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Report

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NOTE

Supplemental Figures 1 and 2 can be found at: http://www.landesbioscience.com/journals/cc/supplement/kumarCC4-11.pdf

ABSTRACT

Hydroxyurea (HU) is a competitive inhibitor of ribonucleotide reductase that is used for the treatment of myeloproliferative disorders. HU inhibits DNA replication and induces apoptosis in a cell type-dependent manner, yet the relevant pathways that mediate apoptosis in response to this agent are not well characterized. In this study, we employed the human myeloid leukemia 1 (ML-1) cell line as a model to investigate the mechanisms of HU-induced apoptosis. Exposure of ML-1 cells to HU caused rapid cell death that was accompanied by hallmark features of apoptosis, including membrane blebbing, phosphatidylserine translocation, and caspase activation. HU-induced apoptosis required new protein synthesis, was induced by HU exposures as short as 15 min, and correlated with the accumulation of p53 and induction of the p53 target gene PUMA. p53 induction in ML-1 cells was ATR dependent and downregulation of p53 through RNAi delayed HU-induced apoptosis. HU did not induce p53 or induce apoptosis in Molt-3 leukemia cells, even though exposure to HU induced a comparable level of DNA damage and robustly activated the ATR pathway. The microtubule inhibitor nocodazole suppressed HU-induced p53 accumulation in ML-1 cells suggesting that a microtubule-dependent event contributes to p53 induction and apoptosis in this cell line. Our findings outline an HU-induced cell death pathway and suggest that activation of ATR is necessary, but not sufficient, for stabilization of p53 in response to DNA replication stress.

INTRODUCTION

Hydroxyurea (HU) has been used for the treatment of chronic myelogenous leukemia and other myeloproliferative disorders including polycythemia vera and essential thrombocytosis for more than 30 years.[1] HU is a competitive inhibitor of ribonucleotide reductase (RNR), a tetrameric enzyme comprised of two regulatory (M-1) and two catalytic (M-2) subunits that catalyzes the rate-limiting step in the production of reduced deoxyribonucleoside triphosphates (dNTPs).[2] HU belongs to the anti-metabolite class of drugs that block key steps in DNA biosynthesis and are widely used to treat cancer. Clinically relevant examples include the nucleoside analogues 5-fluorouracil, an inhibitor of thymidylate synthase, and gemcitabine, which inhibits RNR and DNA polymerase alpha.[3-4] Although each of these agents inhibits DNA synthesis, they also have unique effects on DNA metabolism that contribute to their unique cytotoxicity profiles in vitro and anti-cancer properties in vivo.[5]

Despite its clinical utility and well-characterized biochemical mechanism of action, the pathways through which HU exerts its cytotoxic effects in mammalian cells are less well understood. The p53 tumor suppressor has been implicated as a cell-type-dependent determinant of HU sensitivity. p53 expression was associated with apoptosis in IL-3-dependent bone marrow cells and EBV immortalized lymphoblasts, but other studies have failed to show a cause-and-effect link between p53 status and HU sensitivity.[6-9] Interestingly, an earlier report showed that HU induced a transcriptionally inert form of p53 in human RKO colon carcinoma cells, though this may have been a cell-type dependent phenomenon.[10-11] Stalled replication forks (RFs) arising secondary to dNTP depletion are a likely source of pro-apoptosis signals in HU-treated cells. Stalled RFs are recombination substrates that can be converted into cytotoxic DNA double-strand breaks (DSBs).[12-14] Studies using bacterial systems have shown that the homologous recombination (HR) pathway of DNA repair is essential for suppression of DSBs and for the reactivation of stalled RFs following exposure to replication inhibitors.[15] HR is also important for the maintenance and restart of stalled RFs in mammals; defects in proteins required for HRR, including the RecA ortholog RAD51 are associated with accumulation of spontaneous

DSBs during DNA replication.[16] Members of the RecQ helicase family of proteins, including BLM, are also important for recovery from DNA replication stress, perhaps through the suppression of deleterious recombination events.[17]

The ATR (ATM-Rad3-related) protein kinase is a key regulator of cellular responses to HU and other forms of DNA replication stress. ATR belongs to the PI3K-related kinase gene superfamily that also includes the functionally related kinase, ATM (ataxia-telangiectasia-mutated). ATR and ATM are serine/threonine-glutamine-directed kinases that possess highly overlapping substrate specificities.[18] Known substrates for ATR and ATM include p53 and BRCA1, the downstream effector kinases CHK1 and CHK2, and a host of other proteins that participate in DNA repair and cell cycle checkpoint regulation.[18] ATM is a critical regulator of the cellular response to DSBs that is mutated in the cancer susceptibility/neurodegeneration syndrome ataxia-telangiectasia. Inactivating mutations in ATM cause defects in the ionizing radiation (IR)-induced G_1/S, intra-S phase and G_2/M phase cell cycle checkpoints and profound radiosensitivity.[18] ATR has also been implicated in the G_2/M checkpoint.[19-20] However, its most critical functions relate to its role as a regulator of DNA replication. ATR is an essential gene in mice that is also required for viability at the cellular level.[20-22] ATR-deficient cells exhibit high levels of spontaneous and replication stress-induced DSBs that lead to loss of viability.[23-24] ATR suppresses premature replication origin firing and enforces the S-M checkpoint, which delays mitosis in the presence of unreplicated DNA.[25-29] These combined functions define ATR as a critical determinant of cell survival following DNA replication stress.

