Inhibitor of apoptosis protein-1 promotes tumor cell survival in mesothelioma


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Malignant pleural mesothelioma (MPM) is a highly lethal pleural neoplasm that is often resistant to chemotherapeutic drugs, including cisplatin, and for which little is known regarding carcinogenic pathways. We used differential display to compare gene expression patterns in mesothelioma, normal pleura and normal lung, in order to better understand MPM pathobiology, and to search for genes that may facilitate drug resistance in this cancer. The human inhibitor of apoptosis protein-1 gene (IAP-1/MIHC/cIAP2) was discovered to be highly expressed in MPM. We confirmed overexpression of IAP-1 mRNA and protein in 39 additional human MPM tumor specimens and 3/5 (60%) MPM cell lines by multiple methods, including real time quantitative reverse transcription–PCR and western blot analysis. Using an antisense targeting approach, we found that attenuation of IAP-1 mRNA levels decreases baseline cell viability and increases the sensitivity of MPM cell lines to cisplatin by nearly 20-fold. Reduced IAP-1 gene expression also results in a concordant increase of the pro-apoptotic cleavage product of caspase 9 and a reduction in the number of viable tumor cells. Our observations strongly suggest that IAP-1 is at least partly responsible for promoting carcinogenesis and mediating resistance to cisplatin in many MPM tumors and that further study of this apoptotic pathway is warranted.

Introduction

Malignant pleural mesothelioma (MPM) is a mesodermally derived, asbestos-related cancer that arises in the pleura and relentlessly expands into adjacent structures ultimately resulting in the death of the patient. The mechanisms of carcinogenesis in MPM are poorly understood and, unlike lung cancer, aberrant expression of either p53 or Ras is not believed to be a factor in MPM pathobiology (1–3). Approximately 3000 patients are diagnosed with MPM in the US annually; worldwide incidence of the disease is projected to continue to rise through the next two decades (4–6). There are three major histologic subtypes of MPM: epithelial, sarcomatoid and mixed. Most patients diagnosed with MPM have the epithelial subtype and generally enjoy a somewhat longer survival than patients with non-epithelial subtypes. With no treatment, the median survival of patients presenting with MPM is between 4 and 12 months. Chemotherapy or radiation therapy alone or in combination are not generally effective and the majority of patients will die within a year regardless of treatment (7). Patients with early MPM who have good cardiorespiratory reserve are candidates for trimodality therapy, which includes extrapleural pneumonectomy followed by cisplatin chemotherapy and radiation therapy. Patients with more advanced disease and/or poor reserve are usually treated with one of a variety of chemotherapeutic regimens, the standard response to which ranges between 20 and 25%. Cisplatin is commonly used because it is well tolerated and can act as a radiation sensitizer. Cisplatin is also the preferred drug in a number of treatment protocols that involve intracavitary chemotherapy because it is easily inactivated intravascularly with sodium thiosulfate. Unfortunately, even the best candidates for aggressive treatment have only 40% 5 year survival, and the majority of patients will succumb to MPM much earlier (6,8,9). More effective chemotherapeutic drugs, or methods of sensitizing MPM to current drugs, are desperately needed. Enhancement of the cytotoxic effects of cisplatin-based chemotherapy in MPM by defining and overcoming inherent drug resistance is an important aspect in the effort to affect better treatment options for this disease.

Inhibitor of apoptosis protein-1 (IAP-1), also known as MIHC/cIAP2, is a member of a large family of genes that promote cell survival after apoptotic stimuli. IAP-1 is thought to play a role in carcinogenesis by protecting the tumor cell from apoptosis and promoting tumor development, is expressed by multiple types of tumor cell lines (10) and has been suggested to facilitate resistance of some lung cancer cell lines to gemcitabine (11). IAPs, in general, function primarily by inhibiting the apoptotic action of caspases either by preventing proteolytic cleavage of caspase proforms and/or inhibiting activated caspases directly (12,13). IAP-1 inhibits the mitochondrial pathway of apoptosis at the step following cytochrome c release by binding directly to caspase 9 proform, thus preventing its activation. IAP-1 can also inhibit the death receptor pathway initiated by association of TNF-α to cell surface receptors followed by caspase 8 activation (12).

