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# The Levels of MDM2 Protein Are Decreased by a Proteasome-Mediated Proteolysis Prior to Caspase-3-Dependent pRb and PARP Cleavages

MDM2 is a substrate of caspase-3 in p53-mediated apoptosis. In addition, MDM2 mediates its own ubiquitination in a RING finger-dependent manner. Thus, we investigated whether MDM2 is degraded through a ubiquitin-dependent proteasome pathway in the absence of p53. When HL-60 cells, p53 null, were treated with etoposide, MDM2 was markedly decreased prior to caspase-3-dependent retinoblastoma tumor suppressor protein (pRb) and poly (ADP-ribose) polymerase (PARP) cleavages. Moreover, down-regulation of MDM2 level was not coupled with its mRNA down-regulation. However, the level of MDM2 was partially restored by proteasome inhibitors such as LLnL and lactacystin, even in the presence of etoposide. Our results suggest that, in the p53 null status, MDM2 protein level is decreased by proteasome-mediated proteolysis prior to caspase-3-dependent PARP and pRb cleavages.

Key Words: *HL-60 Cells; Ubiquitin; Apoptosis*

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## INTRODUCTION

Cells undergoing p53-mediated apoptosis promote caspase-3-like activities, which result in the cleavage of the MDM2 oncoprotein and other apoptotic substrates such as poly (ADP-ribose) polymerase (PARP) and retinoblastoma tumor suppressor protein (pRb) (1-3). Furthermore, Pochampally et al. reported that p53 can regulate a proteolytic activity specific for the caspase cleavage site of MDM2, before the onset of apoptosis and activation of other apoptotic caspases (4). However, recently, Fang et al. reported that MDM2 has a capacity to function as a ubiquitin protein ligase (E3) both for itself and p53, which suggests that MDM2 level is regulated by a proteasome-mediated pathway (5). Thus, even though MDM2 is cleaved by the MDM2-specific caspases or caspase-3 like activities, it is possible that the proteasome-mediated pathway degrades MDM2 in the absence of caspases activation by p53. Human acute leukemia cell lines (HL-60 cells; p53 null) have proven particularly informative in the studies on chemotherapy-associated apoptotic proteolytic events (6-9). In this study, we focused mainly on the MDM2 levels of HL-60 cells immediately following etoposide treatment, and whether the MDM2 is degraded through the proteasome-mediated pathway prior to the activation of caspase-3.

## MATERIALS AND METHODS

### Materials

Caspases inhibitor, z-VAD-FMK, was purchased from Enzyme System Product (CA, U.S.A.), and proteasome inhibitors, such as LLnL and lactacystin, were purchased from Calbiochem (CA, U.S.A.). For induction of apoptosis, etoposide was purchased from Sigma (St. Louis, MO, U.S.A.).

### Cell culture

HL-60 cells were cultured in RPMI-1640 medium (Gibco BRL Laboratories, Grand Island, NE, U.S.A.) supplemented with 10% fetal bovine serum. For the induction of apoptosis, etoposide was dissolved in dimethyl sulfoxide, and then added into culture medium in various concentrations for the indicated period of time.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the RNAzol™ B (Biotecx laboratories, Houston, TX, U.S.A.) according to the manufacturer's instructions and quantitated by spectrophotometer. One microgram of total RNA was

reverse transcribed using M-MLV reverse transcriptase (Promega Co., Madison, WI, U.S.A.). The PCR reaction was carried out under the conditions recommended by the manufacturer's instructions (TaKaRa Co., Otsu, Japan). Briefly, 50 μL of a reaction mixture including 2.5 units of *Taq* polymerase (Takara Co.), 5 μL of 10×buffer, 1.5 mM $MgCl_2$, 200 μm dNTPs, 1 μL of first-strand cDNA, and 25 pmol of each primer, was subjected to 28 PCR cycles (denaturation at 94℃ for 1.5 min, annealing at 58℃ for 1 min, and polymerization at 72℃ for 1 min). The following primer pairs were used for PCR amplification: 1) GAPDH (sense; 5'-ATCAC TGCCA CTCAG AAGAC-3', antisense; 5'-CTTGC TCTCA GTATC CTTGC-3'); 2) MDM2 (sense: 5'-AATCA TCGGA CTCAG GTACA-3'; antisense: 5'-GTCCA GCTAA GGAAA TTTCA GG-3').

