

PROLIFERATION, APOPTOSIS, AND MANGANESE SUPEROXIDE DISMUTASE IN MALIGNANT MESOTHELIOMA

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Proliferation and apoptotic indices of tumour cells may have important prognostic significance. Manganese superoxide dismutase (MnSOD), an important anti-oxidant enzyme, has been shown to decrease proliferation of malignant cells transfected with the MnSOD gene. The aim of the present study was to investigate the indices of cell proliferation and apoptosis and their prognostic significance in human mesothelioma and to assess the effect of MnSOD on the proliferation and apoptosis of the mesothelioma cells expressing high constitutive MnSOD activity. Tissue sections from 35 subjects with malignant pleural mesothelioma were studied for cell proliferation by Ki-67 immunohistochemistry and for apoptosis by the TUNEL assay. In additional experiments, 2 mesothelioma cell lines expressing either low (M14K) or high (M38K) MnSOD levels were assessed for proliferative and apoptotic responses to epirubicin. The median proliferation and apoptotic indices of the mesothelioma tissue were 8.2% and 0.75%, respectively. Patients with a high proliferation (>8%) or apoptotic index (>0.75%) showed a worse prognosis ($p < 0.001$). MnSOD expression was inversely correlated with cell proliferation ($p = 0.02$). Our cell line experiments indicated that cells expressing high MnSOD levels were more resistant to apoptosis and showed lower proliferation when exposed to epirubicin *in vitro*. These findings show that high proliferation and apoptosis are associated with a poor prognosis of mesothelioma and that a high MnSOD level is associated with low proliferation of tumour cells. Furthermore, experiments with cultured mesothelioma cells suggest the importance of MnSOD in the proliferation and apoptosis caused by drug exposure. Int. J. Cancer 88:37–43, 2000.

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Malignant mesothelioma represents a resistant tumour (Ong and Vogelzang, 1996) which arises from the mesothelial cells of pleural or peritoneal cavities. The pathogenesis of this disease is associated with exposure to asbestos fibres in most cases (Kane, 1996). Exposure to asbestos fibres leads to generation of reactive oxygen metabolites in the cells (Kamp *et al.*, 1992) and to induction of several early-response genes such as *c-fos* and *c-jun*, which are closely associated with cell proliferation (Janssen *et al.*, 1994). Asbestos fibres induce both proliferation and apoptosis of mesothelial cells (Kane, 1996; Broaddus *et al.*, 1996; Bérubé *et al.*, 1996), which can be attenuated by anti-oxidants (Kane, 1996; Janssen *et al.*, 1994).

We have previously shown that the level of MnSOD, an essential mitochondrial superoxide-scavenging anti-oxidant enzyme, is high in mesothelioma tissue and in cultured mesothelioma cell lines when compared to healthy pleura or non-malignant mesothelial cells (Kinnula *et al.*, 1996; Kahlos *et al.*, 1998, 2000). Manganese superoxide dismutase (MnSOD) is induced by multiple cytokines, most importantly by TNF- α , and by asbestos fibres both *in vivo* and *in vitro* (Kinnula *et al.*, 1995). The role of MnSOD in malignant tumours is poorly understood, and conflicting results have been reported. Its expression is high, not only in mesothelioma but also in brain tumours and colorectal cancer (Cobbs *et al.*, 1996; Janssen *et al.*, 1998), whereas it is low in most other tumours investigated (Oberley and Oberley, 1997). Several studies have also shown that transfection of the MnSOD gene into tumour cells with low MnSOD expression suppresses tumour cell growth both *in vitro* and *in vivo*, with a consequent reduction in tumour cell growth and frequency of metastases (Li *et al.*, 1995; Safford *et al.*, 1994). Therefore, it has been postulated that MnSOD is a tumour suppressor and that transfection

of the MnSOD gene might be used in cancer therapy (Oberley and Oberley, 1997). However, increased anti-oxidant enzyme expression in cancer cells may protect them against anti-cancer drugs and radiotherapy (Sinha and Mimnaugh, 1989).

Proliferation and apoptosis of tumour cells may have important prognostic significance, and MnSOD may have important effects on tumour cell growth, programmed cell death (apoptosis), and prognosis of patients with malignant diseases. MnSOD is a mitochondrial enzyme, and mitochondria play an important role in the early phase of apoptosis (Susin *et al.*, 1998). It has also been suggested that MnSOD over-expression may suppress apoptosis by altering the redox state of these organelles (Manna *et al.*, 1998; Zwacka *et al.*, 1998). In a previous study, we showed that MnSOD was elevated in the mitochondrial compartment of mesothelioma cells and that mitochondrial superoxide production was lower in these mesothelioma cells than in non-malignant mesothelial cells with low MnSOD activity (Kahlos *et al.*, 1999). Furthermore, we and others have shown that mesothelioma cells are resistant to apoptosis by unknown mechanisms other than those mediated by the Bcl-2 protein family (Narasimhan *et al.*, 1998; Soini *et al.*, 1999). Thus, tumour growth, proliferation, and apoptosis may have effects on the prognosis of mesothelioma patients and may be associated with MnSOD levels of the tumour.

In the present study, the extent of cell proliferation, apoptosis, and necrosis was investigated in human mesothelioma biopsies. These results were assessed in relation to the reactivity of MnSOD reported previously (Kahlos *et al.*, 2000). Furthermore, *in vitro* experiments were conducted with 2 mesothelioma cell lines expressing either low or high MnSOD activity.

