The MDM2 Oncoprotein Promotes Apoptosis in p53-Deficient Human Medullary Thyroid Carcinoma Cells*

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ABSTRACT
The MDM2 oncoprotein has been shown to inhibit p53-mediated growth arrest and apoptosis. It also confers growth advantage to different cell lines in the absence of p53. Recently, the ability of MDM2 to arrest the cell cycle of normal human fibroblasts has also been described. We report a novel function for this protein, showing that overexpression of MDM2 promotes apoptosis in p53-deficient, human medullary thyroid carcinoma cells. These cells, devoid of endogenous MDM2 protein, exhibited a significant growth retardation after stable transfection with mdm2. Cell cycle distribution of MDM2 transfectants (medullary thyroid tumor (MTT)-mdm2) revealed a fraction of the cell population in a hypodiploid status, suggesting that MDM2 is sufficient to promote apoptosis. This circumstance is further demonstrated by annexin V labeling. MDM2-induced apoptosis is partially reverted by transient transfection with p53 and p19ARF. Both MTT and MTT-mdm2 cells were tumorigenic when injected into nude mice. However, the percentage of apoptotic nuclei in tumor sections derived from MDM2-expressing cells was significantly higher relative to that in the parental cell line. MDM2-mediated programmed cell death is at least mediated by a down-regulation of the antiapoptotic protein Bcl-2. Protein levels of caspase-2, which are undetectable in the parental cell line, appear clearly elevated in MTT-mdm2 cells. Caspase-3 activation does not participate in MDM2-induced apoptosis, as determined by protein levels or poly(ADP-ribose) polymerase fragmentation. The results observed in this medullary carcinoma cell line show for the first time that the product of the mdm2 oncogene mediates cell death by apoptosis in p53-deficient tumor cells. (Endocrinology 141: 420–429, 2000)

MEDULLARY THYROID carcinoma (MTC) is a neuroendocrine tumor of the parafollicular C cells that accounts for up to 10% of all thyroid tumors (1). One fourth of all MTC appear to be genetically determined and are associated with inherited clinical syndromes (multiple endocrine neoplasia 2A and 2B and familial MTC). The remaining cases of MTC are sporadic and therefore occur as the consequence of somatic alterations caused by both genetic and epigenetic factors (2). Established cell lines from human and animal MTC tumors provide a valuable system to analyze genes involved in the development of this neoplasia. Human medullary thyroid tumor cells (MTT), recently characterized in our laboratory (3), show all of the major properties described for MTC cells. They immunoreact with specific calcitonin antibodies (our unpublished observations) and express somatostatin and somatostatin receptors 2, 3, 4, and 5 (4). The transformed phenotype of these cells is at least due to a loss of expression of the tumor suppressor gene p53 and a genetic deletion involving exon 11 of the ret protooncogene (3). The oncogenic potential of the murine double-minute-2 (mdm2) gene was originally detected in spontaneously transformed murine fibroblasts (5). Thereafter, genetic amplification of the mdm2 gene was detected in different human tumors and cell lines (6, 7). More recently, the oncogenic function of the mdm2 gene product (MDM2) has also been determined in transgenic mice expressing MDM2 in the mammary gland. These animals, which show major alterations of the cell cycle, have a high incidence of breast tumors (8). Coimmunoprecipitation experiments determined that MDM2 physically interacts with the p53 tumor suppressor gene product (9), leading to the idea that, as described for proteins such as the simian virus 40 large T antigen or the papillomavirus E6 protein, the oncogenic potential of MDM2 is based on its ability to bind to and inactivate p53. Thus, the inactivation of p53 function by MDM2 results in the abrogation of both p53-mediated cell cycle arrest and apoptosis. Recent findings indicate that inactivation of p53 by MDM2 occurs by promoting the degradation of the tumor suppressor protein through the ubiquitin-proteasome pathway (10, 11). In addition, the discovery that p53 is able to transcriptionally activate the expression of mdm2 (12) led to the hypothesis that a feedback autoregulatory loop provides a precise time frame for p53 signaling to regulate the cell cycle. MDM2 also interacts with other proteins important in the regulation of cell cycle transition, such as the retinoblastoma gene product, the TATA-binding protein, the transcription factor E2F, and the INK4a-ARF tumor suppressor gene product p19ARF (13–15). Recently, studies in NIH-3T3 fibroblasts revealed that
MDM2 arrests the cell cycle, causing a specific inhibition of Go/G1-S transition (16). In the present report we demonstrate that MDM2 is sufficient to promote apoptosis in the MTT cell line. Transfection of these cells with mdm2 resulted in the isolation of clones that constitutively express MDM2. These clones exhibit a growth retardation compared with the parental cell line. Cell cycle analysis and annexin V labeling show a significant fraction of these MDM2 transfectants undergoing apoptosis, thus providing a direct link between MDM2 expression and programmed cell death.

