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Survivin is highly expressed and promotes cell survival in malignant peritoneal mesothelioma

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Abstract. *Background:* The biology of malignant peritoneal mesothelioma (MPM) is largely unknown. In the present study, we assessed the expression of survivin and other members of the inhibitors of apoptosis proteins (IAP) family (IAP-1, IAP-2 and X-IAP) in a series of 32 MPM surgical specimens and investigated the effects of survivin knockdown in an established MPM cell line. *Methods:* Expression of different IAPs was measured by immunohistochemistry. MPM cells were transfected with a small-interfering RNA (siRNA) targeting survivin mRNA and analyzed for survivin expression, growth rate, and ability to undergo spontaneous and drug (cisplatin, doxorubicin)-induced apoptosis. *Results:* Survivin expression was observed in 29 (91%) surgical MPM specimens, whereas the positivity rate for the other IAPs ranged from 69% to 100%. Transfection of MPM cells with the survivin siRNA induced a marked inhibition of survivin protein expression, a time-dependent decline in cell growth and an enhanced rate of spontaneous and drug-induced apoptosis, with a concomitant increase in the catalytic activity of caspase-9. *Conclusion:* Our results show for the first time that survivin, as well as other IAPs, is largely expressed in clinical MPMs and suggest that strategies aimed at down-regulating survivin may provide a novel approach for the treatment of the malignancy.

Keywords: Survivin, IAPs, peritoneal mesothelioma, siRNA, apoptosis, caspase-9

1. Introduction

Malignant peritoneal mesothelioma (MPM) is a rare primary peritoneal surface malignancy accounting for 10–15% of all malignant mesotheliomas [31]. Patients usually present with progressive ascites at a relatively late stage and their prognosis is poor, with a median overall survival of about 1 year [38]. The therapeutic approach to the disease has not yet been standardized. Treatment options with systemic chemotherapy failed to demonstrate a significant impact on survival. Since the disease remains in the abdominal cavity for most of its natural history [7], regional chemotherapy approaches with direct intraperitoneal drug instillation have been developed to selectively increase the drug concentration in the tumor-bearing area [21]. Recently, a unique strategy combining cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) has been shown to be a potentially effective salvage therapy for patients with MPM [36], although randomized studies are needed to establish the actual benefit of this treatment.

The biology of MPM is largely unknown, and the cellular and molecular mechanisms responsible for the proliferative potential and the relative resistance of MPM cells to therapy have not yet been elucidated. Since apoptotic cell death is the major mode by which chemical and physical anticancer agents kill tumor cells, it is likely that dysregulation of the apoptotic pathways plays a role in sustaining the chemoresistance of MPM cells, as already demonstrated for malignant pleural mesothelioma cells [12]. Previous in-

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vestigations have in fact shown overexpression of anti-apoptotic proteins belonging to the bcl-2 family (bcl-2 and bcl-X_L) and the inhibitors of apoptosis proteins (IAP) family (IAP-1 and survivin) in pleural mesothelioma cell lines and surgical specimens [14,18,25,39, 40]. Through the use of antisense-mediated inhibition approaches, these studies also demonstrated a cytoprotective role of such proteins against spontaneous [18, 40] and anticancer drug-induced apoptosis [14,26,40].

The understanding of the MPM biology relevant to the apoptosis-resistant phenotype could open new opportunities for the design of novel therapeutic strategies targeting the molecular determinants of treatment resistance of this malignancy. For this purpose we analyzed in the present study the expression of survivin and other IAP family members, including IAP-1 (also known as MIHC/cIAP2), IAP-2 (also known as MIHB/cIAP1), and X-IAP (also known as MIHA/hILP), by immunohistochemistry in formalin-fixed paraffin-embedded specimens of MPMs obtained from 32 patients uniformly treated at a single institution by CRS and HIPEC. Moreover, to validate survivin as a potential therapeutic target in MPM, we evaluated the effects of siRNA-mediated survivin down-regulation on proliferative potential, ability to undergo spontaneous apoptosis, and sensitivity to anticancer drugs of an established MPM cell line.

Table 1
Patient and tumor characteristics

	No. of patients (%)
Total	32
Age (years)	
Median	52
Range	29–73
Gender	
Female	21 (65.6)
Male	11 (34.4)
Histologic subtype	
Malignant	29 (90.6)
•Epithelioid, tubulopapillary/solid	12/14
•Biphasic	3
Borderline	3 (9.4)
•Well differentiated	1
•Multicystic	2
Preoperative systemic chemotherapy	
Yes	15 (46.9)
No	17 (53.1)
Carcinomatosis extent	
PCI $\leqslant$ 25%	4 (15.5)
PCI $>$ 25%	28 (87.5)
Completeness of cytoreduction	
CC-0 or CC-1	25 (78.1)
CC-2 or CC-3	7 (21.9)

PCI, peritoneal cancer index.

2. Materials and methods

2.1. Patients and tissue collection

The case series under investigation included 32 patients with MPM clinically, histopathologically and immunohistochemically diagnosed at the Istituto Nazionale Tumori (INT) of Milan, Italy, between 1996 and 2004. All patients signed an informed consent form by which they agreed to donate to INT the left-over tissue after completion of the histologic diagnosis, and for all of them archival paraffin and – whenever possible – frozen blocks of MPM specimens were collected from the tissue bank of the INT Department of Pathology to analyze the expression of apoptosis- and proliferation-related markers. For all the cases the diagnosis of mesothelioma cases was clinically and pathologically proved. All patients underwent CRS and HIPEC [19] according to the clinical treatment protocol approved by INT Review Board and Independent Ethics Committee. The patient characteristics are summarized in Table 1. There were 21 women and 11 men, with a median age of 52 years (range, 29–73). Twenty-nine patients presented at diagnosis with MPM and were part of a recently published clinicopathologic study of 35 patients treated locoregionally at our Institute [23], and 3 patients had a borderline peritoneal histologic subtype. Overall, 17 of the 32 patients had not been treated previously while the remaining 15 had received preoperative systemic chemotherapy and underwent second-line treatment following locoregional progression. At the time of last follow-up, 11 patients were alive with no evidence of disease, 9 were alive with disease, and 12 died of cancer-related causes.

Seven normal peritoneum specimens were also obtained from patients who underwent surgery for non-oncologic diseases.

2.2. Immunohistochemistry

Serial sections of the paraffin-embedded tumor and normal tissue specimens fixed in 10% buffered formalin were obtained to evaluate the immunohistochemical expression of IAP family members and the pro-apoptotic Smac/DIABLO protein, and of antigens

Table 2
Antibodies used in the study

Antigen	Clone	Dilution	Antigen retrieval	Source
Survivin, cytoplasm	Full length	1:2000	PC, CB, 6 min	Abcam (Cambridge, UK)
Survivin, specific for nuclear form	32.1	1:925	PC, CB, 6 min	Abcam
IAP-2 (MIHB/cIAP1)	Ab-1 (aa 26–38)	1:2000	PC, CB, 6 min	Oncogene (San Diego, CA)
IAP-1 (MIHC/cIAP2)	Ab-1 (aa 17–28)	1:2000	AM, 121°C, 10 min	Oncogene
X-IAP (MIHA/hILP)	2F1 (c-terminal)	1:75	PC, CB, 60 min	Medical & Biological Laboratories Co. (Woburn, MA)
Smac/DIABLO	Ab-3 (aa 188–199)	1:2000	WB, TRS, 95°C, 60 min	Oncogene
Ki-67	MIB-1	1:75	PC, CB, 6 min	Dako Corporation (Glostrup, Denmark)
CK18-caspase cleavage product	M30, mouse IgG_{2b}	1:1500	PC, CB, 15 min	Roche Molecular Biochemicals (Mannheim, Germany)

WB, water bath; PC, pressure cooking; AM, autoclave machine; TRS, target retrieval solution; CB, citrate buffer.

associated with cell proliferation (measured by MIB-1) and apoptosis (identified using the M30 antibody that specifically recognizes a caspase-cleaved cytokeratin 18). Antibodies, clones, dilutions, pretreatment conditions, and sources are listed in Table 2. We used the DAB substrate detection system (Dako, Carpenteria, CA) for all antibodies, maintaining appropriate positive and negative controls throughout; all slides were slightly counterstained with hematoxylin. The percentage of immunoreactive cells was determined by two independent observers scoring at least 1000 cells in each sample. We graded immunoreactivity quantitatively as the percentage of reactive tumor cells over the total number of tumor cells. Slides were independently scored by 2 authors (Au.C. and R.E.), and the assessment of all staining results was blinded to knowledge of the clinical outcome of patients. Taking into consideration: (1) the absolute range of immunoreactivity at nuclear and cytoplasmic level, (2) the consistency of immunostaining in serial sections from the same specimens and (3) the reproducibility of immunostaining scoring between different investigators, we defined as cutoff between absent-weak and moderate-high expression the value of 5% and 25% for nuclear and cytoplasmic immunostaining, respectively.

