

UvA-DARE (Digital Academic Repository)

Expression of CuZn- and Mn-superoxide dismutase in human colorectal neoplasms

van Driel, B.E.M.; Lyon, H.; Hoogenraad, D.C.J.; Anten, S.; Hansen, U.; van Noorden, C.J.F.

Published in:
Free radical biology & medicine

DOI:
[10.1016/S0891-5849\(97\)00102-0](https://doi.org/10.1016/S0891-5849(97)00102-0)

[Link to publication](#)

Citation for published version (APA):

van Driel, B. E. M., Lyon, H., Hoogenraad, D. C. J., Anten, S., Hansen, U., & van Noorden, C. J. F. (1997). Expression of CuZn- and Mn-superoxide dismutase in human colorectal neoplasms. *Free radical biology & medicine*, 23, 435-444. DOI: 10.1016/S0891-5849(97)00102-0

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <http://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

**Original Contribution****EXPRESSION OF CuZn- AND Mn-SUPEROXIDE DISMUTASE IN HUMAN COLORECTAL NEOPLASMS**BERNARD E. M. VAN DRIEL,* HANS LYON,[†] DIRK C. J. HOOGENRAAD,* SANDER ANTEN,*
ULLA HANSEN,[†] and CORNELIS J. F. VAN NOORDEN**Academic Medical Center, University of Amsterdam, Laboratory of Cell Biology and Histology, 1100 DE Amsterdam, The Netherlands, and [†]Department of Pathology, Københavns Kommunes Hvidovre Hospital, University of Copenhagen, Denmark

(Received 2 October 1996; Revised 8 January 1997; Accepted 8 January 1997)

Abstract—Decreased intracellular SOD protein levels and activity have been related with malignancy in the past. To investigate their relevance in the carcinogenic process in the colon, we determined quantitatively CuZn-SOD and Mn-SOD levels and total SOD activity by histochemical means in human normal colorectal mucosa, adenomas, and carcinomas. Protein levels and activity were significantly decreased in carcinomas. CuZn-SOD protein levels, but not Mn-SOD levels or total SOD activity were related with differentiation grade and to a lesser extent with Dukes stage. Moderately differentiated carcinomas and Dukes stage A carcinomas showed lowest levels. Some carcinomas expressed elevated levels of CuZn-SOD and this was an indication of poor survival. It is concluded that decreased SOD expression is not a prognostic marker and seems to be a secondary phenomenon rather than directly linked with the carcinogenic process. © 1997 Elsevier Science Inc.

Keywords—Colorectal carcinoma, CuZn-SOD, Mn-SOD, SOD activity, Free radical, Prognosis, Immunohistochemistry, Quantitative histochemistry, Image analysis

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals, and singlet molecular oxygen are generated under normal conditions in probably all cell types as by-products in electron transfer reactions.¹ In principle, these ROS are catabolized efficiently by antioxidant systems that are present in cells. In cellular prooxidant states, intracellular concentrations of ROS are increased, presumably because cells either overproduce these activated forms of oxygen or do not scavenge ROS efficiently.² The enzyme antioxidant pathway of both prokaryotic and eukaryotic organisms essentially consists of two steps. The first step is dismutation of $O_2^{\bullet-}$ to H_2O_2 and oxygen by superoxide dismutase (SOD), and the second step is conversion of H_2O_2 to H_2O , which is catalyzed by glutathione peroxidase (Gpx) and/or catalase.³

SOD is considered to be essential for all oxygen metabolizing cells.⁴ There are two main forms of intracellular SOD in eukaryotic cells. Copper and zinc-containing SOD (CuZn-SOD) is found primarily in the cytoplasm,⁵ whereas manganese-containing SOD (Mn-SOD) is localized in the mitochondrial matrix.⁶ Both forms catalyze dismutation of $O_2^{\bullet-}$. Differences in physiological roles of CuZn-SOD and Mn-SOD in mammals are not known yet, but it is likely that these roles are not exactly the same because the enzymes are located in distinct compartments and cross membranes poorly.⁷ The differences in regulation of synthesis of both SOD enzymes also suggest functional dissimilarity.⁸

There is evidence that decreased intracellular SOD levels are related to malignant transformation. First, CuZn-SOD and Mn-SOD activity is decreased in many malignant tissues as compared with corresponding normal tissue.^{9–15} Second, induction of elevated levels of both CuZn-SOD¹⁶ and Mn-SOD¹⁷ by transfection of cultured malignant cells causes a return to the nonmalignant phenotype. Third, increased cellular

Address correspondence to Prof. Dr. C. J. F. Van Noorden, Laboratory of Cell Biology and Histology, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

levels of ROS, including $O_2^{\bullet-}$ and H_2O_2 are carcinogenic, whereas malignant transformation is prevented by the antioxidants SOD, catalase, and butylated hydroxytoluene.²

On the basis of the hypothesis that carcinogenesis is a result of loss of control of cellular proliferation and differentiation, it has been assumed that Mn-SOD and CuZn-SOD play a role in cellular differentiation. Direct evidence for this hypothesis has been provided by Hansberg *et al.*¹⁸ and St. Clair *et al.*,¹⁹ who showed that cellular Mn-SOD levels are positively related with differentiation grade and by Oberley *et al.*²⁰ who showed that CuZn-SOD activity is low in regenerating liver and hepatomas.

The main objective of the present study was to determine CuZn-SOD and Mn-SOD protein levels and total SOD activity *in situ* in epithelia of human normal colorectal mucosa, adenomas, and carcinomas using quantitative immunohistochemical and enzyme histochemical methods. Because we wanted to study SOD levels in epithelial cells only and avoid interference by other tissues such as connective tissue, we have applied quantitative immunohistochemistry in combination with quantitative enzyme histochemistry. We investigated whether there was a correlation between SOD expression and either malignancy or differentiation grade of colorectal carcinomas. Because of a possible role of SOD in malignant transformation, we correlated CuZn-SOD and Mn-SOD protein expression and total SOD activity with survival and clinicopathological parameters for prognosis of colorectal carcinomas, such as Dukes staging,²¹ presence of local lymph node metastases,²² and vascular invasion.²³

EXPERIMENTAL PROCEDURES

Tissue specimens

Sixty samples of human colorectal carcinomas with adjacent histologically normal mucosa were obtained from the Pathology Department, Kobenhavns Kommunes Hvidovre Hospital, University of Copenhagen, Hvidovre, Denmark. Five samples of tubular adenomatous polyps, and five samples of normal mucosa, taken at least 5 cm from the sites of the carcinomas were obtained from the Pathology Department, University Hospital Maastricht, Maastricht, The Netherlands. After resection, the tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until used. Colorectal cancer patients were followed up to 56 months after surgical resection.