Materials and Methods

Cell culture

The human MTC cell line MTT (3) was maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mm glutamine, 100 mg/ml sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin. The human follicular thyroid carcinoma cell lines FRO, ARO, and NPA were provided by Dr. J. A. Fagin (University of Cincinnati, Cincinnati, OH) and Dr. Juillard (University of California, Los Angeles, CA). They were maintained in the same conditions as those used for the MTT cells. Human breast cancer MCF-7 cells were grown in DMEM supplemented with 10% FBS, 2 mm glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Plasmids and transfections

pCMDM2 was constructed by ligation of the human mdm2 complementary DNA (cDNA) (6) containing the complete open reading frame into the BamHI site of the pCDNA3 eukaryotic expression vector (Invitrogen BV, Leek, The Netherlands). DNAs (pCMDM2 and pCDNA3) were transferred into MTT cultures (10^6 cells/plate) using lipofectin, vitrogen BV, Leek, The Netherlands). DNAs (pCMDM2 and pCDNA3) were also used. For establishment of constitutive expression vectors for selection. Nuclear extracts from transient experiments were collected individually or pooled and expanded to generate cell lines. Unless indicated otherwise, reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD).

Cell growth and tumorigenicity assays

Cells (2 \times 10^5) were seeded in 6-cm plates, and the number of viable cells was determined every 24 h for 4 consecutive days by the trypan blue dye exclusion test. Experiments were performed in triplicate. For tumorigenicity assays, 5 \times 10^5 cells from each cell line were trypsinized, collected in 100 \mu l PBS, and injected sc into nude mice. Tumor formation was monitored weekly, and tumorigenicity was scored as the number of tumors per site after 4 weeks.

Detection of apoptosis

To determine cell cycle distribution, asynchronous cultures were trypsinized and fixed in 70% ethanol. Cells were pelleted, resuspended in PBS, and stained with propidium iodide. Stained samples were analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Histograms containing at least 10,000 events were generated using Lysis II software (Becton Dickinson and Co.).

Apoptosis was also monitored by annexin V labeling and fluorescence microscopy (19). Cells were washed with PBS and then treated with annexin V-fluorescin (Roche Molecular Biochemicals, Mannheim, Germany) for 15 min. After a 488-nm excitation, green fluorescence was visualized and recorded at 515 nm. Phase contrast microscopic images from the same preparations were also obtained.

Apoptotic cells from tumor sections were identified by TUNEL (terminal deoxynucleotidyltransferase-mediated deoxy-UTP-biotin nick end labeling) staining (20), with minor modifications, as previously described (21).

Northern analysis

Total RNA was extracted from guanidinium isothiocyanate cell lysates (22) with phenol-chloroform and isopropanol precipitation. RNA samples (20 \mu g) were separated by 1% agarose electrophoresis under denaturing conditions (1.1 M formaldehyde and 50% formamide) and transferred to Nitrofilter-S (Schleicher & Schuell, Inc., Keene, NH). Prehybridization and hybridization were performed at 42 C for 6 and 24 h, respectively, in a buffer containing 50 mM Na_2HPO_4 (pH 6.5), 5 X SSC (standard saline citrate), 0.2% SDS, 5 X Denhardt’s solution, and 50% formamide. Blots were washed three times at room temperature in 2 X SSC-0.1% SDS and twice at 42 C in 0.1% SSC-0.1% SDS. A 1.6-kb human mdm2 probe, obtained after SalI/BamHI digestion of pCMMD2, was used for hybridization. DNA fragments were purified using Gene clean (BIO 101, La Jolla, CA) and labeled with [α-32P]deoxy-CTP by random priming. Specific activity was usually about 5 \times 10^6 cpm/μg.

To assess equal loading of the samples, the same blots were hybridized with a β-actin probe.