2.3. RNA extraction and reverse-transcription polymerase chain reaction

Total RNA was extracted from frozen tumor and normal tissue samples using the Trizol reagent (Invitrogen, San Giuliano Milanese, Italy) according to the manufacturer's instructions. Total RNA (0.5 μg) was reverse transcribed in the presence of random hexamers using the GeneAmp Reverse Transcription-PCR Core Kit (Applied Biosystems, Foster City, CA). To analyze survivin expression, the resultant cDNA was amplified using specific primers designed based on the nucleotide sequence of survivin gene: the sense primer was 5′-AGCCCTTTCTCAAGGACCAC-3′ and the antisense primer was 5′-TGACAGATAAGGAACCTG CA-3′. Cycling conditions were as follows: initial denaturation at 95°C for 2 minutes followed by 30 cycles at 95° for 1 minute, 62°C for 30 seconds, 72°C for 30 seconds, and 72°C for 7 minutes. A fragment corresponding to β-actin was coamplified and used as the standard of the amplification reaction. The amplified products were separated by agarose gel electrophoresis and analyzed by a ScanJET IIcx/T scanner (Hewlett Packard, Milan, Italy).

2.4. Oligonucleotides

A 21-mer oligonucleotide (GGACCACCGCAUCUCUACAdTdT) covering bases 45–65 downstream of the translational start codon of human survivin cDNA was used as the targeting sequence [5]. A 21-mer oligonucleotide (GGUCGUCGGCUACUUCUACdTdT) that had no significant homology to any known human mRNA in the databases was used as control. Survivin siRNA and control siRNA were purchased from MWG Biotech (Ebersberg, Germany).

2.5. Cell culture and transfection procedures

A human peritoneal mesothelioma cell line (STO) recently established in our laboratory was used in the study [43]. Cells were maintained in culture as a monolayer in DMEM F-12 (BioWhittaker, Verviers, Belgium) medium containing 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 in air. For transfection experiments, cells were seeded in complete

medium in 25-cm^2 plastic flasks at a density of 5×10^5 cells. Cells were transfected after 48 h with survivin siRNA or control siRNA (100 nM final concentration) using oligofectamine (10 μl/flask) reagent in 2 ml of Opti-MEM medium (both from Invitrogen). Eight h after transfection, 3 ml of DMEM F-12 containing 10% fetal bovine serum was added to each flask. Control cells were exposed only to oligofectamine. At different intervals after transfection (from 48 to 96 h), cells were collected by trypsinization and used in the different assays.

For combination experiments with cisplatin (Bristol-Myers, Evansville, IL) and doxorubicin (Pharmacia SpA, Milan, Italy), drugs were dissolved and diluted in 0.9% NaCl solution immediately before use. At the end of the 8-h transfection with each siRNA, cells were exposed for 1 h to cisplatin or doxorubicin and cultured for an additional 72 h at 37°C.

2.6. Western immunoblotting

Total cellular lysates obtained from control and siRNA-transfected cells were separated on a 15% SDS sodium dodecylsulfate-polyacrylamide gel and transferred to nitrocellulose. The filters were blocked in PBS with 5% skim milk and then incubated overnight with the primary antibodies to survivin, IAP-1, IAP-2 and X-IAP (AbCam, Inc., Cambridge, UK). The filters were then incubated with the secondary peroxidase-linked whole antibodies (Amersham Biosciences Europe GmbH, Freiburg, Germany). Bound antibodies were detected using the enhanced chemoluminescence Western blotting detection system (Amersham Biosciences). A β-actin monoclonal antibody (AbCam) was used on each blot to ensure equal loading of protein on the gel. Results were quantified by densitometric analysis.

2.7. Cell growth inhibition assay

To assess the antiproliferative activity of siRNAs, at different intervals after transfection adherent cells were trypsinized and counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). The percentages of adherent viable cells were determined by the Trypan blue dye exclusion test. Each experimental sample was run in triplicate. The results were expressed as percentage variation in the total number of adherent cells in siRNA-transfected cells compared to control cells.

2.8. Apoptosis analysis

Samples of 1×10^6 cells were fixed in 70% (v/v) ethanol and stained with solution A containing 50 μg/ml propidium iodide, 50 mg/ml RNAse, and 0.05% (v/v) Nonidet P40 (NP40) for 30 min at 4°C. The fluorescence of stained cells was measured by a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). Flow-cytometric sub-$G_{0/1}$ peak was detected on DNA plots using the CellFit software according to the SOBR model (Becton Dickinson). After propidium iodide staining, an aliquot of the cell suspension was spotted onto slides and assessed for typical apoptotic nuclear morphology (nuclear shrinkage, condensation and fragmentation) under a fluorescence microscope with appropriate filter combinations. The percentage of apoptotic cells was determined by two independent observers scoring at least 500 cells in each sample. The catalytic activity of caspase-9 was determined by means of the Caspase-9/Mch6 Fluorometric Protease Assay Kit (MBL, Ltd). Total protein and the specific fluorogenic substrate (leu-glu-his-asp-7-amino-4-trifluoromethylcoumarin, LEHD-AFC) were mixed for 1 h at 37°C. Hydrolysis of LEHD-AFC was monitored by spectrofluorometry at 505 nm.

2.9. Statistical analysis

The relationship between the level of expression of IAP family members or pro-apoptotic Smac/DIABLO and clinicopathologic features was analyzed with the Wilcoxon's rank-sum and Kruskal–Wallis tests. The degree of association between the expression of survivin and the other apoptosis-related (IAP-1, IAP-2, X-IAP, Smac/DIABLO, and apoptotic index as defined by M30 immunoreactivity) and proliferation-related markers (Ki-67 as detected by MIB-1 immunostaining) was tested using Spearman's correlation coefficient. For survival analysis, the investigated biomarkers were considered as continuous as well as dichotomous variables using as cutpoint to categorize low- versus highly-expressing tumors the median value of their frequency distribution. Survival was calculated from the date of surgery to the date of death or the time of last follow-up, for censored cases. The estimated survival curve distribution was calculated by the Kaplan–Meier method, and compared using the log-rank test. Student's t test was used to analyze the differences between control and siRNA-transfected cells in terms of survivin protein expression, cell growth, rate of apoptosis, and catalytic activity of caspase-9. Differences were considered statistically significant at $P < 0.05$ (two-sided) in all statistical testing.