The S-phase checkpoint functions of ATR are mediated, in part, through phosphorylation and activation of its effector kinase, CHK1.[30-32] CHK1, in turn, phosphorylates the CDC25A phosphatase leading to its ubiquitylation and degradation.[33] CHK1-mediated phosphorylation also negatively regulates the CDC25C phosphatase by promoting its association with 14-3-3 proteins and nuclear export.[34-35] Downregulation of CDC25A and CDC25C leads to inhibition of the CDK2 and CDK1 cyclin-dependent kinases, which mediate progression through S phase and mitosis, respectively.[36] Thus, CHK1 elicits S-and G_2/M phase arrests, in part, by inactivation of CDK2 and CDK1. CHK1 is also implicated in the DNA damage-induced phosphorylation of p53 on Ser-20, which relieves p53 repression by MDM2.[37] Defects in CHK1 partially phenocopy ATR deficiency and are associated with premature mitosis, premature origin firing, defective homologous recombination, and sensitivity to anti-metabolites.[38-45] Finally, a recent study described reduced or undetectable expression of CHK1 in a subset of aggressive leukemias suggesting that downregulation of CHK1 may occur during leukemogenesis.[46]

In this report we have explored the cellular response to HU in a myeloid leukemia cell line (ML-1) derived from a patient with acute myelogenous leukemia.[47] ML-1 cells expresses wild-type p53 and have been used as a model for studying DNA damage-induced cell cycle checkpoint activation and for investigating the anti-leukemic potential of nucleoside analogues.[48-49] While screening a panel of leukemia cell lines for sensitivity to HU, we made the discovery that ML-1 cells are remarkably sensitive to this drug. Here, we characterize the HU sensitivity of ML-1 cells and explore the mechanism of HU-induced apoptosis. Our study provides evidence that an ATR-p53 pathway functions in a pro-death capacity in response to HU and that activation of ATR is necessary, but not sufficient, for p53 induction in response to DNA replication stress.

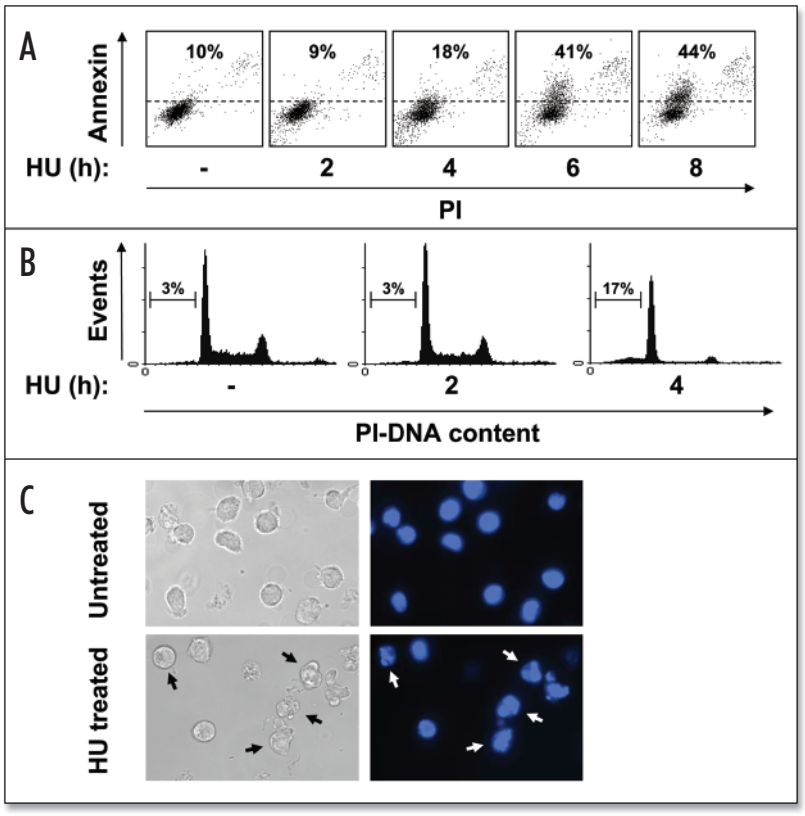

Figure 1. ML-1 myeloid leukemia cells are hypersensitive to HU. (A and B) ML-1 cells were either left untreated or treated with 3 mM HU for the indicated times. (A) The cells were stained with annexin V-FITC and PI and analyzed by flow cytometry. The percentages of annexin-positive cells are displayed in each panel. (B) The cells were fixed in ethanol, stained with PI, and analyzed by flow cytometry to measure DNA content. The percentage of cells exhibiting a sub-2N DNA content is shown in each panel. (C) Apoptotic morphology of HU-treated ML-1 cells. ML-1 cells were allowed to adhere for an hour to poly-lysine coated glass bottom dishes. Subsequently, cells were either left untreated or treated with 3 mM HU for 4 h and then stained with Hoecsht 33342 to visualize cell nuclei. Cells displaying an apoptotic, budded morphology are denoted by arrows.

METHODS

Cell culture and antisera. All cell lines used in this study were maintained in RPMI containing 10% FBS and 10 mM HEPES. ML-1 cells were obtained from Dr. Scott Kaufmann (Mayo Clinic). HPB-ALL and Karpas 45 cell lines were kindly provided by Dr. Shigeki Miyamoto (University of Wisconsin-Madison). Antibody suppliers included: Oncogene Research (α-RPA32), Santa Cruz Biotechnology (α-CHK1 (G-4), α-p53 (DO-1), Upstate Cell Signaling Solutions (α-tubulin, α-PARP, α-PUMA), and R&D Systems (α-CHK1-pS317, α-p53-pS20). The ATR antibody has been previously published.[50] Zeocin (Invitrogen) was used at a concentration of 0.5 mg/ml. Nocodazole (Sigma) was made as a 0.5 mg/ml stock in DMSO and added to a final concentration of 0.5 μg/ml 30–60 min prior to HU addition.

Protein analysis. A number of ML-1 nuclear proteins, including ATR, CHK1, RPA32 and p53 were resistant to extraction with our standard cell