MATERIAL AND METHODS

Patients and tumour specimens

Histopathologically typical cases of malignant pleural mesothelioma were retrieved from the files of the Department of Pathology, Oulu University Hospital. Thirty-five patients (mean age 62 years, 30 men and 5 women) with malignant pleural mesothelioma were included. Biopsy material was fixed in 10% neutral formalin and embedded in paraffin. Mesotheliomas were classified into epithelial, sarcomatoid, or biphasic subtypes (WHO, 1981). Clinical follow-up information was obtained from patient records. Clinical characteristics of these patients are presented in Table I. Four patients had received therapy before the tissue biopsies were obtained: 1 had received bleomycin intrapleurally (patient 19, Table I), 2 had received anti-cancer drugs (vincristine-cyclophosphamide [patient 13] or toremifene [patient 8]), and 1 had received radiation therapy (patient 35).

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TABLE I—CHARACTERISTICS OF THE MESOTHELIUM PATIENTS IN THE STUDY

Patient no	Age/Sex*	Histological subtype**	Asbestos exposure†	Smoking history‡	Survival (months)§
1	75/M	epithelial	no	non-smoker	124
2	70/F	epithelial	no	smoker	2
3	63/M	epithelial	yes	smoker	9
4	73/M	epithelial	yes	smoker	14
5	64/M	epithelial	yes	smoker	1
6	54/M	epithelial	na	smoker	1
7	78/M	epithelial	yes	smoker	1
8	70/M	epithelial	yes	smoker	4
9	63/M	epithelial	na	smoker	5
10	59/M	epithelial	yes	non-smoker	54
11	57/M	epithelial	na	smoker	3
12	51/M	epithelial	no	non-smoker	1
13	46/M	epithelial	yes	smoker	4
14	67/M	epithelial	no	na	37
15	78/M	epithelial	yes	smoker	21
16	68/M	epithelial	yes	smoker	5
17	56/M	epithelial	yes	na	9
18	66/M	epithelial	yes	non-smoker	0
19	50/M	epithelial	yes	smoker	7
20	73/M	epithelial	na	smoker	40
21	59/M	epithelial	yes	na	8
22	79/M	epithelial	yes	non-smoker	12
23	57/M	biphasic	yes	smoker	24
24	57/F	biphasic	na	non-smoker	21
25	70/M	biphasic	no	non-smoker	14
26	68/M	biphasic	na	non-smoker	2
27	42/F	sarcomatoid	na	na	3
28	59/M	sarcomatoid	na	smoker	2
29	63/M	sarcomatoid	na	smoker	1
30	52/M	sarcomatoid	na	smoker	2
31	33/F	sarcomatoid	no	non-smoker	96
32	71/M	sarcomatoid	yes	smoker	5
33	67/M	sarcomatoid	no	non-smoker	5
34	55/F	sarcomatoid	no	non-smoker	0
35	73/M	sarcomatoid	na	smoker	0

*F, female; M, Male; –**WHO 1981; –†established from the patient history; –na, data not available; –‡smoker, current smoker or ex-smoker; na, data not available; –§from the diagnosis.

Cell cultures and exposures

Mesothelioma cell lines M38K (high MnSOD) and M14K (low MnSOD) (Kahlos *et al.*, 1998) were originally established from the tumour tissue of untreated patients (Pelín-Enlund *et al.*, 1990). Cells were grown in RPMI-1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.03% L-glutamine (all from Life Technologies, Paisley, UK) at 37°C in a 5% CO₂ atmosphere. For cytotoxicity studies, cells were seeded on 75 cm² flasks (Nunc; Nalge Nunc, Roskilde, Denmark) and grown for 24 hr prior to exposure to 4-epirubicin (Farmorubicin; Pharmacia, Milan, Italy). After 48 hr exposure to different concentrations of epirubicin (0 to 1 µg/ml, 0 to 1.7 µM), cells were detached with trypsin and centrifuged together with the cells floating freely in the culture medium. For immunocytochemistry, cell pellets were fixed in 10% neutral formalin overnight, after which the formalin was removed and melted 2% agar was laid over the pellets. Agar blocks were further embedded in paraffin. Sections (4 µm thick) were cut from the cell blocks and processed for detection of apoptosis and proliferation, as described later.

RT-PCR assay for MnSOD

Expression of MnSOD mRNA was investigated by RT-PCR. Oligonucleotide primers were selected according to the MnSOD DNA sequence data published earlier (Ho and Crapo, 1988). Total cellular RNA was extracted from the cells using a kit for RNA isolation (RNeasy; Qiagen, Hilden, Germany). RNA (1 µg) was reverse-transcribed with 100 U Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Paisley, UK) and 10 pmol of the anti-sense primer (5'-CTGAAGAGCTGTCTGGGCTGT-3')

at 42°C for 45 min in 20 µl reaction mixture containing 0.5 U RNase inhibitor (5 Prime→3 Prime, Boulder, CO), 5 mM DTT, 400 µM dNTP, 75 mM KCl, 3 mM MgCl₂, and 50 mM Tris-HCl. cDNA was PCR-amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) using 1 U Taq polymerase (Laboratory Products, Sidcup, UK) and 10 pmol of the sense primer (5'-ATGTTGAGCCGGGCAGTGTGC-3') in a 100 µl reaction volume containing 1.5 mM MgCl₂, 16 mM (NH₄)₂SO₄, 67 mM Tris-HCl, and 0.01% Tween 20. The thermal profile involved denaturation at 95°C for 1 min, primer annealing at 65°C for 1 min, and extension at 72°C for 2 min. To determine the range of exponential PCR amplification of the target MnSOD mRNA, varying numbers of PCR cycles were tested (not shown). Exponential amplification of MnSOD mRNA was observed at cycles 21–28; therefore, 26 PCR cycles were performed. PCR products were electrophoresed in an ethidium bromide-stained 2% agarose (Pronadisa; Alcobendas, Madrid, Spain) gel and visualized under UV light. The amplification product in the MnSOD RT-PCR was 255 bp in length. A semi-quantitative adaptation of the MnSOD RT-PCR was used to estimate the amount of MnSOD transcripts expressed in the samples. The method is based on usage of a housekeeping gene, the gene for β-actin (Nakajima-Iijima *et al.*, 1985), as an external standard. The protocol in the β-actin RT-PCR was the same as that described for MnSOD, except that the number of PCR cycles was reduced to 20. The oligonucleotide primers in the β-actin RT-PCR were 5'-CTCCTTAATGTCACGCACGATTTC-3' (anti-sense) and 5'-GTGGGGCGCCCAAGGCACCA-3' (sense), and the amplification product was 540 bp in length.