3. Results

3.1. Expression of apoptosis- and proliferation-related markers in human peritoneal mesothelioma and clinicopathologic features

Tumor cells showed a cytoplasmic staining pattern for the IAP family members IAP-1 and X-IAP and for the pro-apoptotic Smac/DIABLO, whereas for survivin and IAP-2 also nuclear staining was detectable (Fig. 1A–D). The intensity of the immunoreactivity signal was usually homogeneous within a given case and the rate of stained tumor cells varied depending on the evaluated molecular marker (Table 3), whereas for each individual marker immunoreactivity was generally independent of the clinical situation (untreated or pretreated cases). The subcellular localization of survivin was usually either cytoplasmic or nuclear, and cells with survivin coexpressed in the nucleus and cytoplasm were rarely observed. On the whole, the proportion of cytoplasmic and nuclear positive cells ranged from 0 to 100% (median value of expression 60%) and from 0 to 20% (median value of expression 1.5%), respectively, with no qualitative or quantitative correlation between nuclear and cytoplamic immunoreactivity. In fact, according to the threshold values, 19 of the 32 MPMs (59%) contained cells in which

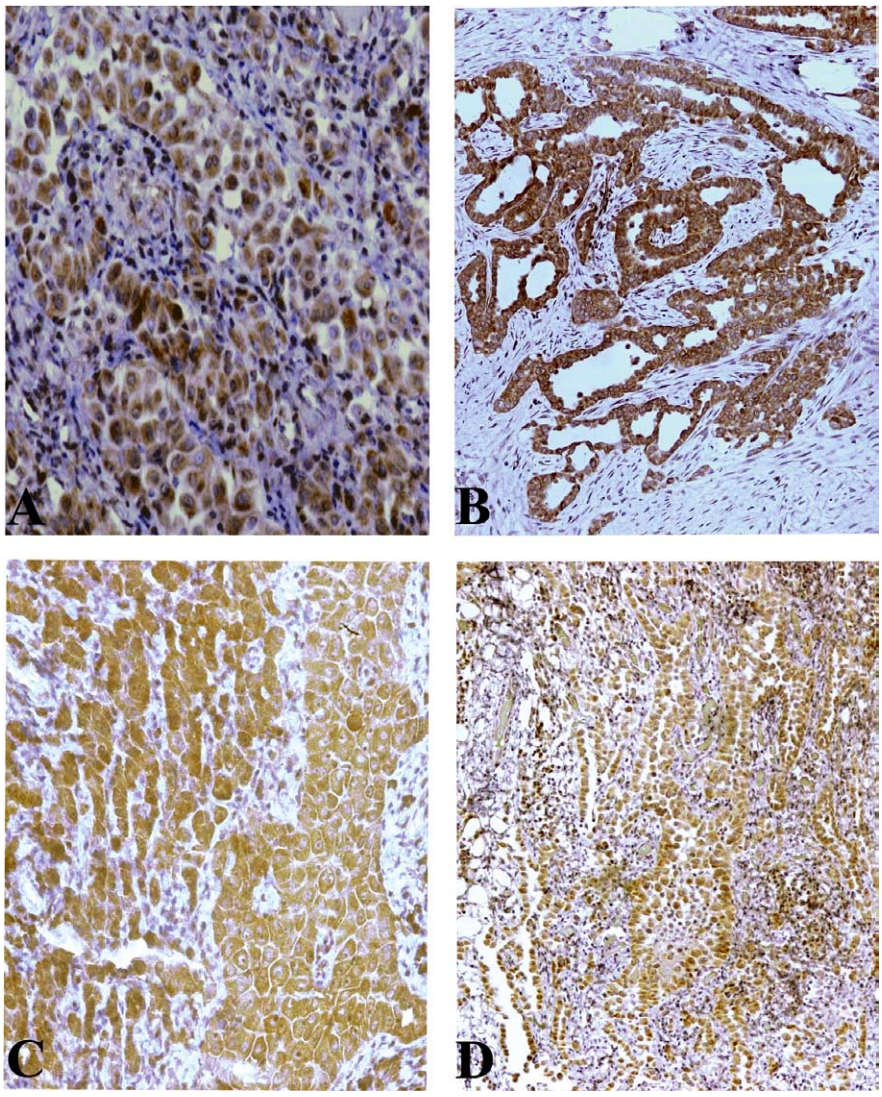

Fig. 1. Immunohistochemical expression of survivin (A), IAP-2 (B), IAP-1 (C) and X-IAP (D) in MPM specimens (amplification: A, C: 200X; B, D: 100X).

Table 3
Staining characteristics for IAP family members, Smac/DIABLO, apoptotic and proliferation indices in peritoneal mesothelioma

	Median expression, % (range, %)		
	Overall series (32 cases)	Previously untreated (17 cases)	Previously treated with systemic chemotherapy (15 cases)
Survivin, full length*	60 (0–100)	70 (10–100)	60 (0–100)
Survivin, specific nuclear form	1.5 (0–20)	2.2 (0–20)	1.5 (0–13.2)
IAP-2*	90 (30–100)	90 (60–100)	90 (30–100)
IAP-1	95 (40–100)	90 (70–100)	100 (40–100)
X-IAP	50 (0–100)	50 (0–100)	30 (0–100)
Smac/DIABLO	5 (0–90)	0 (0–90)	20 (0–90)
AI, CK18-caspase cleavage product	0.45 (0–5.8)	0.80 (0–5.0)	0.40 (0–5.8)
PI, Ki-67	10 (0–50)	5 (1–30)	10 (0–50)

AI, apoptotic index; PI, proliferation index.

* Cytoplasmic/nuclear subcellular distribution.

survivin was moderately/highly expressed only in the cytoplasm, 2 samples (6%) contained cells in which survivin was moderately/highly expressed only at the nuclear level, and 5 samples (16%) contained a population of moderately/highly survivin expressing cells at the cytoplasmic and/or nuclear level. In the remaining 6 cases no survivin immunoreactivity was seen.

While IAP-2 showed cytoplasmic and nuclear staining, the expression of IAP-1 and X-IAP was exclusively cytoplasmic, and the overall median fraction of mesothelioma cells positive for IAP-1, IAP-2 and X-IAP immunostaining was 90% (range, 40–100%), 95% (range, 30–100%) and 50% (range, 0–100%), respectively. Immunoreactivity for Smac/DIABLO was present in the cytoplasm and the overall median fraction of positive cells was 5% (range, 0–90%). It is worth mentioning that in half of the 32 evaluated mesotheliomas no positive cells to Smac/DIABLO were detectable. The percentage of moderately/highly expressing peritoneal mesotheliomas was 100% for IAP-2 and IAP-1, and 68.7% and 34.4%, respectively for X-IAP (22/32 cases) and Smac/DIABLO (11/32 cases). The expression of the different IAPs was also determined by immunoistochemistry in 7 normal peritoneum specimens. A lack of survivin and X-IAP expression was observed in all tested lesions, whereas the presence of IAP-1 and IAP-2 was simultaneously found in 2 lesions (data not shown). Moreover, due to the availability of frozen tissue, for 12 MPM and 7 normal peritoneum specimens it was also possible to assess the expression of survivin mRNA by RT-PCR (Fig. 2). The presence of survivin transcript was observed in 11 cases (91.6%) that also expressed survivin protein, as assessed by immunohistochemistry. The remaining case was negative for survivin expression at both mRNA and protein levels. No survivin transcript was consistently observed in all normal peritoneum samples (Fig. 2).

The CK18-caspase cleavage product staining in tumor samples was exclusively cytoplasmic, and the number of positively stained tumor cells ranged from 0 to 5.8% with a median expression of 0.45%. The frequency of Ki-67-positive nuclei varied from 0 to 50%, with a median value of 10%. When exploratory analyses of the relationships between the various molecular markers were performed, the only positive correlation was observed between survivin, nuclear localization, and spontaneous apoptosis (r_s [Spearman correlation coefficient] $= 0.49$, $P = 0.0047$) or proliferation index ($r_s = 0.57$, $P = 0.0006$), as well

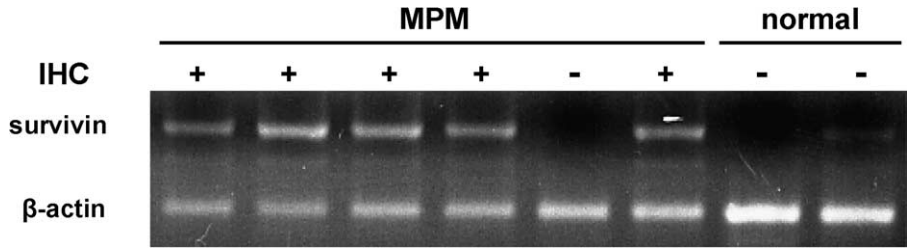

Fig. 2. Survivin mRNA expression as detected by RT-PCR in a representative series of MPM and normal peritoneum specimens. The presence (+) or absence (−) of survivin, as detected by immunohistochemistry (IHC), is reported for each sample.

as X-IAP and spontaneous apoptosis ($r_s = 0.45$, $P = 0.0104$) or proliferation index ($r_s = 0.48$, $P = 0.0055$), and these findings were maintained regardless of the previous clinical situation. Additionally, a strong inverse association was observed between X-IAP and Smac/DIABLO expression ($r_s = -0.744$; $P < 0.0001$). Moreover, no correlation was observed between the expression of the investigated molecular markers and age and gender of the patient, tumor histology, preoperative systemic chemotherapy, extent of carcinomatosis, and degree of cytoreduction.

