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Superoxide dismutase in gastric adenocarcinoma: is it a clinical biomarker in the development of cancer?

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Abstract

Gastric cancer is the second most common cancer worldwide. The involvement of reactive oxygen species (ROS) in the pathogenesis of gastric malignancies is well known. Many human tumours have shown significant changes in the activity and expression of superoxide dismutase (SOD), which might be correlated with clinical–pathological parameters for the prognosis of human carcinoma. The aim of this study is the detection of MnSOD and CuZnSOD activity and their expression in gastric adenocarcinoma and healthy tissues. Gastric samples (adenocarcinoma and healthy tissues) harvested during endoscopy or resected during surgery were used to determine MnSOD and CuZnSOD activity and expression by spectrophotometric and Western blotting assays. The total SOD activity was significantly higher ($p < 0.05$) in healthy mucosa with respect to gastric adenocarcinomas. No differences were found in MnSOD activity and, on the contrary, CuZnSOD activity was significantly lower ($p < 0.001$) in cancer samples with respect to normal mucosa. The rate of MnSOD/CuZnSOD activity in adenocarcinoma was over ninefold higher than that registered in healthy tissues ($p < 0.05$). Moreover, in adenocarcinoma MnSOD activity represented the 83% of total SOD with respect to healthy tissues where the ratio was 52% ($p < 0.001$). On the contrary, in cancer tissues, CuZnSOD activity accounted for only 17% of the total SOD ($p < 0.001$ if compared with the values recorded in normal mucosa). After immunoblotting, MnSOD was more expressed in adenocarcinoma with respect to normal mucosa ($p < 0.001$), while CuZnSOD was similarly expressed in adenocarcinoma and healthy tissues. The SOD activity assay might provide a specific and sensitive method of analysis that allows the differentiation of healthy tissue from tumour tissue. The MnSOD to CuZnSOD activity ratio, and the ratio between these two isoforms and total SOD, presented in this preliminary study might be considered in the identification of cancerous from healthy control tissue.

Keywords: *Superoxide dismutase (SOD), SOD activity, SOD expression, gastric cancer, prognostic marker*

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Introduction

Gastric cancer is the second most common cancer worldwide. Ninety per cent of all tumours of the stomach are malignant, and gastric adenocarcinoma comprises 95% of the total number of malignancies (Dicken et al. 2005). Reactive oxygen species (ROS) such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) have emerged as highly toxic agents responsible for a wide variety of tissue damage (Halliwell & Gutteridge 1999). The involvement of these ROS in the pathogenesis of gastric diseases first became evident from the study on gastric mucosal injury. A growing body of experimental and clinical evidence suggests that gastric mucosal damage by ethanol, non-steroidal anti-inflammatory drugs (NSAID), and *Helicobacter pylori* is mediated through ROS (Das et al. 1997). *H. pylori* and auto-immune gastritis are also the most common causes that create an environment conducive to gastric inflammation. If gastritis persists, gastric atrophy occurs followed by intestinal metaplasia, which in turn may lead to dysplasia. The term 'adenoma' is applied when dysplastic proliferation produces a macroscopic protruding lesion, which is morphologically described as adenoma (Fenoglio-Preiser et al. 2000). ROS are mutagenic compounds known to lead to DNA damage, favour cell transformation, and contribute to the development of a variety of human malignant diseases, including cancer. In addition, chronic inflammatory stress, in which local oxidant burst is increased, is known to be associated with increased cancer risk. Furthermore, several environmental carcinogens can directly generate free radicals and activate inflammatory cells to produce ROS *in vivo* (Kinnula & Krapo 2004). Endogenous metabolic activity (mitochondria, cytochrome P450), environmental stimulus (inflammatory cytokines, chemotherapeutics, drugs), and xenobiotic detoxification systems lead to ROS production (Halliwell & Gutteridge 1999). In response to these events, cells can acquire multiple genetic alterations, including inactivation of tumour suppressor genes and activation of oncogenes that cause malignant conversion and escape from normal growth control (Kinnula & Krapo 2004). Under normal condition, ROS are relatively harmless, but when they are produced excessively or during deficient antioxidant defence, the oxidant-antioxidant balance is disturbed and the metabolites become toxic, which may lead to initiation and promotion of cancer (Janssen et al. 1999). All these events are in part mediated by oxidants and/or changes in cellular redox state in the direction of increased oxidation. To minimize the damaging effect of ROS, the aerobic organism has evolved several antioxidant defences (Michiels & Remacle 1988). Nevertheless, ROS attack many biological macromolecules including cellular protein, lipids (peroxidation), and nucleic acids (strand breaks) (Finkel & Holbrook 2000).

