High levels of MDM2 are not correlated with the presence of wild-type p53 in human malignant mesothelioma cell lines

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Summary Prior analysis of 20 human mesothelioma cell lines for p53 status revealed only two mutations and one p53 null cell line, although p53 expression was detected in most cell lines. In addition, mRNA and protein expression of the retinoblastoma gene product in human mesothelioma cell lines is similar to normal controls. We have tested for p53 induction after exposure to ionising radiation and demonstrate this induction and, to a lesser extent, p21^{WAF1} induction, in both normal mesothelial cells and p53-positive mesothelioma cell lines. We postulated that high levels of MDM2 might alter p53 and retinoblastoma tumour-suppressor function in mesothelioma. However, Southern blot analysis for mdm2 indicated that no amplification had occurred in 18 mesothelioma cell lines tested. Steady-state mRNA and protein levels also did not indicate overexpression. These results indicate that high levels of MDM2 are not responsible for inactivating the functions of wild-type p53 or the retinoblastoma gene product during the pathogenesis of malignant mesothelioma.

Keywords: tumour suppressor; mesothelioma; carcinogenesis

Mesothelioma, a cancer involving disregulation of mesothelial cell growth, has been linked epidemiologically to asbestos fibre exposure (Wagner 1960; Wagner and Berry, 1969; Craighead and Mossman, 1982). The 20-50 year duration of the latency period for this malignancy suggests that it is generated by a multistep process induced by fibre exposure and involving chromosome breakage, rearrangement and deletions (Barrett, 1991; Hei et al., 1992), aneuploidy (Lechner et al., 1985) and mutations secondary to damage by reactive oxygen species (Mossman et al., 1986). At a molecular level, the pathogenesis of the disease is likely to involve modulation of tumour-suppressor pathways that are critical for normal cellular regulation.

Alterations in the p53 tumour-suppressor gene have been identified as the most frequent genetic events in a wide variety of human cancers (Hollstein 1991; Levine et al., 1991; Vogelstein and Kinzler, 1992). In addition, retinoblastoma (Rb) gene dysfunction has been implicated in carcinogenesis (Benedict et al., 1990). The importance of p53 and Rb tumour-suppressor function is shown by the evolution, in DNA tumour viruses, of proteins which disrupt the function of these molecules. Interaction of p53 and Rb with SV40 T antigen (Farmer et al., 1992; Mietz et al., 1992; Jiang et al., 1993; DeCaprio et al., 1988), adenovirus E1a and E1b (Yew and Berk, 1992; Whyte et al., 1988), and papillomavirus E6 and E7 (Mietz et al., 1992; Dyson et al., 1989) has been shown to block wild-type functions of these molecules. Indeed, in recent work showing the presence, in mesothelioma specimens, of SV40 T antigen (Carbone et al., 1994), the authors suggest that latent SV40 infection of mesothelial cells may contribute to development of mesothelioma.

In contrast, the cell lines studied in this report have been shown previously to be negative for SV40 T antigen in a study in which all cells were stained with the polyclonal antibody to SV40 T, Pab 416, as an isotype-matched negative control for the p53 antibodies, Pab 1801 and 122 (Metcalf et

al., 1992). That study established, by sequencing, in most cases, exons 2-11 of the p53 gene, that the cell lines with wild-type p53 genes (18/20), as well as two normal samples examined, expressed a detectable level of p53 protein. One hypothetical explanation for this observation involves overexpression of a cellular protein, which can inhibit the function of the p53 protein.

A candidate cellular protein, mouse double-minute 2 (MDM2), was cloned from the tumorigenic cell line, 3T3DM, and shown to have oncogenic potential (Cahilly-Snyder et al., 1987; Fakharzadeh et al., 1991). MDM2, through its N terminus (Chen et al., 1993; Oliner et al., 1993), binds to the transactivation domain of p53 and interferes with its activity as a transcriptional activator (Zauberman et al., 1993; Finlay, 1993; Momand et al., 1992). Furthermore, induction of transformed foci in rat embryo fibroblasts by overexpression of ras and MDM2 is reduced by 50% by co-transfection with a wild-type p53 vector, and transformed cells express low levels of the wild-type p53 protein (Finlay, 1993). Overexpression of mdm2, in the presence of wild-type p53, has been demonstrated in metastatic osteosarcomas (Ladanyi et al., 1993), a low percentage of non-small-cell lung carcinomas (Marchetti et al., 1995), a subset of human malignant gliomas (Reifenberger et al., 1993) and human leukaemias (Bueso-Ramos et al., 1993; Quesnel et al., 1994; Zhou et al., 1995).