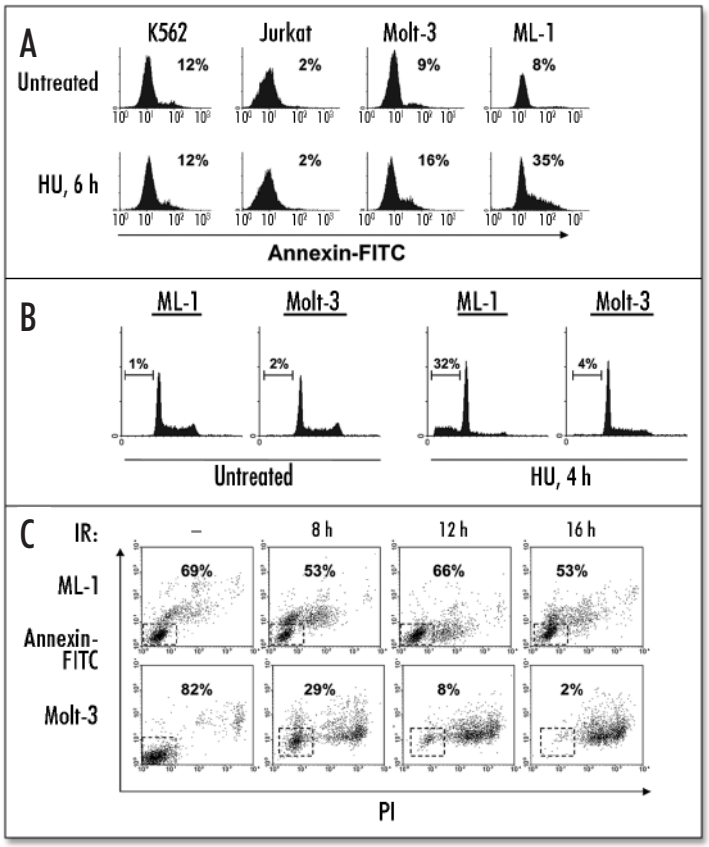

Figure 2. Differential sensitivities of ML-1 and Molt-3 cells to HU and IR. (A) Exponentially growing K562, Jurkat, Molt-3, and ML-1 cells were either left untreated or exposed to 3 mM HU for 6 h. The cells were stained with annexin V-FITC and PI and analyzed by flow cytometry. The percentage of annexin V-positive, apoptotic cells is shown in each panel. (B) ML-1 and Molt-3 cells were either left untreated or HU-treated for 4 h. Cells were fixed with ethanol, stained with PI and analyzed by flow cytometry. The percentage of apoptotic cells showing sub-2N DNA content is shown in each panel. (C) Differential sensitivities of ML-1 and Molt-3 cells to IR. ML-1 or Molt-3 cells were left untreated or exposed to IR (10 Gy) and harvested at the indicated times. The cells were stained with annexin V-FITC and PI and analyzed by flow cytometry. Cell viability data is presented in each panel.

lysis buffer containing 25 mM HEPES, 300 mM NaCl, and 0.5% NP-40. Therefore, all extracts were prepared by boiling in SDS-PAGE sample loading buffer as follows: the cells were washed once in PBS and resuspended to a density of 1 x 10^7 cells/ml in PBS. An equal volume of 4X sample loading buffer (100 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.008% bromophenol blue) was added and the samples were heated at 100°C for 10 min. One hundred microliters of each sample were separated on 10% SDS-PAGE gels and transferred to Immobilon-P PVDF membranes (Millipore). Membranes were blocked in Tris-buffered saline containing 0.2% Tween-20 (TBS-T) and 5% dried milk and incubated overnight at 4°C with the indicated primary antibodies diluted in blocking solution. After washing with TBS-T, the blots were incubated with HRP-conjugated sheep anti-mouse or goat anti-rabbit secondary antibodies (Jackson) and developed using SuperSignal chemilluminescent substrate (Pierce).

Cell cycle analysis and apoptosis assays. For propidium iodide (PI) staining, 1 x 10^6 ML-1 cells were resuspended in 300 µl of PBS. The cells were fixed by the addition of 700 µl of ice-cold 100% ethanol while vortexing. The fixed cells were resuspended in PBS containing 20 µg/ml PI, 50 µg/ml RNAse A, and 0.2% Tween-20 and analyzed by flow cytometry. The data were plotted using the WinMDI shareware package (Stanford University). Annexin V staining was performed using the ApoAlert system (Clontech) according to the manufacturer's conditions. The caspase inhibitor cocktail used in this study contained 50 µM each of zVAD-FMK, YVAD-FMK, and DEVD-FMK (Bachem Bioscience), which were added 1 h prior to HU exposure.

Microscopy. For the phospho-H2AX analysis, ML-1 cells were treated with buffer or 3 mM HU and collected 1 h or 4 h later. The cells were cytospun onto glass slides and, fixed with 4% paraformaldehyde-PBS, and permeabilized in PBS containing 0.2% Triton-X 100 (PBS-T) for 10 min. The cells were then washed with PBS and blocked for 30 min in PBS containing 3% BSA and 2% goat serum prior to immunostaining overnight at 4°C with 2 µg/ml of α-phospho-H2AX antibody diluted in blocking solution. The cells were washed three times in PBS-T and incubated for 1 h at room temperature with 0.4 µg/ml FITC-conjugated goat anti-mouse IgG (Caltag). The cells were washed twice with PBS-T, once in PBS, and mounted using SlowFade reagent (Invitrogen). A Carl Zeiss Axiovert 200 fluorescence microscope was used to visualize the samples.

RNA interference. Approximately 2 x 10^6 ML-1 cells were electroporated with 20 µl of 20 µM scrambled control siRNA or siRNA targeted to ATR or p53.[51] The p53 siRNA was provided as a SmartPool mixture of four annealed oligonucleotides (Dharmacon). Electroporation was carried out with a single 325V, 10 ms pulse using an ECM 80 Electro Square Porator (BTX). The cells were transferred to growth medium and allowed to recover for 48 h prior to drug treatment and analysis.

RESULTS

ML-1 cells are highly sensitive to HU. ML-1 is a p53-positive human myeloid leukemia cell line that has been used as a model to study apoptosis and cell cycle checkpoint induction by genotoxic stress.[52-53] To examine whether ML-1 cells undergo apoptosis in response to HU, we exposed the cells to the drug for 2-8 h and then stained the cells with annexin V-FITC. Flow cytometric analysis revealed that ML-1 cells underwent extensive apoptosis in response to HU. Apoptosis was detectable by 4 h after treatment and was maximal by 6 h post-HU exposure, when approximately 40% of the cells stained positive for annexin V (Fig. 1A). HU-induced apoptosis manifested as an accumulation of cells exhibiting a hypodiploid DNA content and a corresponding decrease in the S phase population, which is consistent with the mechanism of HU as an S-phase-specific cytotoxic agent (Fig. 1B). Bright field and fluorescence microscopy of HU-treated ML-1 cells revealed highly characteristic signs of apoptosis, including membrane blebbing and nuclear fragmentation (Fig. 1C). The DNA replication inhibitor aphidicolin also induced apoptosis in ML-1 cells with kinetics that were nearly indistinguishable from HU (data not shown).

