Tumor Promotion by Mdm2 Splice Variants Unable to Bind p53

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Abstract

The Mdm2 oncoprotein physically associates with p53 and antagonizes its tumor suppressor functions. Previous studies indicate that some tumors express alternatively or aberrantly spliced Mdm2 variants that are unable to bind p53, but whether these actively contribute to carcinogenesis or are a byproduct of cancer progression has been unclear. In this study, we examined the ability of full-length Mdm2 and several tumor-derived splice variants to modulate tumor development in Eµ-myc transgenic mice. We report that several tumor-derived Mdm2 splice variants promote tumorigenesis in a manner that is comparable with full-length Mdm2. Our results imply that the current paradigm for understanding Mdm2 action during oncogenesis is incomplete, and its splice variants contribute to human cancer.

Introduction

Mdm2 is a negative regulator of the p53 pathway that inhibits p53-mediated transactivation and promotes degradation of p53 by ubiquitin-mediated proteolysis (1). Mdm2 (Hdm2 in humans) is also an important oncogene and is amplified and/or overexpressed in many human cancers (2). Several reports have noted that human and murine tumors can also express alternatively or aberrantly spliced Mdm2 variants not found in normal tissue and that, paradoxically, lack the p53 binding domain and fail to interact with p53 (reviewed in Ref. 3). Although their presence in tumors is striking, it has been unclear whether these Mdm2 variants are causally involved in tumorigenesis or whether they simply reflect genetic chaos within the tumor. In vitro studies directed toward understanding the activity of Mdm2 splice variants have been inconclusive. Although initial studies suggested that certain tumor-derived Hdm2 variants could promote the formation of transformed foci in 3T3 cells (4), subsequent studies have failed to recapitulate these activities and, in fact, suggest that many of these variants can be antiproliferative (5). Because these antiproliferative activities are not intuitively compatible with oncogenesis, the appearance of these variants in spontaneous tumors remains a conundrum. In this study, we examine the oncogenic potential of several Mdm2 splice variants in vivo and show that some of these variants can be as oncogenic as full-length Mdm2.

Materials and Methods

Genes and Gene Transfer. The amino acid sequence of human lymphoma-derived Hdm2 variants (6) were aligned with the murine Mdm2 sequence, and the homologous regions of deletions were identified. Mega-primer PCR (7) was used to construct the murine cDNAs homologous to the human variants. An HA epitope tag was added to the NH2 terminus of each cDNA by PCR to facilitate experimentation in the absence of multiple antibody epitopes. Each cDNA was again cloned into the MSCV-green fluorescent protein retrovirus in an identical translational initiation context, and the integrity of the cDNA was confirmed by sequencing. Generation of ecotropic retroviruses and isolation and infection of HSCs were performed as described previously (8).

Briefly, fetal livers were harvested from Eµ-myc transgenic mice at embryonic days 13.5 to 15.5 and were grown with cytokine support for 2 days. High-titer transiently produced retrovirus was filtered to remove potentially contaminating retroviral producer cells and used to infect cells four to five times over a 2-day period, each time supplementing the added media with growth factors. Infection efficiency was determined by flow cytometry for the coexpressed green fluorescent protein.

Immunoprecipitations, Immunoblots, and Immunofluorescence. Immunoprecipitations were performed using wild-type MEFs transduced with Mdm2 and its variants using 150 µg of protein from each sample and an anti-HA antibody (12CA5; Covance; Ref. 9). Immunoprecipitates or whole-cell extracts were subject to immunoblotting after standard SDS-PAGE using antibodies directed against p53 (Novocastra; CM5, 1:1000) or the HA epitope (Covance; 12CA5, 1:1000) antibodies, followed by enhanced chemiluminescence detection (Amersham). Immunofluorescence detection of Mdm2 was performed on samples fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 (in PBS supplemented with 3% BSA), and stained with an anti-HA antibody (12CA5, 1:100). Cells were also stained with 4,6-diamidino-2-phenylindole at a concentration of 0.1 µg/ml in PBS. For fluorescence detection, we used an Axioskop 50 immunofluorescence microscope (Zeiss, Thornwood, NY).

