Different Sensitivity of the Transforming Growth Factor-β Cell Cycle Arrest Pathway to c-Myc and MDM-2*

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Stacy W. Blain‡ and Joan Massagué§
From the Cell Biology Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Recently, the oncoprotein MDM-2 was implicated in the transforming growth factor-β (TGF-β) growth inhibitory pathway by the finding that prolonged, constitutive expression of MDM-2 in mink lung epithelial cells could overcome the antiproliferative effect of TGF-β (Sun, P., Dong, P., Dai, K., Hannon, G. J., and Beach, D. (1998) Science 282, 2270–2272). However, using Mv1Lu cells conditionally expressing MDM-2, we found that MDM-2 does not overcome TGF-β-mediated growth arrest. No detectable changes were observed in various TGF-β responses, including cell cycle arrest, activation of transcriptional reporters, and TGF-β-dependent Smad2/3 nuclear accumulation. This finding was in direct contrast to the effect of forcing c-Myc expression, a bona fide member of the TGF-β growth inhibitory pathway, which renders cells refractory to TGF-β-induced cell cycle arrest. Our results suggest that an MDM-2-dependent increase in cell cycle progression may allow the acquisition of additional mutations over time and that these alterations then allow cells to evade a TGF-β-mediated growth arrest. Our conclusion is that, whereas c-Myc down-regulation by TGF-β is a required event in the cell cycle arrest response of epithelial cells, MDM-2 is not a direct participant in the normal TGF-β antiproliferative response.

Transforming growth factor-β (TGF-β) inhibits cell proliferation in many cell types by blocking progression through the G1 phase of the cell cycle (1–3). This antimitogenic response generally involves inhibition of G1-phase cyclin-dependent kinases (Cdk2, Cdk4, and Cdk6) and rapid down-regulation of c-Myc expression. Although the specific mechanisms that inactivate G1 Cdkks appear to vary between cell types, the down-regulation of c-Myc is observed in most cell types (1, 4). c-Myc has a short half-life, and the TGF-β-dependent down-regulation of c-Myc RNA results in a rapid loss of protein (5–7), as well as the ability of c-Myc to act as a transcriptional activator of genes required for the G1-S phase transition (reviewed in Refs. 8–10). The importance of c-Myc down-regulation is highlighted by the observation that exogenous c-Myc expression renders a cell resistant to the antiproliferative action of TGF-β (1, 4). c-Myc down-regulation has been linked directly with G1 Cdk inactivation, as enforced c-Myc expression in mink lung epithelial cells (Mv1Lu) blocks the TGF-β-dependent induction of the Cdk4/6 inhibitor, p15 (4).

Recently, MDM-2 has been implicated in the TGF-β pathway by the finding that prolonged, ectopic expression of MDM-2 in cell culture could overcome the antiproliferative effect of TGF-β (11). In this assay, MDM-2 appeared to allow Mv1Lu cells to survive prolonged TGF-β exposure to permit colony formation. This resistance appeared to occur in a p53-independent manner, which correlated with increased RB protein phosphorylation and reduced function of the E2F transcription factor. MDM-2 is a negative regulator of p53, known to directly interact with and mediate the degradation of this tumor suppressor gene product (12, 13). Overexpression of MDM-2 has been shown to stimulate the transactivation functions of E2F, presumably through its direct interactions with RB or E2F/DP1 (14, 15). Thus, MDM-2 appears to be an important regulator of both the p53 and the RB cell cycle regulatory pathways (13, 16). We wanted to ascertain whether, similar to c-Myc, MDM-2 was a bona fide member of the TGF-β growth inhibitory pathway or whether other affects by MDM-2 on the cell cycle were the cause of this apparent resistance to TGF-β-mediated growth suppression. By analyzing inducible MDM-2 cell lines, we demonstrate that, unlike c-Myc, MDM-2 is not a direct participant in the TGF-β antiproliferative response, suggesting that the resistance to TGF-β-mediated growth suppression might be secondary to other MDM-2 effects on cell cycle progression.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The human MDM-2 cDNA was cloned into the XbaI site of the pHUD10-3 hygromycin vector (17). The mink lung epithelial cell line, Mv1Lu-TA (17), was maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS) plus 0.5 mg/ml G418. Mv1Lu-tTA cells were transfected with pHUD10-3 hygromycin-MDM-2 using the Lipofectin procedure according to the manufacturer’s protocol (Life Technologies, Inc.). MDM-2-inducible clones were selected as described previously (17). Two clones, TMDMA and TMDMB, were further subcloned by end-dilution to obtain the cell lines analyzed in this study. The TM2 cells, expressing c-Myc, have been previously described (4). Tet cell lines were selected and maintained in minimal essential medium plus 10% FBS, 0.5 mg/ml G418, 0.3 mg/ml hygromycin, and 1 μg/ml tetracycline.