Preparation of cryostat sections

Serial cryostat sections (6 μm thick) were cut at a cabinet temp of -25°C on a motor-driven cryostat

(Bright, Huntingdon, UK) at a low but constant speed. The sections were mounted on clean glass slides and stored at -80°C until used.

Histopathology

Pathological examination was performed with the use of haematoxylin-eosin stained sections of each specimen. Serial sections were used for quantitative immunohistochemical and enzyme histochemical analysis of the content of CuZn-SOD and Mn-SOD protein and total SOD activity. SOD expression was correlated with differentiation grade, survival of the patient, presence of local lymph node metastases or angio-invasion, and Dukes stage.²¹ Dukes classification is as follows: in Dukes stage A, the local colorectal carcinoma does not penetrate the muscularis mucosa; in Dukes stage B, penetration through the muscularis mucosa occurs; in Dukes stage C, cancer cells are found in the local lymph nodes; in Dukes stage D, metastases at distance are found.

Immunohistochemistry

Commercially available polyclonal antibodies against CuZn-SOD and Mn-SOD (The Binding Site, Birmingham, UK) were utilized. Antisera raised against CuZn-SOD have been proven not to crossreact with antisera raised against Mn-SOD.^{10,24} Cryostat sections were air dried overnight. For CuZn-SOD and Mn-SOD immunohistochemistry, sections were fixed in freshly prepared phosphate-buffered formaldehyde (4% w/v; 2 min at room temp) and a graded ethanol series (50–70–90–96–90–70–50%; each step for 1.5 min at room temp). After one rinse in phosphate-buffered saline (PBS; pH 7.4), endogenous peroxidases were blocked by an incubation for 15 min in PBS containing 0.3% H_2O_2 and 0.1% sodium azide. Sections were rinsed in PBS and incubated in PBS containing 10% normal rabbit serum (20 min at room temp). Then, sera were poured from the sections and the sections were incubated for 60 min at room temp for detection of CuZn-SOD and Mn-SOD with horseradish peroxidase-labeled sheep antihuman antibodies diluted 1:100 (0.05 mg protein/ml), according to instructions of the supplier. Control incubations in which nonimmune sheep serum or PBS replaced the primary antibody were included in every run. In addition, immunostaining for both SODs was performed on cryostat sections of kidney, in which glomeruli served as SOD-negative controls and tubuli as SOD-positive controls.²⁵ Sections were rinsed in PBS and then incubated with horseradish peroxidase-labeled rabbit antisheep immunoglobulins (Dako, Glostrup, Denmark) diluted

1:200 in PBS containing 10% human serum with blood group AB (30 min at room temp). After three rinses in PBS, sections were incubated for 10 min at room temp in 50 mM Tris-HCl buffer (pH 7.6) containing 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) and 0.3% H₂O₂, rinsed in distilled water, counterstained lightly with hematoxylin, and mounted in glycerol jelly.

Enzyme histochemistry

SOD activity was detected histochemically as described by Frederiks and Bosch.²⁶ Briefly, the incubation medium to demonstrate SOD activity consisted of 10% polyvinyl alcohol (PVA; weight average M_r 70,000–100,000; Sigma), 100 mM Tris-maleate buffer, pH 7.6, 10 mM cerium chloride (Fluka Chemie, Buchs, Switzerland) and 0.5 mM hypoxanthine (Merck, Darmstadt, Germany). An aqueous solution containing 15 mIU xanthine oxidase (phosphate free, isolated from cow milk; Boehringer, Mannheim, Germany) was spread onto clean glass slides over an area of ± 1 cm². The xanthine oxidase films on the slides were air dried for 5 min at room temp. Cryostat sections from the cryostat knife were subsequently mounted on top of the enzyme film, air dried (5 min) and covered with the incubation medium for 15 min at 37°C. Afterwards, sections were rinsed in hot distilled water (60°C) to stop the reaction immediately. Final reaction product was visualized by incubating the sections for 30 min at room temp in 50 mM sodium acetate buffer at pH 5.3 containing 42 mM cobalt chloride, 100 mM sodium azide, 1.4 mM 3,3'-diaminobenzidine (Sigma) and 0.6 mM H₂O₂. After rinsing, the sections were mounted in glycerol jelly and immediately analyzed. Storage in darkness at 4°C minimized fading of the final reaction product.²⁷ Control incubations were performed as described by Frederiks and Bosch.²⁶

Cytophotometry

CuZn-SOD and Mn-SOD expression were measured as absorbance of polymerized DAB at 480 nm as described by Van Noorden and Frederiks²⁸ using a Vickers M85a scanning and integrating cytophotometer (Vickers Instruments, York, UK). Final reaction product generated by SOD activity was measured at 575 nm.²⁹ Per section, five measurements were taken of epithelial cells in either normal, adenomatous, or malignant tissue. A magnification of $\times 40$ was used and the area scanned was 80 μ m². Of each biopsy, three serial sections were used for detection of both CuZn-SOD and Mn-SOD protein levels or total SOD activity. Results are shown in two ways: (1) as mean ab-