In this study, we performed differential display analysis comparing human tissue specimens of MPM, normal lung and normal pleura to identify genes that are differentially expressed in MPM. IAP-1, one of 60 genes identified, was overexpressed in MPM tumor tissues compared with normal tissues. We chose to investigate IAP-1 further because of its potential involvement in MPM carcinogenesis and in mediating tumor chemoresistance. We confirmed IAP-1 mRNA overexpression in five MPM cell lines and 39 additional tumor specimens using quantitative reverse transcriptase–PCR (RT–PCR). Using antisense targeting strategies, we demonstrated that attenuation of IAP-1 gene expression in cell lines results in reduced baseline cell viability and heightened sensitivity to cisplatin-induced cytotoxicity. Our results strongly suggest

Abbreviations: IAP-1, apoptosis protein-1 gene; MPM, malignant pleural mesothelioma; RT–PCR, reverse transcription–PCR.
that IAP-1 plays an important role in mediating MPM carcino-
genesis and contributes to the inherent resistance of MPM to
cisplatin.

Materials and methods

Cell lines, tissues and clinical database

Established MPM cell lines were kindly provided by Jonathan A. Fletcher,
MD, Department of Pathology, Harvard Medical School, Boston,
Massachusetts. These cell lines were coded for research purposes in such a
way that no information could be derived about the individual
patients from their cell lines or lung adenocarcinoma
(H2L) and lung squamous cell carcinoma (520) were used as additional
controls, and were purchased from American Type Culture Collection
www.atcc.org). Cells were grown in a humidified (5% CO2) incubator under
RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing
10% fetal bovine serum and antibiotics. Discarded, flash frozen, normal lung,
normal pleura and MPM surgical specimens were freshly collected from
patients undergoing thoracic surgical procedures at Brigham and Women’s
Hospital. All tissues were obtained from patients who did not receive pre-
operative treatment. The following standard tissue banking procedures were
followed throughout. Once brought to the Hospital Tumor Bank, tissues were
sliced into 3 mm2 portions and assigned an identifier to catalog their position in
the original specimen. Hematoxylin–eosin stained slides were generated from
each specimen in the Tumor Bank and reviewed by a pathologist for tumor
text and histological subtype. MPM tissues used in this study contained >50%
tumor cells. Linked clinical, epidemiological, outcome and pathological
data were obtained for all patients who contributed tumor specimens and rendered anonymous to protect patient confidentiality. Studies
utilizing human tissues were approved by and conducted in accordance with the
policies of the Institutional Review Board at Brigham and Women’s Hospital.

Differential display

Differential display was performed as described previously (14). RNA was
extracted from frozen specimens of normal lung, normal pleura and MPM
(epithelial subtype), each from a different patient, using a Qiagen RNA
extraction kit (Qiagen, Chatsworth, CA). Differential display was performed using
an RNAmage kit (GenHunter, Nashville, TN); 24 different primer
combinations were tested (three anchor primers and eight arbitrary primers).
Gels were visualized by autoradiography and 60 bands were selected repre-
senting mRNAs that appeared to be differentially expressed. These bands
were excised from the gel, purified (QiAgulcet Gel Extraction kit, Qiagen),
re-amplified by PCR and subcloned into the pCR-TRAP cloning vector
(GenHunter). All inserts were subjected to double-strand DNA sequencing
utilizing an ABI 377 DNA Sequencer. Resulting sequences were analyzed
(using a BLAST search) by comparison to reported sequences deposited in

In situ hybridization

To confirm differential expression of genes identified, fluorescent in situ
hybridization was performed on frozen cryosections from 61 MPM specimens
and three specimens each of normal lung and normal pleura. Cryosections
were cut to 6 µm, fixed in 4% paraformaldehyde for 4 h at 4°C, and treated
with 30% sucrose for 16 h at 4°C. The entire sequence of each gene fragment
was transcribed with T7 and SP6 RNA polymerase (Roche Molecular
Biochemicals, Indianapolis, IN) to generate both sense and antisense RNAs.
The in vitro transcription was performed with 50 ng cDNA, fluorescein-12-
dUTP, T7, SP6, Klenow polymerase or T7 RNA polymerase, as described by the manufacturer.
Labeled nucleotides were separated on a Sephacel G-50 column (Gene.Expressions
Systems, Waltham, MA). Labeled RNAs were collected from columns into
RNAse-free tubes, precipitated and resuspended in 50 µl 0.1 M diithiothreitol
(DTT). Nucleic acid and fluorescein label concentration was estimated using
spectrophotometric analysis at 260 and 495 nm, respectively. Processed tissue
cryosections were re-hydrated in 70% ethanol for 10 min and incubated in
DNSase-free RNase (100 µg/ml in 2× SSC) for 1 h at 37°C followed by
treatment with 0.2 M HCl for 20 min. Following pre-hybridization at 37°C for
2 h, hybridization was performed with a Uni-in-situ GeneLock kit
(GeneExpression Systems) and 10 ng denatured labeled RNA probe at 37°C for
15 h. Following hybridization, the probe was removed and the samples
washed with high stringency conditions. Finally, a drop of Slow-Fade reagent
(Molecular Probes, Eugene, OR) was added to the sections, which were
mounted and stored in the dark at 4°C until visualization with a Bio-
Rad MRC 1024 laser-based confocal microscope (Bio-Rad Laboratories,
Hercules, CA).