Antioxidant enzyme superoxide dismutase (SOD) represents the first line of cellular defence against free radicals; in fact, it is the only enzyme able to dismute superoxide anion, which is highly toxic in cells (McCord & Fridovich 1969, Fridovich 1975, Farber et al. 1990). The enzyme antioxidant pathway consists of two important steps: the first is the dismutation of $O_2^{\bullet -}$ to H_2O_2 and oxygen by SOD; and the second is conversion of H_2O_2 to H_2O, which is catalysed by glutathione peroxidase and/or catalase (Van Driel et al. 1997). MnSOD and CuZnSOD are the two major isoforms of SOD localized in mitochondria and cytosol, respectively, and actively involved in the protection mechanisms against oxidative stress (McCord & Fridovich 1969, Fridovich 1975). Since many human tumours have shown significant changes in the activity and expression of SODs (Kinnula & Krapo 2004), this enzyme might be

correlated with clinical–pathological parameters for the prognosis of human carcinoma.

Moreover, a lack of detailed analyses of SOD expression, and particularly MnSOD and CuZnSOD activities in gastric tumour tissues justifies this research aimed at defining the role of SODs in gastric cancer.

This preliminary study was performed to assess MnSOD and CuZnSOD activity and expression in gastric adenocarcinoma with respect to healthy tissue by spectrophotometric assays and Western blotting techniques as specific and sensitive clinical tests.

This biological approach is promising to develop a new method for early diagnosis and early detection of gastric cancer. It also provides clues to understanding the molecular mechanism of cancer progression. In fact, advances in modern aspects of tumour biology can introduce new prognostic factors. Although the detailed mechanism of gastric cancer development remains uncertain, enhancement in the understanding of its molecular biology in recent years has led to a better perspective on the molecular epidemiology, carcinogenesis, and pathogenesis of gastric cancer. More importantly, it has become possible to use molecular markers in differential diagnosis, prognostic evaluation, and specific clinical interventions.

Materials and methods

Chemicals

The reagents utilized for SOD determinations were obtained from Sigma Aldrich (St Louis, MO USA), Roche Diagnostics (Basel, CH) and Merck & Co (White House Station, NJ USA). Protein determination was performed with a Bio-Rad DC Protein Assay kit (Hercules, CA USA). For electrophoresis and Western blotting detection, minigels, specific buffers, molecular weight markers, and a chromogenic immunodetection kit were purchased from Invitrogen (Pasley UK); the polyvinylidene difluoride (PVDF) membranes were from Bio-Rad (USA), the primary antibodies were obtained from Stressgen (Victoria, British Columbia CAN) and the Cu/Zn-SOD standard was from Sigma Aldrich.

Sample collection and tissue preparation

We prospectively included a total of 14 gastric specimens (five adenocarcinoma and nine gastric normal gastric mucosa) harvested from patients after gastrectomy or during endoscopy, immediately frozen in liquid air, and stored at -70°C until the subsequent processing. Demographical, clinical, and pathological data were collected, evaluated, and registered by a physician. The design was single-blinded for the biochemical evaluation. The study population consisted of five men and nine women, with an average age of 55.8 ± 16.8 years (range 33–80 years). Of them, nine (three men, mean age 51.7 ± 16.8 years) were normal subjects undergoing endoscopy for dyspepsia, and five (two male, mean age 68.3 ± 10.4 years) were affected by adenocarcinomas. No statistical differences were found for demographic characteristics.