Immunoblotting and Kinase Assays—Antibodies against human MDM-2 (sc-965) and p53 (sc-99) were obtained from Santa Cruz Biotechnology. Antibodies against Cdk4, Cdk2, and p27 have been described previously (4, 17, 18). The TMDM cell lines were grown to near confluence and then split 1:3 into medium plus or minus 1 μg/ml tetracycline. After a 20-h incubation, the cells were harvested by trypsinization. Cell pellets from tet cells were lysed according to published procedure (19). They were immunoprecipitated with the appropriate antibodies for 3–16 h at 4 °C. The immunoprecipitates or aliquots (0.2 mg protein) of cell lysate were separated on SDS-polyacrylamide
gel electrophoresis and transferred to polyvinylidene difluoride (Immobilon-P) membranes. The blots were probed with the appropriate primary antibody followed by anti-mouse IgG secondary antibody (Pierce) prior to visualization by enhanced chemiluminescence (ECL or ECL plus, Amersham Pharmacia Biotech). Alternatively, the immunoprecipitates were used in RB kinase assays as described previously (4).

Cell Cycle Analysis—After a 20-h incubation with or without 1 mg/ml tetracycline, the cells were treated for 20 more hours with TGF-β (R & D Systems, Minneapolis) in the presence of 10% FBS. The preparation of stained nuclei was carried out by hypotonic lysis of cells in 0.03% Nonidet P-40, 10 mM NaCl, 1 mg/ml sodium citrate, plus ethidium bromide (25 μg/ml) and RNase (10 μg/ml) at room temperature for 30 min. After the addition of 80 mM citric acid, 250 mM sucrose, and 40 μg/ml ethidium bromide, nuclei were either analyzed immediately using a FACSscan (Becton Dickinson) or stored at 4 °C for later analysis. Parallel cell cultures were assayed for [32P]dideoxyuridine incorporation during the last 3 h. Data are the averages of triplicate determinations and are plotted as a percentage relative to the cpm incorporated in the presence of 1 μg/ml tetracycline and no TGF-β.

Reporter Assays—As reporters we used p3TP-lux (20) and the pSBE4-lux (21), shown to respond to TGF-β and Smad2/3 signaling, respectively. TMDM and TM2 cells were transiently transfected with p3TP-lux and pSBE4-lux using DEAE-dextran as described previously (22). Cells were split 24 h later into medium with or without tetracycline and then treated 16 h later plus or minus TGF-β in 10% serum. Luciferase assays were carried out 24 h later using the Promega luciferase assay kit and a Berthold luminometer.

Indirect Immunofluorescence—Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.2% Trition X-100 in PBS for 10 min. They were incubated with 10% FBS/PBS for 20 min before being incubated with 1 μg/ml affinity-purified Smad2/3 antibody (23) in 3% bovine serum albumin/PBS for 1 h. The cells were washed with PBS and incubated with biotin-conjugated goat anti-rabbit secondary antibody at 5 μg/ml for 45 min. After more PBS washes, they were incubated with streptavidin/fluorescein isothiocyanate at 20 μg/ml for 15 min. Coverslips were mounted with Vectashield (Vector).

Colony Formation Assay—4000 cells were seeded in 6-well dishes into medium with or without tetracycline. 24 h later, cells were treated with 0 or 50 μg TGF-β. The medium was replaced every other day for 8 days, and then the cells were stained with methylene blue. The post-tet

**Fig. 1.** Mv1Lu cell lines with inducible MDM-2 expression and initial analysis of cell cycle progression. A, clonal tet-MDM2 cell lines (TMDMA and TMDMB) were maintained in medium containing 1 μg/ml tetracycline and then grown in the absence of tetracycline for 18 h before harvesting. Lysates were immunoprecipitated with anti-human MDM-2 antibodies followed by anti-human MDM-2 Western immunoblotting. Parental Mv1Lu cells were also analyzed in the presence and absence of tetracycline. B, lysates were analyzed by direct Western immunoblotting with anti-p53 antibodies and quantitated by densitometry. C, TMDMA and TM2 cells were grown in the absence of tetracycline (MDM-2 on and Myc on) or in the presence of 1 μg/ml tetracycline (MDM-2 off and Myc off) for 18 h before the addition of 200 pM TGF-β in the presence of 10% FBS. Cells were harvested for flow cytometric analysis of DNA content after 18 h in the presence or absence of TGF-β. The percentage of cells in the G1 phase at this time is indicated.
passage cells were derived from the parental cells maintained for several passages in the absence of tetracycline. The colony formation assay was then repeated using these cells.