We explored the interaction between pro- and anti-apoptotic proteins characterized in the present series of peritoneal mesotheliomas. An inverse association was consistently found, regardless of the cutpoint used to define overexpressing tumors (the specific median value of immunostaining for each IAPs or the unique cutpoint of 25% immunostained cells) between Smac/DIABLO expression and IAP coexpression. Overexpression of all 4 IAP family members (which was apparent in 16 of 32 MPMs) was associated with down-regulation of Smac/DIABLO expression. In fact, in the 16 cases overexpressing survivin, IAP-1, IAP-2 and X-IAP, the median expression value of the pro-apoptotic Smac/DIABLO protein was 0% (range, 0–50%), and it increased to 40% (range, 0–90%) in the 14 cases in which 3 IAPs were expressed, and to 60–70% in the 2 cases in which coexpression of 2 IAPs was observed ($P = 0.0032$).

At present, with the exception of a trend in favor of a longer median survival time for patients presenting with slowly (Ki-67 $< 10\%$) than with rapidly proliferating MPMs (51 month versus 27 month), in this series of MPM patients subjected to CRS and HIPEC, survival proved not to be affected by the expression of survivin, IAP-1, IAP-2, X-IAP and Smac/DIABLO, considered singly and in association, or by the apoptotic index. No further improvement in the association between clinical outcome and the biomarkers under investigation was obtained by considering biomarker expression as a continuous variable, even after its logarithmic transformation.

3.2. *siRNA-mediated down-regulation of survivin causes a decline in cell growth and induction of apoptosis in peritoneal mesothelioma cells*

To gain insight into the role of survivin in sustaining the growth of peritoneal mesothelioma cells, we used an RNA-interference–based strategy to down-regulate its expression in an established peritoneal mesothelioma cell line (STO). Western blotting experiments carried out in cells collected at different intervals after a 8-h transfection with 100 nM survivin-specific siRNA (Fig. 3A) showed a significant reduction of survivin protein abundance compared with control, oligofectamine-exposed cells. Such inhibition was highest (around 80%; $P < 0.01$) at 48 and 72 h after transfection and still appreciable, although to a lesser extent (around 50%; $P < 0.02$), at 96 h (Fig. 3B). Conversely, transfection of STO cells with the survivin-specific siRNA did not interfere with the expression of other IAPs, including IAP-1, IAP-2 and X-IAP, as assessed at 72 h (Fig. 3C). Transfection of STO cells with a control, unrelated siRNA failed to significantly modulate the expression of the different IAPs at any time point considered (Fig. 3A, B).

We further evaluated the effects of siRNA-mediated survivin down-regulation on the proliferative potential of STO cells. Transfection with survivin-specific siRNA resulted in a time-dependent inhibition of cell growth (Fig. 4) , which was appreciable 48 h after transfection (-40% with respect to oligofectamine-exposed cells, $P < 0.05$) and increased progressively over time. Specifically, growth inhibition of around 80% ($P < 0.01$) was observed at 96 h (Fig. 4). Conversely, transfection with the control, unrelated siRNA did not appreciably affect STO cell growth (Fig. 4).

We tested by flow cytometry whether the cytotoxic effect consequent upon survivin down-regulation was

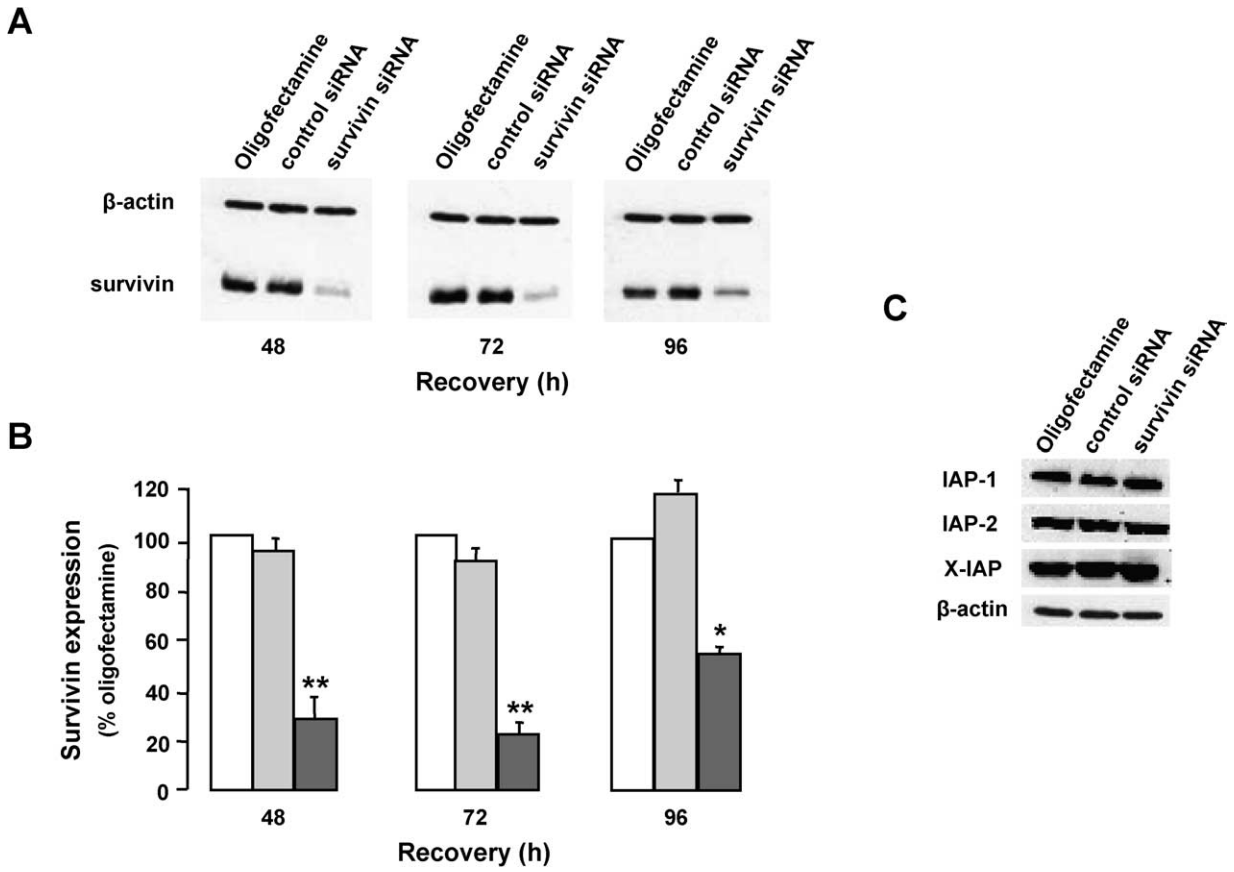

Fig. 3. (A) Representative Western blotting experiments illustrating survivin expression in STO cells exposed to oligofectamine alone or transfected with control siRNA and survivin siRNA. β-actin was used as control for protein loading. (B) Densitometric quantification of survivin band intensities in oligofectamine-exposed cells (empty column) and cells transfected with the control siRNA (gray column) or the survivin siRNA (black column). Data represent mean values $\pm$ SD of 3 independent experiments. $^{*}P < 0.02$; $^{**}P < 0.01$; Student's t test. (C) A representative Western blotting experiment illustrating the expression of other anti-apoptotic proteins (IAP-1, IAP-2 and X-IAP) in STO cells 72 h after transfection of with the survivin-specific siRNA.

due to the induction of apoptosis, and observed the appearance of an apoptotic sub-$G_{0/1}$ peak in cells transfected with the survivin siRNA but not the control siRNA nor in oligofectamine-exposed cells. A modest peak (4.1% of the overall cell population) was already appreciable 48 h after transfection; it progressively increased over time to account for 39.9% of the overall cell population at 96 h (Fig. 5A).

