basal proliferating part, which may be indicative of a differential function of each SOD isotype within this tissue. In the gastric and esophageal carcinomas, the Mn-SOD was strongly expressed in both the malignant tumor cells and in the stromal cells, which might indicate a high physiological activity of these cells. These findings also confirm the results of the ELISA measurements and concur with similar assessments as reported by Izu et al. (18, 19). The expression of Cu/Zn-SOD in the gastric and esophageal carcinomas, which was confined to the tumor cells, was less evident compared with normal tissues.

To assess their clinical relevance, the Mn-SOD and Cu/Zn-SOD content of the normal gastric mucosa and carcinomas was evaluated in relation to the major clinicopathological parameters. There was only a minor relation between Mn-SOD and gender of the patients, whereas no other relevant associations were observed. This indicates that the SOD expression is independent from patient- and tumor-related factors, which are important characteristics of a putative prognostic factor. In the evaluation of the prognostic value of the SOD levels, univariate analyses revealed that a high Mn-SOD content of the carcinomas and a low Mn-SOD content of the normal mucosa were significantly associated with a relatively poor survival of the patients. Consequently, a high carcinoma:normal mucosa ratio of the Mn-SOD content was also significantly related to a poor survival. Our previous study concerning colorectal cancer patients showed similar results, i.e., a high Mn-SOD level of carcinomas was associated with a relatively poor survival of the patients (17). The stage of the tumor is considered to be an important determinant of the prognosis of patients with gastric cancer (50–55). However, recognition of additional prognostic factors independent from the classic parameters could be of clinical significance (56–61). Adjusting the separate Mn-SOD variables to the clinicopathological parameters in the multivariate analysis revealed that a high carcinoma:normal mucosa Mn-SOD ratio remained significantly and independently associated with a relatively poor overall survival.

The exact underlying biological mechanism of this paradoxical and diametrical association is unknown, but the (dis)balance between ROM production and antioxidant defenses seems to be the most important (patho)physiological factor. Taking this into account, a relatively high Mn-SOD content of the carcinomas could stimulate the growth of the tumor cells by protecting them against high toxic ROMs concentrations, while maintaining a sufficient metabolically active oxygen signal to activate growth pathways. Indeed, it has been shown that low concentrations of ROMs can stimulate cell growth in vitro (62–65), induce the expression of some proto-oncogenes, e.g., c-fos and c-myc, and switch on DNA synthesis (62, 64, 66). In addition, recent in vitro studies indicated that transfection of the Mn-SOD gene into cancer cells results in interference in the activity of several transcription factors and modulates cytokine- and chemotherapeutic agent-induced processes, such as apoptosis, cell transformation, and proliferation (67, 68). Furthermore, a high Mn-SOD content could protect tumor cells against the lethal effect of several anticancer therapies, which exert at least part of their anticarcinogenic effect by producing high concentrations of ROMs (11–14). Only six of our patients received additional (adjuvant) therapy, consisting of chemotherapy (four patients) and radiotherapy (two patients), but they did not differ from those who had not received an additional therapy with respect to the SOD levels and overall survival.

In the normal mucosa, a relatively low Mn-SOD content was found to be associated with a poorer survival of the patients. This low SOD content may provide an insufficient protection against the carcinogenic effect of ROMs, which are known to play a role at all stages of tumorigenesis (3, 6–10, 69). Dreher et al. (70) proposed that whereas low ROM levels can stimulate cell division and promote tumor growth, intermediate levels of oxidative stress are most effective in cancer initiation. Perhaps in the normal mucosa, a low Mn-SOD content could result in these intermediate, carcinogetic levels of oxidative stress. In
that respect, it is relevant to mention that our group previously showed that *Helicobacter pylori*-related gastritis, which is recognized as an important pathogenic factor in gastric carcinogenesis (71), is also associated with an increased Mn-SOD level and with a slight decrease in Cu/Zn-SOD in the gastric mucosa (24). In addition, successful *H. pylori* eradication resulted in a normalization of the (Mn-)SOD levels (72). Others have shown that *H. pylori* infection is accompanied by a high production of ROMs by attracted and activated phagocytes, and this is considered to be one of the major causes of the mucosal damage (73, 74). Furthermore, because *H. pylori* infection is also associated with increased oxidative DNA damage in human gastric mucosa (75, 76), a high production of ROMs in *H. pylori*-infected gastric mucosa might play an important role in transforming the chronic gastritis into gastric carcinoma. A relatively high Mn-SOD level can thus be regarded as a defensive response by the host to protect the gastric mucosa against high, toxic ROMs concentrations. An inadequate or relatively low Mn-SOD level might render the normal gastric mucosa more vulnerable to oxidative stress and prone to tumor development, and it is apparently indicative of a poor clinical outcome of the patient.

Finally, the above described association between the Mn-SOD level and clinical outcome of patients with gastrointestinal cancer might also be genetically determined. This hypothesis is corroborated by the report of Ambrosone et al. (77), who showed that the recently discovered genetic polymorphism in the mitochondrial targeting sequence of Mn-SOD is associated with an increased risk for the development of breast cancer. Further studies will have to elucidate whether the genetic variability of Mn-SOD affects the expression and activity of the enzyme and is related to the susceptibility of patients to develop (gastrointestinal) cancer.

In conclusion, esophageal and gastric carcinomas are characterized by an increased Mn-SOD antigen level compared with their corresponding normal tissue, similar to that previously shown for colorectal carcinomas. In contrast, the Cu/Zn-SOD content of esophageal and gastric carcinomas was decreased. Furthermore, a high carcinoma:normal mucosa Mn-SOD ratio in gastric cancer patients is associated with a relatively poor overall survival, which is independent from nine clinicopathological parameters. This ratio could thus be used as an independent prognostic parameter to predict the clinical outcome of patients with gastric cancer, which might enable better treatment planning.

REFERENCES


Superoxide Dismutases in Gastric and Esophageal Cancer and the Prognostic Impact in Gastric Cancer


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