Cell survival studies

The sensitivity of MPM cell lines to cisplatin was determined as described
previously (15) with minor modifications. Cells in exponential growth phase
(30–60% confluent) were exposed to concentrations of cisplatin [cis-platinum
(II)diammine dichloride, Sigma, St Louis, MO] ranging from 2 to 250 µM in
RPMI medium without serum for 1 h at 37°C. Control studies were always
performed concurrently where cisplatin was omitted from the medium. Cells
were washed three times with PBS, treated with trypsin, and plated onto 6
well tissue culture plates at a density of 10 000 cells/well. (Cisplatin-exposed
or control cells were plated in three wells for each experiment, and all
experiments were repeated at least three times.) At 24 h post-plating, cells in
individual wells were collected with trypsin and counted. The average number
of surviving cells was expressed as a fraction relative to control cell numbers.
Survival curves in individual experiments were plotted and I50 values (the theoretical concentration
required to kill 50% of cells) were obtained as a relative measure of
drug sensitivity.

Northern blot analysis

Northern blot analysis was performed according to standard protocols (16) as
previously described (17). Total RNA (20 µg) was fractionated in 2.2 M
formaldehyde/1% agarose gels, visualized using ethidium bromide staining
to assess RNA degradation and transferred to Magnacharge nylon membranes
(Fisher Scientific, Atlanta, GA) via overnight capillary transfer in 10× SSC
buffer. Membranes were UV cross-linked and incubated in pre-hybridization
buffer (Sigma) for 30 min at 42°C. Probes were constructed for IAP-1 and
GAPDH by PCR amplification of MPM cDNA using the following primers
synthesized by Invitrogen Life Technologies: IAP-1 (5‘-TTTGGGG-
AATCTGGAGATGA-3’ and 5‘-CGGATGAACTCTGTTGTTT3’), GAPDH
(5‘-GAAGCTGAGGTCGAGGTGCTG-3’) and 5‘-GAAGATGTGATCGAGGT-
ATTTC-3’). Probes were labeled with α[32P]dCTP (New England Nuclear,
Boston, MA) using a random priming labeling kit (Sigma) and hybridized to the
membrane overnight at 42°C. Blots were washed under high stringency
conditions and visualized by exposure to X-ray film.

Western blot analysis

Subjects to protein visualization were lysed at 4°C in a buffer containing
20 mN Tris–HCl (pH 8), 137 mM NaCl, 2 mM EDTA, 10% glycerol,
1% Nonidet P-40, 200 mM sodium orthovanadate, 1 mM DTT, 1 mM
phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin and 5 mg/ml aprotinin.
Protein quantity was estimated using the Bio-Rad Protein Assay. For western
blot analysis, aliquots of cell lysates were hybridized with a protein solubiliza-
tion stock buffer consisting of 250 mM Tris–HCl (pH 7.4), 2% SDS, 30%
glycerol, 10% β-mercaptoethanol and 0.01% bromophenol blue (pH 6.8) to
a final concentration of 2 µg protein/µl and denatured by boiling for 7–10 min.
Samples containing 100 µg protein per lane were separated on pre-cast 10%
Tris–glycine polyacrylamide gels (Novex, San Diego, CA) and transferred
onto Hybond ECL nitrocellulose membranes (Amersham, Arlington Heights,
IL) according to standard procedures (16). All subsequent incubations
were carried out at room temperature. Nitrocellulose membranes were
incubated for 1 h in a blocking buffer consisting of 5% non-fat dry milk in
PBS (136 mM NaCl, 2.7 mM KCl, 10 mM Na2PO4 and 1.76 mM K2HPO4,
pH 7.2). Polyclonal antibodies to IAP-1 (Santa Cruz Biochemicals, Santa
Cruz, CA) and caspase 9 (Oncogene Research Products, Boston, MA) were
diluted 1:5000 in PBS at a density of 10 000 cells/cm2. (Cisplatin-exposed
or control cells were plated in three wells for each experiment, and all
experiments were repeated at least three times.) At 24 h post-plating, cells in
individual wells were collected with trypsin and counted. The average number
of surviving cells was expressed as a fraction relative to control cell numbers.
Survival curves in individual experiments were plotted and I50 values (the theoretical concentration
required to kill 50% of cells) were obtained as a relative measure of
drug sensitivity.