The mdm2 gene, in mouse, has been shown to produce several sets of proteins (Olson et al., 1993; Barak et al., 1993) resulting from alternative splicing (Wu et al., 1993; Haines et al., 1994), and/or alternative promoter usage (Barak et al., 1994). It has been shown that initiation of translation from the third and fourth initiation codons results in molecules unable to bind to p53 (Olson et al., 1993; Haines et al., 1994). The conservation of a p53-responsive element leading to alternative transcripts has been demonstrated in the human mdm2 gene (Zauberman et al., 1995), but its protein products remain to be characterised.

It has been suggested that MDM2 species that bind to p53 may be involved in an autoregulatory feedback loop (Wu et al., 1993; Perry et al., 1993; Barak et al., 1994; Picksley and Lane, 1993). This feedback control would function by stimulation of mdm2 transcription by p53 from the p53-dependent promoter in intron 1, as opposed to the

constitutive, upstream promoter (Wu et al., 1993; Barak et al., 1994; Zauberman et al., 1995). Increased levels of MDM2 protein reduce p53 stimulation of the p53 response element in the mdm2 gene (Wu et al., 1993), and inhibit the ability of irradiated colorectal carcinoma (RKO) and osteosarcoma (OSA-C1) cell lines to arrest in the G_1 stage of the cell cycle (Chen et al., 1993).

Previous work from this laboratory has demonstrated that both Rb mRNA and protein are expressed in malignant mesothelioma cell lines and primary cultures of NHM cells (Van der Meeren et al., 1993). In addition, the presence of immunohistochemically detectable Rb protein in paraffin-embedded tissue from human mesothelioma specimens and normal human mesothelium has been reported (Ramael et al., 1994). These observations suggest that a downstream inactivator of Rb, as well as p53, could be crucial in mesothelial carcinogenesis. MDM2 has now been shown to bind to Rb as well as p53 (Xiao, ZX et al., 1995). The function of Rb would be additionally compromised by the binding of MDM2 to the transcription factors E2F1/DP1 with consequent enhancement of their activation functions (Martin et al., 1995). Thus, disruption of cellular home-ostasis, secondary to overexpression of mdm2, could lead to disregulated cell growth by: (1) inactivation of p53 tumour-suppressor activity; (2) interference with E2F sequestration on Rb; and (3) activation of the S phase promoting transcriptional factors, E2F1/DP1.

We, therefore, proposed that overexpression of the mdm2 gene product in malignant mesothelioma could result in a functional inhibition of both the p53 and the Rb tumour-suppressor pathways, leading to diminished control of the G_1 checkpoint, even in the presence of functional p53 and Rb. A previous immunohistochemical study of mesothelioma demonstrated detectable MDM2 in six out of ten p53-positive tumour specimens (Segers et al., 1995). We examined the contribution of mdm2 gene amplification and/or protein overexpression to mesothelial carcinogenesis by evaluating mdm2 DNA content, as well as steady-state levels of mRNA and protein in 18 of the mesothelioma cell lines previously characterised for p53 (Metcalf et al., 1992) and comparing these values with those obtained from primary cultures of normal human mesothelial (NHM) cells.

Materials and methods

Cell lines and culture conditions

Human mesothelioma cell lines were cultured as described previously (LaVeck et al., 1988). Detailed information on the history and derivation of the M prefix cell lines has been reported previously (Metcalf et al., 1992). Primary cultures of NHM cells were obtained from patients with non-malignant disease and initiated as described (LaVeck et al., 1988). OSA-Cl, a sarcoma cell line lacking p53 mutation (Leach et al., 1993) but exhibiting a 30- to 40-fold mdm 2 gene amplification as well as overexpression of both mdm2 mRNA and protein (Oliner et al., 1992), was generously supplied by Dr Bert Vogelstein. This cell line, as well as human bronchial fibroblastic (HBF) cell strains, was maintained in Hut medium (Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum; BioFluids, Rockville, MD, USA). Normal human bronchial epithelial (NHBE) cells were grown in LHC9 medium (BioFluids Inc.) as described previously (Lechner and LaVeck, 1985).

Exposure of cells to ionising radiation and immunohistochemical analysis of p53 and p21^{WAF1}

NHM cells and mesothelioma cell lines were seeded in eight-well chamber slides. Logarithmically growing cells were subjected to 3.5 Gy of ionising radiation for 1 min and returned to the incubator for 3 h. Cells were then fixed in 2% paraformaldehyde [in phosphate-buffered saline (PBS)],

followed by methanol treatment. For simultaneous demon-stration of p53 and p21^{WAF1} expression, cells were doubly labelled with CM-1 antibody (1:200 dilution, Signet Laboratories, Dedham, MA, USA; rabbit IgG) and monoclonal anti-p21^{WAF1} antibody (1:50 dilution, Oncogene Science, Uniondale, NY, USA; mouse IgG), followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and Texas red-conjugated anti-mouse IgG antibodies (1:300 dilution; Vector Laboratories, Burlingame, CA, USA). Nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI).