RESULTS AND DISCUSSION

Mv1Lu derivatives expressing a human MDM-2 cDNA under negative control of the tetracycline transactivator (24) were generated, and two independent clones (TMDMA and TMDMB) that expressed exogenous MDM-2 in tetracycline-free medium were chosen for further analysis (Fig. 1A). To verify that the exogenously expressed MDM-2 was functional, we examined the levels of endogenous p53 after induction of MDM-2 (Fig. 1B). In both clones, the level of p53 in the presence of MDM-2 was approximately one-third the level seen in its absence, suggesting that the exogenous MDM-2 was expressed to levels sufficient to elicit a biological response.

The addition of TGF-β to Mv1Lu cells caused G1 arrest as detected by FACS analysis (Fig. 1C). A similar G1 arrest by TGF-β was seen in the TMDM cells (Fig. 1C and data not shown), maintained in the presence of tetracycline (MDM-2 off). In the absence of tetracycline (MDM-2 on), a decrease in the G1 population of the TMDM cells was observed, suggesting that the overexpression of MDM-2 altered the typical cell cycle distribution of Mv1Lu cells. However, the addition of TGF-β in the absence of tetracycline still caused G1 arrest. As a control, we compared the effects of TGF-β on TM2 cells, a Mv1Lu derivative which expresses exogenous human C-Myc under tetracycline control (4). Enforced expression of C-Myc in the TM2 derivative which expresses exogenous human C-Myc under tetracycline (Fig. 1C), as had been shown previously (4). In the absence of tetracycline, the TM2 cells also had a reduced G1 content, suggesting that c-Myc expression also altered the typical cell cycle distribution.

As FACS analysis only enabled us to examine the cell cycle profile at a fixed time point, we assayed for [125I]deoxyuridine incorporation in order to examine the rate of DNA synthesis (Fig. 2A). In the presence of tetracycline (MDM-2 and c-Myc off states), TGF-β inhibited [125I]deoxyuridine incorporation in Mv1Lu, TMDMA, TMDMB, and TM2 cells. In the absence of tetracycline (MDM-2 and c-Myc on states), the TMDMA, TMDMB, and to a lesser extent, TM2 cells all had increased [125I]deoxyuridine incorporation, suggesting an increase in proliferative activity under these conditions. More importantly, [125I]deoxyuridine incorporation in the TMDMA and TMDMB cells with MDM-2 on was still inhibited by the addition of TGF-β, whereas TM2 cells with c-Myc on were not inhibited even at TGF-β concentrations as high as 500 pM (Figs. 2A and 4).

The above described results suggest that the expression of human MDM-2 is able to affect the cycling of the TMDM cells. The cells may pass through G1 faster, resulting in a larger S phase population at any given time point. To investigate this phenomenon, we examined a variety of G1 cell cycle components in the TMDM cells in the presence and absence of tetracycline (Fig. 2B and data not shown). Although the levels of p27 and Cdk4 were unchanged, the level of Cdk2 in the absence of tetracycline was increased. Additionally, the amount of the active, phosphorylated form of Cdk2 (Fig. 2B, cdk2*) was increased in the absence of tetracycline approximately 3-fold. This increase correlated with a corresponding 3-fold increase in Cdk2-associated RB kinase activity, which could account for the faster transit through G1 by the TMDM cells. Cyclin E-Cdk2 complexes appear rate-limiting for G1 progression, and forced expression of cyclin E has been shown to induce premature S-phase entry (25–27). Although c-Myc has been reported to activate cyclin E-Cdk2 complexes by as-yet-debated mechanisms (28), the level of Cdk2 and the amount of Cdk2-associated RB kinase activity appears unchanged in TM2 cells as previously shown (4), suggesting a difference between the c-Myc and MDM-2-dependent increase in cell cycle transit in Mv1Lu cells.

Despite the increase in Cdk2-associated RB kinase activity detected in TMDM cells in the absence of tetracycline, the addition of TGF-β under these conditions inhibited this kinase activity (Fig. 2B). Thus, unlike the TM2 cells, the TMDM cells were not able to overcome TGF-β-mediated arrest, suggesting that MDM-2 does not directly interfere with the TGF-β antiproliferative response. To further clarify this point, we examined other TGF-β responses to ascertain whether they were perturbed by MDM-2 expression (Fig. 3). The function of the TGF-β signal transduction proteins, Smad2 and Smad3, as transcription factors requires their accumulation in the nucleus in response to TGF-β. Recently, it was suggested that
overexpression of MDM-2 might inhibit the nuclear import of ectopically expressed Smad proteins (29). However, these observations were made with overexpressed proteins in the absence of TGF-β. As determined by Smad2/3 indirect immunofluorescence of TMDM cell lines, the TGF-β-induced nuclear accumulation of endogenous Smad2/3 appears unperturbed by MDM-2 overexpression, suggesting that MDM-2 does not affect this central event in TGF-β signal transduction (Fig. 3A).

