Genotoxic Stress–Induced Expression of p53 and Apoptosis in Leukemic Clam Hemocytes with Cytoplasmically Sequestered p53

Stefanie Böttger, Emily Jerszyk, Ben Low, and Charles Walker

Department of Zoology, Center for Marine Biology and Marine Biomedical Research Group, University of New Hampshire, Durham, New Hampshire

Abstract

In nature, the soft shell clam, *Mya arenaria*, develops a fatal blood cancer in which a highly conserved homologue for wild-type human p53 protein is rendered nonfunctional by cytoplasmic sequestration. In untreated leukemic clam hemocytes, p53 is complexed throughout the cytoplasm with overexpressed variants for both clam homologues (full-length variant, 1,200-fold and truncated variant, 620-fold above normal clam hemocytes) of human mortalin, an Hsp70 family protein. *In vitro* treatment with etoposide only and *in vivo* treatment with either etoposide or mitoxantrone induces DNA damage, elevates expression (600-fold) and promotes nuclear translocation of p53, and results in apoptosis of leukemic clam hemocytes. Pretreatment with wheat germ agglutinin followed by etoposide treatment induces DNA damage and elevates p53 expression (893-fold) but does not overcome cytoplasmic sequestration of p53 or induce apoptosis. We show that leukemic clam hemocytes have an intact p53 pathway, and that maintenance of this tumor phenotype requires nuclear absence of p53, resulting from its localization in the cytoplasm of leukemic clam hemocytes. The effects of these topoisomerase II poisons may result as mortalin-based cytoplasmic tethering is overwhelmed by *de novo* expression of p53 protein after DNA damage induced by genotoxic stress. Soft shell clam leukemia provides excellent *in vivo* and *in vitro* models for developing genotoxic and non-genotoxic cancer therapies for reactivating p53 transcription in human and other animal cancers displaying mortalin-based cytoplasmic sequestration of the p53 tumor suppressor, such as colorectal cancers and primary and secondary glioblastomas, though not apparently leukemias or lymphomas. [Cancer Res 2008;68(3):777–82]

Introduction

In normal mammalian cells, p53 suppresses the formation of tumors by arresting the cell cycle or by apoptosis in response to genotoxic stress–induced DNA damage (1). Levels and activity of p53 increase in response to DNA damaging agents (2–4). Transcription of p53-regulated genes yields proteins that can edit and repair DNA and/or promote apoptosis based on the degree of DNA damage. Loss of cell cycle control results upon inactivation of p53 and loss of p53 transcriptional activity, and can lead to development and progression of tumors (1). Nontranscriptional induction of apoptosis can also occur through binding of mitochondrially directed p53 and inactivation of Bcl2 or other anti-apoptotic proteins (5, 6).

In this study, we use genotoxic stress–induced DNA damage to promote expression and to reverse cytoplasmic sequestration of p53, and to restore its apoptotic function in a naturally occurring diffuse tumor of the hemocytes of the soft shell clam. In leukemic clam hemocytes, a highly conserved clam homologue for human p53 (Map53; Genbank accession number AF253323) is rendered nonfunctional by sequestration in the cytoplasm by both variants of the clam homologue for human mortalin (*Mya arenaria* mortalin, full-length and truncated; Genbank Accession numbers AY326398 and EF576660, respectively; refs. 7, 8). Truncated clam mortalin is missing exon 3 that includes the ATP-binding and ATPase domain required to reverse p53 binding. We hypothesize that normal transcriptional functions of p53 in tumor suppression are silenced when clam mortalin proteins are overexpressed. For instance, transcriptionally controlled editing and repair of DNA and induction of apoptosis would not be initiated by p53 that cannot enter the nucleus, bind DNA, and direct the expression of relevant genes. Similarly, ubiquination dictated by Mdm2 binding to the transactivation domain (TAD) of p53 or by CIP1 down-regulation of G_1 cyclin-/cyclin-dependent kinase–dependent phosphorylation and activation of p53 cannot occur. Cytoplasmic-sequestered p53 might still direct nontranscriptional initiation of apoptosis at the mitochondria if mortalin binding does not interfere with this pathway (9).

Although cytoplasmic sequestration has not yet been recorded for human leukemias or lymphomas, a subset of naturally occurring but unrelated human cancers do retain p53 in the cytoplasm (e.g., undifferentiated neuroblastoma, breast, retinoblastoma, colorectal adenocarcinomas, and glioblastoma; refs 10–12), and this phenotype can be induced in mouse NIH 3T3 and human HeLa cells (13–16). In all of these cases, wild-type p53 is inactivated because it is retained in the cytoplasm. A variety of molecular mechanisms have been linked to cytoplasmic sequestration in these human cancers, such as truncation of the nuclear localization motif receptor protein importin α (17), overactive nuclear export by an Mdm2 dependent pathway (18), and cytoplasmic tethering by foreign (viral; ref. 19) or local cytoplasmic proteins (9, 15, 20). In human colorectal cancer cells and leukemic clam hemocytes, mortalin is responsible for cytoplasmic tethering when the latter protein is overexpressed (8, 21). Dundas et al. (12) indicate that high levels of mortalin expression are correlated with poor clinical outcome for colorectal cancer. A recent study of primary and secondary glioblastomas suggests that mortalin and/or other tethering molecules (e.g., cullin 7 or PARC) may be also be responsible for cytoplasmic sequestration in these cancers.