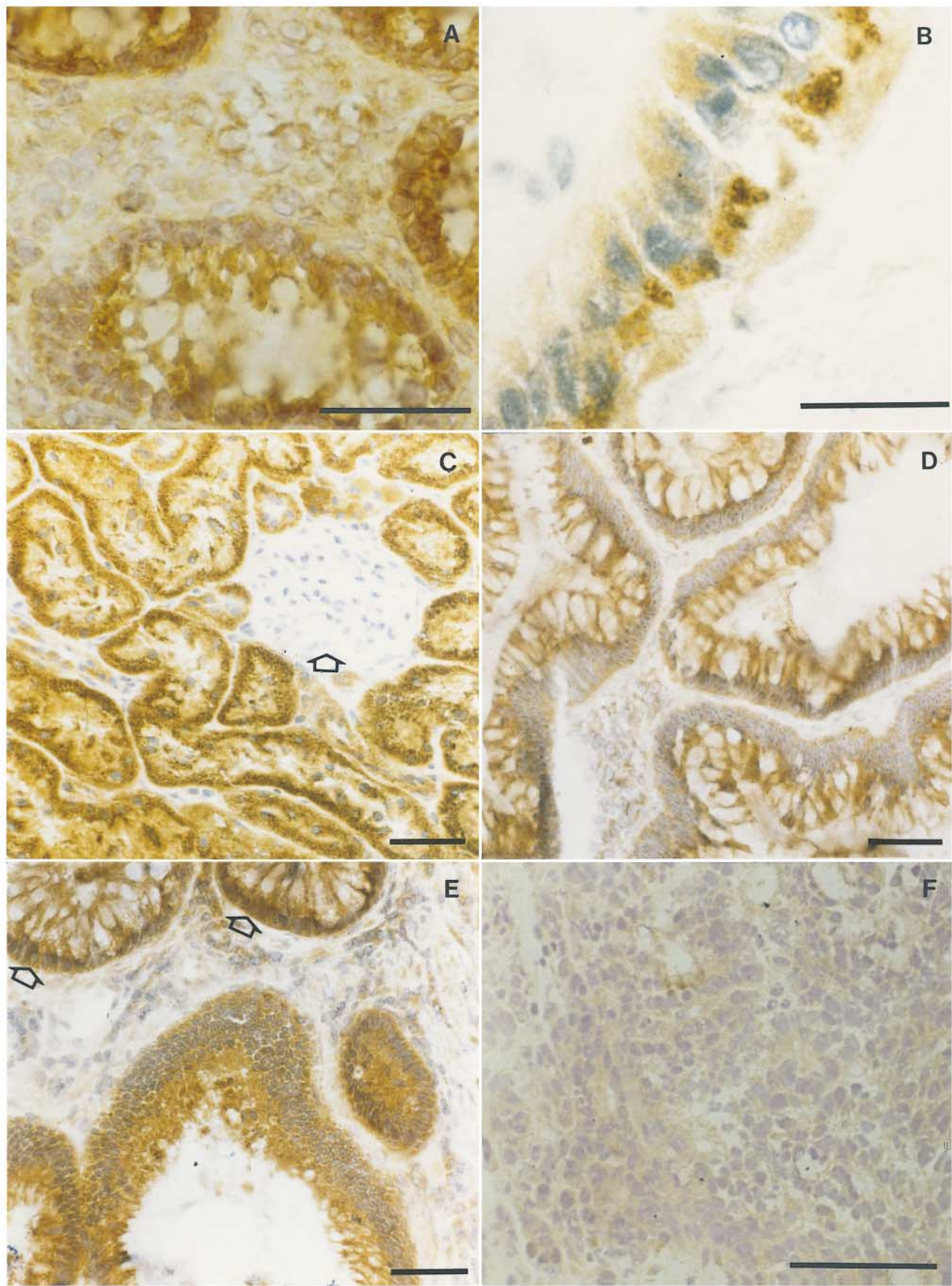
sorbance values and (2) as the ratio of SOD expression in the neoplasm and surrounding normal mucosa [$\times 100(\%)$]. The use of ratios avoids potential methodological errors in quantification such as variations in section thickness, fixation procedures despite the fact that these were all controlled as rigidly as possible (see below).

Validation of the quantification procedure

The enzyme histochemical method was validated for quantitative purposes by Frederiks and Bosch.²⁶ For immunohistochemical analysis, all tissues were treated identically. Exposure to all reagents, including DAB and H₂O₂, was kept constant. Tissue sections of normal mucosa, adenomas, and adenocarcinomas were immunostained at the same time to reduce interassay variability. These rigidly controlled incubation procedures ensured that the amount of final reaction product reflected the amount of immunoreactive protein in individual tissues.^{30–33} The peroxidase reaction in the presence of DAB and H₂O₂ can be used validly for quantitative purposes.^{33–35} We have tested the validity of the immunohistochemical procedures for quantification of CuZn-SOD and Mn-SOD expression by variation of section thickness (4–12 μ m) in preliminary experiments. The amount of final reaction product generated specifically by peroxidase as marker for both SODs (test minus control) was proportional with section thickness (data not shown). On the basis of these results, it was concluded that quantification of polymerized DAB was a valid method to measure in situ levels of CuZn-SOD and Mn-SOD.

Statistical analysis

Statistical comparisons of CuZn-SOD and Mn-SOD levels and SOD activity in colorectal carcinomas and adjacent normal mucosa were performed with non-parametric paired Mann-Whitney test statistics. Comparisons of SOD expression with differentiation grade were performed with a Kruskal-Wallis ANOVA test and unpaired Mann-Whitney test statistics. Differences were considered significant at an α level ≤ 0.05 . Multiregression analysis was performed with JPM software (SAS Institute, Cary, NC). For survival analysis, we assigned patients to subgroups on the basis of histopathological criteria such as Dukes stage, presence of angio-invasion, or presence of lymph node metastases. Furthermore, patients were separated on the basis of immunohistochemical parameters. CuZn-SOD expression was divided into two groups: a mean absorbance value ≤ 6.6 and > 6.6 . Mn-SOD expression was also divided into two groups: a mean absorbance



value ≤ 5.3 and > 5.3 . These cutoff levels were chosen in order to obtain two groups of equal size. Allocation of the patients to more than two groups made survival analysis unreliable because of the small number of patients per group. Kaplan-Meier survival curves were calculated and tested for significance by univariate log rank statistics. These curves included only colorectal cancer-related deaths as events. The independent prognostic value of parameters was tested using the multivariate Cox regression model. The level of significance was set at p -values < 0.05 , and the domain of the correlation coefficient (r) was set at $r \leq -.3$ and $r \geq .3$. Nonparametrical correlation coefficients were calculated by applying Spearman correlation analysis.