To further verify that programmed cell death resulted from transfection of survivin siRNA, we assessed the presence of cells with an apoptotic nuclear morphology by fluorescence microscopy after cell staining with propidium iodide (Fig. 5B). At all time points considered, apoptosis was observed in a negligible fraction (always <3%) of cells transfected with the control siRNA and in oligofectamine-exposed cells. Conversely, the rate of apoptosis in cells transfected with the survivin siRNA markedly increased with time to reach 38.5% ($P < 0.01$) of the overall cell population at 96 h (Fig. 5B).

At the molecular level, siRNA-mediated inhibition of survivin expression in STO cells coincided with a significantly increased catalytic activity of caspase-9. Specifically, the enzyme's activity, as assessed by in vitro hydrolysis of the specific fluorogenic substrate LEHD-AFC, was 2.6-fold and 4.1-fold higher ($P < 0.01$) in STO cells transfected with the survivin siRNA than in oligofectamine-exposed cells at 72 h and 96 h after transfection, respectively (Fig. 5C). Conversely, no appreciable difference in caspase-9 activation was found in cells transfected with the control siRNA compared to oligofectamine-exposed cells (Fig. 5C).

Finally, to test whether the basal level of survivin plays a role in the *in vitro* sensitivity of peritoneal

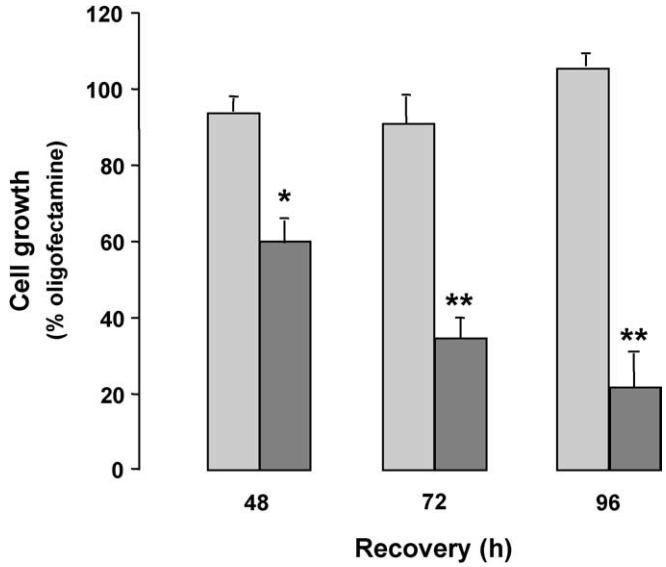

Fig. 4. Effects of siRNA-mediated survivin down-regulation on in vitro growth of STO cells. Data are expressed as percentage values of cell growth in cells transfected with the control siRNA (gray column) or the survivin siRNA (black column) as compared to that of control cells exposed to oligofectamine alone. Data represent mean values ± SD of 3 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; Student's t test.

mesothelioma cells to anticancer drugs, we examined the effect of survivin down-regulation on the apoptotic response to cisplatin and doxorubicin 72 h after treatment. Exposure to different concentrations of cisplatin (from 7.0 to 70 μM) induced a dose-dependent increase in the percentage of apoptotic cells, which accounted for 1.5% to 36.1% of the overall cell population in oligofectamine-exposed cells. Such an increase was slightly higher, although not significantly different, in cells transfected with the control siRNA. Conversely, the extent of cisplatin-induced programmed cell death was significantly ($P < 0.01$) higher in cells exposed to the survivin siRNA than in cells transfected with the control siRNA or treated with oligofectamine and accounted for 30.5% to 85.9% of the overall cell population (Fig. 6A). A dose-dependent increase in caspase-9 catalytic activity was also observed following cisplatin exposure, and such an enhancement was significantly ($P < 0.02$) higher in cells transfected with the survivin siRNA than in those exposed to the control siRNA or oligofectamine (Fig. 6B).

Results obtained after a 1-h exposure to different doxorubicin concentrations (from 0.03 to 3.0 μM) showed a dose-dependent enhancement in the percentage of apoptotic cells, which was similar in cells exposed to oligofectamine or transfected with the control siRNA and accounted for 1% to 24.5% of the overall cell population. However, a significantly ($P < 0.05$) higher induction of apoptotic response (ranging from 9.9% to 63.2%, as a function of drug concentration) was found in cells transfected with the survivin siRNA (Fig. 6A). Also in this case, a dose-dependent activation of caspase-9 activity was consistently observed, although the extent of such an activation was significantly ($P < 0.02$) higher in cells transfected with the survivin siRNA than in those exposed to the control siRNA or oligofectamine (Fig. 6B).

4. Discussion

Expression of the IAP family member survivin has been documented in the majority of human tumor types [2], and in several instances high levels of the protein were shown to be predictive of tumor progression [1] and/or resistance to chemotherapy [42]. As regards mesotheliomas, studies have been limited to malignant pleural mesothelioma. Specifically, Xia et al. [40] showed survivin overexpression in 12 of 12 clinical specimens analyzed; more recently, Falleni et al. [11] reported significantly increased levels of survivin mRNA in pleural malignant mesotheliomas compared to normal/fibrotic pleural tissues.

In the present study the pattern of expression of survivin and other IAP family members (IAP-1, IAP-2 and X-IAP) has been analyzed for the first time in a large series of MPMs from patients entered a locoregional treatment protocol carried out in a single insti-

