The Pathway Regulating MDM2 Protein Degradation Can Be Altered in Human Leukemic Cells

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Abstract

The MDM2 protein regulates the functional activity of the p53 tumor suppressor through direct physical association. Signals that control MDM2 expression are poorly understood but are likely to play an important role in the regulation of p53 activity. We show here that the half-life of MDM2 protein is shorter in proliferating than in quiescent peripheral blood mononuclear cells. We also demonstrate that MDM2 protein half-life is extended in some, but not all, p53 mutant human leukemic cell lines. In at least one of these p53 mutant lines, increased MDM2 protein stability is associated with higher amounts of MDM2 protein. Moreover, we demonstrate that MDM2 protein accumulates to a much greater extent in proteasome inhibitor-treated cells containing unstable MDM2 than in cells possessing stable MDM2. These results demonstrate that MDM2 expression is regulated by events that control the stability of the protein and suggest that the normal regulation of MDM2 turnover can be altered in tumor cell lines.

Introduction

The transforming potential of the MDM2 oncprotein is activated by overexpression (1, 2), and high levels of MDM2 protein are present in some human tumor cells (3). The primary mechanism by which MDM2 overexpression is thought to induce cellular transformation is through its ability to bind to the p53 tumor suppressor and block p53 activity (4). MDM2 can inhibit p53 activity by binding to the acidic activation domain of p53 (5) and by targeting p53 for degradation via the proteasome (6, 7). Because the transforming activity of MDM2 has been attributed to the overproduction of protein (1, 2), it is important to understand the mechanisms that regulate MDM2 protein expression and how these mechanisms may be altered in human tumor cells. It is well established that p53 is itself a key regulator of MDM2 transcription (8–11). Besides those signals that induce p53 transcriptional activity (e.g., DNA damage), very little is known about other cellular signals that may regulate MDM2 protein expression. In the work presented here, we have investigated how MDM2 protein stability may be differentially regulated in quiescent and growth stimulated human PBMCs. We have also examined the possibility that the normal regulation of MDM2 turnover can be altered in tumor cell lines.

Materials and Methods

Cell Culture. All lines were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Inc.). The M8166 (T-cell) line possesses wt p53 alleles, whereas the CEM (T-cell), HEL (erythroleukemia), Jurkat (T-cell), MOLT4 (T-cell), and Raji (B-cell) lines possess mutant p53 alleles (12). PBMCs were isolated with Ficoll-Paque (Pharmacia) according to the manufacturer’s instructions. Quiescent PBMCs were cultured in complete RPMI 1640 and used for studies immediately after isolation from normal donors. To generate a population of actively proliferating T cells, quiescent PBMCs were seeded at 2 x 10⁶ cells/ml in complete RPMI 1640 supplemented with 1% PHA-M (Life Technologies, Inc.). After 3 days, recombinant IL-2 (Sigma) was added into the media to a final concentration of 1 unit/ml. Cells were counted every other day and maintained at 1 x 10⁶ cells/ml in IL-2-containing media. Once cells possessed a doubling time of ~48 h, they were used for the appropriate studies.

Cycloheximide and MG115 Treatment of Cells. Cells were seeded at 2 x 10⁶ cells/ml in their respective culture medium containing 75 µg/ml of cycloheximide or 10 µg/ml MG115. Cells were harvested at different time points after cycloheximide treatment and pelleted by centrifugation. For Western analysis, cell pellets were stored at −80°C prior to extraction of protein.

Protein Extraction and Western Analysis. Cells were lysed in TENN buffer [50 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 0.5% NP40, and 150 mM NaCl] supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml soy trypsin inhibitor, and 1 µg/ml pepstatin A. Lysates were clarified by centrifugation, and the protein concentration was determined by the Bradford method (Bio-Rad). Samples were mixed with 3 x SDS-PAGE sample loading buffer, boiled, separated on SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes. Blots were probed with 0.5 µg/ml p53 (DO-1; Calbiochem), 3 µg/ml MDM2 (IF2; Calbiochem), and actin (0.1 µg/ml, Sigma) antibodies as described previously (12). Signals were visualized by using horseradish peroxidase-conjugated antibodies (sheep anti-mouse for MDM2 and p53, Amersham; goat-anti rabbit for actin, Boehringer Mannheim) and enhanced chemiluminescence (DuPont NEN).

RNA Isolation and RNase Protection. Total RNA was isolated using RNeazol B (Tel-Test, Inc.) as per manufacturer’s instructions. RNase protection using an MDM2 probe that measures the levels of MDM2-P1 and MDM2-P2 transcripts was performed as described previously (12), except that 7.5 µg of total RNA were used in hybridization reactions.