Requests for reprints: Charles Walker, Department of Zoology, Center for Marine Biology and Marine Biomedical Research Group, The University of New Hampshire, Durham, NH 03824. Phone: 603-862-2111; Fax: 603-862-3784; E-mail: cwwalker@christa.unh.edu.

doi:10.1158/0008-5472.CAN-06-0968

In the current study, we use leukemic clam hemocytes for *in vitro* and *in vivo* assays to evaluate cytotoxicity, intracellular localization of p53, DNA damage, and apoptosis after treatment with two genotoxic DNA topoisomerase II poisons, etoposide, and mitoxantrone. Because we were investigating a blood disease in the clam, these topoisomerase II poisons were originally chosen from the standard agent database at the National Cancer Institute because they are highly cytotoxic to human lymphoblastic leukemia cell lines (22) and have shown effectiveness against human leukemias and lymphomas.[1] Importantly, these topoisomerase II poisons do have significant genotoxic effects on a variety of human cancers but have never been evaluated in terms of their effects on cancers resulting from mortalin-based cytoplasmic sequestration.

Understanding the molecular basis for this negative regulatory pathway for p53 might lead to therapies for *any* human cancer where cytoplasmic sequestration is mediated by mortalin. As Martins et al. (23) point out, "reinstatement of p53 function is an attractive tumor-specific therapeutic strategy, but it will only work if tumors harbor persistent p53-activating signals that engage growth inhibition or death." In the present study, we show that clam leukemia cells have an intact p53-activating pathway leading to apoptosis, and that maintenance of this diffuse tumor requires nuclear absence of p53 dictated by its localization in the cytoplasm.

Materials and Methods

Clams. Soft shell clams (*Mya arenaria*; n = 150–200) were collected at the lowest tides of each month from sand flats on Marsh Island in New Bedford Harbor at Fairhaven, Massachusetts (41° 38.0′ N 70° 55.0′ W) and maintained at the University of New Hampshire Coastal Marine Laboratory, New Castle, New Hampshire. For biopsy, a small aliquot of hemolymph (10 μL) was removed from the pericardial sinus and incubated for 2 h at 8°C. Clams were classified with a Zeiss IM inverted microscope as normal (0%, round, nonmotile leukemic clam hemocytes; 100%, attached normal clam hemocytes), early incipient leukemic (1%–50%, leukemic clam hemocytes), late incipient leukemic (50%–99%, leukemic clam hemocytes), or fully leukemic (100%, leukemic clam hemocytes). In 51 collections made over a 5-year period, the average number of clams that was 100% leukemic was 5.64%; the range was 0–11%. Only fully leukemic clams were used for the experiments presented in this study.

Antibody for p53. A polyclonal antibody to *Mya arenaria* p53 protein (Map53 rabbit anticlam polyclonal) was raised against a peptide synthesized from a highly immunogenic region in the core sequence of clam p53 and including part of DNA binding domain V (Map53 23-mer CACPGRDRKADERGSLPPMVSGG). The polyclonal antibody obtained was successfully screened for its ability to recognize clam p53 in Western blot analysis of both *in vitro* expressed clam proteins and whole cell extracts collected *in vivo* from leukemic and normal clam hemocytes (7).

Treatment of leukemic clam hemocytes with leptomycin B *in vitro*. To determine if overactive nuclear export is responsible for cytoplasmic sequestration of p53, leukemic clam hemocytes were treated with leptomycin B, which blocks CRM1-mediated nuclear export. Hemolymph was removed from the pericardial sinus of fully leukemic (100%) clams. Blood was divided into eight tubes for time 0, 4, 8, and 24 h with two treatments per time (control and leptomycin B treatment). Leptomycin B treatments only received a final concentration of 115 nmol/L leptomycin B, control treatments received normal clam medium without the drug. All tubes were maintained on a rotator at 8°C for the required length of time. At each time point (time 0, 4, 8, and 24 h), nuclear and cytoplasmic protein extractions (NEPER; Pierce) were performed and clam p53 distribution was determined using Western blotting.

Treatment of leukemic clam hemocytes with topoisomerase II poisons *in vitro*. Hemolymph was removed from the pericardial sinus of fully leukemic (100%) clams and was diluted with clam culture medium (24) to 4×10^5 leukemic clam hemocytes mL^{-1}. Hemolymph and topoisomerase II poisons (0–0.07 mmol/L mitoxantrone and 0–0.1 mmol/L etoposide) were diluted with clam culture medium to a final concentration of 1×10^5 leukemic clam hemocytes mL^{-1} in 1.5 mL microcentrifuge tubes (Fisher Scientific). Samples of 400 mL were removed at time 0, 6, and 18 h; *in vivo* — to approximate drug concentrations used *in vitro* (0.07 mmol/L mitoxantrone and 0.075 mmol/L etoposide), hemolymph volumes were estimated based on clamshell size, and the appropriate concentrations of drugs were injected immediately after the zero time biopsy. Additional biopsies were performed at 8, 20, and 24 h on 800 μL of hemolymph removed from the pericardial sinus.