In Vitro Assays. p53+/+ MEFs expressing E1A/eras [clone C8 (10)] were transduced with full-length Mdm2 or its variants by retrovirus-mediated gene transfer. Infected cell populations were plated at 1 × 105 cells/well in 12-well tissue culture plates and assessed for cell death 24 h after Adriamycin treatment by trypan blue exclusion. Oncogenic transformation was assessed by the ability of these cells to form colonies in soft agar. Here, C8 cells were transfected with p53 or its variants and were plated in 0.035% agar at 1 × 105 cells/well in a 12-well dish. Colonies were allowed to form for 2 weeks, and additional agar was added to support cells after 1 week. Colonies were visualized by Giemsa (0.02%) staining.

Adaptive Transfer and Tumor Analysis. Adoptive transfer experiments were performed as described (8) using C57BL/6 recipient mice 6–8 weeks of age. Briefly, retrovirally infected HSCs were harvested and washed in cold PBS. Approximately 2 × 106 viable cells were injected into the tail veins of lethally irradiated mice (~9 Gy total body dose; 137Ce source) that were maintained under pathogen-free conditions. Tumor development was monitored by biweekly palpation of lymph nodes, and data were analyzed using the log-rank test performed on Prism software (GraphPad Software, Inc.). Lymph node enlargements of >5 mm in the longest dimension were considered palpable tumors. Histological samples were removed postmortem and fixed in buffered formalin for 24 h, after which they were stored in PBS until embedded in paraffin and processed as described in detail elsewhere (11).

1 The abbreviations used are: HA, hemagglutinin; HSC, hematopoietic stem cell; MEF, mouse embryo fibroblast; MSCV, murine stem cell virus.
Results and Discussion

To determine whether Mdm2 splice variants could have oncogenic activity in vivo, we examined their ability to modify tumor behavior in the Eμ-myc transgenic mice, a mouse model of human non-Hodgkin’s B-cell lymphoma (12). We reasoned that these animals would provide an excellent system for testing the potential impact of Mdm2 splice variants on tumor behavior: (a) Mdm2 splice variants have been observed in human lymphomas (6) and spontaneously arise in Eμ-myc lymphomas (13); and (b) this model is acutely sensitive to perturbations in the p53 pathway (8, 13, 14). Hence, full-length Mdm2 is predicted to accelerate lymphomagenesis, and thus tumors overexpressing Mdm2 provide a baseline for comparing the potency of each variant. Finally, chimeric animals expressing transgenes in the lymphoid compartment can be rapidly produced by retroviral-mediated delivery of genes into HSCs isolated from Eμ-myc transgenic mice, followed by adoptive transfer into nontransgenic recipient animals (8). Consequently, this approach allowed us to directly compare the in vivo effects of Mdm2 and its splice variants while avoiding the confounding founder effects associated with germ-line transgenic mice.

We generated a series of murine Mdm2 cDNAs corresponding to the Hdm2 splice variants that have been detected in human lymphomas (6) and tested their ability to associate with p53 (Fig. 1). These vectors, along with full-length Mdm2 (Mdm2FL), were introduced into p53+/− MEFs expressing the E1A and ras oncogenes (10) using retrovirus-mediated gene transfer. As expected, Mdm2Δ28–280 and Mdm2Δ28–298, and Mdm2Δ28–390 did not physically associate with p53 in coimmunoprecipitation assays, although full-length Mdm2 readily did so (Fig. 1B; data not shown). We also were unable to detect an association between Mdm2Δ76–480 and p53, which differs from reports for its Hdm2 counterpart (4). However, in contrast to the other Mdm2 variants (Fig. 1C), Mdm2Δ76–480 was difficult to detect by immunoblotting, and therefore we could not rule out the possibility that it retained residual p53 binding activity. However, this variant, which has a predicted molecular weight of 9,000 and lacks the Mdm2 nuclear localization signal, was expressed diffusely throughout the cell as measured by immunofluorescence staining (Fig. 1D).