RESULTS

Crypts in normal mucosa showed uniform immunohistochemical CuZn-SOD and Mn-SOD staining in all epithelial cells (Fig. 1A). Staining was absent in the mucus in goblet cells. CuZn-SOD and Mn-SOD expression in the lamina propria was confined to interstitial mononuclear cells resembling leukocytes (Fig. 1A, D, and E). CuZn-SOD and Mn-SOD immunostaining was restricted to the cytoplasm, whereas nuclei were always unstained (Fig. 1). Staining of CuZn-SOD was diffuse (Fig. 1A), in contrast with that of Mn-SOD, which was more granular (Fig. 1B). Control reactions, in which the primary antibodies were replaced by nonimmune sheep serum or PBS, were negative. In the kidney, which served as a control, glomeruli were always negative for both enzymes, whereas tubuli showed positivity (Fig. 1C). Representative localization patterns of both SOD enzymes in adenomatous and malignant tissue are shown in Fig. 1D–F. Variation in SOD immunostaining in three consecutive sections of the same biopsy was acceptable, because the intraindividual standard error of the mean per section ranged from 0.9% up to 11.4% (median value: 7.8%) for Mn-SOD and from 2.0% up to 12.3% (median value: 9.8%) for CuZn-SOD. Localization of SOD activity in normal mucosa was not restricted to intracellular compartments, but was also present at extracellular sites, such as at the surface of epithelial cells and in connective tissue (Fig. 2). In contrast with the protein levels of CuZn-SOD and Mn-SOD, which

were uniformly distributed over all columnar epithelial cells of crypts, total SOD activity was higher in epithelial cells at the surface of the crypts. The amounts of CuZn-SOD and Mn-SOD protein and total SOD activity in epithelia of normal mucosa, adenomas, and adenocarcinomas of the colorectum are shown in Table 1.

CuZn-SOD protein levels

Expression of CuZn-SOD in adenomas (Fig. 1D) did not differ significantly from that in normal mucosa (Table 1). Colorectal carcinomas showed a significantly lower expression of CuZn-SOD than adjacent normal mucosa ($p < .0001$). Fifty out of 60 colorectal carcinomas (83%) showed decreased amounts of CuZn-SOD. In these carcinomas, the mean CuZn-SOD expression was reduced by 22% when paired comparisons were made with normal mucosa in the same biopsies. The remaining 10 colorectal carcinomas (17%) showed a mean increased amount of CuZn-SOD of 11% (range: 2.3–32.2%, median value: 8.9%) in comparison with normal adjacent mucosa. The mean expression of CuZn-SOD in poorly differentiated carcinomas (82% of the mean value in normal mucosa) was significantly higher than in moderately differentiated (68%) and in highly differentiated (66%) carcinomas ($p = .005$ and $p = .03$, respectively). Expression of CuZn-SOD in highly differentiated carcinomas did not differ significantly from that in moderately differentiated carcinomas. CuZn-SOD expression in colorectal carcinoma was significantly correlated with differentiation grade by Spearman correlation analysis ($r = .34$, $p = .009$).

A positive correlation ($r = .64$, $p = .0001$) between CuZn-SOD expression in colorectal carcinomas and adjacent histologically normal mucosa was also found.

Mn-SOD protein levels

Normal mucosa and adenomas showed similar staining intensities for Mn-SOD (Table 1; Fig. 1B and E). Mn-SOD expression in carcinomas was significantly lower than in normal adjacent mucosa ($p < .0001$). Fifty-six out of 60 (93%) colorectal carcinomas con-

Fig. 1. Immunolocalization of CuZn-superoxide dismutase (CuZn-SOD) and Mn-superoxide dismutase (Mn-SOD). Bar = 50 μ m. (A) Homogeneous CuZn-SOD expression in colonic epithelium. (B) Granular Mn-SOD expression in surface epithelial cells of the colon. (C) Immunolocalization of CuZn-SOD in rat kidney. CuZn-SOD immunoreactive proteins are absent in glomeruli (arrow), but present in epithelial cells of tubuli. (D) Immunolocalization of CuZn-SOD in a human adenoma of the colon. CuZn-SOD immunoreactive proteins are diffusely distributed in the cytoplasm of epithelial cells. (E) Immunolocalization of Mn-SOD in a human adenoma and normal crypts of the colon. Epithelial cells of normal crypts (arrows) show similar intensity of staining as the cells of the adenoma. (F) CuZn-SOD expression in a poorly differentiated colorectal carcinoma. Expression is low in cancer cells. A similar staining pattern was observed for Mn-SOD.

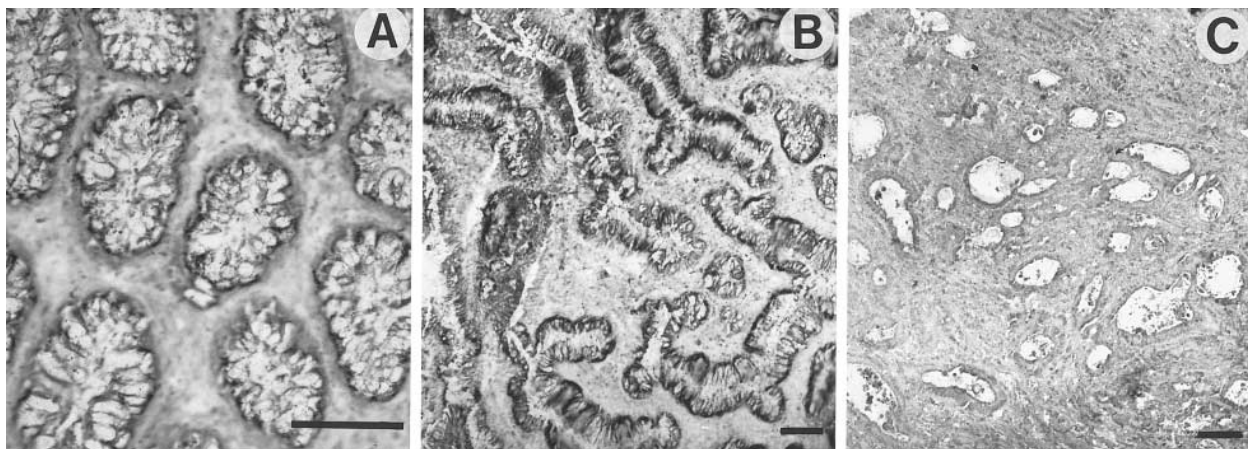


Fig. 2. Histochemical localization of superoxide dismutase (SOD) activity. Bar = 100 μ m. (A) SOD activity in normal colonic epithelium. (B) SOD activity in an adenoma. Epithelial cells of the neoplastic glands show similar activity as the cells in normal mucosa. (C) SOD activity in an adenocarcinoma. Activity is low in epithelial cells.

tained diminished amounts of Mn-SOD in comparison with adjacent normal mucosa.