Immunohistochemistry

Tumour cell proliferation was evaluated using a monoclonal mouse anti-human Ki-67 antibody (MAb MIB-1; Zymed, San Francisco, CA). A polyclonal rabbit antibody for human recombinant MnSOD was a gift from Dr. J.D. Crapo (National Jewish Medical Center, Denver, CO) (Kinnula *et al.*, 1994; Kahlos *et al.*, 2000). Sections (4 µm thick) were cut from a representative paraffin block or a cell block. Sections were first deparaffinised in xylene and rehydrated in descending ethanol series. To enhance the immunoreactivity, sections were incubated in 10 mM citrate buffer (pH 6.0), boiled in a microwave oven for 2 min at 850 W, and boiled again for 8 min at 350 W. Endogenous peroxidase activity was eliminated by incubation in 0.1% hydrogen peroxide in absolute methanol for 10 min. After incubation with primary antibodies (anti-Ki-67 dilution 1:50 for 1 hr, anti-MnSOD dilution 1:1,000 for 2 hr) at room temperature, a biotinylated secondary anti-mouse or anti-rabbit antibody was applied (dilution 1:300 and 1:1,000, respectively), followed by avidin-biotin-peroxidase complex (all from Dakopatts, Glostrup, Denmark). The colour was developed using 3,3'-diaminobenzidine, and the sections were lightly counterstained with haematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Replacement of the primary antibodies with PBS at pH 7.2 and normal rabbit serum was used as the negative control.

XTT cytotoxicity assay

The microculture tetrazolium dye colorimetric assay (XTT; Boehringer-Mannheim, Mannheim, Germany) was used to investigate the overall cytotoxic effect of epirubicin on mesothelioma cells. Cells were seeded on a 96-well (2 × 10⁴ cells/well) microtitre plate (Falcon, Becton Dickinson, Lincoln Park, NJ), and after incubation for 24 hr under conditions similar to those described previously for the cell cultures, cells were exposed to epirubicin for 48 hr. At the end of the exposure, 50 µl of the XTT labeling mixture were added to each of the wells and the spectrophotometric absorbance of the samples was measured at 492 nm with a microculture plate reader (Victor; Wallac, Turku, Finland). Viability is expressed as relative absorbance, *i.e.*, 1 – (absorbance of exposed wells/absorbance of control wells). Three independent experiments were performed, each in quadruplicate.

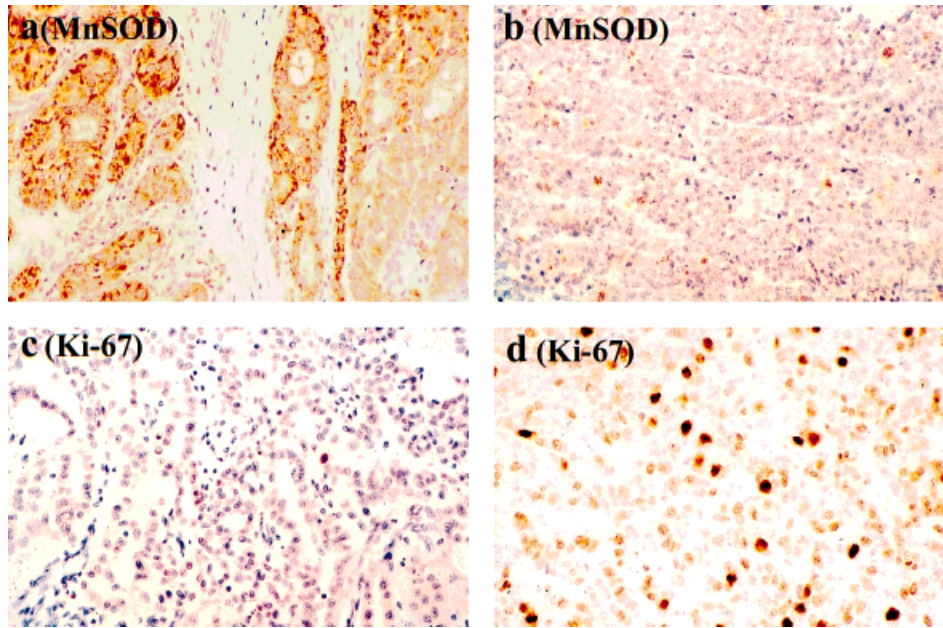


FIGURE 1 – Immunostaining of MnSOD (a) and Ki-67 (c) in a patient with epithelial malignant mesothelioma showing high MnSOD in association with low cell proliferation. In another patient with epithelial mesothelioma, low MnSOD expression (b) is associated with a high proliferation index (47%) (d) (Magnification $\times 142$).