Previous studies indicate that loss of p53 function can suppress apoptosis induced by DNA-damaging agents, promote the immortalization of MEFs, and facilitate oncogenic transformation (10, 15, 16). Although full-length Mdm2 can recapitulate the effects of p53 loss in these assays, studies assessing the ability of Mdm2 variants to produce similar effects have produced conflicting results (4, 5, 17). Therefore, we performed several experiments to determine whether the murine versions of tumor-derived Hdm2 splice variants we produced had oncogenic activity in vitro.

To determine whether the Mdm2 splice variants could modulate chemotherapy-induced apoptosis, we treated the onco- gene-transformed MEFs expressing Mdm2FL or its variants with Adriamycin and examined cell viability 24 h later. Consistent with its ability to antagonize p53 function, Mdm2FL suppressed drug-induced cell death, although not to the same extent as complete loss of p53 (Fig. 2A; data not shown). In contrast, none of the Mdm2 variants were antiproliferotic, and if anything, each slightly enhanced drug-induced cell death (Fig. 2A, open symbols). This is consistent with previous reports indicating that Mdm2 variants can have antiproliferative activity in vitro (5, 17) and affect our ability to maintain expression of these variants in cells for extended periods (data not shown).

We next examined the ability of the Mdm2 splice variants to promote cellular immortalization or oncogenic transformation. To assess their immortalization potential, Mdm2 or its splice variants were introduced into wild-type MEFs and examined for their ability to form colonies at clonogenic density. In this assay, wild-type MEFs senesce and do not form colonies, whereas p53-deficient MEFs form colonies with high efficiency. Only Mdm2FL was able to increase the clonogenic potential of MEFs, whereas none of the variants were able to do so (data not shown). To assess their transforming potential, p53+/− MEFs expressing E1A and ras with or without an Mdm2 variant were plated in soft agar, where the ability to form colonies is considered a surrogate for tumorigenic potential. In this assay, p53 loss dramatically increases the growth of these cells in soft agar (data not shown; see Refs. 16, 18). Once again, Mdm2FL, but none of the variants, promoted colony formation (Fig. 2B). Notably, these results are in contrast to initial reports indicating that the human versions of these variants could promote focus formation when expressed in NIH 3T3 cells (4). In our assays, none of the Mdm2 splice variants showed any oncogenic properties in vitro.

To examine the activity of Mdm2 and its variants in vivo, we isolated HSCs from fetal livers of Eμ-myc mice on embryonic days 13.5 to 15.5 and introduced a control vector, full-length Mdm2, or individual splice variants into these cells by retrovirus-mediated gene transfer. Our infection efficiencies ranged from 5 to 20% and did not vary significantly between the constructs examined (data not shown). Infected cell populations were injected into the tail veins of lethally irradiated syngeneic mice, and animals were monitored for successful reconstitution of their hematopoietic compartment and the appearance of disease. Each Mdm2 variant was tested in multiple experiments in parallel with the control vector (MSCV) and full-length Mdm2.

Consistent with previous reports (11), tumors arising in mice re-
splice variants can contribute to tumor development in a relevant in vivo model and, as such, imply that their appearance in human tumors is causally linked to carcinogenesis. Indeed, despite their apparent lack of oncogenic activity in vitro, most Mdm2 splice variants were nearly as potent as full-length Mdm2 in facilitating the development of aggressive lymphomas in vivo. These observations, in conjunction with reports correlating Hdm2 splice variants with poor prognosis or advanced tumor stage in patients with breast (19), brain (20), and bone (21) cancer suggest that at least some Hdm2 splice variants might be important in determining tumor behavior. Ironically, most clinical studies that have examined the involvement of Hdm2 in human carcinogenesis detect Hdm2 by immunohistochemistry. Because many variants lack epitopes recognized by standard antibodies, their overall frequency in cancers may be substantially underestimated.