Real time quantitative RT–PCR

IAP-1 mRNA levels were quantified in normal pleural tissue, MPM cell lines
and tumor specimens using real time quantitative RT–PCR. PCR reactions were
optimized and performed precisely following the manufacturer’s recommended protocol
for the Applied Biosystems 7700 Sequence Detection System (Applied Biosystems).
Total RNA (2 µg) was reverse-transcribed into cDNA using Taq-Man
Reverse Transcription reagents and random hexamers as the primer (Applied
Biosystems). PCR reactions were set up in a 25 µl reaction volume using
SYBR Green PCR Master Mix (Applied Biosystems). Optimized primers
(from above) amplifying portions of IAP-1 and GAPDH were originally
designed according to recommended specifications (Applied Biosystems) and
used at a final concentration of 900 nM in the reaction mixture. PCR
amplification was performed in a 96 well format using optical plates and
covers (Applied Biosystems) in an Applied Biosystems 7700 Sequence
Detector. To confirm the absence of non-specific amplification in PCR
reactions, no-template controls containing H2O substituted for template were run in all wells.
A melting point dissociation curve was automatically generated after every experiment to
confirm the presence of a single PCR species in all experimental wells. The comparative Ct
method was used to obtain quantitative values for gene expression levels in all samples (Applied
Biosystems, see http://www.applied-
biosystems.com for details). This method normalizes expression levels between samples using another housekeeping gene (i.e. GAPDH) as a reference to standardize for different starting template amounts.

Vector construction
A 2.2 kb fragment of IAP-1 cDNA containing the entire coding sequence (nucleotides 2725–4925) was PCR-amplified from MPM cDNA using the following primers: 5'-TCCCTTTTCTCCCCATCA-3' and 5'-TGGCTTGATGTCCCGGAAGC-3'. The PCR product was purified (Wizard® PCR Prep, Promega, Madison, WI) and verified by gel electrophoresis and restriction digest. The resulting fragment was TA-cloned into the pcDNA3.1/V-5-His plasmid vector (Invitrogen Life Technologies) as recommended by the manufacturer. Resulting clones were analyzed for orientation by direct DNA sequencing across the insert using primers supplied by the manufacturer (Invitrogen Life Technologies). The complete sequence of the insert was confirmed using sequencing primers and internal primers (from RT–PCR). MPM cell line 94-589 was stably transfected with IAP-1 antisense-expressing vector using LipofectAMINE PLUS reagent (Invitrogen Life Technologies). Transfected cells were selected with G418 (300 μg/ml, Invitrogen Life Technologies), and several clones analyzed for IAP-1 mRNA levels by quantitative RT–PCR. All clones expressed similarly low levels of IAP-1 mRNA. One was chosen for further analysis.

Immunohistochemistry
Indirect immunoperoxidase analysis of IAP-1 protein was performed on 6 μM MPM cryosections fixed for 10 min in 95% ethanol at −20°C. IAP-1 detection was performed using an accelerated avidin/biotin peroxidase procedure per the manufacturer’s recommended protocol (Vectastain ABC Elite rabbit kit, Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was quenched by incubation in a 0.3% solution of H2O2 in methanol for 5 min. Blocking of non-specific activity was accomplished using an avidin/biotin blocking kit and diluted serum of the secondary antibody species (Vector Laboratories). Polyclonal antibodies to IAP-1 (Santa Cruz Biochemicals) were diluted 1:200 in PBS and incubated on tissue sections overnight at 4°C. IAP-1 antibodies were replaced with PBS as a negative control for every batch. Sections were developed in dianinobenzidine (DAB kit, Vector Laboratories) and lightly counterstained with Gill’s hematoxylin. Images were captured using an Olympus T041 microscope and color print film.

Densitometry and data analysis
Quantification of multiple western blots (n = 3) was accomplished using a densitometric-based analysis performed on scanned fluorograms using Kodak Digital Science ID software (v. 2.02). A two-tailed unpaired t-test was used to generate P values and determine the significance of all quantified differences in pixel density. GraphPad Prism v.3.02 (GraphPad Software, San Diego, CA) was used for regression analysis of survival curves (to extrapolate IC50 values) and to assess quantified differences in IAP-1 mRNA levels among tumor samples using a two-tailed Mann–Whitney test. The degree of correlation between patient survival and IAP-1 gene expression levels was examined using Spearman correlation calculations. All differences were determined to be statistically significant if P < 0.05.