3'-End labelling of DNA in apoptotic cells

Apoptosis was determined morphologically in mesothelioma tissue and in mesothelioma cells by *in situ* labelling of the 3' ends of the DNA fragments (TUNEL) using the ApopTag apoptosis detection kit (Oncor, Gaithersburg, MD). Sections from the tissue and cell blocks were dewaxed in xylene, rehydrated in ethanol, and incubated with 20 $\mu\text{g/ml}$ proteinase K (Boehringer-Mannheim) at room temperature for 15 min. Incubation in 2% hydrogen peroxide in PBS was performed to block endogenous peroxidase activity, after which slides were exposed to a terminal transferase enzyme and digoxigenin-labelled nucleotides. Thereafter, slides were incubated with an anti-digoxigenin antibody labelled with peroxidase, and the colour was developed with diaminobenzidine–hydrogen peroxide. Samples were lightly counterstained with haematoxylin.

Light microscopic evaluation

Each section was evaluated blindly by 2 authors. The extent of cell proliferation was calculated as the percentage of Ki-67-positive tumour cells. Apoptotic cells with clearly labeled nuclei and smaller apoptotic bodies were counted. At least 500 consecutive tumour cells were counted in each sample, and the percentages of positive cells were expressed as proliferation or apoptotic indices. The extent of necrosis was assessed by a morphometric method described previously (Eerola *et al.*, 1997). Briefly, a Nikon (Tokyo, Japan) Labophot light microscope was employed connected *via* a video camera (Panasonic F10, Osaka, Japan) to a TV monitor (Sony, Tokyo, Japan) equipped with a net of 50 points adherent to the screen. The total magnification for counting the volume density was $\times 40$, and all available sections from the tumours were counted in each case. Evaluation of MnSOD staining intensity has been reported previously (Kahlos *et al.*, 2000). In that study, MnSOD was assessed semi-quantitatively by grading the staining intensity of the tumour cells in mesothelioma sections and simultaneously estimating the proportion of positive cells of all tumour cells of the section. This semi-quantitation was expressed as negative (0), weak (+1), moderate (+2), or intense (+3) according to the staining intensity in tumour cells and the proportion of positive tumour cells.

Detection of caspase 3 activation by Western blotting

The apoptotic response of mesothelioma cells to epirubicin was also assessed by detecting caspase 3 activation using polyclonal

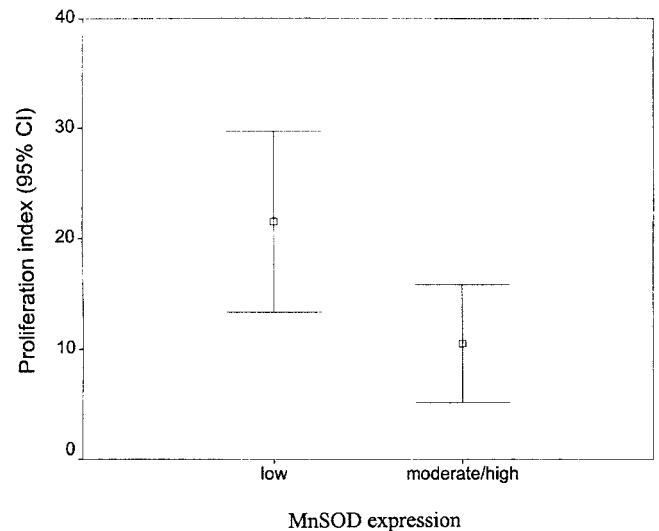


FIGURE 2 – Proliferation index of mesothelioma is higher in mesothelioma cases with low MnSOD expression than in cases with moderate or high MnSOD expression (95% CI, confidence interval; $p = 0.02$ by *t*-test).

rabbit anti-human caspase 3 antibody (CPP32; PharMingen, San Diego, CA). According to the manufacturer, the antibody detects both the 32 kDa inactive pro-enzyme and the fragmented active unit of 17 kDa. Cell pellets were suspended in sterile water, and the protein concentration was measured using the Bio-Rad (Hercules, CA) protein assay. From each cell sample, 75 μg of protein were mixed with the electrophoresis sample buffer, boiled at 95°C for 5 min, and applied to a 12% SDS-polyacrylamide gel. The gel was electrophoresed for 1.5 hr (90 V) and the protein transferred onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham, Arlington Heights, IL) in a Mini-Protean II Cell (Bio-Rad). Blotted membranes were incubated with the primary antibody (1:2,000) for 1 hr, followed by incubation with a