The mean Mn-SOD expression in these colorectal carcinomas was reduced by 27%. Only 4 out of 60 (7%) colorectal carcinomas showed a mean increase in Mn-SOD expression of 6% (range: 1.1–11.6%, median value: 6.1%). Homogeneous distribution patterns were observed, irrespective of differentiation grade. No correlation was observed between Mn-SOD levels and differentiation grade. A correlation was not found either between Mn-SOD expression in colorectal carcinomas or the adjacent normal mucosa.

SOD activity

In analogy with CuZn-SOD and Mn-SOD protein levels, total SOD activity was similar in adenomas and normal mucosa (Table 1; Fig. 2A and B). Without an

exception, colorectal carcinomas showed significantly lower SOD activity than adjacent normal mucosa ($p < .0001$). The mean decrease in SOD activity in carcinomas was 55% when paired comparisons were made with normal mucosa. The positive correlation ($r = .59$, $p < .0001$) that was found between SOD activity in colorectal carcinomas and adjacent normal mucosa was comparable with that of CuZn-SOD protein levels. Total SOD activity in carcinomas was not related with differentiation grade.

Prognostic significance of SOD expression in colorectal carcinomas

Kaplan-Meier survival curves revealed significantly poorer survival rates in relationship with angio-invasion ($p = .004$; Fig. 3A), higher Dukes stages ($p = .03$; Fig. 3B), and lymph node metastases ($p = .02$;

Table 1. Cu/Zn-SOD and Mn-SOD Protein Levels and Total SOD Activity in Human Colorectal Neoplasms

Classification	Mean Absorbance \pm SD ^a	<i>p</i> -Value ^b
CuZn-SOD		
Normal mucosa (A)	0.14 \pm 0.03	A vs. B: NS ^c A vs. C: <i>p</i> < 0.0001
Adenomas (B)	0.13 \pm 0.03	
Adenocarcinomas (C)	0.11 \pm 0.03	
Mn-SOD		
Normal mucosa (D)	0.12 \pm 0.02	D vs. E: NS D vs. F: <i>p</i> < 0.0001
Adenomas (E)	0.10 \pm 0.03	
Adenocarcinomas (F)	0.09 \pm 0.08	
SOD activity		
Normal mucosa (G)	0.71 \pm 0.12	G vs. H: NS G vs. I: <i>p</i> < 0.0001
Adenomas (H)	0.71 \pm 0.18	
Adenocarcinomas (I)	0.33 \pm 0.11	

^a SD: standard deviation.

^b Mann-Whitney test, $\alpha = 0.05$.

^c NS: not significant.

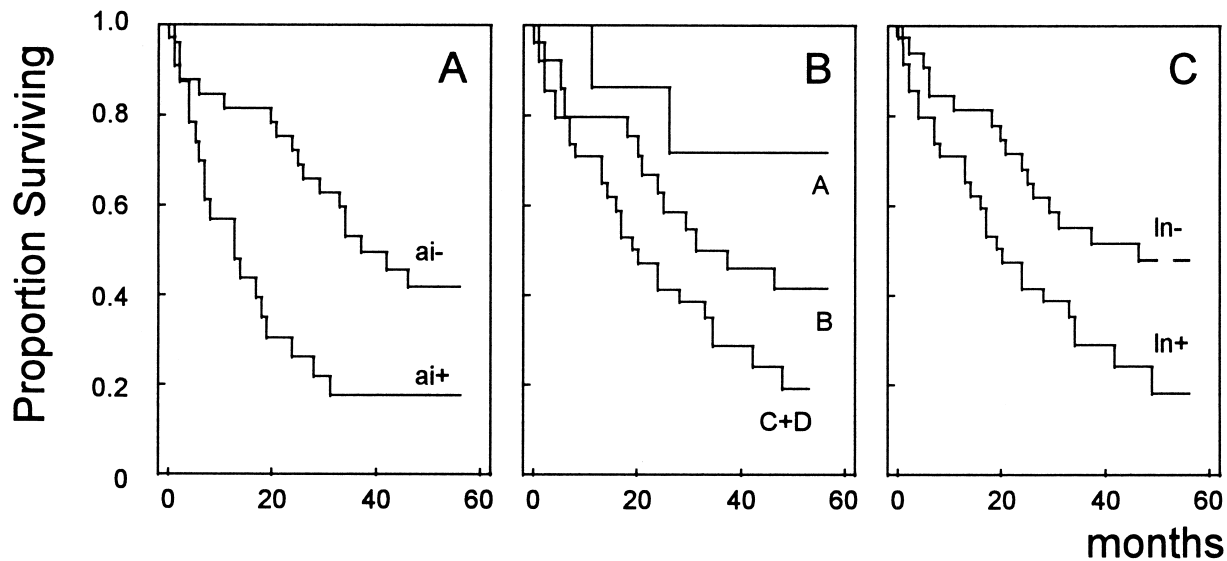


Fig. 3. Kaplan-Meier survival curves on the basis of (A) presence (ai+) or absence (ai-) of angio-invasion; (B) Dukes classification (A = Dukes stage A; B = Dukes stage B; C + D = Dukes stages C and D); (C) Presence (ln+) or absence (ln-) of lymph node metastases.

Fig. 3C). Differentiation grade of the carcinoma and age of the patients were not related with survival. We established the relationship between protein levels of both forms of SOD and total SOD activity and these histopathological parameters for prognosis. Dukes A carcinomas showed a somewhat higher loss of CuZn-SOD expression (35%) than Dukes B carcinomas (18%) and Dukes C/D carcinomas (20%) ($p = .07$ and $p = .06$, respectively; Table 2). No differences in

CuZn-SOD expression were observed between Dukes B and Dukes C/D carcinomas. Dukes A carcinomas never showed increased expression of CuZn-SOD, whereas 20% of the patients in Dukes B and C/D stages did. Neither Mn-SOD expression nor SOD activity were significantly different in the Dukes stages.

Multiple regression analysis did not result in a model for prediction of the dependent variables on the basis of any of the independent variables, using the difference in CuZn-SOD or Mn-SOD expression or total SOD activity between colorectal carcinomas and adjacent normal mucosa or absolute SOD expression as dependent variable and Dukes stage, lymph node metastases, angio-invasion, differentiation grade, sex, and age as the independent variables.