Results
Identification of genes that are differentially expressed in MPM tumors
In an attempt to identify genes that mediate carcinogenesis in MPM, we compared patterns of gene expression using differential display in three human tissues: MPM (epithelial subtype), normal lung and normal pleura (Figure 1). Thirty bands representing differentially expressed genes were selected for sequence analysis. Band intensities (i.e. expression levels) were highest in pleura for 17 genes, in lung for 16 genes and MPM for 27 genes. Expression levels were below the detection limit for 13 genes in pleura, 10 genes in lung and 15 genes in MPM, given that no bands were visible under these circumstances. One-third of the sequenced bands had substantial homology to known genes (Table 1). The majority of the remainder genes were overexpressed in MPM and were highly homologous to expressed sequence tags (ESTs) isolated from a variety of tumor and/or fetal cell libraries. We chose fragment 39 (human IAP-1) for further analysis because of its known physiological role in apoptosis. Tissue-specific differences in mRNA expression levels for many genes identified by differential display were confirmed using fluorescent in situ hybridization (Figure 2) and northern blot analysis (17).

Expression of IAP-1 in human MPM tumor specimens
We quantified (using real time quantitative RT–PCR) the levels of IAP-1 mRNA in 39 MPM samples consisting of tumor specimens of epithelial (n = 18), mixed (n = 19) and sarcomatoid histology (n = 2). IAP-1 mRNA was detected in all 39 samples examined and overall showed a 90-fold median increase (range 2.5–1000-fold) relative to the average IAP-1 mRNA expression level in samples of normal pleura (n = 3). Tumors of epithelial histology generally possessed higher levels of IAP-1 mRNA compared with those of non-epithelial histology. Thirteen of 19 samples (68%) with mRNA levels above the median values were of the epithelial subtype. The median increase in IAP-1 mRNA levels in epithelial subtype tumors (181-fold) was significantly (P = 0.0028) higher than that for the non-epithelial tumors (64-fold). We next examined whether IAP-1 mRNA levels correlated with patient survival considering recent reports of a significant correlation in acute myeloid leukemia patients between clinical outcome and expression levels of XIAP (another IAP family member) (10). However, we did not find a significant correlation between IAP-1 gene expression and patient survival (P = 0.43) or any other clinical parameter. This is not surprising because the specimens tested were not standardized to any clinical parameters such as stage or treatment modality. Immunohistochemical analysis of MPM tumor cryosections confirmed the presence of IAP-1 protein in these samples. Relative IAP-1 protein levels in MPM tumor subtypes mirrored results of quantitative RT–PCR (Figure 3). IAP-1 protein levels were substantially lower and unevenly distributed among tumor cells in samples of non-epithelial (i.e. mixed) histology (Figure

Fig. 1. Representative differential display autoradiogram showing gene expression patterns in tumor, normal lung and normal pleura. Three reaction sets using different anchor primers (AP3, AP4, AP5) are shown for normal pleura (P), normal lung (L) and tumor (T). Arrows indicate examples of bands corresponding to differentially expressed genes.
Table I. Sequence homology of differential display bands to known genes

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Relative expression levels were qualitatively estimated from autoradiogram band intensities and increase from 1 to 3 (0, no band visible).

Fig. 2. Fluorescent in situ hybridization analysis confirms expression patterns identified by differential display. The relative abundance of two mRNA species is shown in cryosections of lung, pleura and tumor tissues using probes constructed from Fragment 12 (EFEMP1) and Fragment 39 (IAP-1). All panels, original magnification ×25. For each gene: left panels, phase contrast photomicrographs; right panels, fluorescent photomicrographs.

3A). In mixed samples, approximately one-quarter of all tumor cells exhibited IAP-1 staining. Furthermore, IAP-1 immunoreactive tumor cells (arrows, Figure 3A) were typically contained in well-circumscribed clusters, suggesting a clonal origin for these cells. Conversely, high levels of IAP-1 protein were observed in nearly all tumor cells in samples of epithelial histology (Figure 3B). Tumor cells in these cryosections also possessed relatively uniform IAP-1 protein levels as seen in the homogenous quality of the staining intensity across multiple samples. In all MPM tumors studied, stromal cells (e.g. ‘S’, Figure 3B) never expressed detectable levels of IAP-1 protein.

Expression of IAP-1 in human MPM cell lines
After confirming the presence of high levels of IAP-1 mRNA and protein in human tumor specimens, we investigated the potential physiological role of this gene in MPM through

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IAP-1 expression in mesothelioma

**Fig. 3.** Indirect immunohistochemical analysis of IAP-1 protein in representative human MPM tumors. (A and B) Tissue cryosections from mixed and epithelial subtype MPM tumors, respectively, stained with a polyclonal antibody specific for IAP-1 protein (brown chromagen). The majority of tumor cells in mixed subtype tumors did not express detectable levels of IAP-1 protein (A). The few IAP-1 positive cells in these tumors (black arrows) typically consisted of well-circumscribed clusters, perhaps indicative of a clonal origin. Conversely, abundant IAP-1 protein was observed in nearly all tumor cells from MPM epithelial histology samples (B). For all samples, irrespective of histology, no IAP-1 protein was detected in the tumor stromal component (S). Original magnification ×50.