RNA isolation and Northern blot analysis

RNA was isolated from logarithmically growing cells by the method of Chomczynski and Sacchi (1987) and subjected to Northern blot evaluation as described previously (Metcalf et al., 1992). Filters were prehybridised at 65°C in Hybrisol I (Oncor, Gaithersburg, MD, USA) for a minimum of 2 h and then hybridised overnight with a random-primed $\alpha^{32}P$-labelled 900 bp (-312 to approximately $+600$ relative to ATG) XhoI restriction fragment from the MDM C14-2 clone of the human mdm2 gene, kindly supplied by Dr Bert Vogelstein (Oliner 1992). The filters were washed once at room temperature with 2 × standard saline citrate (SSC), 1% sodium lauryl sulphate (SDS), and then at 65°C twice with 2 × SSC, 1% SDS, twice with 1 × SSC, 1% SDS, twice with 0.5 × SSC, 1% SDS, and once at room temperature with 0.1 × SSC. RNA loading and integrity were verified by either concurrent or sequential probing of the filters with a random-primed $\alpha^{32}P$-labelled 900 bp PstI restriction fragment of a clone of the rat glyceraldehyde-3-phosphate dehydro-genase (GAPDH) gene (Fort et al., 1985), generously provided by Dr Marc Piechaczyk. Several exposures of each membrane were scanned on a Molecular Dynamics laser densitometer (Sunnyvale, CA, USA). Volumes for each band of mdm2 RNA were calculated and normalised to volumes for the GAPDH band in the same lane on the same filter. These ratios were then compared with mdm2/GAPDH ratios for the VAMT-1 mesothelioma cell line. Since VAMT-1 cells are null for p53 expression (Metcalf et al., 1992), these cells were chosen to provide a constitutive MDM2 expression level (Zauberman et al., 1995) for comparison. Interestingly, the MDM2 expression in this cell line was equivalent to that in the normal HBF cell strain used for protein normalisation. All expression ratios are arbitrary numbers and have not been normalised to an absolute concentration standard.

Protein extraction and Western blot analysis

Lysates were prepared, from logarithmically growing cells, as the supernatant fraction of a 30 min, 4°C centrifugation at 38 600 × g in a buffer containing 50 mM Tris, pH 7.4, 150 mM sodium chloride, 1% (v/v) Triton-X-100, 1% deoxycholic acid (v/v), 0.1% SDS, 0.1 mM dithiothreitol and 0.57 mM phenylmethylsulphonyl fluoride. Routinely, samples contain-ing 100 μg of protein, determined using the BCA Reagent (Pierce, Rockford IL, USA), were separated by 7.5% Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P (Millipore, Bedford, MA, USA) membranes, probed with the anti-human MDM2 AB-1 (Oncogene Science, Uniondale, NY, USA) and detected by chemiluminescence using the Renaissance (Dupont/NEN, Boston, MA, USA) reagents and protocol. This anti-MDM2 antibody recognises an epitope in the amino terminus between residues 26 and 150 and would detect the p53-binding protein species (Olson et al., 1993; Haines et al., 1994). The membranes were reprobed, without stripping, with an anti-Rb rabbit polyclonal IgG, RB(C-15) (Santa Cruz Biotech, Santa Cruz, CA, USA). Protein loading for OSA-Cl was reduced to 25 μg. A series of dilutions for OSA-Cl lysate, 10–100 μg (data not shown), established the linear range for protein densitometry and demonstrated that the Rb signal per μg lysate protein was similar for all mesothelioma cells tested. Since the Rb protein product is expressed at similar

levels in mesothelioma cell lines and NHM cells (Van der Meeren et al., 1993), Rb was used as the internal control for protein loading. MDM2/Rb ratios were calculated as described for mRNA and normalised to those obtained for HBF cells without conversion to an absolute concentration standard.

DNA isolation and Southern blot analysis

DNA was extracted, digested with EcoRI, and analysed by Southern blotting (Maniatis et al., 1982). Membranes were probed with an α^{32}P-labelled mdm2 cDNA fragment, as described for RNA analysis. DNA loading and integrity were verified by reprobing with a random primed α^{32}P-labelled 900 bp Sau3AI fragment of the human single copy gene, J_H, generously provided by Dr Philip Leder (Ravetch et al., 1981a). The 4.6 kb and/or 8.8 kb bands of mdm2 DNA were normalised to values for the 22 kb J_H band in the same lane on the same filter. These ratios were then compared with mdm2/J_H DNA content in HBF cells as described for Northern blot analysis.