We next compared the HU sensitivity of ML-1 to other leukemic cell lines. For this analysis we employed p53-deficient (K562, Jurkat) and p53 wild-type (Molt-3) cell lines. Each cell line was exposed to 3 mM HU for 6 h and apoptosis induction measured by annexin V staining and flow cytometry. ML-1 cells were more sensitive to HU than either of the p53-deficient cell lines, which failed to undergo appreciable apoptosis during the 6 h time course of the experiment (Fig. 2A). Although Molt-3 cells were susceptible to HU, they were quantitatively less sensitive than ML-1 cells; the viability of ML-1 cells decreased by 27% during the experiment, whereas the viability of Molt-3 cells decreased by 7%. Similar results were obtained when hypodiploid DNA content was used as a parameter of HU-induced apoptosis. A 4 h treatment with HU induced hypodiploid DNA in 32% of ML-1 cells, but only 4% of Molt-3 cells (Fig. 2B). The virtual absence of apoptosis in the p53-deficient K562 and Jurkat cell lines following HU exposure is consistent with a key role for p53 in this process. However, the fact that p53-wild-type ML-1 and Molt-3 cells exhibit differential survival in response to HU indicates that p53 functional status is not the sole determinant of sensitivity to this drug.

The HU sensitivity of ML-1 cells could reflect a general hypersensitivity of this cell line to genotoxic stimuli. To test this hypothesis, we compared the apoptosis profiles of ML-1 and Molt-3 cells exposed to IR. In comparison to HU, 10 Gy of IR was a relatively weak inducer of apoptosis in ML-1 cells. Whereas HU induced apoptosis within 4 h, IR-induced apoptosis was not observed until 6-8 h after exposure, at which time approximately 25% of the

cells were annexin V-positive (Fig. 2C and data not shown). The fraction of irradiated cells undergoing apoptosis remained nearly constant over the next 8 h. IR was a much stronger stimulus of apoptosis in Molt-3 cells. Molt-3 viability decreased from 82% to 29% 8 h after exposure to 10 Gy of IR, and further declined to 2% after 16 h (Fig. 2C). These results suggest that ML-1 cells are not generally hypersensitive to genotoxic stress, but instead may harbor specific defects that confer susceptibility to DNA replication inhibitors.

Characterization of HU-induced apoptosis in ML-1 cells. Given the rapidity of HU-induced apoptosis, we were interested in determining the minimum length of drug exposure required to induce cell death. Remarkably, exposure to HU for as little as 15 min was sufficient to increase the fraction of annexin V-positive cells after 4 h (Fig. 3A). Increasing the HU exposure time to 1 h led to a corresponding increase in the apoptotic fraction, whereas longer exposures did not further augment cell death. This result indicates that the commitment phase of apoptosis in ML-1 cells occurs within 1 h of HU exposure and that ML-1 cells are unable to recover from a transient DNA replication block.

To further characterize HU-induced apoptosis in ML-1 cells, we measured the caspase-mediated cleavage of poly(ADP-ribose) polymerase (PARP), which serves as a reliable marker for genotoxin-induced apoptosis.[54] Using antibodies that recognize full-length PARP and its major caspase cleavage product, we observed that the majority of PARP migrated as the full-length, noncleaved form in asynchronously growing ML-1 cells (Fig. 3B). Exposure to HU resulted in PARP cleavage that was detectable at the 4 h time point. Both PARP cleavage and cell blebbing were blocked by pan-specific caspase inhibitors (Fig. 3C and data not shown), indicating that HU-induced caspase activation is causal for apoptosis.

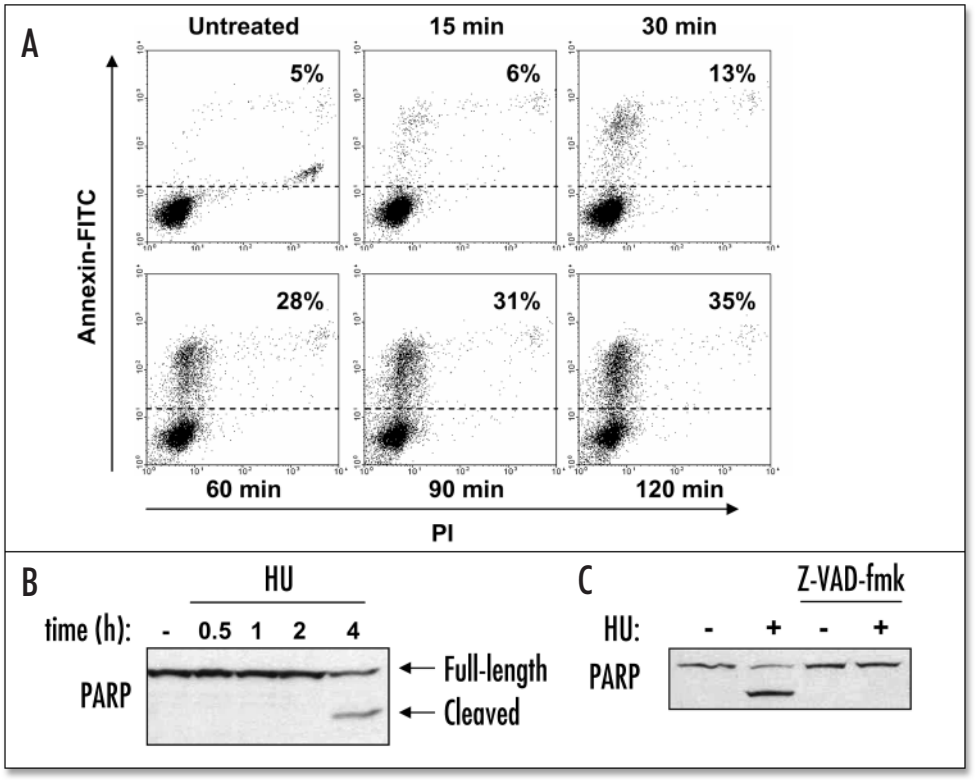

Figure 3. Characterization of HU-induced apoptosis. (A) Induction of apoptosis by pulse exposures to HU. ML-1 cells were either left untreated or treated with 3 mM HU for the indicated times. Cells were washed and left to recover for 4 h. Cells were subsequently stained with annexin V-FITC and PI and analyzed by flow cytometry. The percentage of apoptotic cells is shown in each panel. (B) Time course of PARP cleavage following exposure to HU. ML-1 cells were either left untreated or treated with 3 mM HU for the indicated times. Cell extracts were immunoblotted with α-PARP antibody. (C) HU-induced apoptosis is inhibited by caspase inhibitors. ML-1 cells were preincubated with a mixture of caspase inhibitors (see Methods) prior to exposure to HU for 4 h. PARP cleavage was measured by immunoblotting.