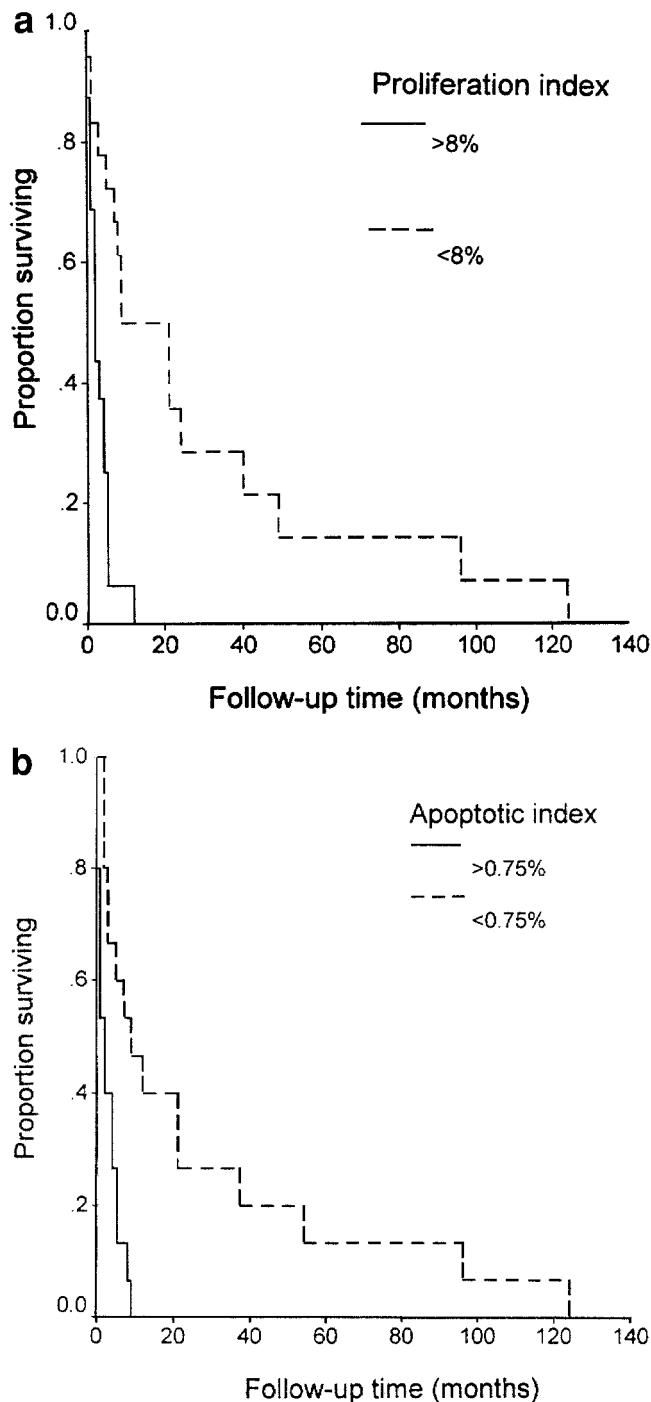


FIGURE 3 – Prognosis in relation to tumour cell proliferation by Ki-67 staining (a) and apoptosis by the TUNEL method (b).

donkey anti-rabbit antibody conjugated to horseradish peroxidase (diluted 1:5,000) for 1 hr (Amersham). The ECL system was used for detection of the protein, and the luminol excitation was imaged on X-ray film (Kodak Biomax MR; Rochester, NY). β -Actin expression was detected to confirm loading homogeneity by re-probing the same membranes (after stripping them of bound antibodies) with a mouse anti-actin MAb (1:20,000) followed by a sheep anti-mouse antibody conjugated to horseradish peroxidase (1:5,000) (Amersham) and detecting the protein as described for caspase 3.

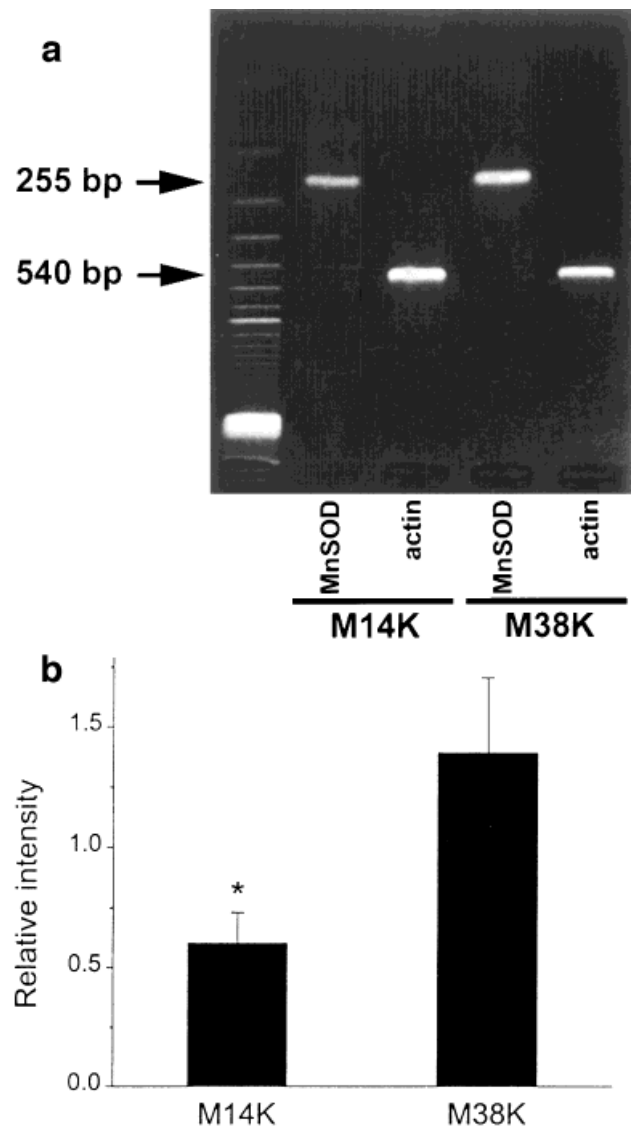


FIGURE 4 – MnSOD mRNA (255 bp) expression detected by RT-PCR is higher in M38K mesothelioma cells than in M14K mesothelioma cells (a). MnSOD mRNA (b) is estimated in relation to β -actin mRNA (540 bp), measured simultaneously from the same RNA sample under similar assay conditions ($n = 4$, $*p = 0.02$ by Mann-Whitney U -test).

Statistical methods

SPSS (7.5) for Windows (Chicago, IL) was used for statistical analyses. The results are expressed as means \pm SD. Comparisons between 2 groups were performed using the independent samples t -test or the Mann-Whitney U -test. The significance of associations was determined using Fisher's exact probability test. Survival analysis was performed by the Kaplan-Meier method, and the significance of associations was tested by the log-rank and Breslow tests. $P < 0.05$ was considered statistically significant.