Results

Although the mesothelioma cell lines investigated here have been characterised for p53 expression (Metcalf et al., 1992), assays indicating biological function have not been reported. Therefore, we tested the ability of p53 to be induced by DNA damage, as demonstrated in most cell types (Kuerbitz et al., 1992; Lu and Lane, 1993). Selected mesothelioma cell lines with a basal level of detectable, wild-type p53 (Metcalf et al., 1992) were compared with NHM and the p53 null cell line, VAMT-1, for induction of nuclear p53 and p21^{WAF1} in response to ionising radiation. Figure 1 presents the data, which are illustrated photographically in Figure 2 for NHM cells and the mesothelioma lines, M33K and VAMT-1. The M33K cell line is representative of mesotheliomas that express wild-type p53 at a low, but detectable, level in the absence of T antigen, while VAMT-1 cells are null for p53 expression. Both M33K and VAMT-1 are wild-type in exons 2–11 by sequence analysis (Metcalf et al., 1992). Cells were scored as positive for induction, when p53 or p21^{WAF1} showed nuclear accumulation as defined by the DAPI stain. In both NHM and M33K cells, ionising radiation induces nuclear accumulation of p53 and p21^{WAF1}. Marked cells in NHM and M33K panels illustrate positive, nuclear accumulation of the induced protein. The lower marked cell in the M33K was scored positive for p53 and negative for p21^{WAF1} induction. The VAMT-1 cells illustrate the appearance of background staining for both proteins. Figure 1 displays the data obtained by counting cells showing nuclear staining on either irradiated or control slides. Five of the six mesothelioma lines analysed here showed significant, colocalised (Figure 1) induction of p21^{WAF1} as well as p53. Values for irradiated VAMT-1 cells and control M24K cells were not significant. Interestingly, the M28K cell line did not demonstrate nuclear p53 in control or irradiated specimens, suggesting that this p53-expressing cell line may have a defect in induction of the protein by DNA damage. The positive response of five of six mesotheliomas supports the hypothesis that the p53 tumour suppressor is functional as a DNA damage sensor (transcription factor) in the majority of mesothelioma cells.

To investigate the possibility that amplification of the mdm2 gene, and subsequent elevation of mdm2 expression, could result in interference with the p53 and Rb tumour-suppressor pathways in malignant mesothelioma, 18 malignant mesothelioma cell lines were assayed for mdm2 gene content relative to HBF by Southern blot analysis (Table I). None of the samples showed mdm2 gene rearrangements. The mdm2 gene content of mesothelioma cell lines relative to HBF, ranged from 1.0 to 1.7 (Table I), suggesting that the mdm2 gene is not amplified in malignant mesothelioma cell lines.