Insight into the mechanism of apoptosis can be gleaned from assessing the requirement for de novo protein synthesis. To determine whether protein synthesis was required for HU-induced apoptosis, ML-1 cells were preincubated with the protein synthesis inhibitor cycloheximide (CHX, 5 μg/ml) for 30 min prior to the addition of HU. Cells were then incubated for an additional 4 h and stained with annexin V-FITC and PI. The percentage of annexin V-positive cells increased from 2% to 9% following exposure to CHX alone, indicating that CHX is mildly toxic to ML-1 cells (Fig. 4A). However, CHX clearly antagonized HU-induced apoptosis. The annexin V-positive fraction declined from 29% in cells treated with HU, to 11% in cells treated with CHX and HU, which is similar to the level of apoptosis in cells treated with CHX alone. New protein synthesis is therefore required for HU-induced cell death in ML-1 cells.

HU-induced apoptosis correlates with p53 induction. The p53 tumor suppressor plays a critical role in apoptosis induction by genotoxic stimuli by facilitating the expression of pro-apoptosis genes including *BAX*, *PUMA*, *NOXA*, and factors that promote oxidative cellular damage.[55] We therefore sought to determine whether induction of p53 correlated with apoptosis in HU-treated ML-1 cells. ML-1 or Molt-3 cells were mock treated or exposed to HU over a 4 h time course and cell extracts analyzed by SDS-PAGE and immunoblotting with a α-p53 antibody. Exposure of ML-1 cells to HU resulted in the induction of p53 that was detectable within 2 h of drug treatment and was maximal by the 4 h time point, at which time the cells demonstrated features of apoptosis (Fig. 4B). The level of p53 induction by HU was slightly lower than the level obtained following exposure to 10 Gy of IR. However, when one considers that the effects of HU are restricted to the 30–40% of ML-1 cells in S phase, the relative potencies of the two agents as inducers of p53 are comparable. In contrast, the induction of p53 in HU-treated Molt-3 cells was weak, even though p53 was strongly induced by IR in this cell line (Fig. 4B). Thus, the induced levels of p53 in ML-1 and Molt-3 cells correlate with the relative sensitivities of these cell lines to HU. We also examined the expression of the p53-dependent target gene PUMA following HU exposure. PUMA protein levels exhibited transient induction 1 h after exposure to HU, suggesting its enhanced expression may precipitate apoptosis in ML-1 cells (Fig. 4C).

ATR-dependent induction of p53 is required for HU-induced apoptosis. The degree of p53 induction in ML-1 and Molt-3 cells correlated with their relative sensitivities to HU; ML-1 cells strongly induced p53 and were sensitive to HU, whereas Molt-3 cells weakly induced p53 and were comparatively resistant to HU. We therefore sought to directly test the importance of p53 for apoptosis induction and to establish the upstream requirements for p53 induction. To test whether p53 contributed to HU-induced apoptosis, we electroporated ML-1 cells with control siRNA or p53-specific siRNA and then challenged the cells with HU or vehicle 48 h later. Immunoblotting using α-p53 and α-PARP antibodies demonstrated that the basal and HU-induced levels of p53 were efficiently suppressed by the p53 siRNA (Fig. 5A). In contrast, the p53 siRNA did not block the expression or HU-induced phosphorylation of the 32 kDa subunit of replication protein A (RPA32). The suppression of p53 correlated with a dramatic reduction in the amount of cleaved PARP at the 4 h time point, which strongly suggests that p53 is required for apoptosis induction.

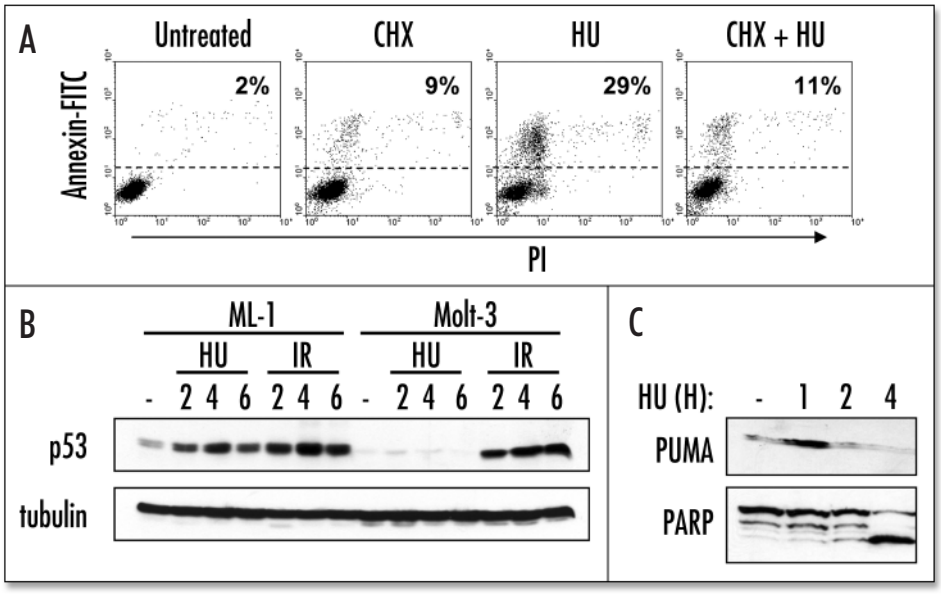

Figure 4. Differential p53 induction correlates with the HU sensitivities of ML-1 and Molt-3 cells. (A) HU-induced apoptosis required new protein synthesis. ML-1 cells were preincubated with the protein synthesis inhibitor cycloheximide (CHX, 5 μg/ml) for 30 min prior to the addition of HU or vehicle. After 4 h, cells were costained with annexin V-FITC and PI and analyzed by flow cytometry. The percentages of apoptotic cells are shown in each panel. (B) Time courses of p53 induction in response to HU or IR. ML-1 or Molt-3 cells were either left untreated or exposed to HU (3 mM) or IR (10 Gy) for the indicated times. Cell extracts were immunoblotted with α-p53 or α-tubulin antibodies. (C) HU-induced accumulation of PUMA in ML-1 cells. ML-1 cells were exposed to 3 mM HU for the indicated lengths of time. Cell extracts were immunoblotted with α-PUMA and α-PARP antibodies.