Two different transcriptional reporter constructs, p3TP-lux and pSBE4-lux, were used to assess TGF-β transcriptional activation (Fig. 3B). p3TP-lux contains three repeats of a PAI-1 sequence responsive to TGF-β and is a classical reporter used to assess TGF-β activation (20). pSBE4-lux contains eight repeats of a four-base pair sequence that binds TGF-β-activated Smad3 and Smad4, and thus it is a specific indicator of Smad activation (21). In TMDMA and TMDMB cells maintained in the presence or absence of tetracycline, the addition of 100 pM TGF-β stimulated the p3TP-lux reporter by approximately 10-fold (Fig. 3B, left). TGF-β also stimulated the pSBE4-lux reporter in these cells to similar extents in the presence or absence of tetracycline, albeit the fold stimulation was somewhat higher in the TMDMA cells (Fig. 3B, right). Expression of c-Myc in the TM2 cells reduced the induction of both reporters by half when compared with the induction observed in its absence.

Thus, in the TMDM cells expressing levels of MDM-2 sufficient to elicit an effect on p53 levels and cell cycle progression, no detectable changes were observed in various TGF-β responses, including cell cycle arrest, activation of transcriptional reporters, and TGF-β-dependent Smad2/3 nuclear accumulation. This finding was in direct contrast to the effect of forcing c-Myc expression in the TM2 cells, which, in addition to altering cell cycle progression, renders cells refractory to TGF-β-induced cell cycle arrest. In contrast to previous results (11), we did not obtain any colonies in long-term TMDMA or TM-DMB cultures overexpressing MDM2 in the presence of TGF-β (Fig. 4 and data not shown); this was consistent with our previous results, suggesting that the TMDM cells were not refractory to TGF-β-mediated growth inhibition. In fact, the TMDM cells were indistinguishable from parental Mv1Lu cells maintained in the presence and absence of TGF-β (Fig. 4).

Despite the fact that cells expressing c-Myc are resistant to short term TGF-β exposure (48 h or less), as described above, the number of TM2 colonies obtained in the colony formation assay in the presence or absence of TGF-β during the 8-day time frame was very low (Fig. 4). This finding was also in direct contrast to previous results (11), where cells overexpressing c-Myc grew to confluency during the course of their experiment and numerous TGF-β-resistant colonies were detected. Our results, however, were not unexpected, as others have shown that prolonged exposure to high levels of c-Myc causes apoptosis unless the cells acquire additional mutations in the p19ARF or p53 genes, which are, coincidentally, the upstream and downstream members of the MDM-2 pathway (30, 31). The results with c-Myc suggest that the difference between the two studies may be because of the use of constitutive versus inducible expression systems. Our cell lines that conditionally express c-Myc or MDM-2 are always maintained in the presence of tetracycline (c-Myc and MDM-2 off states), thus decreasing the selective pressure for mutations that might arise through
prolonged exposure to c-Myc or MDM-2. Similar to c-Myc, we propose that an MDM-2-dependent increase in cell cycle progression may allow the cell lines used in the previous study (11) to acquire mutations during prolonged exposure to overexpressed MDM-2, which might secondarily circumvent TGF-β cell cycle arrest signals.

To verify this hypothesis, we maintained our TM2 and TMDMA cultures for several passages in the absence of tetracycline (c-Myc and MDM-2 on states), effectively converting them to constitutive c-Myc- or MDM-2 expressing lines. We then repeated the colony formation assay (Fig. 4, bottom). Significant apoptosis was observed in the TM2 cells during the initial 8-day passaging, with 90% of the cells dying (data not shown). However, the resulting population was now able to survive high levels of c-Myc expression and grow to confluency as a 8-day passaging, with 90% of the cells dying (data not shown). However, the resulting population was now able to survive high levels of c-Myc expression and grow to confluency as a 8-day passaging, with 90% of the cells dying (data not shown). However, the resulting population was now able to survive high levels of c-Myc expression and grow to confluency as a 8-day passaging, with 90% of the cells dying (data not shown). However, the resulting population was now able to survive high levels of c-Myc expression and grow to confluency as a 8-day passaging, with 90% of the cells dying (data not shown). However, the resulting population was now able to survive high levels of c-Myc expression and grow to confluency as a 8-day passaging, with 90% of the cells dying (data not shown). However, the resulting population was now able to survive high levels of c-Myc expression and grow to confluency as a 8-day passaging, with 90% of the cells dying (data not shown).

TGF-β is a required event in the cell cycle arrest response of epithelial cells, MDM-2 is not a direct participant in the normal TGF-β antiproliferative response.

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