Since overexpression of mRNA could lead to increased levels of protein expression, four primary cultures of NHM cells, one primary culture of HBF cells and 18 malignant mesothelioma cell lines (Table I, Figure 3) were evaluated for steady-state levels of mdm2 mRNA. All of these specimens revealed a single message of 5.5 kb, consistent with the size of mdm2 mRNA previously reported (Ladanyi et al., 1993; Oliner et al., 1992) (Figure 3). The presence of multiple mRNA species (Zauberman et al., 1995) was not detected in these cells. Analysis of the four NHM revealed a range of mdm2 mRNA levels with three samples from 1.4 to 3.6 as much mRNA as VAMT-1, and one outlier at a 10.5-fold excess (Table I). Nothing in the donor history or culture characteristics of this NHM culture explained this observation. Steady-state levels of MDM2 mRNA in the malignant mesothelioma cell lines ranged from 1.1 to 5.1, relative to VAMT-1 (Figure 3, Table I). Figure 3 illustrates MDM2 steady-state mRNA levels in representative mesothelioma (lanes 1–5), NHM (lanes 6 and 7), HBF (lane 8) and overexpressing OSA-C1 (lane 9) cells.
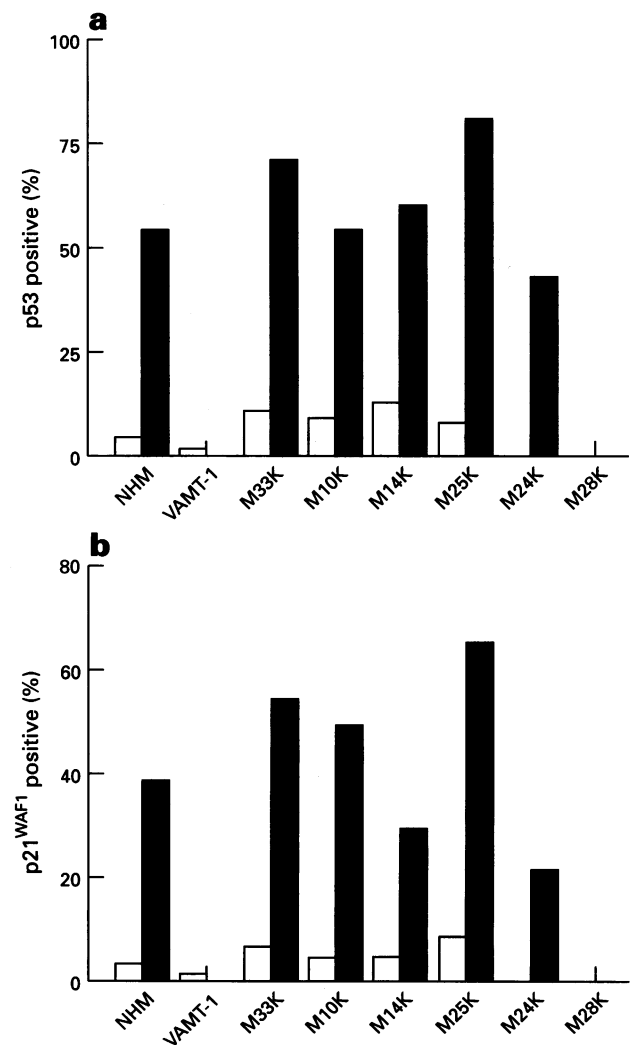
Figure 1 Induction of (a) p53 and (b) p21^{WAF1} in NHM and mesothelioma cell lines. The specified cell cultures were treated (■) or mock treated (□) with 3.5 Gy for 1 min and then incubated at 37°C for 3 h. Cells were treated as detailed in Materials and methods, and the p53- and p21^{WAF1}-positive cells were enumerated visually on a fluorescence microscope. Values are presented as the percentage of total cells counted. These totals were: NHM$^+$, 530; NHM$^-$, 800; VAMT-1$^+$, 202; VAMT-1$^-$, 309; M33K$^+$, 201; M33K$^-$, 310; M10K$^+$, 296; M10K$^-$, 471; M14K$^+$, 220; M14K$^-$, 268; M25K$^+$, 108; M25K$^-$, 256; M24K$^+$, 175 M24K$^-$, 319; M28K$^+$, 174; M28K$^-$, 393.
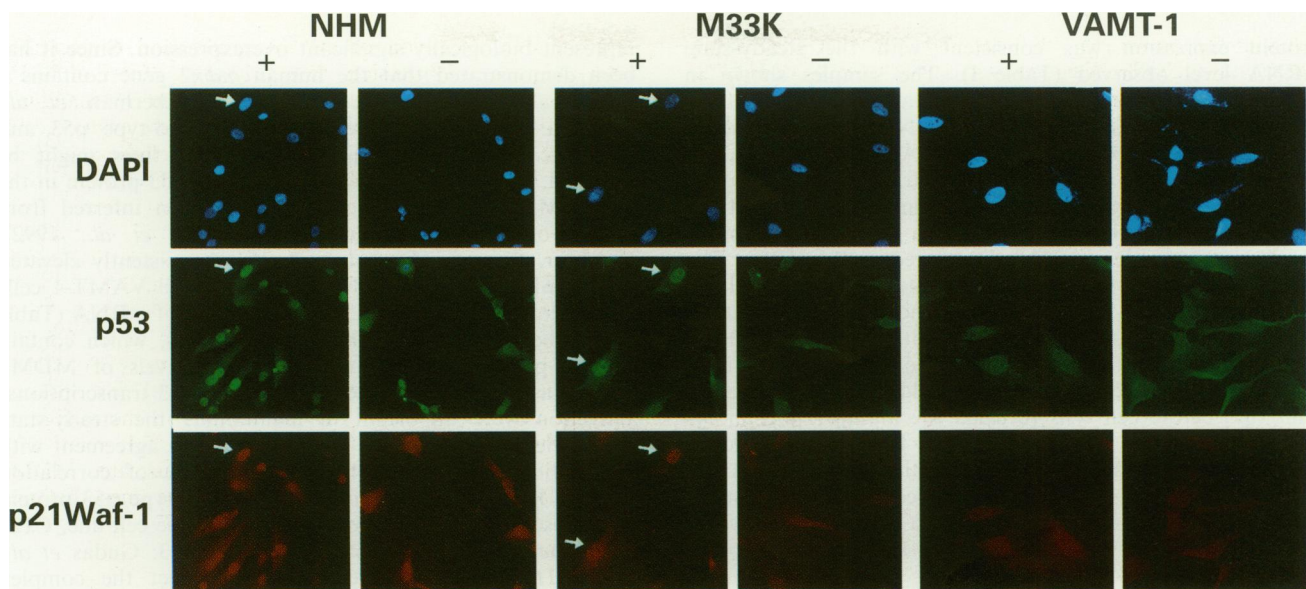
Figure 2 p53 and p21^{WAF1} are induced in NHM and mesothelioma cells by ionising radiation. Nuclei were visualised by staining with DAPI, p53 with fluorescein and p21^{WAF1} with Texas red. Irradiated cultures and mock-irradiated controls are illustrated for NHM, M33K and VAMT-1. +, Cells which received 3.5 Gy for 1 min; −, mock-irradiated control cells. Arrows in the p53 and p21^{WAF1} panels for NHM illustrate induced cells. In the M33K panels, both marked cells are positive for p53 induction, while positive induction is illustrated by the upper cell and negative induction by the lower cell in the p21^{WAF1} panel. The VAMT-1 cells, which do not express p53 protein, are included to illustrate the appearance of background membrane staining.