**Fig. 4.** Expression of IAP-1 mRNA and protein in MPM cell lines. (A) IAP-1 mRNA expression levels in multiple MPM cell lines are expressed as a fold increase relative to normal pleura. The relative sensitivity of each cell line to cisplatin (CDDP) is seen in IC50 values. Error bars, SEM. (B) Detection of IAP-1 mRNA from three MPM cell lines and two (control) lung cancer cell lines (H23 and 520) was performed using northern blot analysis and GAPDH as a loading control (lower box). As expected, two bands were detected ~5.0 and 6.0 kb in length. (C) Detection of IAP-1 protein in two representative MPM cell lines (94-589 and 98-483) using western blot analysis and β-actin as a loading control (lower box).

in vitro studies in human MPM cell lines. We first measured IAP-1 mRNA expression levels in five established MPM cell lines utilizing real time RT–PCR (Figure 4A). As for human tissues, we expressed IAP-1 levels relative to normal pleura as normal mesothelial cells are not amenable to long-term culture and our primary goal was identification of MPM cell lines with high levels of IAP-1 mRNA relative only to other MPM cell lines. The cell lines used in this study originated from founder cells in pleural effusions from patients with MPM tumors of epithelial (n = 1, cell line 97-246), mixed (n = 3, cell lines 98-542, 98-483 and 94-589) and sarcomatoid histology (n = 1, cell line 92-428). Relative to normal pleura, IAP-1 mRNA is expressed at significantly higher levels in 3/5 (60%) cell lines: 98-483 (P = 0.0043), 92-482 (P = 0.0004) and 94-589 (P = 10−5). We next explored the potential relationship between IAP-1 mRNA levels and drug resistance in MPM cells lines. Following exposure of cell lines to concentrations of cisplatin ranging from 2 to 250 μM, we plotted survival curves and calculated IC50 values (the theoretical concentrations required to kill 50% of cells) as a relative measure of drug sensitivity. We found, with a single exception (98-483), that levels of IAP-1 mRNA appear to be directly proportional to cisplatin resistance (Figure 4A). The cell line with the greatest amount of IAP-1 mRNA (94-589) was, on average, almost four times as resistant to cisplatin-induced cytotoxicity as the cell lines with the least amount of IAP-1 mRNA (98-542 and 97-246), as determined from relative IC50 values. Levels of IAP-1 mRNA in 94-589 were nearly 15-fold higher (relative to normal pleura) than in the two cell lines with the lowest levels of detectable IAP-1 mRNA. IAP-1 mRNA in cell lines was confirmed to be of the expected transcript sizes (18). Northern blot analysis of the three cell lines with significantly elevated levels of IAP-1 mRNA revealed a major band at 5.2 kb and a minor band at 6.0 kb (Figure 4B). As an additional control, northern blot analysis was also performed on cell lines established from lung adenocarcinoma (H23) and lung squamous cell carcinoma (520) as IAP-1 is highly expressed in lung. IAP-1 protein was detected in all five MPM cell lines using western blot analysis and a representative blot is shown for two of the three cell lines with significantly elevated mRNA levels (94-589 and 98-483, Figure 4C). IAP-1 protein levels were visibly higher in 94-589 compared with 98-483, suggesting that transcription and translation are coupled for this gene in MPM cells.

**IAP-1 mediates MPM tumor cell survival**

We created stable cell lines transfected with a vector expressing IAP-1 in the antisense orientation to directly examine the role of this gene in facilitating inherent drug resistance of MPM cells. We chose 94-589 cells for this purpose because this cell line possessed the highest levels of IAP-1 mRNA. Expression of IAP-1 mRNA was dramatically attenuated in all four clones...
evaluated containing the antisense vector. We also observed that many of these cells did not persist in culture and displayed morphological evidence of apoptosis, unlike cells transfected with the vector alone, which were indistinguishable from parental cells. In the clone chosen for further analysis, we measured an ~3-fold decrease in IAP-1 mRNA levels compared with parental cells (Figure 5A). Northern blot analysis confirmed these observations, and cells transfected with vector alone did not display any significant (P > 0.05) difference in IAP-1 mRNA levels compared with parental cells (data not shown). Decreased levels of IAP-1 mRNA in transfected cells was accompanied by a concordant decrease in protein levels (Figure 5B). Transfected cells were also more sensitive to cisplatin-induced cytotoxicity, as seen in the ~20-fold decrease in IC50 values extrapolated from survival curves (Figure 5C). There was no discernible difference in IC50 values for cells transfected with vector alone compared with parental cells (data not shown).