### Western blot analysis

Cells were lysed in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, PMSF (10 μg/mL), aprotinin (10 μg/mL), leupeptin (10 μg/mL), 5 mM phenanthroline, and 28 mM benzamidine-HCl] for 30 min on ice. Lysates were clarified by centrifugation. Lysates were quantitated using the Bradford assay (Life Science Co., CA, U.S.A.) with bovine serum albumin as a reference standard. Thirty-five μg of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to immobilon-P transfer membrane (Millipore Co., MA, U.S.A.). After incubation with primary antibodies, proteins were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies, followed by ECL according to the manufacturer's instructions (Amersham Life Science Co., Bucks, U.K.). Primary antibodies against Rb (PharMingen Co., CA, U.S.A.), caspase-3 (Santa Cruz Co., CA, U.S.A.), MDM2 (Santa Cruz Co.), PARP (Enzyme System Product Co., CA, U.S.A.), and β-actin (Santa Cruz Co.) were applied at optimized concentrations.

### Flow cytometric analysis

For flow cytometric analysis of DNA content, approximately $10^6$ cells were fixed in 80% ethanol at -20℃ for 24 hr. Ethanol-fixed cells were stained with propidium iodide (PI) staining solution [PI (50 μg/mL), RNase A (0.1 mg/mL), 0.1% NP-40, 0.1% trisodium citrate] for 30 min, and then analyzed by a Becton Dickinson FACS analyzer.

### DNA fragmentation assay

Cells were harvested at the indicated times by centrifugation and lysed in ice for 20 min by the addition of 20 μL lysis buffer consisting of 20 mM EDTA, 100 mM Tris (pH 8.0), and 0.8% (w/v) sodium lauryl sarcosine. Two μL of RNase A (5 mg/mL) and 20 μL of proteinase K (10 mg/mL) were added and incubated at 37℃ for 1 hr and 2 hr, respectively. Total lysates were loaded onto 1.5% agarose gel and separated at 50 mV for 2 hr. DNA fragments were visualized after staining with ethidium bromide by translumination with UV light.

### Enzymatic cleavage activity of caspase-3

Cells were lysed in lysis buffer [50 mM Tris (pH 7.5), 0.03% NP-40, 1.0 mM DTT] for 30 min on ice. After centrifugation at 12,000 rpm for 30 min at 4℃, the supernatant was quantitated using the Bradford assay kit with bovine serum albumin as a reference standard. Lysates (20 μg) were incubated with 0.2 mM ac-DEVD-*p*NA (Enzyme System product Co.) in a total volume of 0.1 mL. Assays were performed twice, and the results were presented as the average increase in absorbance at 405 nm.

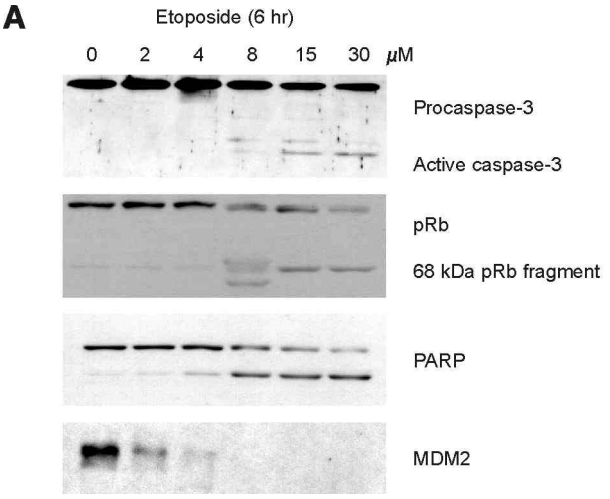
## RESULTS

### Etoposide-induced apoptosis in HL-60 cells

To determine adequate concentration of etoposide inducing apoptosis, HL-60 cells were cultured in the presence of various concentrations of etoposide for the indicated period of time. Fig. 1A shows that distinct apoptotic DNA ladders were observed above 8 μM concentration of etoposide. Consistent with this data, PARP and pRb cleavage occurred at above 8 μM concentration of etoposide concomitantly with caspase-3 activation (Fig. 1B). Surprisingly, MDM2 was markedly decreased prior to these changes under 8 μM concentration.

### Caspase-3-independent down-regulation of MDM2

To confirm caspase-3-independent down-regulation of MDM2, HL-60 cells were co-treated with etoposide and pan-caspase inhibitor, z-VAD-FMK. Fig. 2A shows that MDM2 was also markedly decreased even in the presence of z-VAD-FMK, and in the absence of PARP, pRb, or caspase-3 changes, suggesting that MDM2 down-regulation is an early event in the apoptotic process, and that it is mediated by caspase-3-independent pathway. When we examined caspase-3 activity in the same condition, consistently with the protein level, caspase-3-like activity was not increased 1 hr after the etoposide treatment (Fig. 2B).