RESULTS

The proliferation index of mesothelioma tissue as determined by Ki-67 immunohistochemistry was $14.1 \pm 13.2\%$ (range 1–47%, median 8.2%). The apoptotic index of the mesothelioma tumours assessed by the TUNEL method was $1.1 \pm 1.2\%$ (range 0–4.8%, median 0.75%), whereas necrosis was detected in $8.3 \pm 17\%$ (range 0–70%, median 1%) of the tumour areas. A representative

staining of high and low MnSOD in mesothelioma is shown in Figure 1. More complete data on MnSOD expression in malignant mesothelioma have been published elsewhere as part of another study which compares the immunoreactivity of MnSOD in adenocarcinoma and mesothelioma (Kahlos *et al.*, 2000). Altogether, 24 of 35 cases showed moderate or strong MnSOD reactivity.

MnSOD expression in mesothelioma was associated with cell proliferation (Fig. 2) ($p = 0.02$ by *t*-test) but not with apoptosis or necrosis ($p = 0.456$ and $p = 0.161$ by *t*-test, respectively). Tumours displaying moderate or high MnSOD expression showed lower proliferation than tumours displaying low MnSOD immunoreactivity (Figs. 1, 2). High proliferation and apoptotic indices were associated with more prominent necrosis in mesothelioma tissue ($p = 0.035$ and $p = 0.002$, respectively by Fisher's exact test). Exclusion of the 4 patients with prior therapy did not change these associations, though the proliferation index of patients without prior therapy was higher (mean 15.2, median 13.4%). High proliferation (>8%) or apoptotic index (>0.75%) ($p < 0.001$ by log-rank test) (Fig. 3) and a high percentage of necrosis (>1%) were associated with worse prognosis ($p = 0.027$ by log-rank test). The prognostic associations were still significant if the patients with prior therapy were excluded. History of past asbestos exposure or smoking was not associated with the degree of tumour cell proliferation or apoptosis (data not shown). In previous studies, we have also shown that the MnSOD level in mesothelioma tumours is not associated with previous exposure to asbestos fibres (Kahlos *et al.*, 1998, 2000).

Given that biopsies of untreated patients showed only an estimation of spontaneous cell proliferation and apoptosis, further experiments were conducted with 2 mesothelioma cell lines which have been shown to express either the highest (M38K) or lowest (M14K) MnSOD activity of our mesothelioma cell lines (Kinnula *et al.*, 1996; Kahlos *et al.*, 1998). Semi-quantitative RT-PCR analysis confirmed a higher expression of MnSOD mRNA in M38K than in M14K mesothelioma cells (Fig. 4). Exposure to epirubicin for 48 hr reduced the viability (assessed by XTT assay) of M14K cells from 86% to 38%, the corresponding values for M38K cells being 96% and 85% (Fig. 5a). Proportions of spontaneously proliferating cells were 46% for M14K cells and 51% for M38K cells. In M14K cells, low concentrations of epirubicin (0.1 and 0.2 $\mu\text{g/ml}$) paradoxically induced an increase in proliferation up to 64%, whereas this value decreased with higher concentrations (Fig. 5c). In M38K cells, however, the proliferation index decreased in a dose-dependent manner (Fig. 5c). The spontaneous apoptotic indices of the unexposed M14K and M38K cells were 3.1% and 2.5%, respectively. Epirubicin induced a marked apoptotic response (up to 66%) in a dose-dependent manner only in M14K mesothelioma cells when assessed by the TUNEL method and by caspase 3 activation (Figs. 5b, 6a,c). Only 8% of M38K cells showed apoptosis after exposure to the highest concentration of epirubicin, and no caspase 3 activation could be detected (Fig. 5b, 6b,d).

DISCUSSION

Our results show that both high proliferation and apoptotic indices are markers of poor prognosis in mesothelioma. Expression of MnSOD is usually high in this tumour (Kahlos *et al.*, 1998, 2000), and this high expression appears to be associated with lowered spontaneous cell proliferation and marginally improved prognosis. Existing data on the prognostic role of tumour cell apoptosis in cancer are conflicting (Soini *et al.*, 1998). In non-small-cell carcinoma, enhanced apoptosis predicts shortened survival (Törmänen *et al.*, 1995). Our results show that high apoptosis (>0.75%) is associated with poor prognosis also in mesothelioma. Our findings with proliferation indices are in agreement with results on mesothelioma showing that a high proliferation index measured by Ki-67 staining is associated with a poor prognosis (Beer *et al.*, 1998). Further, our results with mesothelioma cells suggest that MnSOD may have an important role in both proliferation and apoptosis of these cells when exposed to cytotoxic drugs.