By means of Spearman correlation analysis, we observed an inverse correlation between age of the patients and CuZn-SOD protein levels in normal mucosa adjacent to the carcinomas ($r = -.46$, $p = .0002$). This correlation was not observed for Mn-SOD protein levels or total SOD activity. The mean age of the patients was 73 ± 9.5 years (range: 41–88 years, median value: 75 years).

We also related expression of both SODs with survival using Kaplan-Meier curves. We did not find differences in survival on the basis of absolute CuZn-SOD levels in the carcinomas (Fig. 4A). However, all 10 patients with carcinomas showing increased CuZn-SOD expression in comparison with adjacent normal mucosa died within 24 months, whereas patients who survived up to 56 months never showed an increased expression. Mn-SOD overexpression was not related

Table 2. Percentage CuZn-SOD and Mn-SOD Protein Levels and Total SOD Activity in Human Colorectal Carcinomas, When Compared to Adjacent Normal Mucosa (=100%)

	<i>n</i> ^a	Percentage Expression (%) Mean \pm SD ^b	<i>p</i> -Value ^c
CuZn-SOD			
Dukes stage			
A (I)	7	64.9 \pm 15.8	I vs. II: $p = 0.07$
B (II)	24	81.9 \pm 20.8	II vs. III: NS ^d
C/D (III)	29	80.1 \pm 17.5	I vs. III: $p = 0.06$
Mn-SOD			
Dukes stage			
A (I)	7	81.5 \pm 15.3	I vs. II: NS
B (II)	24	69.7 \pm 18.1	II vs. III: NS
C/D (III)	29	73.2 \pm 17.8	I vs. III: NS
SOD activity			
Dukes stage			
A (I)	7	58.5 \pm 4.9	I vs. II: NS
B (II)	24	55.1 \pm 12.9	II vs. III: NS
C/D (III)	29	53.7 \pm 13.4	I vs. III: NS

^a Number of patients.

^b SD: standard deviation.

^c Mann-Whitney test, $\alpha = 0.1$.

^d Not significant.

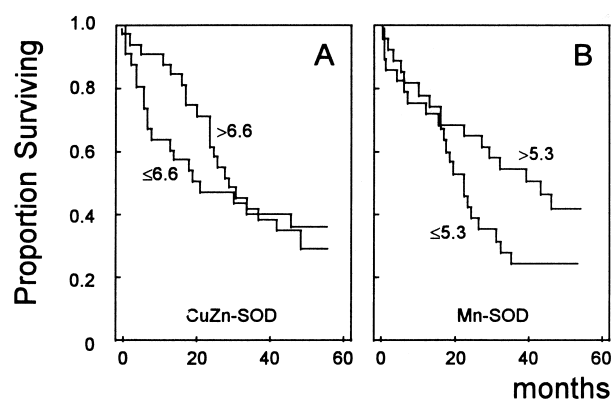


Fig. 4. Kaplan-Meier survival curves on the basis of (A) CuZn-SOD expression and (B) Mn-SOD expression. Survival of patients with low expression of CuZn-SOD (≤ 6.6 AU) or Mn-SOD (≤ 5.3 AU) did not differ significantly from that of patients with high CuZn-SOD (> 6.6 AU) or Mn-SOD (> 5.3 AU) expression.

with survival, since two out of four patients with Mn-SOD overexpression survived for at least 48 months (data not shown). There was a tendency of poorer survival in the group of patients showing a low Mn-SOD expression (≤ 5.3 AU) in comparison with higher Mn-SOD expression (> 5.3 AU), although this difference was not statistically significant ($p = .08$; Fig. 4B). Total SOD activity in carcinomas was not related with survival.

DISCUSSION

It has been suggested that loss of SOD activity may account at least partly for the behavior of cancer cells.⁹ In the present study, most of the colorectal carcinomas were found to be characterized immunohistochemically by decreased amounts of cytoplasmic CuZn-SOD and mitochondrial Mn-SOD and total SOD activity when compared with adjacent normal mucosa. Quantitative differences in expression of CuZn-SOD and Mn-SOD or SOD activity were not detected between adenomas of the colon and normal adjacent mucosa. These findings implicate that loss of CuZn-SOD and Mn-SOD is a rather late event in the carcinogenetic process, which becomes significant when neoplasms have turned into malignancy. The intriguing aspect of the loss of CuZn-SOD is the fact that in well-differentiated and moderately differentiated as well as in Dukes stage A, the decrease is largest. A functional explanation for this highly significant phenomenon is hard to give.

CuZn-SOD and Mn-SOD expression was never detected immunohistochemically at extracellular sites, which is in agreement with the findings of Dobashi *et al.*²⁵ Therefore, the extracellular final reaction product

that we found at the surface of epithelial cells and in connective tissue (Fig. 2) must be produced by the extracellular form of SOD (EC-SOD).^{36,37} This was reported as well by Frederiks and Bosch.²⁶

Mulder *et al.*³⁸ found a significant increase in CuZn-SOD levels in homogenates in the sequence of normal mucosa-adenoma-carcinoma. A decrease in CuZn-SOD expression was observed in only 18% of the colorectal carcinomas, whereas we found decreased expression in 83% of the cases. The presence of a lamina propria that is largely negative for SOD expression (Fig. 1A) may have caused an underestimation of the actual amounts of CuZn-SOD present in epithelial cells of normal mucosa and adenomas when using homogenates. The decreased total SOD activity in epithelia in carcinomas *in situ* that we found (Table 1) supports this explanation.

Both upregulation and downregulation of Mn-SOD have been described for human renal adenocarcinomas.³⁹ Elevated expression of Mn-SOD and other antioxidants was hypothesized to be related to poor survival, but we did not observe poor survival to be related with Mn-SOD overexpression. In fact, we found a tendency of low Mn-SOD expression to be related with poor survival. Increased levels of CuZn-SOD were only observed in patients who died within 24 months, whereas an increase never occurred in biopsies of patients surviving up to 56 months. Decreased levels of CuZn-SOD expression were not related with survival. Total SOD activity was not related with survival either, which is not surprising because we detected all SOD activity including CuZn-SOD, Mn-SOD, and EC-SOD activity. SOD activity was decreased in colorectal carcinomas when compared with adjacent normal mucosa without an exception. Apparently, in the individual cases of either elevated CuZn-SOD or Mn-SOD levels, this increase did not compensate for the decrease in the other forms of SOD.