Results

MDM2 and p53 Half-Life Analysis in Quiescent and Growth-stimulated PBMCs. To determine whether the stability of MDM2 protein may be differentially regulated in quiescent versus proliferating cells, the half-life of MDM2 protein was measured in both untreated and PHA/IL-2-treated PBMCs. Freshly isolated PBMCs from normal individuals are comprised predominantly of quiescent T cells (~70%), whereas the treatment of PBMCs with PHA and the culturing of these cells with IL-2 will generate a relatively pure population of cycling T cells. MDM2 protein half-life was measured by determining the level of protein at various time points after treatment with the protein synthesis inhibitor cycloheximide. Interestingly, Fig. 1a shows that the level of MDM2 protein declined much more rapidly in cycloheximide-treated, growth-stimulated cells than in unstimulated cells. The half-life of MDM2 in growth-stimulated
The possibility that alterations in the regulation of MDM2 protein stability may contribute to high levels of MDM2 protein in human tumor cells. We first performed MDM2 Western blot analysis on a series of leukemic cell lines. Although no lines expressed a high level of MDM2 protein (>10-fold above the level of MDM2 protein measured in PHA/IL-2-stimulated PBMCs), we were surprised to find similar amounts of MDM2 protein in wt and mutant p53-containing lines and that the p53 mutant Jurkat line expressed the highest amount of MDM2 protein (Fig. 2a). This line expressed six times more MDM2 protein (as determined by densitometric analysis) than that measured in PHA/IL-2-stimulated PBMCs.

The expression profile of MDM2 in leukemic lines was not expected, considering that the majority of these lines possess mutant p53 and should possess low amounts of MDM2-P2 transcripts (12); p53-regulated transcripts that have been shown to be translated at high efficiencies in vitro and in cells in culture (14, 15). To verify the absence of these transcripts in mutant p53 lines, RNase protection analysis was performed. As expected, those lines with mutant p53 did not possess MDM2-P2 transcripts (Fig. 2b). Fig. 2b also shows that all lines possessed similar amounts of MDM2-P1 transcripts; MDM2 transcripts that are regulated by the p53-independent promoter of the MDM2 gene. These results raise the possibility that MDM2-P2 transcripts are not translated more efficiently in lymphoid cells. However, it is also conceivable that the half-life of MDM2 protein is longer in some of the lines with undetectable amounts of MDM2-P2 transcripts, but expressing higher or similar level of MDM2 protein as wt p53-containing lines. To address this, the half-life of MDM2 protein was measured in several mutant p53 lines. All lines analyzed possessed similar doubling times, and half-life studies were performed with cells in their logarithmic stage of growth. Fig. 3 shows that the half-life of MDM2 protein was clearly longer in the HEL, CEM, Jurkat, and MOLT4 lines than in the Raji line or in PHA/IL-2-stimulated PBMCs. It is unlikely that the difference in MDM2 half-life measured in the lines is due to an unequal inhibition of protein synthesis by cycloheximide because this compound was found to inhibit incorporation of
protein levels in the Raji line (after treatment. Fig. 4 demonstrates a greater fold increase in MDM2 protein that are regulated by growth stimulation (24). Thus, MDM2 protein stability may be associated with another mechanism that can contribute to the overproduction of MDM2 protein in human tumor cells. It is, however, unlikely that stabilized MDM2 is sufficient for the inhibition of p53 function in the cell lines analyzed here because the p53 was mutated in all lines possessing MDM2 with a long half-life. Additionally, the expression levels of MDM2 lacking the p53 binding region have been shown to transform NIH3T3 cells (19), and overexpression of MDM2 has been documented to alter cell cycle control pathways in p53 null cells in vivo (20). Recently, it has also been documented that MDM2 overexpression can block the growth-inhibitory activities of TGF-β1 via a p53-independent mechanism (21) and that p53 null transgenic mice that overexpress MDM2 develop a different spectrum of tumors than mice that are only null for p53 (22). Moreover, although rare, alterations in both MDM2 and p53 expression have been detected in primary human tumor cells (23). Therefore, it is conceivable that stabilized MDM2 may provide a selective growth advantage in p53 mutant cells by altering p53-independent growth control pathways. It is, however, possible that alterations in MDM2 turnover do not contribute at all to the transformation process but are a consequence of alterations in other critical growth control molecules that are important in the regulation of MDM2 stability. It has been reported recently that the alternative translation product of the human CDKN2A locus (p14ARF) is regulated by p53 and can regulate MDM2 stability (24). Thus, MDM2 protein stability may be associated with either p53 or p14ARF expression status. Interestingly, both the Jurkat (stable MDM2) and Raji (unstable MDM2) lines possess undetectable levels of p14ARF protein (25) and harbor mutant p53 alleles. It is therefore likely that the stability of MDM2 will not be associated

Discussion

p53 and MDM2 expression is regulated at multiple levels by mitogenic signals. Growth stimulation of human lymphocytes has previously been shown to induce an overall increase in both p53 RNA and p53 protein (17, 18). MDM2 RNA (specifically, those transcripts that are regulated by p53) and MDM2 protein have also been shown to be up-regulated in growth-stimulated cells (12). We show here that the stability of MDM2 protein is regulated by growth stimulation signals as well. Interestingly, we show that MDM2 protein has a shorter half-life in growth-stimulated PBMCs than in quiescent PBMCs. This may appear to be inconsistent with an overall increase in the amount of MDM2 protein. However, considering that MDM2 expression can be regulated at multiple levels, the increase in MDM2 protein measured in stimulated cells is probably due to the fact that growth stimulation induces a greater fold increase in MDM2 transcription and/or translation than turnover of MDM2 protein.