Table I Analysis of MDM2 in human mesothelioma cell lines

Cell line	p53[b]	mRNA	MDM2[a] DNA	Protein
M33K	Wt (2−11)	4.0	1.0	4.6
M24K	Wt (2−11)	5.1	1.4	4.2
M25K	Wt (2−11)	2.1	1.0	5.8
M20	Wt (2−11)	2.7	1.1	2.3
M32K	Wt (2−11)	1.8	1.1	3.8
M28K	Wt (2−11)	2.1	1.0	2.3
VAMT-1	Null (2−11)	1.0	1.1	0.1
M14K	Wt (2−11)	4.5	1.0	4.5
M19	Wt (2−11)	3.9	1.0	4.0
M9K	Wt (5−9)	4.0	1.2	3.9
Hut290	Wt (4−11)	2.3	1.0	2.1
Hut28	Wt (2−11)	1.5	1.2	4.2
M10K	Wt (2−11)	1.5	1.3	1.2
M15K	Mt (4−11)	1.6	1.1	2.1
JMN	Mt (4−11)	1.3	1.0	1.1
DND	Wt (2−11)	1.0	1.0	1.5
MT3	Wt (5−9)	3.0	1.2	4.4
M14M	Wt (2−11)	4.7	1.7	5.2
HBF	ND	1.0	1.0	1.0
OSA-Cl	ND	21	35.4	50

[a]Autoradiograms from several different exposures of membranes were analysed by densitometry. Values were calculated as described in Materials and methods and normalised to HBF3898 for DNA and protein, and to VAMT-1 for mRNA values. [b]Exons sequenced. Wt, wild-type; mt, mutant; ND, not done.

Figure 3 Steady-state MDM2 mRNA levels in human mesothelial cells and mesothelioma cell lines. Total cellular RNA (20 μg) was analysed by Northern blotting as described in Materials and methods. The membranes were hybridised either serially or in combination with probes for MDM2 and GAPDH. Ratios of mRNA levels are shown in Table I relative to VAMT-1 (lane 1), a p53 null mesothelioma cell line. The data shown are illustrative of both malignant mesothelioma cell lines (lanes 1−5), primary cultures of NHM (lane 6, NHM 1 = UMD10348 and lane 7, NHM 2 = GU931028), primary fibroblastic cells (lane 8 = HBF3898) and the overexpressing OSA-Cl line (lane 9).