Previous *in vitro* studies with transfected MnSOD gene have indicated that MnSOD can reduce tumour growth and proliferation

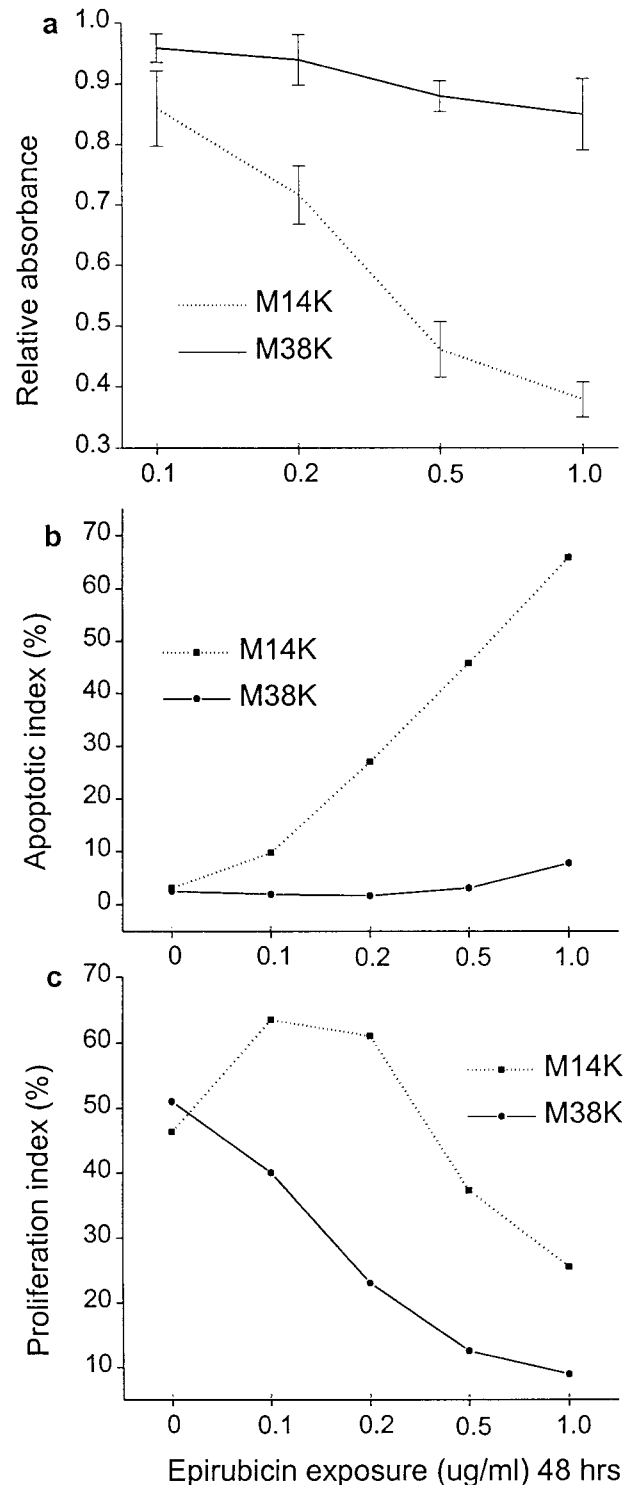


FIGURE 5 – Cell viability, apoptosis, and proliferation in mesothelioma cells treated with different concentrations of epirubicin. After exposure for 48 hr, cell viability (a) was reduced significantly more in M14K than in M38K mesothelioma cells as assessed by the XTT method (mean of 3 individual experiments, each done in quadruplicate). The extent of apoptosis (b), assessed by the TUNEL technique, was increased dose-dependently in M14K cells with increasing concentrations of epirubicin, whereas the effect was minimal in M38K cells (mean of 2 experiments). Proliferation index (c), assessed by Ki-67 immunostaining, showed that cell proliferation in M14K cells was increased with low and decreased with high concentrations of epirubicin, whereas in M38K cells proliferation was reduced dose-dependently (mean of 2 experiments).