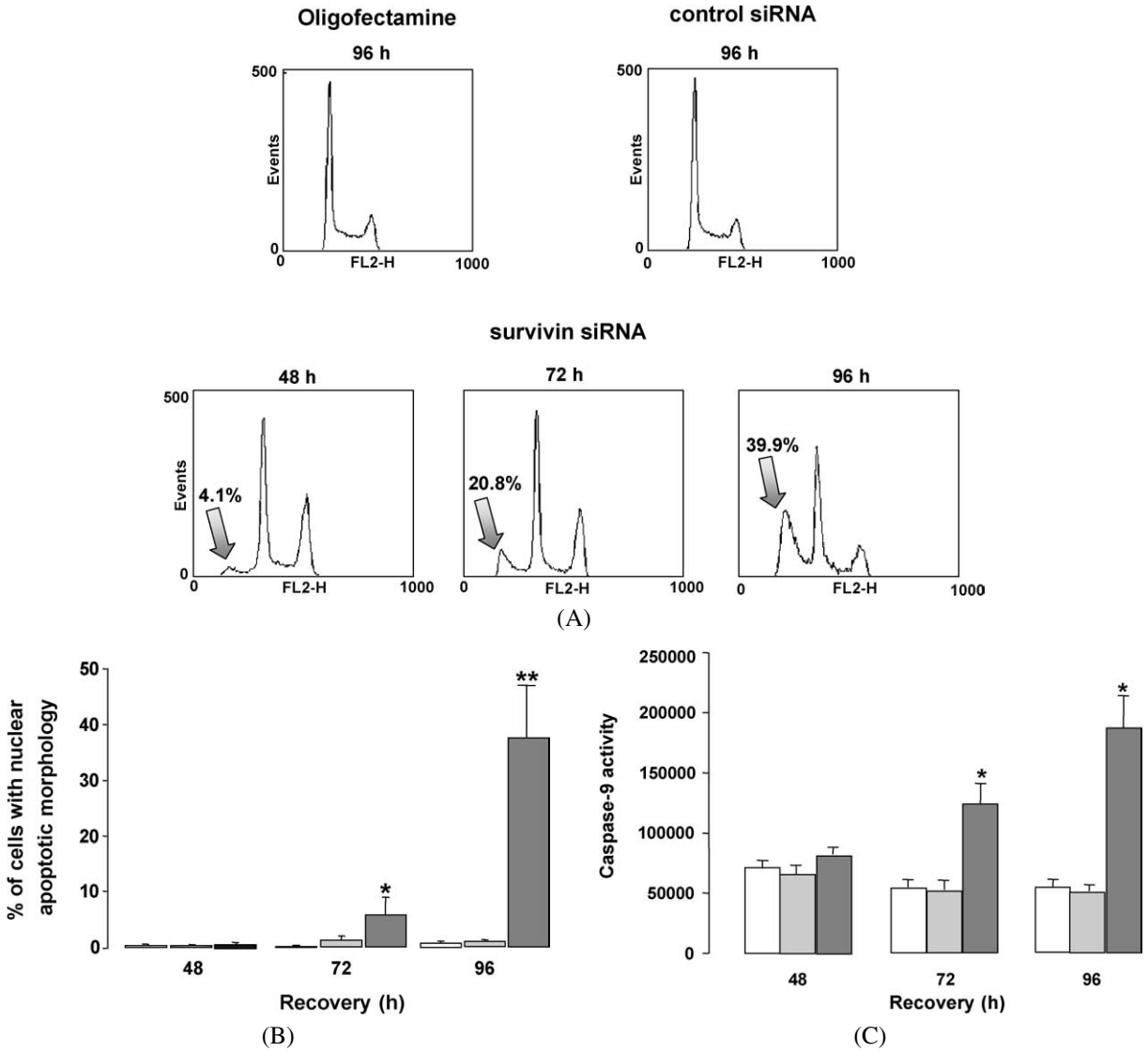

Fig. 5. Effects of siRNA-mediated survivin down-regulation on the apoptotic rate of STO cells. (A) Flow-cytometric analysis of STO cells stained with propidium iodide at different intervals after transfection with control siRNA and survivin siRNA. The sub-G_1 cell population percentages are reported in the bottom left corner of the histogram. (B) The percentage of cells with an apoptotic morphology with respect to the overall population as assessed by fluorescence microscopy in STO cells exposed to oligofectamine alone (empty column) and transfected with the control siRNA (gray column) or the survivin siRNA (black column). Data represent mean values ± SD of 3 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; Student's t test. (C) Catalytic activity of caspase-9 as determined by hydrolysis of the fluorogenic substrate LEHD-AFC in control cells exposed to oligofectamine alone (empty column) and transfected with the control siRNA (gray column) or the survivin siRNA (black column). Data are expressed as relative fluorescence units and represent mean values ± SD of 3 independent experiments. $^{*}P < 0.01$; Student's t test.

tution. Results from immunohistochemical analysis indicate that IAPs are upregulated in a high percentage of tumors, ranging from 69% to 100% as a function of the different proteins. Specifically, survivin protein expression was seen in 90.6% of the lesions. This finding was consistent with the presence of survivin mRNA in 11 out of 12 (91.6%) lesions for which frozen material was available. Moreover, the frequently observed simultaneous overexpression of the different IAPs in individual specimens suggests that this family of anti-apoptotic proteins is heavily dysregulated in MPMs. A lack of survivin and X-IAP expression was consistently found in the normal peritoneum samples examined. Conversely, IAP-1 and IAP-2 were simultane-

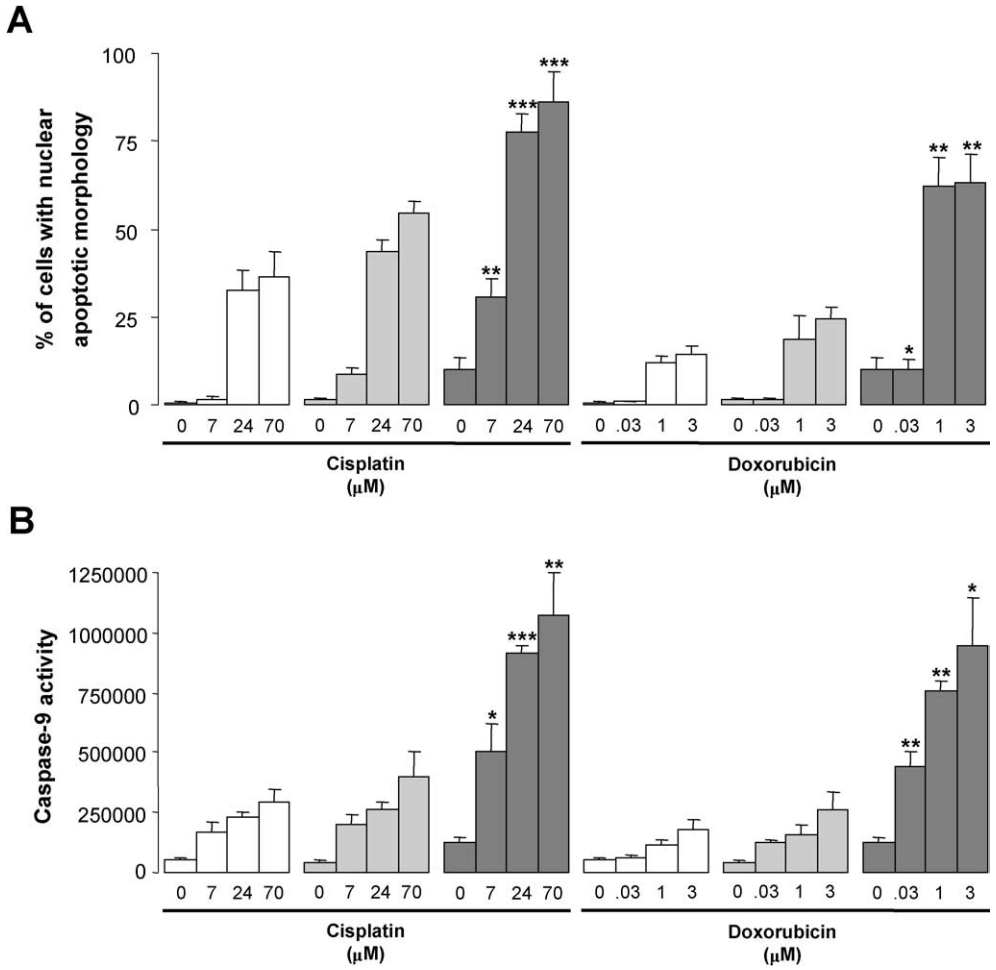

Fig. 6. Effects of siRNA-mediated survivin down-regulation on the apoptotic response of STO cells to cisplatin and doxorubicin. (A) The percentage of cells with an apoptotic morphology with respect to the overall population as assessed by fluorescence microscopy in STO cells exposed to oligofectamine alone (empty column) and transfected with the control siRNA (gray column) or the survivin siRNA (black column) in the absence or presence of different cisplatin or doxorubicin concentrations. Data represent mean values ± SD of 3 independent experiments. $^{*}P < 0.05$; $P^{**} < 0.01$; $^{***}P < 0.001$; Student's t test. (B) Catalytic activity of caspase-9 in control cells exposed to oligofectamine alone (empty column) and transfected with the control siRNA (gray column) or the survivin siRNA (black column) in the absence or presence of different cisplatin or doxorubicin concentrations. Data are expressed as relative fluorescence units and represent mean values ± SD of 3 independent experiments. $^{*}P < 0.02$; $^{**}P < 0.01$; $^{***}P < 0.001$; Student's t test.

ously present in 2 out of 7 cases. This last finding is in accordance with the documented expression of the two IAPs in a variety of normal adult tissues [37].