RT-PCR amplification

MTC tumor samples were provided by Drs. E. Mato and X. Matias-Guiu (Hospital de la Santa Creu i Sant Pau, Barcelona, Spain). Total RNA from the tumor samples were extracted as described above (22). RNA preparations (1 μg) were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Pharmacia Biotech, Piscataway, NJ) for first strand synthesis. Aliquots of the reactions were then used for PCR amplification using Taq polymerase (Perkin-Elmer Corp., Norwalk, CT). Forward and reverse primers for mdm2 amplification were 5'-GCTGAAGAGGGCTTTGAT-3' and 5'-TGGTGTTAAAGGTAGCCTAAGCT-3'. Amplification was carried out for 40 cycles, and PCR cycle parameters were: denaturation at 94 C for 1 min, annealing at 55 C for 1 min, and extension at 72 C for 1 min. Control amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with the following forward and reverse primers: 5'-GACCCCATCTGCCTCAG-3' and 5'-TTTCCATGGTGTTGTAAG-3'. Amplification was performed in 40 cycles with these PCR cycle parameters: denaturation at 94 C for 1 min, annealing at 62 C for 30 sec, and extension at 72 C for 90 sec. PCR products were separated and visualized in ethidium bromide-stained 2% agarose gels.

Western analysis

Nuclear extracts were obtained as previously described (23). Equal amounts of nuclear proteins (20 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). After blocking membranes with 5% low fat dry milk in Tris-buffered saline-0.05% Tween-20, immunodetection of MDM2 was performed using a commercial monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After incubation with a horseradish peroxidase-conjugated secondary antibody, immunoreactive proteins were visualized by Western blotting luminol reagent (Santa Cruz Biotechnology, Inc.). Starting from total protein extracts, similar protocols were used to detect the apoptosis-related proteins Bcl-2, Bcl-x, caspase-3, and receptor interactin protein (RIP), using antibodies obtained from Transduction Laboratories (Lexington, KY). Poly(ADP-ribose) polymerase (PARP) and actin antibody were purchased from Santa Cruz Biotechnology, Inc.

Statistical analysis

Statistical significance among experimental groups was determined using Student’s t test. Differences were considered significant at P < 0.05.

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Results

The mdm2 protooncogene is not expressed in the MTT cell line

We have previously reported that overexpression of p53 in MTT cells causes a partial G1-specific arrest, as p53 clones are able to partially overcome the G1 block and progress through the cell cycle (3). In this study we have searched for cell cycle regulatory pathways operating in this p53-null cell line and analyzed the participation of the MDM2 oncoprotein.

We initially characterized the expression levels of mdm2 by Northern analysis. To our knowledge, expression of mdm2 had never been tested in any thyroid-derived tumor cell line, so we included a panel with three follicular tumor cell lines (FRO, ARO, and NPA). Total RNA was extracted and hybridized with a (FRO, ARO, and NPA). Total RNA was extracted and hybridized with a mdm2 cDNA probe. As shown in Fig. 1A, a 5.5-kb mdm2 transcript was detected in the three follicular thyroid carcinoma cell lines. The mol wt for mdm2 messenger RNA (mRNA) was as previously described (6). Expression was maximum in FRO cells and was also detected in ARO and NPA. However, expression of mdm2 was absent in MTT cells.

The absence of mdm2 transcripts in the MTT cell line prompted us to analyze whether this observation was restricted to this particular cell line or could be extended to other MTC samples. To address this question, RNA from four MTC tumors was analyzed for the presence of mdm2 by RT-PCR. Positive and negative control experiments included NPA and MTT samples, respectively. Results show that none of the tumors analyzed expressed mdm2, whereas a band of the expected size was amplified from NPA cells. In all cases, the integrity of the RNA samples was confirmed using primers for GAPDH (Fig. 1B).

Expression of mdm2 interferes with MTT cell growth

To analyze the participation of the mdm2 protooncogene in the transformed phenotype of the MTT cell line, we introduced an exogenous mdm2 gene to study the effect on cell proliferation. A mammalian expression vector carrying the human mdm2 cDNA in sense orientation was transfected by lipofection into MTT cell line. A control experiment was performed using empty vector (pCDNA3). G418 was added to the cultures 48 h after transfection, and clonal selection was maintained for 3 weeks. After that period, we observed that the ability of individual colonies to progress was clearly reduced in those cells receiving the mdm2 expression vector. Moreover, outgrowing colonies from mdm2 transfections were clearly smaller than those obtained with the empty vector (Fig. 2, A and B). To quantify this observation, plates were fixed with methanol and stained with crystal violet. As a control for these experiments, two genes previously described to act as negative regulators of cell growth were used: p53, which has been shown to inhibit cell growth in this particular cell line (3), and the INK4a tumor suppressor gene p19ARF, which interferes with cell proliferation in many cell lines (18). Results are summarized in Table 1. Compared with the control transfections, expression of mdm2 decreased colony formation about 3-fold. This reduction was similar to that obtained with the tumor suppressor gene p19ARF. A much greater effect was observed with a p53 expression vector. These results indicate that overexpression of mdm2 has a negative effect on cell growth.