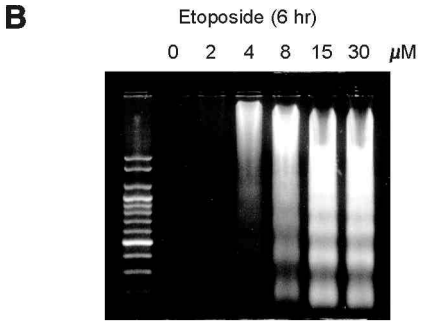
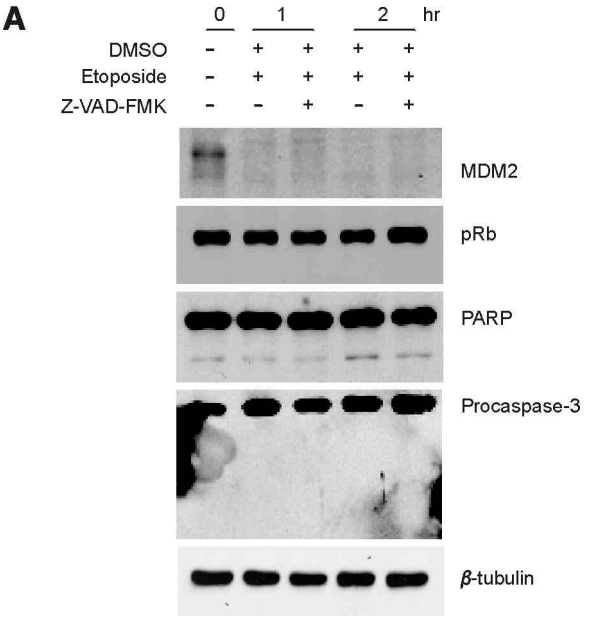




Fig. 1. Immunoblot and DNA fragmentation analysis in etoposide-treated HL-60 cells. HL-60 cells were treated by various concentrations of etoposide for the indicated periods of time. After 6 hr, samples were obtained and subjected to SDS-PAGE followed by immunoblotting (A) or agarose gel electrophoresis (B).



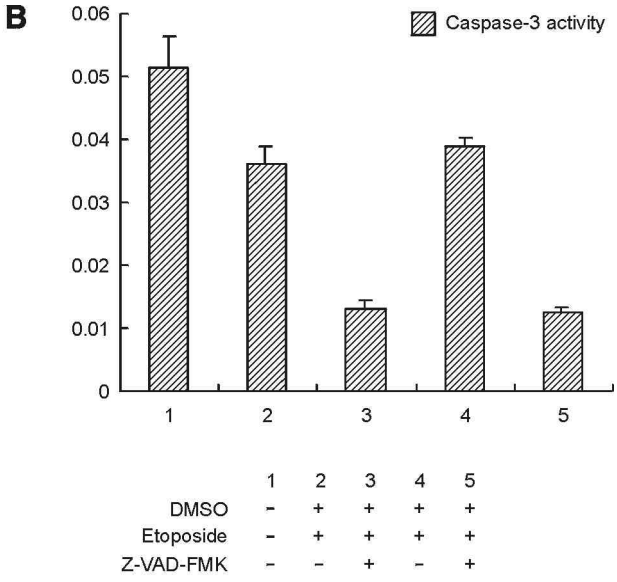




Fig. 2. Effect of caspase inhibitor on the expressions of MDM2, PARP, pRb, and caspase-3. HL-60 cells were treated with z-VAD-FMK (50 μM) in the presence of etoposide (8 μM) for the indicated periods of time. Cell lysates were resolved by 6.5% or 12% SDS-PAGE and subjected to Western blotting with anti-MDM2, anti-PARP, anti-caspase-3, anti-pRb, and β-tubulin antibodies (A). The same results were reproduced by three independent experiments. Caspase-3 like activity was determined using ac-DEVD-pNA, synthetic substrate of caspase-3 (B). Assays were performed twice. Results are presented as the average increase in absorbance at 405 nm.

## MDM2 down-regulation in the absence of apoptosis

We hypothesized that MDM2 level would be decreased within 1 hr of etoposide treatment in the absence of caspase-3 activation. To substantiate this possibility, we determined the expression patterns of MDM2 within 1 hr of etoposide treatment. Expectedly, MDM2 level was rapidly decreased within 1 hr in the absence of distinct PARP cleavage or down-regulation of procaspase-3 (Fig. 3A). Fig. 3B also shows the absence of distinct apoptosis within 1 hr of etoposide treatment. Next, we investigated the levels of MDM2 mRNA in etoposide-treated HL-60 cells. Fig. 3C shows that MDM2 mRNA levels were not markedly lowered in concert with MDM2 protein levels.