Very recently Gordon et al. [15] investigated the expression of different IAPs, including survivin, IAP-1, IAP-2, X-IAP and livin in pleural mesothelioma surgical specimens, and found that at least one IAP was present in all tested lesions. The positivity rates for the different IAPs ranged from 18% to 100% and from 51% to 100% when the gene expression was analyzed at mRNA and protein levels, respectively. It was found that survivin mRNA expression was associated with a relatively unfavourable patient outcome. Conversely, X-IAP or livin mRNA expression was associated with a relatively longer patient survival. However, no data were reported concerning the prognostic values of the expression of the IAPs when assessed at the protein level [15].

Caspases are the executioners of apoptosis in both intrinsic and extrinsic pathways [33]. The activated caspases are subject to inhibition by IAPs through direct binding [9,34]. This inhibitory effect can be abrogated by Smac/DIABLO, a pro-apoptotic factor that is released from mitochondria and, following bind-

ing to IAPs, reactivates initiator and effector caspases by relieving IAP-mediated inhibition [10]. Our results demonstrate for the first time that Smac/DIABLO is down-regulated in the majority (about 65%) of MPMs, mainly those presenting with concomitant up-regulated expression of IAPs. This last finding can be explained at least partially by the evidence that some IAPs such as X-IAP and, to a lesser extent, IAP-1 and IAP-2 exhibit E3 ubiquitin ligase activity and mediates polyubiquitination and consequent degradation of Smac/DIABLO [6,22]. Our data are in line with those obtained in a recent study dealing with the immunohistochemical analysis of Smac/DIABLO expression in different types of human carcinomas and sarcomas and showing a lack of expression of the pro-apoptotic protein in 49% of tumors analyzed [41]. Such results point to an important role of IAP overexpression in preventing apoptosis in MPMs and suggest that resistance to programmed cell death may contribute to the insensitivity of this tumor type to standard therapies.

Since CRS and HIPE have a major impact on the overall survival of patients with diffuse MPM [8,36], it is likely that a larger case series with a longer follow-up is mandatory to define the potential impact of biological markers on patient prognosis. The finding that we did not observe any association between the expression of the different apoptosis-related factors and patient survival could be due, at least in part, to the prevalent deregulation of the apoptotic pathways in the majority of the tumors and in most of the cells within individual tumors, which might affect the possibility to detect a distinct clinical behavior as a function of apoptosis-related markers. Moreover, it is worth mentioning that an association between survivin, or other IAPs, with cancer patient prognosis has been reported in some studies but not in others. Specifically, inconsistent and sometimes contrasting results have been obtained as a function of intracellular localization (nuclear *versus* cytoplasmic) of the protein [20]. However, a selection bias in the cases selected for the present study cannot be excluded, nor can the actual benefit of a potentially effective salvage treatment, which is able to counteract the intrinsic tumor aggressiveness. In fact, it is noteworthy that only a proliferation feature like Ki-67 expression, which may reflect the natural history of the disease, trendily outperformed the other biomarkers under investigation. Moreover, Nonaka et al. [23] recently reported that in a series of 35 MPMs, mitotic index was found to be associated with overall survival and progression free-survival. Conversely a lack of prognostic significance was observed for other biologic markers including epidermal growth factor receptor, p16, and metalloprotease-2 and -9.

In recent years researchers have made considerable efforts to develop strategies for modulating apoptosis in cancer [13]. In this context, approaches to counteract survivin, an IAP family member that acts as a cell survival factor as it is involved not only in the inhibition of apoptosis but also in control of mitotic progression of tumor cells [4], have been proposed with the dual aim to inhibit tumor growth and enhance the response of tumor cells to apoptosis-inducing agents [3]. Taking into account the huge expression of survivin in MPM but not in normal peritoneum and considering that no information is available regarding the effects of the modulation of survivin expression on the proliferative potential and chemosensitivity profile of MPM cells, we developed in the present study an RNA-interference–mediated approach to inhibit survivin expression in an established MPM cell line. Survivin down-regulation was efficiently achieved through the use of a 21-mer siRNA targeting a consensus sequence within exon 1 of survivin mRNA, and the specificity of the inhibitory approach was also confirmed by the lack of any effect following cell transfection with a control, unrelated siRNA. Silencing of the survivin gene resulted in a significant and time-dependent decline in cell proliferation and an increase in the rate of spontaneous apoptosis. These findings corroborate previous evidence obtained in experimental models of pleural mesothelioma and other human tumor types indicating that interference with survivin function by the use of siRNAs [27,36,40] and other molecular antagonists, including antisense oligonucleotides [16,40], hammerhead ribozymes [28,30] and dominant-negative mutants [17,44], leads to increased apoptotic cell death.

In STO cells transfected with survivin-specific siRNAs we also found significant enhancement of the in vitro catalytic activity of caspase-9, which is consistent with the recognized selective role of survivin in antagonizing mitochondrial-dependent apoptosis. However, it is still unclear whether survivin inhibits caspases through direct binding or indirectly, thereby requiring intermediate proteins [32]. In this context, a possible direct interaction of survivin with caspase-9 was reported by O'Connor et al. [24], whereas, more recently, Song et al. [35] suggested an alternative model for indirect inhibition of caspases by survivin based on its ability to physically interact with Smac/DIABLO. Accordingly to Song's model, the capability of survivin to sequester Smac/DIABLO would allow other IAPs to block caspases without being antagonized.

The possibility to restore the susceptibility to programmed cell death as a consequence of survivin gene silencing appears particularly relevant in STO cells since they are characterized by the overexpression of additional anti-apoptotic proteins such as those belonging to the Bcl-2 family, Bcl-2 and Bcl-X_L (data not shown). Interestingly, concomitant expression of anti-apoptotic factors belonging to both the IAP and Bcl-2 families seems to be a common feature in surgical MPM specimens (data not shown).

A number of in vitro and in vivo studies indicated that survivin down-regulation was able to sensitize human tumor cells of different histologic origin to conventional chemotherapeutic drugs with distinct mechanisms of action [3] as well as to ionizing radiation [29]. Here we demonstrated that the level of survivin expression influences the in vitro response of MPM cells to cisplatin and doxorubicin as suggested by the significantly increased apoptotic response observed after sequential treatment with a survivin-specific siRNA and the individual drugs.

This finding has potential clinical implications since it could provide a rational basis for the design of combined therapies, including survivin inhibitors, to improve the responsiveness of MPM to chemotherapy. However, considering the presence of other anti-apoptotic factors besides survivin in MPMs, it is likely that approaches based on the simultaneous targeting of different cytoprotective factors could be used to obtain enhancement of MPM cell sensitivity to agents available in the clinical setting.

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References

[1] D.C. Altieri, Survivin and apoptosis control, *Adv. Cancer Res.* **88** (2003), 31–52.

[2] D.C. Altieri, Survivin, versatile modulation of cell division and apoptosis in cancer, *Oncogene* **22** (2003), 8581–8589.

[3] D.C. Altieri, Validating survivin as a cancer therapeutic target, *Nat. Rev. Cancer* **3** (2003), 46–54.

[4] D.C. Altieri, The case of survivin as a regulator of microtubule dynamics and cell-death decisions, *Curr. Opin. Cell Biol.* **18** (2006), 609–615.

[5] A. Carvalho, M. Carmena, C. Sambade et al., Survivin is required for stable checkpoint activation in taxol-treated HeLa cells, *J. Cell Sci.* **116** (2003), 2987–2998.

[6] E.M. Creagh, B.M. Murphy, P.J. Duriez, C.S. Duckett and S.J. Martin, Smac/Diablo antagonizes ubiquitin ligase activity of inhibitor of apoptosis proteins, *J. Biol. Chem.* **279** (2004), 26906–26914.

[7] M. Deraco, N. Santoro, O. Carraro et al., Peritoneal carcinomatosis: Feature of dissemination, A review, *Tumori* **85** (1999), 1–5.