What could be the functional significance for the differential regulation of p53 and MDM2 expression in quiescent versus proliferating cells? It is probably not a high priority for a nondividing cell to expend energy required for either the synthesis or degradation of proteins involved in controlling damaged-induced cell cycle arrest. However, an actively proliferating cell needs to possess an increased capacity to respond to the potentially harmful effects of cellular damage. One way to increase the capacity for a p53 response is to increase the amount of latent p53. We propose that this is accomplished by events that regulate p53 and MDM2 RNA production (12, 17, 18), as well as MDM2 degradation (see Fig. 1). The various mechanisms controlling p53 and MDM2 expression are likely to play an important role in generating an optimal ratio of MDM2 and p53 proteins that is needed to maintain high levels of latent p53 protein in actively proliferating cells.

It appears that there are multiple mechanisms that can lead to the overproduction of MDM2 protein in human tumor cells, including MDM2 gene amplification (3) and enhanced translation of MDM2-P2 transcripts (15). Besides presenting data that suggest that MDM2 protein stability is differentially regulated in quiescent versus active cycling cells, we also present data that suggest that the normal regulation of MDM2 protein turnover mediated by the proteasome can be altered in human tumor cells. The half-life of MDM2 protein was found to be extended in a number of leukemic cell lines, and MDM2 accumulated to a much lesser degree in proteasome inhibitor-treated cells harboring stable MDM2 than in treated cells possessing unstable MDM2. In at least one of these lines (the Jurkat line), the increased stability of MDM2 protein appears to contribute to a high amount of protein (a 6-fold greater amount of protein than that measured in normal cycling lymphocytes). It is therefore possible that alterations in the regulation of MDM2 turnover via the proteasome may be another mechanism that can contribute to the overproduction of MDM2 protein in human tumor cells. It is, however, unlikely that stabilized MDM2 is sufficient for the inhibition of p53 function in the cell lines analyzed here because the p53 gene is mutated in all lines possessing MDM2 with a long half-life. Alternatively, the expression levels of MDM2 lacking the p53 binding region have been shown to transform NIH3T3 cells (19), and overexpression of MDM2 has been documented to alter cell cycle control pathways in p53 null cells in vivo (20). Recently, it has also been documented that MDM2 overexpression can block the growth-inhibitory activities of TGF-β1 via a p53-independent mechanism (21) and that p53 null transgenic mice that overexpress MDM2 develop a different spectrum of tumors than mice that are only null for p53 (22). Moreover, although rare, alterations in both MDM2 and p53 expression have been detected in primary human tumor cells (23). Therefore, it is conceivable that stabilized MDM2 may provide a selective growth advantage in p53 mutant cells by altering p53-independent growth control pathways. It is, however, possible that alterations in MDM2 turnover do not contribute at all to the transformation process but are a consequence of alterations in other critical growth control molecules that are important in the regulation of MDM2 stability. It has been reported recently that the alternative translation product of the human CDKN2A locus (p14ARF) is regulated by p53 and can regulate MDM2 stability (24). Thus, MDM2 protein stability may be associated with either p53 or p14ARF expression status. Interestingly, both the Jurkat (stable MDM2) and Raji (unstable MDM2) lines possess undetectable levels of p14ARF protein (25) and harbor mutant p53 alleles. It is therefore likely that the stability of MDM2 will not be associated
simply with p53 or p14arf expression status, and that there are other unknown growth control molecules that are important in the regulation of MDM2 stability that are altered in human leukemic cells.

In summary, our results suggest that MDM2 protein expression is regulated by mechanisms controlling the stability of the protein. It will be of interest for future studies to determine whether MDM2 stability is regulated by signals that not only induce proliferation but also inhibit cell growth (i.e., signals that promote senescence, differentiation, and others). Our data also suggest that mechanisms regulating MDM2 stability can be altered in human tumor cell lines. We did perform half-life studies using primary leukemic cells and found that some patients harbor cells possessing MDM2 with a long half-life (data not shown). However, because we could not control for the proliferative rate of tumor cells derived from the various patients (unlike in cell lines), we do not know if the differences in MDM2 stability measured in primary tumors are due to difference in the proliferative rate of the cells or due to alterations in pathways regulating MDM2 stability. More work will obviously be needed to identify the various factors that play a role in the regulation of MDM2 stability, how alterations in these pathways lead to MDM2 stabilizing MDM2 stability. More work will obviously be needed to identify the various factors that play a role in the regulation of MDM2 stability, how alterations in these pathways lead to MDM2 stabilizing MDM2 stability. More work will obviously be needed to identify the various factors that play a role in the regulation of MDM2 stability, how alterations in these pathways lead to MDM2 stabilizing MDM2 stability.

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