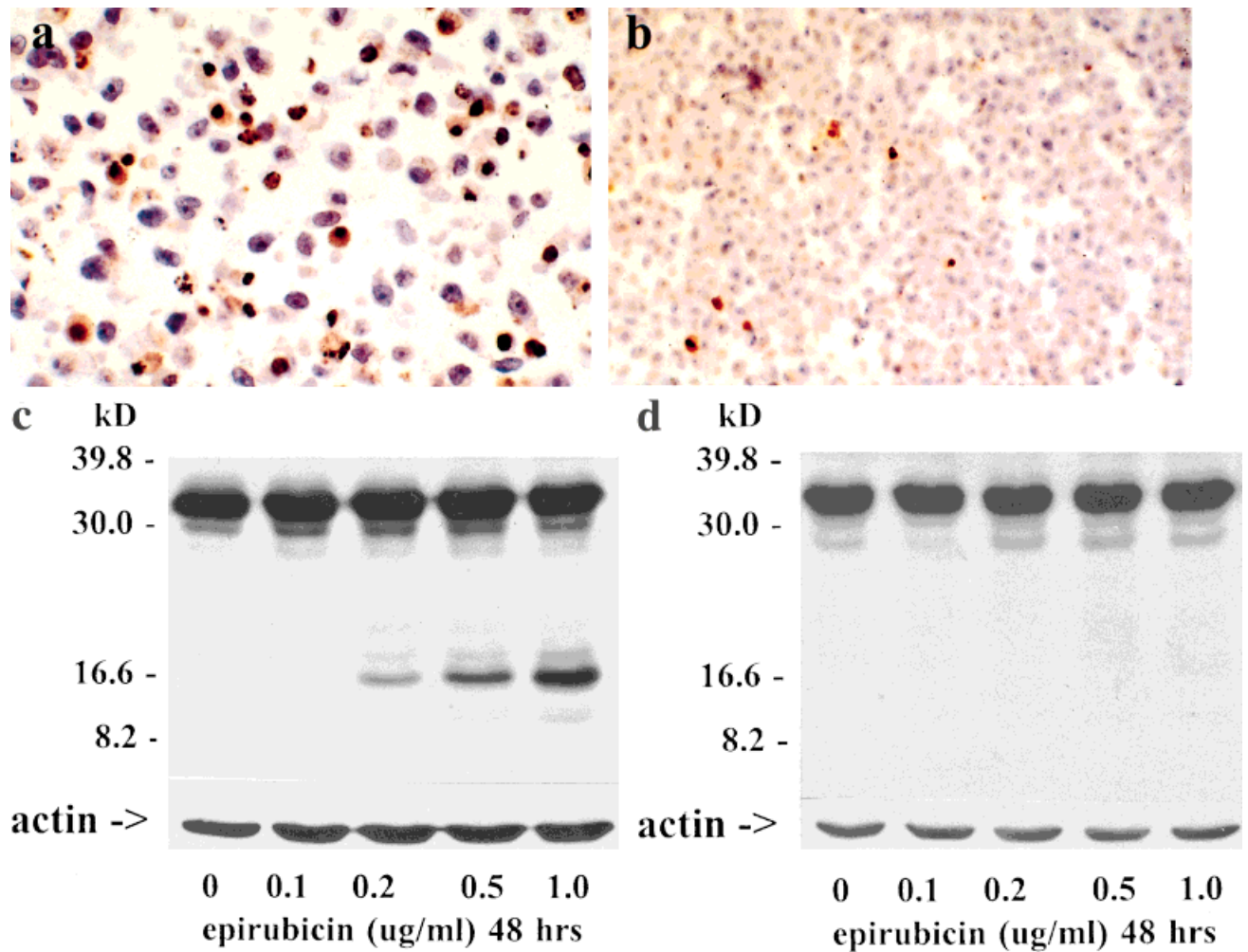


FIGURE 6 – Epirubicin-induced (0.5 $\mu\text{g/ml}$, 48 hr) apoptosis in M14K cells could be detected by the TUNEL method (a) and by detecting caspase-3 pro-enzyme cleavage (c). In M38K cells, the increase in the percentage of apoptotic cells was only minimal (b) and caspase-3 pro-enzyme cleavage could not be detected (d). Caspase-3 pro-enzyme (32 kDa) is cleaved into 17 kDa and 10 kDa fragments when activated during apoptosis, and the antibody used detects both the inactive pro-enzyme and the 17 kDa active fragment (b) [Magnification (a) $\times 284$ and (b) $\times 142$].

(Oberley and Oberley, 1997). However, very little is known about the effects of MnSOD on tumour proliferation, apoptosis, or survival *in vivo*. Our results showing lower proliferation in tumours displaying high MnSOD are consistent with these earlier findings and suggest the importance of this enzyme in tumour biology *in vivo*. The possible prognostic role of MnSOD in cancer is also unknown, but in one study high MnSOD expression in gastrointestinal cancer predicted poor survival (Janssen *et al.*, 1998). In contrast to that study, we have previously shown that prognosis of mesothelioma is dismal regardless of MnSOD levels, yet MnSOD levels might reflect small variation in the survival of these patients (Kahlos *et al.*, 2000).

Anthracyclines, such as epirubicin, are often used in the treatment of malignant mesothelioma. These compounds produce oxidants through redox-cycling reactions and induce apoptosis (Powis, 1989; Wagener *et al.*, 1996). The present study indicates that mesothelioma cells with lower levels of MnSOD (M14K) are more sensitive to epirubicin-induced toxicity and apoptosis than cells with high MnSOD (M38K). This difference was similar with all of the methods used, *i.e.*, morphologically, by the TUNEL technique, and by activation of caspase 3, which is a protease acting as an executor in the late phase of apoptosis (Nunez *et al.*, 1998). Besides MnSOD, other anti-oxidant mechanisms may play an important role in the resistance to apoptosis of M38K cells.

Observations from our laboratory have indicated that M38K cells contain markedly higher levels of catalase, glutathione, and several enzymes of glutathione metabolism than M14K cells (Kahlos *et al.*, 1998; Kinnula *et al.*, 1998). In conclusion, our results show that the high resistance of mesothelioma cells against cytotoxic drugs is associated with low apoptosis and possibly with the high anti-oxidant capacity and MnSOD of the cells.

Both cell lines showed very similar spontaneous proliferation, but proliferation responses of cells exposed to epirubicin were different. A low concentration of epirubicin enhanced the proliferation of M14K cells. These cells are more sensitive against this drug and oxidants, and they contain lower levels of MnSOD and other anti-oxidant enzymes (Kahlos *et al.*, 1998). In agreement, Li *et al.* (1998) showed that inhibition of transcription factors AP-1 and NF- κ B by MnSOD over-expression leads to a decrease of cell proliferation. Furthermore, although toxic at high concentrations, oxidants such as superoxide and hydrogen peroxide have growth-stimulatory effects at low concentrations (Burdon *et al.*, 1990). Thus, increased proliferation of M14K cells by low concentrations of epirubicin is in line with the oxidant effects and relatively low anti-oxidant capacity of these cells, which may cause induction of the transcription factors and subsequent cell growth. Regulation of cell proliferation and apoptosis in cancer cells is therefore complicated and probably dependent not only on the drug but also on

the anti-oxidant capacity of the cells. Cytotoxic drugs not only may cause cell death but also may have a proliferative effect on tumour cells, possibly explaining the poor response against various cytotoxic drugs *in vivo*.

We conclude that high cell proliferation, apoptosis, and necrosis as indices of tumour growth are associated with a worse prognosis of human malignant mesothelioma. High MnSOD expression is associated with low cell proliferation in human malignant mesothelioma, but this association does not explain the poor prognosis

of this disease. Our experiments with mesothelioma cell lines expressing low or high MnSOD, however, showed that MnSOD level may modulate the apoptotic or proliferative response of mesothelioma cells induced by cytotoxic drugs, such as epirubicin.

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