We next attempted to generate stable transfectants expressing mdm2. For this purpose, G418 resistant colonies were isolated and expanded as clonal cell lines, designated MTT-mdm2 clones (c1 to c5). To avoid clonal heterogeneity, pools (-p) from the same transfection assays were also isolated and analyzed in parallel. A control cell line (pC-MTT) was originated by transfection of MTT cells with the empty vector. MTT-mdm2 clones are viable and show major alterations on cell morphology with respect to the parental cell line (Fig. 2, C and D). Whereas asynchronous cultures of MTT cells have a typical criss-cross pattern and fibroblast-like morphology, MDM2 transfectants exhibit lower saturation density values and appear more refringent under phase contrast microscopy. This morphology was different from that produced by other genes tested in the assay that have a negative effect on cell growth. They did not show the cell to cell extensions found in p53-transfected cells (Fig. 2E), or the spindle-shaped morphology of MTT cells transfected with p19ARF (Fig. 2F).

Before further characterization, the expression levels of
mdm2 mRNA in MTT-mdm2 clones were analyzed by Northern blot (Fig. 3A). Specific transcripts corresponding to the exogenous mdm2 were detected in MTT-mdm2 cells, whereas hybridization was absent in those cells transfected with the control vector. To confirm that detected transcripts encoded for a MDM2 protein, nuclear extracts from MTT-mdm2-c1, -c4, and -p1, which showed higher expression levels of mRNA, were isolated, resolved by electrophoresis, and immunoblotted with a specific human MDM2 monoclonal antibody. A polypeptide migrating at 90 kDa was observed in all MTT-mdm2 transfectants (Fig. 3B). Protein accumulation was maximum in MTT-mdm2-c1. These results confirmed the presence of MDM2 and indicated that the exogenous protein is efficiently translocated to the nucleus. To obtain an estimation of the levels of protein achieved in MTT-mdm2 clones, nuclear extracts from MCF-7 were included in the assay. The results show that MDM2 protein levels in MTT-mdm2 clones were comparable to those in cells naturally overexpressing MDM2 (24).

TABLE 1. Effect of mdm2 expression on MTT colony formation

<table>
<thead>
<tr>
<th>Vector</th>
<th>Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCDNA3</td>
<td>400 ± 17</td>
</tr>
<tr>
<td>p19ARF</td>
<td>190 ± 16a</td>
</tr>
<tr>
<td>pC-mdm2</td>
<td>155 ± 23a</td>
</tr>
<tr>
<td>pZ-p53 sense</td>
<td>20 ± 5 a</td>
</tr>
</tbody>
</table>

MTT cells were transfected with 10 μg of the different plasmids using the lipofection technique. G-418-resistant cells were allowed to grow for 3 weeks, then fixed and stained with crystal violet to determine colony number. Experiments were performed in triplicate. Average values and SEs are shown. *Statistically significant vs. pCDNA3 (P ≤ 0.01).

We next quantified the negative effect on cell growth by determining the growth rate of MTT cells transfected with either mdm2 or the control vector. Cells were seeded, and the number of viable cells was determined for 4 consecutive days (Fig. 3C). The results demonstrated a significant growth retardation induced by MDM2. After 4 days in culture, the total cell number of MDM2 transfectants was up to 40% lower than that obtained for the parental cell line. This inhibitory effect, although variable, was observed in both individual clones and pooled cultures.

MDM2 promotes apoptosis in MTT cells

To analyze the cellular mechanisms responsible for MDM2 interference with MTT cell growth, we analyzed cell cycle distribution of MTT cells transfected with mdm2. Asynchronous cultures from those clones positive in the Western blot were collected and analyzed by flow cytometry. Histograms from two individual clones and one pool are shown (Fig. 4), and data summarized in Table 2. Cell cycle distribution of MTT cells, transfected with the control vector, showed values similar to the previously described histograms for the parental, untransfected MTT cells (3). MTT-mdm2 clones consistently showed a fraction of hypodiploid cells (ranging from 35–49%), with a DNA content below 2N (sub-G0/G1). This distribution is characteristic of apoptotic cells (25) and therefore suggests that MDM2 promotes cell death in these cells. It is remarkable that apart from this sub-G0/G1 fraction, the remaining cells are distributed along the cell cycle almost normally, although G2-M values were slightly lower than those measured in MTT cells.