Because CuZn-SOD and Mn-SOD protein levels or SOD activity were not related with the presence of lymph node metastases, or angio-invasion, which all—including Dukes staging—proved to be significantly related to survival in the present study, SOD protein levels or activity alone appear to be of no prognostic value. Because we could not demonstrate any relationship of Mn-SOD expression with either Dukes staging, presence of angio-invasion or lymph node metastases, it was surprising to find a tendency of poorer survival in patients showing low levels (≤ 5.3) of Mn-SOD.

Both up- and downregulation of SOD levels in tumors may have clinical significance. Elevated levels may cause resistance to chemotherapeutic agents⁴⁰ on the one hand and higher effectivity of radiotherapy on the other.⁴¹ Decreased levels may lead to increased in-

cidence of mutations,⁴² increased cell proliferation,^{43,44} and can, in fact, be carcinogenic.^{2,45}

In conclusion, we have found mean decreased protein levels of both CuZn-SOD and Mn-SOD and total SOD activity in carcinomas. There was no evidence of a role of both SODs in the development of colorectal cancer from the results in our study possibly with the exception of elevated levels of CuZn-SOD in carcinomas that was strongly linked with poor survival. One has to realize that evidence for a role of CuZn-SOD and Mn-SOD in the carcinogenetic process has been mainly obtained so far from cell culture experiments, which can never completely reflect or replace the in vivo system. Because of the lack of clear correlations with the development of malignancy, we agree with the suggestion of Punnonen et al.¹³ that the decrease in SOD expression can be very well a secondary phenomenon related to cell differentiation and/or proliferation.

Acknowledgements — The authors are grateful to Dr. A. F. P. M. de Goeij of the Pathology Department of the University Hospital of Maastricht for supplying the adenoma specimens. The pathological examination of the specimens by Dr. J.-Y. Song, the preparation of the manuscript by Mrs. T. M. S. Pierik, and the photographic work by Mr. J. Peeterse are highly appreciated.

REFERENCES

- Halliwell, B.; Gutteridge, J. M. C. *Free radicals in biology and medicine*, 2nd ed. Oxford: Oxford University Press; 1989.
- Cerutti, P. A. Prooxidant states and tumor promotion. *Science* **227**:375–381; 1985.
- Sies, H. Strategies of antioxidant defence. *Eur. J. Biochem.* **215**:213–219; 1993.
- Fridovich, I. Superoxide dismutases. *Annu. Rev. Biochem.* **44**:147–159; 1975.
- McCord, J. M.; Fridovich, I. Superoxide dismutase: An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244**:6049–6055; 1969.
- Weisiger, R. A.; Fridovich, I. Superoxide dismutase. Organelle specificity. *J. Biol. Chem.* **248**:4793–4796; 1973.
- Carlsson, L. M.; Jonsson, J.; Edlund, T.; Marklund, S. L. Mice lacking extracellular superoxide dismutase are more sensitive to hyperoxia. *Proc. Natl. Acad. Sci. USA* **92**:6264–6268; 1995.
- Strålin, P.; Marklund, S. L. Effects of oxidative stress on expression of extracellular superoxide dismutase, CuZn-superoxide dismutase and Mn-superoxide dismutase in human dermal fibroblasts. *Biochem. J.* **298**:347–352; 1994.
- Oberley, L. W.; Buettner, G. R. Role of superoxide dismutase in cancer: A review. *Cancer Res.* **39**:1141–1149; 1979.
- Marklund, S. L.; Westman, N. G.; Lundgren, E.; Roos, G. Copper- and zinc-containing superoxide dismutase, manganese-containing superoxide dismutase, catalase, and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues. *Cancer Res.* **42**:1955–1961; 1982.
- Loven, D. P.; Guernsey, D. L.; Oberley, L. W. Transformation affects superoxide dismutase activity. *Int. J. Cancer* **33**:783–786; 1984.
- Oberley, L. W.; Oberley, T. D. Role of antioxidant enzymes in cell immortalization and transformation. *Mol. Cell Biochem.* **84**:147–153; 1988.
- Punnonen, R.; Kudo, R.; Punnonen, K.; Hietanen, E.; Kuoppala, T.; Kainulainen, H.; Sato, K.; Ahutopa, M. Activities of antioxidant enzymes and lipid peroxidation in endometrium cancer. *Eur. J. Cancer* **29A**:266–269; 1993.
- Durak, I.; Perk, H.; Kavutçu, M.; Canbolat, O.; Akyol, O.; Beşik, Y. Adenosine deaminase, 5' nucleotidase, xanthine oxidase, superoxide dismutase, and catalase activities in cancerous and noncancerous human bladder tissues. *Free Radic. Biol. Med.* **16**:825–831; 1994.
- Oberley, T. D.; Schultz, J. L.; Oberley, L. W. In vitro modulation of antioxidant enzyme levels in normal hamster kidney and estrogen-induced hamster kidney tumor. *Free Radic. Biol. Med.* **16**:741–751; 1994.
- Beckman, J. S.; Minor, R. L.; White, C. W.; Repine, J. E.; Rosen, G. M.; Freeman, B. A. Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. *J. Biol. Chem.* **263**:6884–6892; 1988.
- Church, S. L.; Grant, J. W.; Ridnour, L. A.; Oberley, L. W.; Swanson, P. E. Increased manganese superoxide dismutase expression suppresses the malignant phenotype of human melanoma cells. *Proc. Natl. Acad. Sci. USA* **90**:3113–3117; 1993.
- Hansberg, W.; De Groot, H.; Sies, H. Reactive oxygen species associated with cell differentiation in *Neurospora crassa*. *Free Radic. Biol. Med.* **14**:287–293; 1993.
- St. Clair, D. K.; Oberley, T. D.; Muse, K. E.; St. Clair, W. H. Expression of manganese superoxide dismutase promotes cellular differentiation. *Free Radic. Biol. Med.* **16**:275–282; 1994.
- Oberley, L. W.; Bize, I. B.; Sahu, S. K.; Leuthauser, S. W. H. C.; Gruber, H. E. Superoxide dismutase activity of normal murine liver, regenerating liver, and H6 hepatoma. *J. Natl. Cancer Inst.* **61**:375–379; 1978.
- Dukes, C. E. The classification of cancer of the rectum. *J. Pathol.* **35**:323–332; 1932.
- Brodsky, J. T.; Richard, G. K.; Cohen, A. M.; Minsky, B. D. Variables correlated with the risk of lymph node metastases in early cancer. *Cancer* **67**:322–326; 1992.
- Krasna, M. J.; Klancbaun, L.; Cody, R. P.; Schneibaum, S.; Ben Ari, G. Vascular and neural invasion in colorectal carcinoma: Incidence and prognostic significance. *Cancer* **61**:1018–1023; 1988.
- Crapo, J. D.; McCord, J. M. Oxygen-induced changes in pulmonary superoxide dismutase assayed by antibody titrations. *Am. J. Physiol.* **231**:1196–1203; 1976.
- Dobashi, K.; Asayama, K.; Kato, K.; Kobayashi, M.; Kawaoi, A. Immunohistochemical localization of copper-zinc and manganese superoxide dismutase in rat tissues. *Acta Histochem. Cytochem.* **22**:351–365; 1989.
- Frederiks, W. M.; Bosch, K. S. Localization of superoxide dismutase activity in rat tissues. *Free Radic. Biol. Med.* **22**:241–248; 1997.
- Patel, H. R. H.; Frederiks, W. M.; Marx, F.; Best, A. J.; Van Noorden, C. J. F. A quantitative histochemical study of D-amino acid oxidase activity in rat liver in relationship with feeding conditions. *J. Histochem. Cytochem.* **39**:81–86; 1991.
- Van Noorden, C. J. F.; Frederiks, W. M. *Enzyme histochemistry: A laboratory manual of current methods*. Oxford: Oxford University Press; 1992.
- Frederiks, W. M.; Bosch, K. S.; Van den Munckhof, R. J. M.; Van Noorden, C. J. F. A quantitative histochemical study of xanthine oxidase activity in rat liver using the cerium capture method in the presence of polyvinyl alcohol. *J. Histochem. Cytochem.* **42**:1091–1097; 1994.
- Van der Ploeg, M.; Streefkerk, J. G.; Daems, W. T.; Brederoo, P. Quantitative aspects of cytochemical peroxidase reactions with 3'3-diaminobenzidine and 5,6-hydroxy indole as substrates. In: Wisse, E.; Daems, W. H.; Molenaar, I.; Van Duijn, P., eds. *Electron microscopy in cytochemistry*. Amsterdam: North Holland Publishing Company; 1973:123–126.
- Pool, C. W.; Madlener, S.; Diegenbach, P. C.; Sluiter, A. A.; Van der Sluis, P. Quantification of antiserum reactivity in immunohistochemistry. *J. Histochem. Cytochem.* **32**:921–928; 1984.