The upregulation and activation of p53 in response to genotoxic stress is promoted by ATM and ATR through direct and indirect mechanisms. ATM and ATR directly phosphorylate p53 on Ser-15, which antagonizes interactions between p53 and the E3 ubiquitin ligase HDM2.[18,56] ATM and ATR indirectly promote p53 activation through phosphorylation of the CHK2 and CHK1 kinases, respectively, which, in turn, phosphorylate p53 on Ser-20.[37,57] ATR has previously been implicated in the regulation of p53 in response to IR, UV light, and hypoxic cell stress.[24,50,58-59] Given the importance of ATR as a mediator of HU-induced responses, we used RNAi to test whether HU-induced accumulation of p53 in ML-1 cells was ATR dependent. Electroporation of ML-1 cells with an ATR-specific siRNA, but not a control siRNA, suppressed ATR expression and attenuated p53 induction by HU (Fig. 5B). In contrast, an ATM siRNA did not substantially inhibit the induction of p53 in response to HU (data not shown). This result suggests that ATR contributes to p53 induction in HU-treated ML-1 cells, most likely via direct phosphorylation of Ser-15.

The phosphorylation of p53 on Ser-20 by the CHK1 and CHK2 protein kinases is believed to contribute to p53 induction and activation in response to genotoxic stress.[37,60] The differential induction of p53 between ML-1 and Molt-3 cells could therefore reflect a difference in Ser-20 phosphorylation state. To explore this possibility, we measured the HU-induced phosphorylation of p53 on Ser-20 in both ML-1 and Molt-3 cells. ML-1 cells exhibited p53 Ser-20 phosphorylation within 1 h of HU treatment or 2 h after exposure to the radiomimetic drug zeocin, which induces DSBs (Fig. 5C). In contrast, HU-induced Ser-20 phosphorylation was not detected in Molt-3 cells, even though zeocin strongly induced both Ser-20 phosphorylation and p53 accumulation (Fig. 5C). To test whether the absence of p53 Ser-20 phosphorylation in HU-treated Molt-3 cells was caused by defective activation of the ATR-CHK1 pathway, we measured the ATR-dependent phosphorylation of CHK1 on Ser-317 following HU exposure.[31,51] HU-treatment induced CHK1 phosphorylation in Molt-3 and ML-1 cells, indicating that the ATR-CHK1 pathway is activated in both cell lines. Interestingly, the levels of Ser-317-phosphorylated CHK1, total CHK1 and ATR were greatly reduced in ML cells relative to Molt-3 cells (Fig. 5D). The expression level of both proteins in ML-1 cells was also clearly reduced when compared to the ATR and CHK1 levels in a panel of human leukemic cell lines (Sup. Fig. 1). The importance of the reduced levels of ATR and CHK1 in the context of HU-hypersensitivity is uncertain. However the finding that CHK1 is strongly phosphorylated on Ser-317 in Molt-3 cells suggests that failure to induce p53 phosphorylation is not the result of a gross defect in the ATR-CHK1 pathway.

Nocodazole antagonizes p53 induction and PARP cleavage in HU-treated ML-1 cells. The above results demonstrated that differential induction of p53 between ML-1 and Molt-3 cells is largely responsible for their different sensitivities to HU. The findings also suggested that ATR activation per se was insufficient for p53 induction, and that a second signal is generated in ML-1 cells that potentiates p53 accumulation following HU treatment. One plausible explanation for the robust induction of p53 in response to HU was that stalled DNA replication forks in ML-1 cells are rapidly converted into cytotoxic DSBs, which strongly induce p53.[48] If this hypothesis is correct, then one should be able to detect DSBs prior to caspase activation and accumulation of apoptosis-related strand breaks. To explore this possibility, we examined the ATM/ATR-dependent phosphorylation of histone H2AX. The accumulation of phosphorylated histone H2AX (designated γ-H2AX) at foci of DNA damage provides an indirect measure of DSB formation in mammalian cells.[61] In the absence of HU treatment, the nuclei of ML-1 cells demonstrated a weak and diffuse γ-H2AX immunostaining pattern, which was comparable to the pattern seen in untreated Molt-3 cells. Exposure to HU for 1 h induced similar levels of γ-H2AX immunostaining in ML-1 and Molt-3 cells (Fig. 6A), suggesting comparable levels of DSBs between the cell lines. However, at 4 h post-HU, approximately 50% of the γ-H2AX-positive ML-1 cells demonstrated an intense γ-H2AX staining pattern that was not observed in similarly treated Molt-3 cells (Fig. 6A, denoted by arrows). These cells often exhibited an abnormal nuclear morphology suggestive of apoptosis. Caspase inhibitors inhibited HU-induced H2AX phosphorylation in immunoblotting experiments (Fig. 6B), suggesting that the γ - H2AX^{high} cells were actively undergoing apoptosis. This result is consistent with previous literature showing that apoptosis-dependent DSBs are potent inducers of H2AX phosphorylation.[62] However, the finding that ML-1 and Molt-3 cell lines display comparable levels of γ-H2AX at early time points after HU exposure provides indirect evidence that these cell lines initially accumulate comparable levels of DSBs in response to DNA replication stress.