We further confirmed that hypodiploid cells detected by flow
cytometry corresponded to cells undergoing apoptosis. For that purpose, MTT and MTT-mdm2-cl cells, which exhibited the highest sub-G0/G1 fraction, were collected and treated with annexin V. This protein, which specifically interacts with phosphatidylserine exposed in the outer layer of the plasma membrane, is a valuable marker for detection of apoptotic cells (19).

Data obtained from fluorescence detection of MTT and MTT-mdm2-cl together with the phase contrast microscopic images of the same fields are shown (Fig. 5). Apoptosis was clearly detected in MTT-mdm2 cells (Fig. 5, A and B), whereas it was virtually absent in MTT samples (Fig. 5, C and D). Staining with propidium iodide indicated that necrotic cells were almost ab-
sent in both preparations (not shown). Quantification of different fields indicated that the fraction of apoptotic cells was about 40% of the total cell population, thus providing a good correlation among growth retardation profiles, cell cycle histograms, and apoptosis.

**MDM2 induction of apoptosis is partially reverted by wild-type p53**

In an attempt to understand whether MDM2 induction of apoptosis is related to the p53 defect of MTT cells, a series of transient transfection experiments was performed. Both MTT and MTT-mdm2 (clone c1) cells were seeded and then transfected with an expression vector for wt p53. A parallel experiment was also performed using an expression vector for the p19ARF, and finally, both vectors were also cotransfected. In all cases, cells were collected 72 h after transfection to determine the percentage of apoptotic cells and cell cycle distribution by flow cytometry.

Control experiments with an empty expression vector gave sub-G0/G1 values similar to those obtained previously (Table 1). After transfection with p53, the percentage of the sub-G0/G1 was significantly lower, indicating that the tumor suppressor partially reverts MDM2 induction of apoptosis (Table 3). This observation parallels the increase in the percentage of cells in G0/G1 phase. Similar data were obtained after transfection with p19ARF, although in this case, cells were not clearly arrested in G0/G1. When both constructs were cotransfected, results were comparable to those obtained with p53 alone, indicating that the effect of those genes is not additive.

**Tumors derived from mdm2-expressing cells show an increased number of apoptotic nuclei**

We next evaluated whether the negative effect induced by MDM2 on cell growth and the ability to promote apoptosis were extended when MTT cells were allowed to form tumors in vivo. MTT and MTT-mdm2 (clone 1) cells were injected sc

### TABLE 2. Cell cycle distribution of MTT cells transfected with mdm2

<table>
<thead>
<tr>
<th></th>
<th>Sub-G0/G1</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT</td>
<td>1.57 ± 0.17</td>
<td>47.34 ± 0.20</td>
<td>27.08 ± 0.43</td>
<td>24.01 ± 0.30</td>
</tr>
<tr>
<td>MTT-mdm2-c1</td>
<td>49.63 ± 0.87a</td>
<td>34.94 ± 0.54</td>
<td>9.25 ± 0.13</td>
<td>6.18 ± 0.24</td>
</tr>
<tr>
<td>MTT-mdm2-c4</td>
<td>46.54 ± 0.72a</td>
<td>35.21 ± 0.95</td>
<td>11.65 ± 0.37</td>
<td>6.60 ± 0.19</td>
</tr>
<tr>
<td>MTT-mdm2-p1</td>
<td>33.06 ± 0.25a</td>
<td>41.75 ± 1.18</td>
<td>14.32 ± 0.29</td>
<td>10.87 ± 0.26</td>
</tr>
</tbody>
</table>

Samples were collected and fixed, and cell cycle was analyzed by FACScan as described in Materials and Methods. Experiments were performed by triplicate. Average values and sds are represented.

### TABLE 3. Cell cycle distribution of MTT-mdm2 cells transfected with p53, p19ARF, and p53/p19ARF

<table>
<thead>
<tr>
<th></th>
<th>Sub-G0/G1</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT-mdm2-c1</td>
<td>46.29 ± 0.70</td>
<td>35.06 ± 0.83</td>
<td>12.54 ± 1.09</td>
<td>6.11 ± 0.79</td>
</tr>
<tr>
<td>MTT-mdm2/p53</td>
<td>33.48 ± 0.87a</td>
<td>42.42 ± 0.46</td>
<td>12.44 ± 0.83</td>
<td>11.44 ± 0.64</td>
</tr>
<tr>
<td>MTT-mdm2/p19ARF</td>
<td>36.47 ± 1.21a</td>
<td>37.37 ± 0.63</td>
<td>15.73 ± 0.86</td>
<td>10.64 ± 0.99</td>
</tr>
<tr>
<td>MTT-mdm2/p53 + p19ARF</td>
<td>32.74 ± 1.01a</td>
<td>43.93 ± 0.64</td>
<td>12.10 ± 0.73</td>
<td>11.33 ± 0.82</td>
</tr>
</tbody>
</table>

Samples were collected and fixed, and cell cycle was analyzed by FACScan as described in Materials and Methods. Experiments were performed by triplicate. Average values and sds are represented.