The homogenates were prepared from 50 to 100 mg wet tissue samples in 20 vols of 50 mM phosphate buffer (pH 7.8) with 0.1 mM EDTA and 5 μl/100 mg of tissue of inhibitory cocktail for the proteases. The tissues were homogenized for 2 min (14 000 rpm) on ice in UltraTurrax, sonicated for a few seconds, and centrifuged at 10 000*g*

for 10 min at 4°C. After the centrifugation, SOD activity and expression determinations were carried out on the surnatants.

The study was conducted according to good clinical practice and the Declaration of Helsinki.

Superoxide dismutase activity assay

The superoxide dismutase (SOD) activity was measured in a UV/VIS Uvikon 941 spectrophotometer (Kontron Instruments), temperature controlled to 25 ± 1°C; the assay was performed in quadruplicate.

SOD activity was determined with the xanthine oxidase-cytochrome *c* method according to Crapo et al. (1978). The cytochrome *c* reduction by superoxide anions generated by xanthine oxidase/hypoxanthine reaction was detected at 550 nm. Activity values were expressed in unit per mg of protein, being 1 U of SOD defined as the amount of sample producing 50% inhibition under the assay conditions. The Cu/Zn-containing form of SOD was assayed using the inhibitory effect of 1 mM KCN on SOD activity (Fridovich 1982).

Protein assay

Total soluble protein concentrations in sample extracts was measured according to Lowry et al. (1951), with the microassay procedure and bovine serum albumin (BSA) as a standard. The protein content, in quadruplicate, was determined using 96-well microtiter plates. Extinction was read at 750 nm in a Labsystem MultisKan EX spectrophotometer. Values were reported as mg protein per ml and were used to normalize enzyme activity and expression.

Electrophoresis and Western blotting

SDS-PAGE of tissue extracts were performed in a Invitrogen Xcell SureLock Mini-Cell using minigels (Bis-Tris gels) consisting of 12% running gel with MES running buffer at pH 7.3, under reducing conditions. Samples were added to LDS buffer and reducing agent (0.5 M DTT) and heated for 10 min at 70°C. Apparent molecular weights of immunopositive bands were determined by comparison with pre-stained molecular weight markers. CuZnSOD from bovine erythrocytes was loaded in the gels as standard to identify the SOD isoform. Cytosolic extracts were loaded in the gel in volumes corresponding to the protein content of 30 μg/lane.

After electrophoresis, proteins were transferred, for 1 h, to PVDF membranes in an Invitrogen Xcell SureLock Blot Modul using transfer buffer, pH 7.2. After blotting, the PVDF membranes were treated with the chromogenic Western blot immunodetection kit. Blots were blocked with concentrated casein solution in buffered saline solution and then incubated with the diluted primary specific antibody for 1 h. Polyclonal rabbit anti-human Cu/ZnSOD, diluted 1:7000 and polyclonal rabbit anti-rat Mn-SOD, diluted 1:5000 were used to identify the SOD isoforms. Membranes were washed with a specific antibody wash solution (concentrated buffered saline solution containing detergent, from Invitrogen) and incubated with the secondary antibody solution consisting of alkaline phosphatase-conjugated anti-rabbit IgG for 45 min. Then the blots were visualized using a chromogenic substrate containing BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate) and NBT (nitro blue tetrazolium).

Immunopositive bands were semi-quantified by Quantity One Software (Bio-Rad), and results were expressed in arbitrary units.

Statistical analyses

SOD activity values were expressed as the means ± standard error of the mean (SEM); data from densitometric analyses of immunopositive bands were reported as the means ± SEM.