We explored potential molecular mechanisms of cisplatin-induced cell death in MPM cells (and increased drug sensitivity in transfected cells) by examining levels of caspase 9 protein at multiple time points after exposure to a cisplatin concentration (100 μM) that approximated the IC50 of parental cells (Figure 5D). In parental cells (94-589), caspase 9 proform protein (48 kDa) was constitutively expressed at all time points. The 32 kDa apoptotic fragment was first detected at 1 h post-exposure but did not increase significantly (P > 0.05) by 3.5 h post-exposure. Protein levels of the apoptotic fragment peaked at 6 h post-exposure (2-fold increase relative to 1 h post-exposure, P = 0.015), and were detected through 24 h post-exposure at levels that were indistinguishable from those at 1 h post-exposure (P > 0.05). In the IAP-1 antisense cell line, the 32 kDa apoptotic fragment of caspase 9 was observed in the absence of cisplatin (control lane, ‘C’) and peaked at 1 h post-exposure (3.5-fold increase, P = 0.00024 compared with control). Protein levels of the cleavage product in transfected cells declined slightly but remained significantly elevated compared with control at 3.5 h post-exposure (2-fold increase, P = 0.0036). Although still detectable at 6 and 24 h post-exposure, the apoptotic fragment was not significantly elevated compared with control at these time points (P > 0.05). Morphological evidence of apoptosis in all cell lines mirrored rapid cleavage (i.e. activation) of caspase 9 (data not shown). Up to 50% of tumor cells in the cultures exposed to cisplatin displayed one or more hallmark characteristics of apoptosis, such as membrane blebbing and pyknotic and condensed nuclei. Cells transfected with vector alone did not differ from parental cells in constitutive expression patterns of caspase 9 proform protein nor inducible expression of the apoptotic fragment after cisplatin exposure. These cells did not display visible indications of apoptosis under typical culturing conditions in the absence of cisplatin in contrast to that observed in cells transfected with the IAP-1 antisense construct (data not shown).

Discussion

We have explored patterns of gene expression in MPM using differential display and identified IAP-1 as a gene highly expressed in tumor specimens compared with normal pleura and normal lung. We verified that IAP-1 is significantly overexpressed in a substantial number of MPM tissues, particularly those of the (most common) epithelial subtype.

![Fig. 5. IAP-1 mediates cisplatin resistance in MPM cells via inhibition of caspase 9.](image-url)
The results of this study strongly suggest that IAP-1, and relevant apoptosis-associated pathways, play some role in the neoplastic transformation of many MPM tumors and contribute to the drug-resistant phenotype of this cancer. IAP-1 is a well-characterized gene whose protein product is known to facilitate resistance to apoptosis in many cancers/diseases. Our study represents the first deliberate attempt to examine the role of this gene in MPM, a highly drug-resistant cancer about which little is known regarding basic pathobiology. The stages and pathways of neoplastic transformation in MPM are unclear and do not generally involve many well-studied growth control genes such as p53 and Rb (1–3). Our results highlight a previously unknown mechanism in MPM for evading drug-induced cytotoxic cell death. These results also provide a series of testable hypotheses designed to further add to what is known of MPM carcinogenesis. In addition, the differential expression of IAP-1 protein in MPM subtypes may serve as a molecular marker for MPM, a cancer for which predicting response to therapy is difficult and inexact.

We found IAP-1 to have a functional role in vitro in promoting the inherent drug-resistant phenotype of MPM. Experimental evidence shows that IAP-1 prevents tumor cell death (apoptosis) after exposure to cisplatin by inhibiting the activation (i.e. cleavage) of caspase 9, a known survival promoting mechanism for IAP-1. Whether IAP-1 interacts with additional caspases in the context of MPM is not currently known. We also observed high levels of IAP-1 mRNA in two non-small cell lung cancer cell lines (H23 and 520). These findings are in agreement with those described previously by Bandala et al. (11) using another non-small cell lung cancer line (A549). In that report, resistance to gemcitabine-induced cell death was postulated to involve IAP-1 (11). Thus, it appears probable that IAP-1 facilitates resistance of MPM to cisplatin and perhaps other similar-acting drugs that rely on the activation of apoptotic pathways for their desired cytotoxic effects.