## Effect of proteasome inhibitors on the MDM2 level in the presence of etoposide

Finally, we investigated whether proteasome-mediated proteolysis should be involved in the early down-regulation of MDM2 following etoposide treatment. Fig. 4A

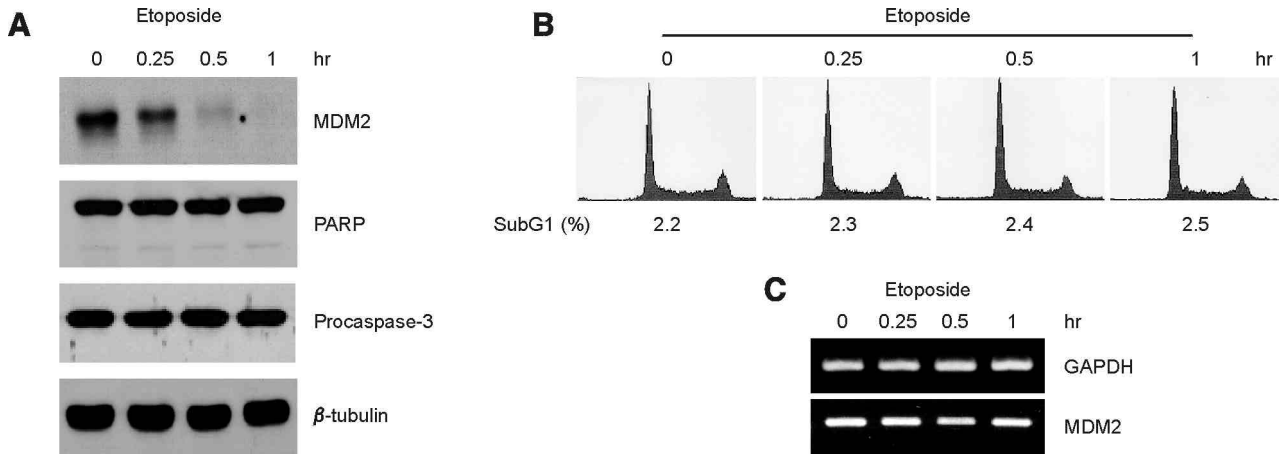



**Fig. 3.** Decrease of MDM2 level in the absence of apoptosis. Note the dramatic decrease of MDM2 level prior to caspase-3-dependent PARP cleavage in etoposide (8 $\mu$M)-treated HL-60 cells (**A**). Flow cytometric analysis was performed using propidium iodide staining solution (**B**). MDM2 mRNA levels were determined by RT-PCR with MDM2-specific primers (**C**).