32. Sternberger, L. A.; Sternberger, N. H. The unlabeled antibody method: Comparison of peroxidase-antiperoxidase with avidin-biotin complex by a new method of quantification. *J. Histochem. Cytochem.* **34**:599–605; 1986.
33. Geerts, W. J. C.; Verburg, M.; Jonker, A.; Das, A. T.; Boon, L.; Charles, R.; Lamers, W. H.; Van Noorden, C. J. F. Gender-dependent regulation of glutamate dehydrogenase expression in periportal and pericentral zones of rat liver lobules. *J. Histochem. Cytochem.* **44**:1153–1159; 1996.
34. Geerts, A.; Roels, F. Quantitation of catalase activity by microspectrophotometry after diaminobenzidine staining. *Histochemistry* **72**:357–367; 1981.
35. Van Duijn, P. Model systems. Principles and practice of the use of matrix-immobilized enzymes for the study of the fundamental aspects of cytochemical enzyme methods. In: Stoward, P. J.; Pearse, A. G. E., eds. *Histochemistry*, vol. 3. Edinburgh: Churchill Livingstone; 1991:433–472.
36. Marklund, S. L. Extracellular superoxide dismutase in human tissues and human cell lines. *J. Clin. Invest.* **74**:1398–1403; 1984.
37. Sandström, J.; Karlsson, K.; Edlund, T.; Marklund, S. L. Heparin-affinity patterns and composition of extracellular superoxide dismutase in human plasma and tissues. *Biochem. J.* **294**:853–857; 1993.
38. Mulder, T. P. J.; Verspaget, H. W.; Janssens, A. R.; De Bruin, P. A. F.; Griffioen, G.; Lamers, C. B. H. W. Neoplasia-related changes of two copper (Cu)/Zinc (Zn) proteins in the human colon. *Free Radic. Biol. Med.* **10**:501–506; 1990.
39. Oberley, T. D.; Sempf, J. M.; Oberley, M. J.; McCormick, M. L.; Muse, K. E.; Oberley, L. W. Immunogold analysis of antioxidant enzymes in human renal cell carcinoma. *Virchows Arch.* **424**:155–164; 1994.
40. Hirose, K.; Longo, D. L.; Oppenheim, J. J.; Matsushima, K. Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation. *FASEB J.* **7**:361–368; 1993.
41. Urano, M.; Kuroda, M.; Reynolds, R.; Oberley, T. D.; St. Clair, D. K. Expression of manganese superoxide dismutase reduces tumor control radiation dose: Gene therapy. *Cancer Res.* **55**:2490–2493; 1995.
42. Moraes, E. C.; Keyse, S. M.; Tyrell, R. M. Mutagenesis by hydrogen peroxide treatment of mammalian cells: A molecular analysis. *Carcinogenesis* **11**:283–293; 1991.
43. Armato, U.; Andreis, P. G.; Romano, F. Exogenous Cu,Zn superoxide dismutase suppresses the stimulation of neonatal hepatocytes' growth by tumor promoters. *Carcinogenesis* **51**:1547–1555; 1984.
44. Murell, G. A. C.; Francis, M. J. D.; Bromley, L. Modulation of fibroblast proliferation by oxygen free radicals. *Biochem. J.* **265**:659–665; 1990.
45. Kennedy, A. R.; Troll, W.; Little, J. B. Role of free radicals in the initiation and promotion or radiation transformation in vitro. *Carcinogenesis* **5**:1213–1215; 1984.

ABBREVIATIONS

AU—arbitrary units
 CuZn-SOD—copper and zinc-containing SOD
 DAB—3,3'-diaminobenzidine tetrachloride
 EC-SOD—extracellular SOD
 Gpx—glutathione peroxidase
 Mn-SOD—manganese-containing SOD
 PBS—phosphate-buffered saline
 PVA—polyvinyl alcohol
 ROS—reactive oxygen species
 SOD—superoxide dismutase