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**FIG. 5. Detection of MDM2-induced apoptosis in MTT cells.** Apoptosis was monitored by annexin V and fluorescence microscopy. Phase contrast microscopy pictures of MTT-mdm2 (A) and MTT cells (C) were analyzed by fluorescence at 515 nm (B and D, respectively).
into nude mice, and tumor formation scored after 4 weeks. As shown in Table 4, both MTT and MTT-mdm2 cells gave rise to tumors in 100% of the cases. Tumors derived from MTT-mdm2 cells emerged later, although this difference was not significant, indicating that the negative interference of MDM2 with cell growth does not reverse the transformed phenotype of these cells.

To rule out the possibility that the lack of effect could be due to a loss of mdm2 expression during tumor development, samples were analyzed for the presence of MDM2 protein by immunohistochemistry. Slide preparations from MTT and MTT-mdm2 tumors were fixed and incubated with MDM2 antibodies. As expected, tumors derived from the parental cell line did not show immunostaining. However, MTT-mdm2 tumors showed positive staining, indicating that MDM2 is efficiently expressed in the tumor (data not shown).

We next examined whether mdm2, expressed in the tumors derived from MTT-mdm2 cells, was also able to promote apoptosis in vivo. For this purpose, tumor sections were analyzed for the presence of apoptotic nuclei by TUNEL assay (Table 3). Tumors derived from the MTT cell line showed a very low percentage of TUNEL-positive cells (0.3%). However, in those tumors derived from MTT-mdm2 cells, the percentage of apoptotic nuclei increased almost 20-fold (5.3%). These results unambiguously confirm the ability of the MDM2 protein to induce apoptosis in MTT cells, both in vivo and in vitro.

**Bcl-2 and caspase-2 participate in MDM2-induced apoptosis**

Molecular mechanisms underlying MDM2-mediated apoptosis in MTT cells were explored. We reasoned that if MDM2 is able to induce apoptosis in MTT cells, specific antiapoptotic pathways operating in the parental cell line should be shut down in those cells transfected with mdm2. To test this hypothesis, we measured protein levels of Bcl-2, a protein that suppresses programmed cell death in many cell lines (26). Using specific antibodies for Bcl-2, we detected an immunoreactive band in the parental MTT cells (Fig. 6). In those clones transfected with mdm2, Bcl-2 protein levels were almost undetectable. Only after long exposure of the autoradiographs could a faint band be visualized, indicating a strong down-regulation of Bcl-2 induced by mdm2. We also measured protein levels of Bcl-x. The bcl-x gene is related to bcl-2, although proteins encoded by this locus can function independently of Bcl-2. Two products, generated by alternative splicing, arise from the bcl-x gene: Bcl-xL and Bcl-xS. Whereas the former also inhibits apoptosis in some cell systems, Bcl-xS promotes cell death (27). In MTT cells, Bcl-x was present in asynchronous cultures and, upon transfection with mdm2, no consistent modifications of Bcl-x were observed. In some clones, a slight up-regulation of Bcl-x was detected, whereas in most cases no major differences were found.

It was recently shown that Bcl-2 regulates apoptotic cascades mediated by caspase-3 and caspase-2 (28), so we analyzed whether any of these cystein proteases could be detected in MTT-mdm2 cells (Fig. 7). We used antibodies for caspase-2 and caspase-3 and probed membranes containing total protein extracts from MTT and MTT-mdm2

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**TABLE 4. Effect of mdm2 expression on tumorigenicity and population of TUNEL-positive cells from the tumors**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Tumors in nude mice</th>
<th>% Apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCDNA3</td>
<td>6/6</td>
<td>0.3</td>
</tr>
<tr>
<td>pcMDM2</td>
<td>6/6</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Tumorigenicity is expressed as the number of tumors/sites injected and was scored 4 weeks after transfection. The percentage of apoptosis was determined by TUNEL assay.

**FIG. 6.** MDM2 down-regulates protein levels of Bcl-2. Protein extracts from MTT cells and MTT cells transfected with mdm2 (clones c1, c4, and p1) were separated by SDS-PAGE and probed with specific antibodies for Bcl-2 and Bcl-x. Equal loading of the samples was assessed using an actin antibody. Mol wt marker migration is indicated.