Finally, since MDM2 protein expression may be controlled at the post-translational level (Landers et al., 1994), the steady-state level of MDM2 protein was studied in the mesothelioma cell lines, five NHM isolates, one culture of HBF cells and two primary NHBE cell strains by Western blot analysis (Table I, Figure 4). A single band of 90 kDa (Figure 3), similar in size to that previously reported for the MDM2 protein product (Leach et al., 1993; Oliner et al., 1992; Barak and Oren, 1992; Momand et al., 1992; Haines et al., 1994) and consistent with the single mRNA species, was observed in all samples tested. The five NHM cell samples demonstrated a protein range of 1.2 to 9.8, relative to HBF (data not shown) with the highest value corresponding to the cell strain with the highest mRNA level. The 18 malignant mesothelioma cell lines exhibited protein levels ranging from 0.1 to 5.8, relative to HBF (Table I). In general, the level of
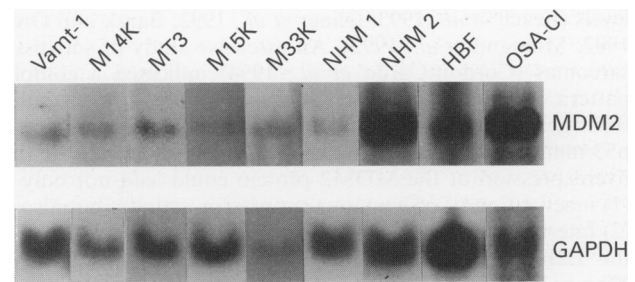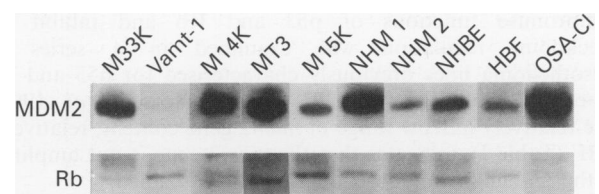

Figure 4 Steady-state protein levels of MDM2 and Rb in normal human cells and mesothelioma cell lines. Total cellular protein (100 μg) was analysed by Western blotting as described in Materials and methods. The membranes were probed with antibody to MDM2 and visualised with chemiluminescence. Membranes were then reprobed with antibody to Rb as detailed in Materials and methods. Representative samples of steady-state protein lysates probed for MDM2 and Rb are shown. Lanes 1−5 are malignant mesothelioma cell lines, lanes 6 and 7 are NHM (NHM 1 = UMD10227, NHM 2 = GU931028), lane 8 is NHBE 3883, lane 9 is HBF3898 and lane 10 is OSA-Cl. Lanes 1−9 contain 100 μg protein, while lane 10 (OSA-Cl) contains 25 μg protein. MDM2 values relative to Rb are reported in Table I.

protein expression was consistent with the steady-state mRNA level observed (Table I). The samples shown in Figure 4 are representative of steady-state protein expression in mesothelioma cell lines (lanes 1-5), NHM (lanes 6 and 7), NHBE (lane 8), HBF (lane 9) and OSA-C1 (lane 10). All of the mesothelioma cell lines demonstrate MDM2 protein levels within the range of normal human cells, except for the VAMT-1 cell line, which shows a 10-fold reduction in MDM2 protein level when compared with HBF. This observation might indicate the presence of p53-stimulated mdm2 transcription in the other cell lines (Zauberman et al., 1995). Reprobing the membranes with anti-Rb antibody revealed a band at approximately 115 kDa (Figure 4). In other experiments (data not shown), addition of phosphatase inhibitors before cell lysis revealed the multiply phosphorylated Rb species in mesothelioma and HBF. Semi-quantitative comparison of Rb protein in mesothelioma cell lines and primary cultures of normal cells revealed similar amounts of the Rb protein product per μg protein analysed, relative to HBF (1.06 ± 0.36 s.d., $n = 18$ and 1.32 ± 0.5 s.d., $n = 8$ respectively).

Discussion

It has been reported that loss of p53 tumour-suppressor activity in a variety of human cancers with low frequency of p53 mutation, has been associated with amplification of the mdm2 gene and/or increases in MDM2 mRNA and protein levels (Leach et al., 1993; Oliner et al., 1992; Barak and Oren, 1992; Momand et al., 1992). An extensive study of soft-tissue sarcomas (Cordon-Cardo et al., 1994) indicated a complex pattern of mdm2 overexpression that did not result solely from gene amplification and existed even in the presence of p53 mutations and overexpression. Current data indicate that overexpression of the MDM2 protein could lead not only to (1) inactivation of p53 tumour-suppressor activity, but also to (2) interference with E2F binding on Rb and (3) activation of the E2F/DP1 transcriptional factors (Haines et al., 1994; Xiao et al., 1995; Martin et al., 1995), resulting in the loss of G_1 checkpoint regulation by p53 and Rb. It has been demonstrated that Rb is expressed in malignant mesothelioma cell lines and tumours (Van der Meeren et al., 1993; Ramael et al., 1994) (Figure 4) and that p53 mutation is an infrequent event (Metcalf et al., 1992). Furthermore, exposure of NHM and mesothelioma lines expressing wild-type p53 to ionising radiation induces nuclear accumulation of p53 and p21[WAF1] in five of six lines tested (Figures 1 and 2). These data indicate that the p53 observed by immunohistochemical staining (Metcalf et al., 1992) is responsive to DNA damage and able to activate the p21[WAF1] gene transcriptionally in most mesothelioma cell lines. Therefore, the possibility that the presence of mdm2 overexpression and/or amplification might be able to compromise functions of p53 and Rb and inhibit G_1 checkpoint regulation was examined in a series of mesothelioma lines previously characterised for p53 and Rb expression (Metcalf et al., 1992; Van der Meeren et al., 1993). The relatively narrow range of mdm2 gene content, relative to HBF (Table I), indicates that the mdm2 gene is not amplified in the malignant mesothelioma cell lines studied.