Cyto genetic analysis of the cell lines used in this study (J.M. Fletcher, personal communication) and of MPM tumors in general (19) reveals a large number of chromosomal deletions, rearrangements and insertions. Despite multiple chromosomal aberrations, these cells grow well and persist in culture without noticeable morphological indications of apoptosis. Assuming that genetic abnormalities are not wholly contained in non-essential stretches of DNA, continued cell survival implies possession of a means to overcome death signals elicited under circumstances of substantial DNA aberrations. In the current study, we show that IAP-1 blocks apoptosis in large numbers of MPM cells after exposure to cytotoxic doses of cisplatin. Based on our results, we believe that IAP-1 may be at least partly responsible for preventing apoptosis and maintaining tumor cell viability in the absence of any drug challenge. IAP-1 probably plays an important role in this process for the following reasons. First, initial identification of IAP-1 antisense transfected clones in our study was difficult because overall cell viability was markedly reduced and morphological evidence of apoptosis was frequently present in these cultures when compared with cells transfected with the vector alone. In creating stable cell lines, we have by definition subcloned a population of cells able to persist in culture with the antisense construct. Even so, transfected cells are significantly more sensitive than parental cells to cisplatin and, given the well-characterized function of IAP-1, it is unlikely that the either the observed decreased baseline cell viability or the increased drug sensitivity results from non-specific effects. Evidence for apoptosis in the absence of any outside influence is seen in the accumulation of the caspase 9 cleavage (i.e. pro-apoptotic) fragment in cells transfected with the antisense construct (and not control transfectants) prior to drug exposure (Figure 4D, lane ‘C’). These observations combine to suggest that IAP-1 acts to promote baseline MPM tumor cell survival under some circumstances.

IAP-1 facilitated drug resistance is presumably one of multiple mechanisms of drug resistance/carcinogenesis in MPM. When IAP-1 expression levels in cell lines are dramatically abrogated, sensitivity to cisplatin increases by nearly a factor of 20 to an IC50 value of 5.8 μM (Figure 5C). Other cell lines not transfected with antisense constructs that have similar levels of IAP-1 mRNA possess IC50 values ~10-fold higher (Figure 4A). Although the exact expression level threshold is not known, it is probable that those cell lines/tumors with the highest levels of IAP-1 would benefit most from therapeutic targeting of this gene/protein. Consequently, we hypothesize that targeting of IAP-1 (and/or associated molecules in relevant signaling/apoptotic pathways) in patients with MPM tumors expressing high levels of this gene will probably improve not only their response to chemotherapy, but may result in some degree of tumor regression independent of other treatments.

It has been suggested that several IAPs (including IAP-1) act as part of a positive-feedback loop that facilitates tumor cell survival in the presence of TNF-α (13, 20). TNF-α can be a potent mediator of apoptosis, yet paradoxically, in some cases this protein can inhibit apoptosis by regulating the transcription of a number of survival promoting genes, such as IAP-1 (20, 21). TNF-α induces expression of IAP-1 mRNA through stimulation of the transcription factor NF-κB, and IAP-1 itself can also stimulate activation of NF-κB, thus resulting in a positive-feedback loop that acts ultimately to prevent apoptosis (22, 23). Evasion of TNF-mediated apoptosis may be of particular significance in MPM. In fact, TNF-α has been proposed as an early asbestos-related tumor marker because significantly increased levels of circulating TNF-α have been detected in individuals exposed to asbestos and who were eventually diagnosed with thoracic malignancies (24). In addition, a substantial inflammatory cell component is often present enveloping MPM tumors (25). Recent results from gene profiling efforts in MPM (using high density oligonucleotide microarrays) performed in our laboratory have revealed that multiple cytokines, including TNF-α, are expressed at high levels in MPM surgical specimens (R. Bueno, unpublished data). Finally, in vitro experimental evidence has been produced to show that MPM cells fail to respond to apoptotic stimuli elicited by either TNF-α, cisplatin or a combination of the two (26,27). It is then reasonable to hypothesize that IAP-1 acts synergistically with TNF-α to promote survival of MPM tumor cells.

This study also provides the necessary rational to explore targeting of IAP-1, perhaps via NF-κB, in designing better treatment strategies for MPM. This approach has been met with some success in managing other neoplasms, such as colorectal cancer (21). Known NF-κB inhibitors include anti-inflammatory and immunosuppressive drugs (e.g. glucocorticoids and aspirin). These compounds have been used with varying degrees of success to treat multiple medical conditions including cancer. Despite the lack of consistent and predictable response of cancers to these drugs, exploring the feasibility of
treating MPM in this manner is worthwhile because it appears that the action of NF-κB is cell type and signal specific and depends largely on the degree of NF-κB activation/inhibition (28). Any improvement in the efficacy of chemotherapeutic drugs in MPM will probably have dramatic clinical consequences as (i) MPM tumors have a high degree of intrinsic drug resistance (particularly towards cisplatin), (ii) cisplatin is an integral part of the most successful treatment regimens to date and (iii) patients with this disease generally have a very poor prognosis.

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