[8] M. Deraco, M. De Simone, C.R. Rossi et al., An Italian Multicentric phase II study on peritonectomy and intra peritoneal hyperthermic perfusion (IPHP) to treat patients with peritoneal mesothelioma, *J. Exp. Clin. Cancer Res.* **22** (2003), 41–45.

[9] Q.L. Deveraux and J.C. Reed, IAP family proteins-suppressors of apoptosis, *Genes Dev*. **13** (1999), 239–252.

[10] C. Du, M. Fang, Y. Li, L. Li and X. Wang, Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition, *Cell* **102** (2000), 33–42.

[11] M. Falleni, C. Pellegrini, A. Marchetti et al., Quantitative evaluation of the apoptosis regulating genes, *Lung Cancer* **48** (2005), 211–216.

[12] D.A. Fennell and R.M. Rudd, Defective core-apoptosis signalling in diffuse malignant pleural mesothelioma: opportunities for effective drug development, *Lancet Oncol*. **5** (2004), 354–362.

[13] U. Fischer and K. Schulze-Osthoff, New approaches and therapeutics targeting apoptosis in disease, *Pharmacol. Rev.* **57** (2005), 187–215.

[14] G.J. Gordon, K. Appasani, J.P. Parcells et al., Inhibitor of apoptosis protein-1 promotes tumor cell survival in mesothelioma, *Carcinogenesis* **23** (2002), 1017–1024.

[15] G.J. Gordon, M. Mani, L. Mukhopadhyay et al., Expression patterns of inhibitor of apoptosis proteins in malignant pleural mesothelioma, *J. Pathol.* **211** (2007), 447–454.

[16] D. Grossman, J.M. McNiff, F. Li and D.C. Altieri, Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma, *J. Invest. Dermatol.* **113** (1999), 1076–1081.

[17] D. Grossman, P.J. Kim, J.S. Schechner and D.C. Altieri, Inhibition of melanoma tumor growth in vivo by survivin targeting, *Proc. Natl. Acad. Sci. USA* **98** (2001), 635–640.

[18] S. Hopkins-Donaldson, R. Cathomas, A.P. Simoes-Wust et al., Induction of apoptosis and chemosensitization of mesothelioma cells by Bcl-2 and Bcl-xL antisense treatment, *Int. J. Cancer* **106** (2003), 160–166.

[19] S. Kusamura, R. Younan, D. Baratti et al., Cytoreductive surgery followed by intraperitoneal hyperthermic perfusion: analysis of morbidity and mortality in 209 peritoneal surface malignancies treated with closed abdomen technique, *Cancer* **106** (2006), 1144–1153.

[20] F. Li, J. Yang, N. Ramnath, M.M. Javle and D. Tan, Nuclear or cytoplasmic expression of survivin: what is the significance?, *Int. J. Cancer* **114** (2005), 509–512.

[21] M. Markman and D. Kelsen, Efficacy of cisplatin-based intraperitoneal chemotherapy as treatment of malignant peritoneal mesothelioma, *J. Cancer Res. Clin. Oncol.* **118** (1992), 547–550.

[22] Y. Morizane, R. Honda, K. Fukami and H. Yasuda, X-linked inhibitor of apoptosis functions as ubiquitin ligase toward mature caspase-9 and cytosolic Smac/DIABLO, *J. Biochem. – Tokyo* **137** (2005), 125–132.

[23] D. Nonaka, S. Kusamura, D. Baratti et al., Diffuse malignant mesothelioma of the peritoneum: a clinicopathological study of 35 patients treated locoregionally at a single institution, *Cancer* **104** (2005), 2181–2188.

[24] D.S. O'Connor, D. Grossman, J. Plescia et al., Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin, *Proc. Natl. Acad. Sci. USA* **97** (2000), 13103–13107.

[25] S.L. O'Kane, R.J. Pound, A. Campbell et al., Expression of bcl-2 family members in malignant pleural mesothelioma, *Acta Oncol*. **45** (2006), 449–453.

[26] M.K. Ozvaran, X.X. Cao, S.D. Miller et al., Antisense oligonucleotides directed at the bcl-xl gene product augment chemotherapy response in mesothelioma, *Mol. Cancer Ther.* **3** (2004), 545–550.

[27] F. Paduano, R. Villa, M. Pennati et al., Silencing of survivin gene by small interfering RNAs produces supra-additive growth suppression in combination with 17-allylamino-17-demethoxygeldanamycin in human prostate cancer cells, *Mol. Cancer Ther.* **5** (2006), 179–186.

[28] M. Pennati, G. Colella, M. Folini et al., Ribozyme-mediated attenuation of survivin expression sensitizes human melanoma cells to cisplatin-induced apoptosis, *J. Clin. Invest.* **109** (2002), 285–286.

[29] M. Pennati, M. Binda, G. Colella et al., Radiosensitization of human melanoma cells by ribozyme-mediated inhibition of survivin expression, *J. Invest. Dermatol.* **120** (2003), 648–654.

[30] M. Pennati, M. Binda, G. Colella et al., Ribozyme-mediated inhibition of survivin expression increases spontaneous and drug-induced apoptosis and decreases the tumorigenic potential of human prostate cancer cells, *Oncogene* **23** (2004), 386–394.

[31] B. Price, Analysis of current trends in United States mesothelioma incidence, *Am. J. Epidemiol.* **145** (1997), 211–218.

[32] J.C. Reed, The survivin saga goes in vivo, *J. Clin. Invest.* **108** (2001), 965–969.

[33] G.S. Salvesen and V.M. Dixit, Caspase activation: the induced-proximity model, *Proc. Natl. Acad. Sci. USA* **96** (1999), 10964–10967.

[34] G.S. Salvesen and C.S. Duckett, IAP proteins: blocking the road to death's door, *Nat. Rev. Mol. Cell Biol.* **3** (2002), 401–410.

[35] Z. Song, X. Yao and M. Wu, Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis, *J. Biol. Chem.* **278** (2003), 23130–23140.

[36] P.H. Sugarbaker, T.D. Yan, O.A. Stuart and D. Yoo, Comprehensive management of diffuse malignant peritoneal mesothelioma, *Eur. J. Surg. Oncol.* **32** (2006), 686–691.

[37] B. Vischioni, P. van der Valk, S.W. Span et al., Expression and localization of inhibitor of apoptosis proteins in normal human tissues, *Hum. Pathol.* **37** (2006), 78–86.

[38] L. Weissmann, R. Osteen, J. Corson et al., Combined modality therapy for intraperitoneal mesothelioma, *Proc. Am. Soc. Clin. Oncol.* **7** (1988), 274.

[39] M. Wu, S. Yuan, A.H. Szporn et al., Immunocytochemical detection of XIAP in body cavity effusions and washes, *Modern Pathol.* **18** (2005), 1618–1622.

[40] C. Xia, Z. Xu, X. Yuan et al., Induction of apoptosis in mesothelioma cells by antisurvivin oligonucleotides, *Mol. Cancer Ther.* **1** (2002), 687–694.

[41] N.J. Yoo, H.S. Kim, S.Y. Kim et al., Immunohistochemical analysis of Smac/DIABLO expression in human carcinomas and sarcomas, *APMIS* **111** (2003), 382–388.

[42] N. Zaffaroni, M. Pennati and M.G. Daidone, Survivin as a target for new anticancer interventions, *J. Cell Mol. Med.* **9** (2005), 360–372.

[43] N. Zaffaroni, A. Costa, M. Pennati et al., Potential of survivin as a new therapeutic target in diffuse malignant mesothelioma of the peritoneum, Abstract n° 4613 presented at the 97th AACR Annual Meeting (April 1–5, 2006 – Washington, USA).

[44] D.E. Zhu, N. Hoti, Z. Song et al., Suppression of tumor growth using a recombinant adenoviral vector carrying the dominant-negative mutant gene Survivin-D53A in a nude mice model, *Cancer Gene Ther.* **13** (2006), 762–770.