All data before statistical analyses were checked for normal distribution. The results obtained were compared by a Student's *t*-test for independent samples to analyse the differences (STAT software); values of $p < 0.05$ were considered as being significantly different.

Results

SOD activity

The mean activity of total SOD was significantly higher ($p < 0.05$) in healthy gastric mucosa (24.4 ± 2.3 U mg^{-1} of protein) with respect to gastric adenocarcinomas (16.4 ± 4.1 U mg^{-1} of protein) (Figure 1).

In adenocarcinoma MnSOD activity showed a marked but not significant decrease compared with healthy tissues (Figure 2), on the contrary CuZnSOD activity was significantly lower ($p < 0.001$) in adenocarcinoma samples compared with normal mucosa (Figure 3).

Some peculiar features appeared when the ratios between the activities of each isoform and of MnSOD/total SOD and of CuZnSOD/total SOD are considered (Table I).

The ratio between MnSOD and CuZnSOD (Table I) was significantly higher (9.16 ± 4.80) in adenocarcinoma cases with respect to healthy gastric mucosa (1.23 ± 0.22) ($p < 0.05$). Moreover the MnSOD represents the 83% than the total SOD (Table I) in adenocarcinoma with significantly different values with respect to the healthy tissue that showed a MnSOD contribute versus total SOD of 52% ($p < 0.001$).

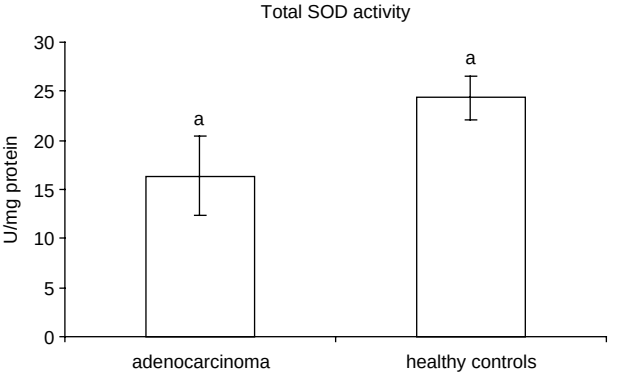

Figure 1. Total SOD activity in adenocarcinoma (five samples) and healthy tissues (nine samples). Data are presented as the mean ± standard error of the mean (SEM). The same letters at the top of the bars indicate significant differences: [a]$p < 0.05$.

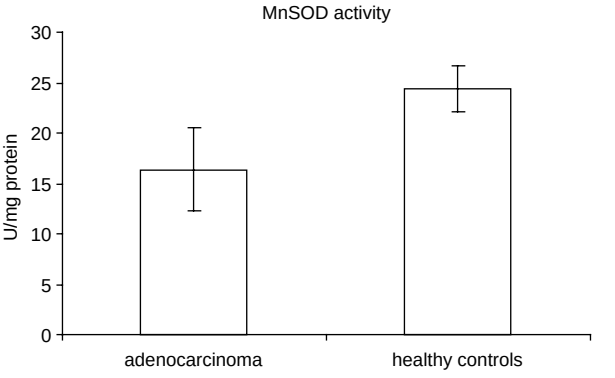

Figure 2. MnSOD activity in adenocarcinoma (five samples) and healthy tissues (nine samples). Data are presented as the mean ± standard error of the mean (SEM).

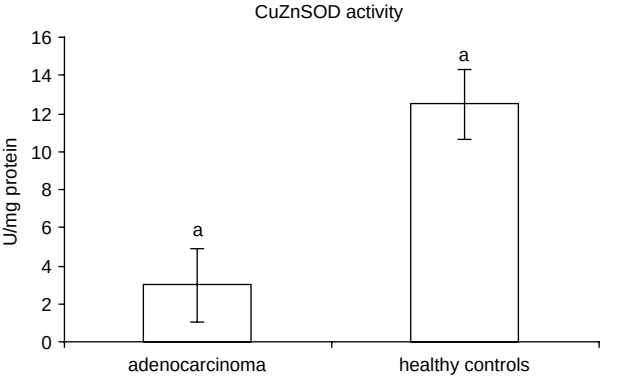

Figure 3. CuZnSOD activity in adenocarcinoma (five samples) and healthy tissues (nine samples). Data are presented as the mean ± standard error of the mean (SEM). The same letters at the top of the bars indicate significant differences: $^{a}p < 0.001$.