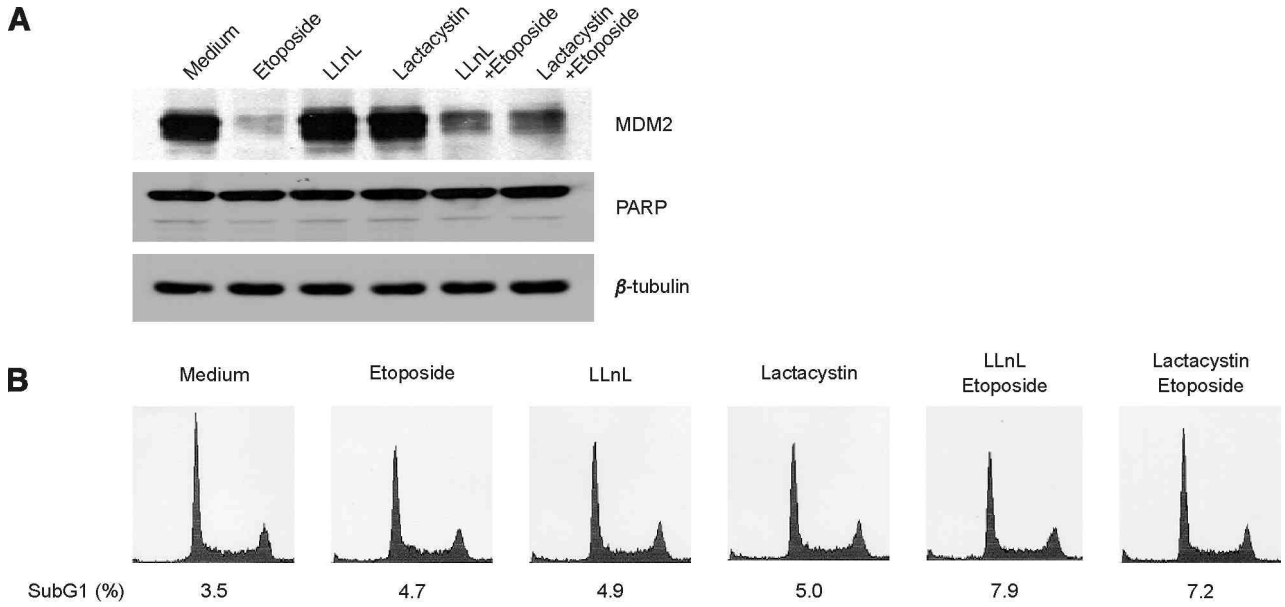



**Fig. 4.** Restoration of MDM2 levels in the presence of proteasome inhibitors. HL-60 cells were cultured with etoposide (8 $\mu$M) in the presence or absence of 50 $\mu$M LLnL or lactacystin for 1 hr, and the MDM2 levels were determined by immunoblot (**A**). Similar results were obtained from three independent experiments. Flow cytometric analysis was performed using propidium iodide staining solution (**B**).

shows that the level of MDM2 was partially restored by the presence of proteasome inhibitors such as LLnL and lactacystin, even in the presence of etoposide. In addition, flow cytometric analysis was performed to determine whether the onset of apoptosis should be delayed in the presence of partially restored MDM2. However, Fig. 4B shows that the onset of apoptosis was not blocked even in the presence of partially restored MDM2. Furthermore, proteasome inhibitors themselves induced apoptosis in HL-60 cells. We also observed that, when incubated with proteasome inhibitors along with etoposide, HL-60 cells rapidly progressed into apoptosis compared to when treated with etoposide only (data not shown).

## DISCUSSION

MDM2, as a 90-kDa phosphoprotein, can regulate p53 stability by targeting the p53 protein for proteasomal degradation (10). Furthermore, p53 can up-regulate MDM2 at transcriptional level (11, 12). Thus, MDM2 and p53 are interconnected by negative or posi-

tive feedback loop. During the p53-induced apoptosis, the MDM2 is also cleaved by a caspase at the aspartic acid residue 361 (13, 14). However, in the absence of p53, the destination of MDM2 during apoptosis is still unknown.

HL-60 cells, p53 null, have been used in the studies on chemotherapy-associated apoptotic proteolytic events (6-9). In the present study, we focused mainly on the MDM2 levels of HL-60 cells immediately following etoposide treatment, and whether the MDM2 is degraded through a ubiquitin-dependent proteasome pathway prior to the apoptotic execution phase in the absence of p53 activation. MDM2 level was rapidly decreased within 1 hr of etoposide treatment without marked down-regulation of MDM2 mRNA level. Even though caspase-3 is highly effective in cleaving MDM2, caspase-3-like activity was not increased 1 hr after the etoposide treatment. Martins et al. also reported that levels of procaspase-3 in HL-60 cells diminished markedly 2-3 hr after etoposide treatment (15). MDM2 can also be a substrate of caspase-8 in apoptotic process. To rule out this possibility, we performed Western blot analysis using anti-MDM2 in HL-60 cells following etoposide treatment, in the presence or absence of z-VAD-FMK. The level of MDM2 was markedly lowered within 1 hr even in the presence of 100 $\mu$M z-VAD-FMK. Furthermore, MDM2 down-regulation occurred prior to PARP and pRb cleavage mediated by caspase-3. Thus, these results suggest that caspase-3 and 8 are not involved in early MDM2 down-regulation, and that its early down-regulation may occur prior to the onset of apoptosis.

Due to its E3 ligase activity, MDM2 level can be regulated by proteasome-mediated pathway (5). We investigated whether proteasome-mediated proteolysis is involved in the early down-regulation of MDM2 following etoposide treatment. The level of MDM2 was partially restored by proteasome inhibitors such as LLnL and lactacystin even in the presence of etoposide. In addition, recent findings suggest that $p14^{Arf}$ sequesters MDM2 into nucleoli and blocks export of MDM2 from nucleus to cytoplasm (16, 17). Thus, it still remains to be elucidated whether $p14^{Arf}$ is involved in the down-regulation of MDM2 in this system.

In conclusion, this study was mainly focused on the change of the MDM2 level in early apoptotic process, and the results suggest that, in the absence of p53, proteasome-mediated proteolysis plays an important role in early down-regulation of MDM2.

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