Microtubule inhibitors have been shown to antagonize topoisomerase poison-induced cell death and we wished to determine whether microtubules were important for apoptosis initiated by HU.[63] To test this possibility, we treated ML-1 cells with the microtubule inhibitor nocodazole (NOC) 1 h prior to the addition of HU and then measured PARP cleavage 4 h later. HU-induced PARP cleavage was inhibited by NOC preexposure, though some cleavage was still observed (Fig. 6C). In addition, the reduction in PARP cleavage observed in HU- and NOC-treated cells was accompanied by a clear reduction in the level of p53 induction. At the cellular level, ML-1 cells treated with HU and NOC showed an approximately two-fold reduction in the hypodiploid fraction relative to cells treated with HU alone (Fig. 6D). Whereas the S phase population was essentially depleted in the HU-treated cells, ML-1 cells exhibiting an S-phase DNA content were still observed following exposure to NOC and HU. Importantly, NOC pretreatment did

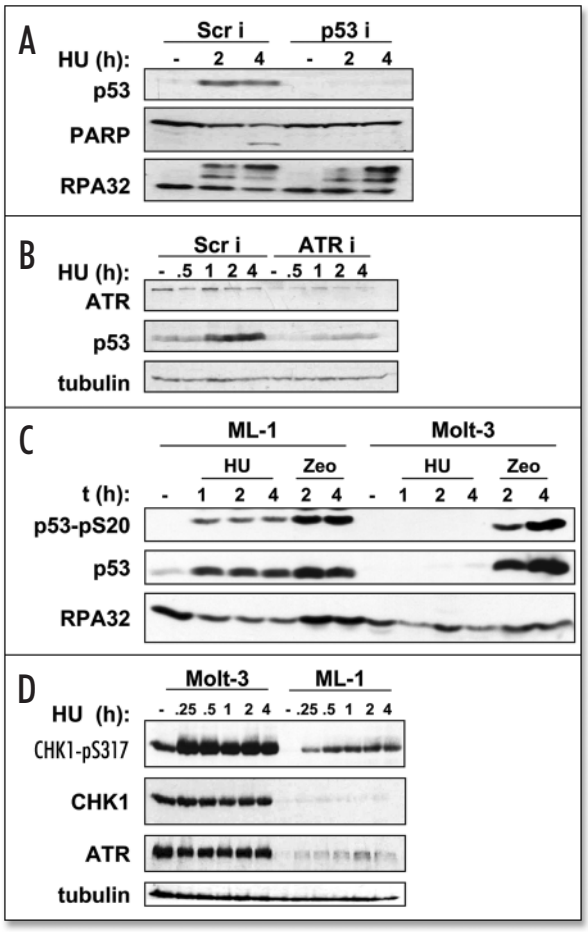

Figure 5. ATR-dependent accumulation of p53 is required for optimal HU-induced apoptosis. (A) p53 is required for HU-induced PARP cleavage. ML-1 cells were electroporated with either control siRNA or siRNA targeted to p53. After 48 h, the cells were treated with HU or vehicle for the indicated times. Cell extracts were then immunoblotted with α -p53, α-PARP and α-RPA32 antibodies. (B) HU-induced p53 accumulation is ATR-dependent. ML-1 cells were electroporated with either control siRNA or ATR-specific siRNA. After 48 h, the cells were either left untreated or HU-treated for the indicated times. Cell extracts were immunoblotted with α-ATR, α-p53 and α-tubulin antibodies. (C) Differential phosphorylation of p53 on Ser-20 in response to HU. ML-1 and Molt-3 cells were exposed to HU (3 mM) or zeocin (Zeo, 0.5 mg/ml) and harvested at the indicated times after treatment. Cell extracts were immunoblotted with antibodies specific for p53, Ser-20-phosphorylated p53 (p53-pS20), and RPA32 as a loading control. (D) Reduced expression of ATR and CHK1 in ML-1 cells. ML-1 and Molt-3 cells were either left untreated or treated with HU for the indicated times. Cell extracts were immunoblotted with α-CHK1-pS317, α-CHK1, α-ATR and α-tubulin antibodies.

not reduce the fraction of ML-1 cells in S phase prior to HU exposure, ruling out the possibility that the protective effects of NOC were attributable to fewer HU-susceptible cells (data not shown). The finding that NOC delays apoptosis implies that mitosis or another microtubule-dependent process contributes to the initiation of apoptosis in ML-1 cells following HU treatment.

DISCUSSION

In this report we have investigated the mechanism of apoptosis induction by HU in ML-1 myeloid leukemia cells. We have shown that HU induces a remarkably rapid program of apoptosis in this cell line; HU exposures as short as 15-30 min are sufficient to induce cell death in approximately one third of S-phase cells, whereas exposures greater than 1 h effectively depleted the entire S phase population within 4–6 h. The inability of ML-1 cells to survive even relatively short pulses of HU or the DNA polymerase α inhibitor aphidicolin, when combined with the observation that these cells were relatively resistant to IR, implies that ML-1 harbors one or more defects in pathways that mediate resistance to DNA replication stress. We have therefore sought out to map the apoptosis pathways initiated following HU exposure, as well as to uncover potential defects responsible for HU hypersensitivity.

HU-induced a classical caspase-dependent apoptotic cell death in ML-1 cells characterized by cytoplasmic membrane blebbing, nuclear fragmentation, and caspase activation within 4 h. Given the rapidity of this response we were surprised to find that new protein synthesis was required for apoptosis induction. Nevertheless, the requirement for new protein synthesis is compatible with a role for a p53-dependent transcriptional program in the initiation of apoptosis and requirement for p53 in this process was directly established using RNAi (Fig. 5A). Although the contribution of p53 to replication inhibitor-induced apoptosis appears to be cell-type dependent, our findings are congruent with earlier work showing that p53 expression is positively correlated with HU sensitivity.[6-7] Other studies have shown that p53 promotes apoptosis by trans-activating promoters of pro-apoptosis regulators including the BH3 domain containing proteins BAX, PUMA, and NOXA.[55] Whereas the levels of BAX were constitutively high in ML-1 cells, PUMA was induced following HU treatment, suggesting that this p53 target gene contributes to apoptosis induction (Fig. 4C and data not shown). However, because 10 Gy of IR also strongly induced p53 in ML-1 cells, but elicited a lower level of cell death, p53-independent pathways must also contribute to apoptosis induction in this cell line.

The differential induction of p53 in ML-1 and Molt-3 cells appears to underlie their distinct sensitivities to DNA replication inhibitors and understanding the basis for this difference should illuminate the mechanism of HU-induced apoptosis. We showed that the HU-induced accumulation of p53 required ATR and correlated with p53 phosphorylation on Ser-20, which is most likely carried out by the HU-inducible, ATR effector kinase, CHK1. Paradoxically, even though ATR is generally viewed as a determinant of cellular resistance to DNA replication stress, our results imply that ATR functions in a pro-apoptosis capacity following HU exposure—at least in the context of the acute response to this drug in ML-1 cells. Transfection of ATR siRNA did not suppress HU-induced PARP cleavage; however the efficacy of the electroporation-based ATR knock-down was relatively poor in ML-1 cells (Sup. Fig. 2) and residual p53 induction, either ATR-dependent or ATR-independent, was likely responsible for the observed cell death. Notably, a pro-apoptosis function of ATR was recently established in studies of HIV Vpr protein-induced apoptosis,[64-65] suggesting that ATR is a cell type- and stimulus-dependent regulator of apoptosis in mammalian cells.