Furthermore, CuZnSOD represents only the 17% than the total SOD (Table I) in adenocarcinoma against the 48% measured in the healthy tissues ($p < 0.001$).

SOD expression

After immunoblotting detection two different immunopositive bands (16 and 23 kDa) were recognized in the human extracts, based upon immunoreactivity with specific

Table I. Ratio of MnSOD to CuZnSOD activities and of the two isoforms to total superoxide dismutase (SOD) activity (U mg^{-1} protein).

MnSOD/CuZnSOD		MnSOD/total SOD (%)	CuZnSOD/total SOD (%)
Adenocarcinomas (five cases)	9.16 ± 4.80^{a}	83.0 ± 5.1^{b}	17.0 ± 5.3^{c}
Healthy controls (nine cases)	1.23 ± 0.22^{a}	52.0 ± 4.5^{b}	48.0 ± 4.5^{c}

Data are presented as the mean ± SEM. The same letters indicate significant differences: $^{a}p < 0.05$; $^{b,c}p < 0.001$.

Mn and CuZnSOD polyclonal antibody (Figure 4, A, B). The former with an apparent molecular weight of 16 kDa corresponded to CuZnSOD, which in humans is a homodimer with a molecular weight of 31.2 kDa (Crapo et al. 1992). The second one, with an apparent molecular weight of 23 kDa, corresponded to the monomeric form of the MnSOD that exists in human as a homotetramer with an individual subunit molecular weight of about 23 kDa (Barra et al. 1984).

Concerning the expression data, MnSOD was significantly more expressed ($p < 0.001$) in adenocarcinoma tissue compared with healthy tissue (Figure 4, A–C). No statistical significant differences were found in the levels of CuZnSOD among tumour