**FIG. 7.** Caspase-2 is up-regulated in MTT cells transfected with mdm2. Protein extracts from MTT cells and MTT cells transfected with mdm2 (clones c1, c4, and p1) were separated by SDS-PAGE and probed with specific antibodies for caspase-2, caspase-3, RIP, PARP, and actin. Mol wt marker migration is indicated.
cells. In the parental cell line, caspase-2 was undetectable. However, it was clearly up-regulated in cell lines transfected with \textit{mdm2}. We next determined protein levels of caspase-3. As shown, we were unable to detect the presence of this protease in extracts from either MTT or MTT-\textit{mdm2} cell lines, although it was clearly visualized in extracts from Jurkat cells (not shown). To further rule out a participation of caspase-3, we determined the proteolytic cleavage of PARP, an enzyme involved in DNA repair, and a common substrate of caspase-3 (29). Total extracts were subjected to Western blot analysis with an antibody against PARP. As expected, only the uncleaved, 115-kDa isoform was observed in both MTT and \textit{mdm2} transfected cells.

Taken together these results point to a mechanism involving caspase-2 and independent of caspase-3. These mechanisms have been described in some apoptotic pathways, such as those mediated by tumor necrosis factor-\textit{a} (TNF\textit{a}) (30). To explore whether a similar mechanism could be acting in MTT-\textit{mdm2} cells, we determined protein levels of RIP (31), an adapter molecule involved in apoptotic pathways involving caspase-2 independently of caspase-3. Total protein extracts from the MTT cell line and from cells transfected with \textit{mdm2} were obtained. Western blot was carried out using an antibody against RIP. As shown in Fig. 7 an immunopositive band corresponding to RIP (74 kDa) was observed. However, we detected the same amount of the immunoreactive band in both MTT and MTT-\textit{mdm2} clones, suggesting that activation of caspase-2 may occur through a different apoptotic pathway.

**Discussion**

The results presented in this study provide evidence for a novel function mediated by MDM2 and indicate for the first time that this oncoprotein promotes apoptosis in a human MTC cell line characterized by the presence of a genetic rearrangement of the \textit{p53} locus (3). These results together with those showing the ability of MDM2 to arrest the cell cycle of normal fibroblasts (16) indicate that the product of the \textit{mdm2} protooncogene may also interfere negatively with cell proliferation. Several studies have previously demonstrated that MDM2 promotes tumorigenesis when it is overexpressed. It has been shown to cooperate with \textit{ras} in the transformation of rat embryo fibroblasts (32) and to induce neoplastic conversion of murine immortalized cells (33). Likewise, MDM2 is able to prevent \textit{p53}-mediated apoptosis in some tumor cell lines (34) as well as \textit{G1} cell cycle arrest even in the absence of \textit{p53} (35).

These opposite functions support the idea that, as previously described for other genes such as \textit{c-myc} (36), E1\textit{A} (37), or cyclin D1 (38, 39), some regulatory proteins could be involved in both tumorigenesis and apoptosis depending on the cellular environment. MDM2 should, then, be considered as a multifunctional regulator of cell cycle progression, whose effect on cell growth may be dependent not only on \textit{p53}, but also on other known or unknown regulatory proteins.

Expression of \textit{mdm2} is found in the three follicular tumor thyroid cell lines tested. It is important to mention that expression of \textit{mdm2} is higher in FRO cells, in which no alterations of \textit{p53} have been described (40). Both ARO and NPA carry a mutation of the \textit{p53} gene and would render protein products for this tumor suppressor unable to \textit{trans}-activate \textit{mdm2}. Nevertheless, differences in \textit{mdm2} expression are not dramatic among the three cell lines tested, suggesting that other regulatory genes, apart from \textit{p53}, participate in \textit{mdm2} transcription. Of special interest is the observation that \textit{mdm2} transcripts are not detected in any of the four MTC tumor samples analyzed. Whether there is a correlation between lack of \textit{mdm2} expression and this particular tumor type requires further investigation and is currently being studied.

Cell growth profiles and cell cycle histograms of MTT-\textit{mdm2} clones indicate that whereas in some cells expression of \textit{mdm2} promotes apoptosis, others not only remain viable, but also progress along the cell cycle. The fact that the same results have been observed in pools and individual clones rule out the possibility of an artifact caused by an inappropriate integration of \textit{mdm2} during transfection. Rather, the effects must be explained considering that in MTT cells, MDM2 may be also activating some of the previously described pathways that favor cell growth (41). It is also possible that the threshold of MDM2 expression dictates the decision of a given cell to either enter the cell cycle or commit programmed cell death. In this regard, it may be important that a correlation was observed between \textit{mdm2} expression levels and the percentage of apoptosis in the asynchronous cultures. Expression is maximum in the individual clones, where a higher percentage of apoptosis is found.