The ATR-CHK1 pathway was activated within minutes of HU exposure in both ML-1 and Molt-3 cells, however, the induction of p53, as well the phosphorylation of p53 on Ser-20, was much more robust in the ML-1 cells. The failure to induce p53 in HU-treated Molt-3 cells, despite robust CHK1 phosphorylation, implies that activation of the ATR-CHK1 pathway is insufficient for p53 induction and that a second signal generated in ML-1 cells promotes full activation of p53. Because prolonged exposures to HU induces

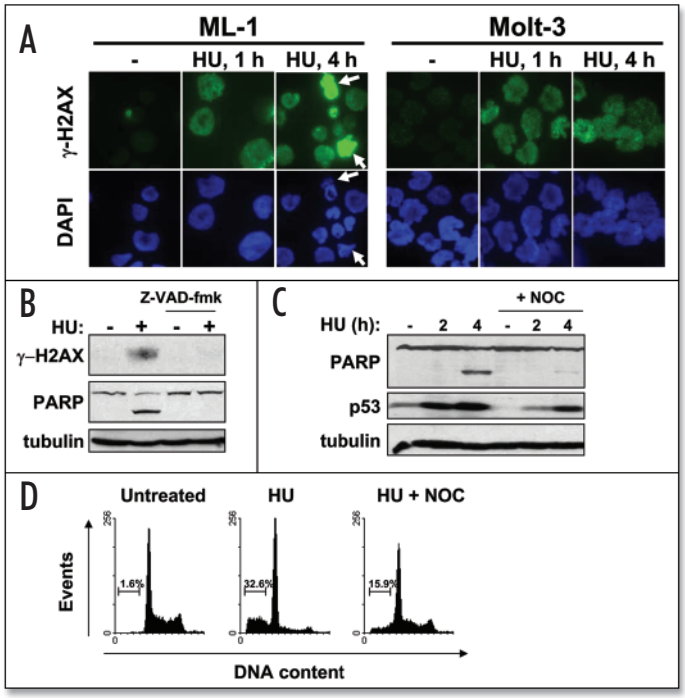

Figure 6. Suppression of HU-induced apoptosis by nocodazole. (A) Comparison of histone H2AX phosphorylation between HU treated ML-1 and Molt-3 cells. ML-1 or Molt-3 cells were left untreated or exposed to HU (3 mM) for 1 h or 4 h. The cells were cytospun onto glass slides, stained with α-γ-H2AX antibody followed by a FITC-conjugated secondary antibody. The cells were counterstained with DAPI to visualize nuclei. Digital images of representative fields are shown (100X). The locations of two apoptotic ML-1 cells are marked with arrows. (B) Suppression of H2AX phosphorylation by caspase inhibitors. ML-1 cells were pretreated with a caspase inhibitor cocktail prior to HU exposure for 4 h. Cell extracts were then prepared and analyzed by immunoblotting with α-γ-H2AX. (C) Nocodazole (NOC) inhibits HU-induced PARP cleavage and p53 induction. ML-1 cells were preincubated with NOC (0.5 μg/ml) for 1 h prior to addition of HU or vehicle. Cells were harvested at the indicated times and extracts analyzed by immunoblotting with α-PARP, α-p53 and α-tubulin antibodies. (D) NOC inhibits hypodiploid DNA formation in response to HU. ML-1 cells were cultured with or without NOC 1 h prior to addition of HU or vehicle for an additional 4 h. Cells were fixed with ethanol, stained with PI and analyzed by flow cytometry. The percentage of apoptotic cells exhibiting sub-2N DNA content is shown in each panel.

DSBs in cultured mammalian cells,[12-13] we initially hypothesized that ML-1 cells accumulate abnormally high levels of cytotoxic DSBs in response to HU. However, ML-1 and Molt-3 cells exhibited qualitatively similar profiles of γ-H2AX staining when exposed to HU for 1 h or less, suggesting comparable levels of DSBs prior to the induction of apoptosis (Fig. 6A). Furthermore, HU-treated ML-1 cells did not exhibit hyperactivation of ATM, which is another sensitive marker for DSB formation[66] (S. Kumar and R. Tibbetts, unpublished data). The available evidence therefore suggests that increased DSBs are not responsible for p53 accumulation or the inductive phase of apoptosis in ML-1 cells after brief exposures to HU. Although the source of the putative second signal for p53 induction is presently unclear, the finding that NOC partially suppressed p53 induction and PARP cleavage suggests a role for microtubules. A potential explanation for the protective effect of NOC is that mitotic events contribute to apoptosis induction. However, the CDK1-dependent phosphorylation of histone H3 on Ser-10 was downregulated in HU-treated ML-1 cells, suggesting that this aspect of the S-M checkpoint is intact (Kumar S, Tibbetts R, unpublished data). It is possible that ML-1 cells initiate lethal mitotic events independent of CDK1 activation or that the protective effect of NOC is unrelated to its effects on the mitotic spindle.

A recent study described reduced or undetectable expression of CHK1 in a subset of aggressive leukemias,[46] and we showed here that ML-1 cells express low levels of ATR and CHK1. The significance of the reduced levels of these factors to the HU sensitivity of this cell line is uncertain; ML-1 cells are refractory to stable transfection with ATR and CHK1 expression vectors and, for now, the link between reduced ATR and CHK1 expression and HU sensitivity is purely correlative. Nevertheless, given that CHK1 is haploinsufficient in mice,[41] the reduced levels of CHK1 and its upstream kinase in ML-1 cells might be predicted to confer an HU sensitive phenotype. Although reduced, the levels of ATR in ML-1 cells are apparently sufficient to support p53 induction and subsequent apoptosis. Future experiments will be required to test whether ectopic expression of ATR or CHK1 can confer HU resistance to ML-1 cells, or if HU sensitivity in these cells is the result of additional defects in replication stress-response pathways.

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