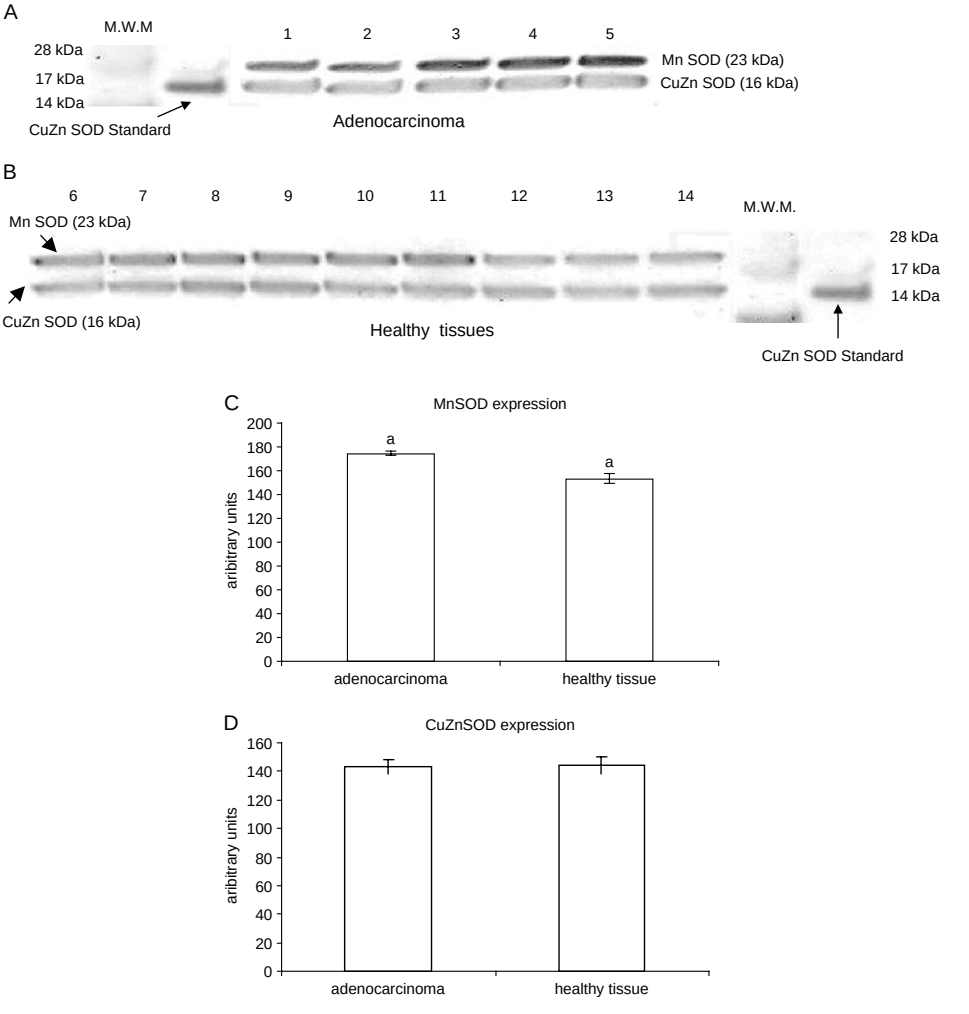

Figure 4. Western blot analysis of MnSOD and CuZnSOD in adenocarcinoma tissues (A) and healthy tissues (B). Five adenocarcinoma (1–5) and nine healthy tissues (6–14) are showed. A total of 30 μg protein/lane were loaded onto 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Densitometric analysis of blots for MnSOD (C) and CuZnSOD (D) were expressed as relative intensity values (arbitrary units). Data are presented as the mean ± standard error of the mean (SEM). The same letters at the top of the bars indicate significant differences: $^{a}p < 0.001$. MWM, molecular weight marker.

tissues and healthy controls after the densitometric scan of the band (Figures 4, A, B, D).

Discussion

The balance between ROS production and antioxidant defence determines the condition of oxidative stress.

Generally MnSOD is characterized by low expression levels in cells, but can be strongly induced both *in vivo* and *in vitro* by multiple stress conditions, such as the presence of cytokines, changes in cell redox state, and hyperoxia (Zelko et al. 2002, Kinnula & Krapo 2004). It has been shown that MnSOD plays a central role in promoting cellular differentiation and tumorigenesis (St Clair et al. 1994). MnSOD is specifically located in the mitochondria, a major site of $O_2^{-\bullet}$ production under hyperoxic conditions (Tsan et al. 1998), where the adaptation of the oxidative metabolism of the cell is allowed (Zhang et al. 1994, Pinteaux et al. 1998).

The present results showed an higher ratio between MnSOD and CuZnSOD activities in adenocarcinoma even of over ninefold higher than that registered in healthy tissues ($p < 0.05$). Interestingly, we observed that in adenocarcinoma tissues compared with tissues from healthy patients, SOD amount was almost due to MnSOD activity ($83.0\% \pm 5.1\%$), while in healthy tissues the two SOD isoforms were represented in similar measures.

The significant increase in MnSOD expression in adenocarcinomas observed in this study was in agreement with other studies in human colorectal cancer (Janssen et al. 1999). Similarly in oesophageal and gastric cancer tissues, Izutani et al. (1996, 1998) reported an increase in MnSOD mRNA expression with a marked increase in tumour necrosis factor (TNF)-α mRNA in gastric cancer (Izutani et al. 1996). High levels of MnSOD mRNA in cancer tissues are probably a necessary protective mechanism against the superoxide radical and TNF-α cytotoxicity (Izutani et al. 1996).

Recent data indicate that the increase in MnSOD levels in colorectal and gastric cancer is an independent prognostic factor for the overall survival (Janssen et al. 1998, 2000).