Steady-state mdm2 protein and mRNA levels were also evaluated in these cell lines. The few NHM samples available ($n = 4$ for mRNA) does not allow sweeping generalisations with respect to the 'normal range' of expression. Three of the four samples showed mRNA levels similar to the tumour cell lines (1.4-3.6 relative to VAMT-1), while one sample showed a 10.5-fold elevated expression (Figure 3, lane 6). mRNA levels in malignant mesothelioma cell lines (Table I) ranged from 1.0 to 5.1 relative to VAMT-1, a variation which was similar to the NHM cells. Previous studies of NHM cells showed that they exhibit broad interindividual variation in other biological properties (LaVeck et al., 1988; Lechner et al., 1989). Therefore, the variation observed is not likely to

represent biologically significant overexpression. Since it has been demonstrated that the human mdm2 gene contains a functional, p53-responsive promoter (Zauberman et al., 1995), a correlation between levels of wild-type p53 and MDM 2 expression in mesothelioma cell lines might be expected. Elevated expression of wild-type p53 protein in the M9K, M32K and DND cell lines has been inferred from results of immunocytochemistry (Metcalf et al., 1992). However, these cell lines do not show consistently elevated mdm2 mRNA levels (Table I). The p53 null VAMT-1 cells show a low level of MDM2 protein but not of mRNA (Table I). Furthermore, the M15K and JMN lines, which contain mutant p53 genes, do not express low levels of MDM2 mRNA as might be expected if wild type p53 transcriptional activation were important in maintaining the steady-state mRNA level of this gene. These data are in agreement with observations of others who report a lack of correlation between levels of MDM2 mRNA and wild-type p53 protein in a subset of human gliomas and the murine cell line, C127 (Reifenberger et al., 1993; Perry et al., 1993; Gudas et al., 1995). This lack of correlation may reflect the complex regulatory interactions, which are being delineated for the genes and gene products involved in G_1 checkpoint regulation.

While loss of function of tumour-suppressor gene products has been implicated in the pathogenesis of a wide variety of cancers, no alteration in expression of either the p53 or Rb gene product has been demonstrated in this laboratory in a high percentage (85% for p53 and 100% for Rb) of malignant mesothelioma cell lines (Metcalf 1992; Van der Meeren et al., 1993). Additionally, the results reported here indicate that disruption of the wild-type p53 and Rb tumour-suppressor pathways by high levels of MDM2 protein is not a major factor in the aetiology of malignant mesothelioma. Thus, it is possible that uncharacterised downstream factors in the p53 and/or Rb tumour-suppressor pathways could be altered during the pathogenesis of malignant mesothelioma.

Recent studies have revealed an increasing number of cellular proteins and DNA-binding proteins that interact with p53. These include XPB and XPD, components of transcription repair complex TFIIH (Wang et al., 1996); CBF, a CCAAT binding factor; heat shock protein 70; replication protein A; SPI, a general transcription factor; TBP, a TATA binding protein; and WT1, the Wilms' tumour gene product (for review see Pietenpol and Vogelstein, 1993). Rb also has been found to interact with cellular proto-oncogenes, cell cycle-related proteins and other transcriptional factors, such as c-myc, N-myc, ATF-2, cdc2 proteins (for review see Goodrich and Lee, 1993), cyclin D2 and cyclin-dependent kinase 4 (CDK4) (Ewen et al., 1993). In addition, alterations of proteins that modulate the activity of these Rb-binding proteins, such as p16[INK4], which inhibits CDK4 phosphorylation of Rb (Ewen et al., 1993), could be cellular components that participate in the loss of cell cycle regulation. Recent data indicate that a large proportion of mesothelioma cell lines (Okamoto et al., 1994; Cheng et al., 1994), as well as primary mesothelioma tumours (Xiao, S et al., 1995; Cheng 1994), have homozygous deletions of the p16 gene. Further studies of p16[INK4] in primary mesothelioma tumours will be necessary to evaluate the physiological significance of the loss of p16[INK4]. In addition, other targets in these tumour-suppressor pathways need to be evaluated to advance the understanding of the pathogenesis of mesothelioma.

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