The results reported here have been observed at both early and late passages. To date, no significant decrease in the expression of \textit{mdm2} has been observed in our cultures. The fact that MDM2 levels are maintained, and apoptosis is also detected at late passages rule out the possibility that the deleterious effect of MDM2 may be limited to early events in the transfection assays, where high amounts of the protein are expressed inappropriately. Rather, we believe that MDM2 physiologically regulates and promotes apoptosis in these cells. Moreover, apoptosis mediated by MDM2 may be partially reverted by exogenous expression of both \textit{p53} and \textit{p19\textsuperscript{ARF}}, as determined by transient transfection analysis. In the case of \textit{p53}, we have previously demonstrated that the tumor suppressor gene causes a \textit{G1} arrest in these cells (3), and here we observed that even in the presence of MDM2, \textit{p53} partially arrest MTT cells in that phase of the cell cycle, thereby preventing them from undergoing apoptosis. On the other hand, the ability of \textit{p19\textsuperscript{ARF}} to partially reverse MDM2-induced apoptosis is in keeping with the observation that the product of the INK4a locus is able to bind to and promote the degradation of MDM2 (14, 15).

In agreement with results published for the MTC cell line TT and MTC tumors (42), we have clearly detected expression of Bcl-2 in MTT cells, suggesting that the Bcl-2 oncoprotein may contribute to the pathogenesis of these tumors and transformed cells. Here we show that apoptosis induced by MDM2 is accompanied by down-regulation of Bcl-2, thus allowing cell death to progress. This together with the activation of caspase-2 suggest that, as previously described for other cell systems (43), both pathways interact. Nevertheless,
in our Western assays with caspase-2 antibodies, we detect immunoreactive forms corresponding to the procaspase form and have been unable to detect any band corresponding to any cleaved form of this protease. Results also show that caspase-3 is not activated in MTMT-mdm2 cells, as 1) this protease is not detected in protein extracts; and 2) fragmentation of PARP, a well-characterized substrate for caspase-3, is not cleaved in MTMT-mdm2 cells. This points to an apoptotic cascade dependent on caspase-2, although caspase-3 independent, such as those described for cell death mediated by TNFα. It has been shown that TNF binding to its receptor results in the clustering of receptor death domains. Then, the adapter protein RIP binds through its own death domain to the clustered receptor death domain, and this complex joins another adapter molecule, RAIDD/CRADD (44, 45). Upon recruitment by CRADD, caspase-2 drives its activation through self-cleavage. Two pieces of evidence suggest that a different pathway is acting in MTMT cells transfected with MDM2. First, protein levels of RIP were similar in control and MDM2-expressing cells, and as mentioned, we have been unable to detect a cleaved form of caspase-2.

Previous reports (8, 14) and the observation described here definitively indicate that the response to MDM2 overexpression is cell specific. Therefore, it is important to determine the cellular environment in which MDM2 is able to induce apoptosis, and in this context, the medullary carcinoma cell line MTT constitutes an excellent system for these studies. As these cells are naturally devoid of p53 (3), other regulatory proteins functionally related to MDM2 should be carefully analyzed. Potential candidates for this analysis include the p53 homolog p73 (46), an antiproliferative protein whose function is modified by MDM2 (47, 48).

References

14th International Symposium of The Journal of Steroid Biochemistry & Molecular Biology

“RECENT ADVANCES IN STEROID BIOCHEMISTRY & MOLECULAR BIOLOGY”
24–27 June 2000 — Québec, Canada

The 14th International Symposium of The Journal of Steroid Biochemistry & Molecular Biology—“Recent Advances in Steroid Biochemistry & Molecular Biology” will be held in Québec, Canada, on 24–27 June 2000. The following topics will be considered:

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5. Enzyme Inhibitors
6. Steroids and the Immune System
7. Steroids and the Menopause

Lectures (approximately 25–30) will be by invitation of the Scientific Organizing Committee and, in addition, there will be a poster section. All poster presentations will be subject to selection by the Scientific Organizing Committee and abstracts (maximum 200 words) must be sent to Dr J. R. PASQUALINI by Monday 14 February 2000 (postmark) (ORIGINAL + 12 copies). For further details, please contact:

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