It is noteworthy that we found that CuZnSOD activity is significantly lower in tumours with respect to healthy controls ($p < 0.001$). The present data showed that the activity ratio of CuZnSOD and total SOD was significantly lower in adenocarcinoma ($17.0\% \pm 5.3\%$) with respect to healthy tissues ($48.0\% \pm 4.5\%$), ($p < 0.001$).

Low levels of CuZnSOD activity were found in colorectal adenomas and carcinomas compared with normal colorectal mucosa while no differences in CuZnSOD expression were identified in colorectal cancer tissues compared with the normal mucosa (Janssen et al. 1998, 1999).

It seems that CuZnSOD expression is stable and its activity is considered to be an internal control for CuZnSOD gene expression (Zelko et al. 2002). In fact, although several authors have considering this enzyme to be constitutively expressed, they have stated that its mRNA levels can be markedly up- and down-regulated by various physiological conditions (Zelko et al. 2002).

Concerning protein expression, the over-expression of SODs, *in vitro,* increases cell differentiation, decreases cell growth, and the proliferation can reverse a malignant phenotype to a non-malignant one (Kinnula & Krapo 2004).

A significant increase in MnSOD expression was found in adenocarcinoma with respect to healthy mucosa, while CuZnSOD expression in adenocarcinoma seems to be comparable with that of healthy tissues.

Mn SOD is a determinant enzyme of cell resistance to pro-oxidant cytokines (TNF-α and IL-1β), and contributes to the survival of cells against ionizing radiation and tumoricidal chemotherapeutic drugs (Kuninaka et al. 2000). Moreover MnSOD expression is induced by cytokines and generally many tumour, included gastric cancer, show an over-expression of cytokines (Bossola et al. 2000).

Recent studies indicate that MnSOD over-expression might cause a ROS increase associate to better tumour cell killing, or it could have a protective role of tumour cells, depending on the type of cells involved and the agents used (Ambrosone et al. 2005). The role of expression of MnSOD in relation to cancer prognosis remain to be clarified (Ambrosone et al. 2005).

In the present study a discrepancy between activity and expression was revealed: no differences were detected in MnSOD activity and CuZnSOD expression while the MnSOD expression and the CuZnSOD activity changed in adenocarcinoma.

The discrepancy between expression and activity of SOD might be probably due to the presence of an inactivator factor of SOD, previously identified in lung carcinomas (Iizuka et al. 1984).

The incongruence between activity and expression might be explained by protein modification and/or associations to several proteins (Halliwell & Gutteridge 1999).

The changes in gene expression seem to be unable to modify the amounts of SOD enzyme protein obtained after Western blotting (Chan et al. 1999). Probably the rapid degeneration of SOD enzymes was due to the high levels of ROS and the presence of SOD isoforms that were less active but still recognized by antibodies (Chan et al. 1999). Moreover, several factors involved in the post-translation control of SOD enzyme biosynthesis have to be considered (Chan et al. 1999).

In the present study, SOD activity and expression assays can provide specific and sensitive methods of analysis that allows the differentiation of healthy tissue from tumour tissue with a very small quantity of tissue (such as biopsy samples). The MnSOD to CuZnSOD activity ratio, and the ratio between these two isoforms and total SOD, presented in this preliminary work, might be more explicative in the identification of cancerous from healthy control tissue.

Since MnSOD is a key factor in cell survival, the MnSOD to CuZnSOD activity ratio, and the ratio between the two isoforms and total SOD, presented in this preliminary study, it might be more important in the identification of cancerous from healthy control tissue. Moreover, the results obtained in single-blind design agree with histological data. This is confirmation of the specificity and sensitivity of the technique utilized in this study as a diagnostic biomarker for gastric cancer.

Further research will be necessary to define the role of MnSOD and CuZnSOD in cancer and at the same time to assess SODs activity and expression as a prognostic marker of gastric cancer.

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