Although the molecular basis for their oncogenic activity is not obvious from known structure-function relationships, it appears independent of their ability to physically associate with p53. Notably, most tumor-derived Mdm2 variants are predicted to interact with full-length Mdm2 (3), and although the opposite occurs in vitro, it remains possible that they potentiate Mdm2 function in some cell types or at certain stages of tumor development. In this regard, we have noted that, similar to Mdm2FL, several Mdm2 splice variants can reduce pressure to inactivate p53 during myc-induced lymphomagenesis, implying that these variants can in some way compensate for p53
deficiency.

constituted with Eµ-myc HSCs transduced with the control vector arose with incomplete penetrance at a relatively long latency (Fig. 3A; 40% tumor incidence at 200 days). As predicted, full-length Mdm2 accelerated lymphomagenesis. Hence, mice receiving Mdm2FL-transduced HSCs developed lymphomas with a higher penetrance and shorter latency than was observed for cohorts receiving vector-transduced controls (Fig. 3A; median onset, 90.5 days; n = 21; P < 0.001). Although Mdm2FL-driven lymphomas were highly aggressive and typically disseminated into nonlymphoid compartments (Fig. 4), neither their overall onset nor dissemination pattern was as pronounced as observed in the absence of p53 (Fig. 3A; median onset, 33 days; n = 41; P < 0.0001 compared with Mdm2FL).

Remarkably, three of four Mdm2 variants also accelerated lymphomagenesis to the similar extent as full-length Mdm2 (Fig. 3B; Mdm2ΔA28−220 n = 13, P = 0.002; Mdm2ΔA28−390 n = 17, P = 0.001; Mdm2Δ76−480 n = 16, P < 0.001 compared with control vector). The only exception was Mdm2ΔA28−220, which did not differ significantly from the control vector (n = 21, P = 0.141). Notably, the lymphomas produced by the oncogenic Mdm2 variants were also aggressive and displayed an overall pathology similar to lymphomas expressing full-length Mdm2 (Fig. 4; data not shown). These results are in stark contrast to our in vitro findings and those reported elsewhere (5, 6) and demonstrate that Mdm2 splice variants can have oncogenic activity when assessed in a relevant in vivo context.

These data provide compelling evidence that tumor-derived Mdm2

![Figure 2: Mdm2 splice variants do not have oncogenic activity in vitro. A, p53+/+ MEFs expressing E1A and ras along with Mdm2 or one of the indicated variants were treated with Adriamycin and assessed for viability 24 h later by trypan blue exclusion. The data represent the means of results from three experiments; bars, SD. Note that Mdm2ΔA28−220 was not tested in this assay. B, the same cell populations described in A were plated in soft agar, and colony formation was assessed 14 days later. Shown is a representative example of three independent experiments plated in triplicate.](image)

![Figure 3: Acceleration of lymphomagenesis by tumor-derived Mdm2 splice variants. A, chimeric transgenics of mice expressing Mdm2FL or a vector control in the hematopoietic compartment were produced after retroviral delivery into HSCs from the fetal livers of Eµ-myc transgenic embryos and subsequent adoptive transfer into nontransgenic recipients. For comparison, p53+/− Eµ-myc mice developing lymphomas are included. Note that virtually 100% of the lymphomas arising from p53+/− Eµ-myc mice lose the remaining wild-type allele and become p53 null (14). The Kaplan-Meier plot shows the percentage of mice that remain tumor free at various times after the adoptive transfer. Tumor onset is defined as the time between reconstitution and the appearance of well-palpable lymph nodes (≈5-mm diameter). B, chimeric transgenics expressing various Mdm2 splice variants were produced as described in A. Kaplan-Meier plots show the tumor onset of mice reconstituted with Eµ-myc HSCs transduced with the control vector (dotted), Mdm2FL (thin line), and the indicated Mdm2 variant (bold lines). Note that the control and Mdm2FL curves are shown in each panel for comparison and reproduce the same data shown in A.](image)
loss. Perhaps these variants might result in the observed antiproliferative effects of Mdm2 while retaining some ability to modulate p53. In this regard, directed mutations designed to abrogate the growth-inhibitory domains of Mdm2 can increase its transforming ability (22). Alternatively, these variants might retain one or more of the several p53-independent activities attributed to Mdm2 (reviewed in Ref. 23). Importantly, the inconsistency between in vitro and in vivo assays implies that the oncogenic activity of these variants is context dependent, which represents a confounding factor for mechanistic studies. Nevertheless, our results imply that the current understanding of Mdm2 action during oncogenesis is incomplete and that further investigation into the in vivo functions of Mdm2 and its splice variants is warranted.

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