Pretreatment of leukemic clam hemocytes with wheat germ agglutinin *in vitro*. To evaluate mechanism of action of p53 and detect any alternative routes leading to apoptosis, we set up four treatments: leukemic clam hemocytes untreated, leukemic clam hemocytes treated with wheat germ agglutinin (WGA) only, leukemic clam hemocytes treated with 0.075 mmol/L etoposide only, and leukemic clam hemocytes pretreated with WGA followed by exposure to 0.075 mmol/L etoposide. Pretreatment of leukemic clam hemocytes with WGA was conducted using FITC-labeled WGA (Sigma) that was transfected into the cells using the Chariot protein delivery system (Active Motif). WGA was allowed to couple with the Chariot compound for 30 min at room temperature to form complexes that were incubated for 1 h with leukemic clam hemocytes suspended in clam culture medium at concentrations of 6×10^5 hemocytes per 100 μL medium. Successful transfection of WGA into leukemic clam hemocytes results in blockage of the nuclear pores, and translocation of p53 into the nucleus should also be blocked. *De novo* or unbound p53 should be directed to mitochondria, resulting in nontranscriptional induction of apoptosis. After transfection, all treatments (control and etoposide treated) were allowed to incubate for 6 h, nuclear and cytoplasmic proteins were separated (NEPER; Pierce), and clam p53 distribution was determined using Western blotting.

Cytotoxicity assay. Before *in vitro* and *in vivo* drug treatments, lactate dehydrogenase (LDH) activity was measured (CytoTox 96 NonRadioactive Cytotoxicity Assay G1780; Promega) using increasing numbers of leukemic clam hemocytes (0–140,000 cells) to test the validity of this assay in the clam model, determine the numbers of leukemic clam hemocytes needed, and adjust environmental conditions (e.g., temperature). Once these variables were determined, we followed the procedure and analyses described by Promega and used in >350 published studies.

Immunocytochemistry. Cytospins of 100 μL of leukemic clam hemocytes from untreated and treated individuals *in vitro* and *in vivo* were fixed and permeabilized by immersion in acetone. These preparations were treated with the clam p53 primary antibody (7). Resulting preparations were developed with a peroxidase-labeled secondary antibody (Vectastain ABC Elite IgG kit; Vector Laboratories). Control cytospins received identical treatment in the absence of primary antibody. After completing the assay, fields of 200 leukemic clam hemocytes were counted and scored into three categories: (*a*) cells with predominantly nuclear clam p53, (*b*) cells with predominantly cytoplasmic clam p53, and (*c*) cells where the intracellular distribution of clam p53 was indeterminate. The nonparametric Wilcoxon-Mann-Whitney test was applied to assess differences between results in catagories *a* to *c* and between time points.

Western blotting. To determine the cellular localization of p53 protein, nuclear and cytoplasmic proteins were extracted from 200 μL of hemolymph containing leukemic clam hemocytes at each time point during treatment with mitoxantrone and etoposide using NE-PER (nuclear and cytoplasmic extraction reagents; Pierce). Total protein was measured spectrophotometrically (Ultraspec 3100) using a modified Lowry procedure (Bio-Rad protein assay). Nuclear and cytoplasmic proteins (37.5 μg) were assayed by Western blotting on 4-15% Tris-HCl Ready gels (Bio-Rad) transferred to a polyvinylidene difluoride membrane (Bio-Rad), treated with the clam p53 primary antibody, and visualized colorimetrically (7).

Comet assay. To detect DNA damage during *in vitro* and *in vivo* drug treatments with topoisomerase II agents (measured as DNA fragmentation

[1] http://www.dtp.nci.nih.gov/docs/compare/examples/topoii.html

and observed as a comet-shaped tail discharged from the nucleus), COMET assays were performed at each time point (Trevigen CometAssay kit 4250-050). Results were scored as percent of DNA-damaged cells in a total of 100 cells.

Apoptosis assays. To document apoptosis, Romanovsky staining (ThermoShandon; Kwik Diff Stain kit) and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL) assay (*in situ* cell death detection kit; AP; Roche) were performed on cytospins of leukemic clam hemocytes at 6 h *in vitro* and 8 h *in vivo*. After staining, hemocytes were mounted under coverslips with Permount (for Romanovsky; Fisher Scientific) or with Vectashield (for TUNEL; Vector Laboratories) and scored at ×600 on a Zeiss Axioplan II microscope (Carl Zeiss, Inc.; ref. 25). For the Romanovsky assay, cells were designated as apoptotic when intracellular vacuoles were observed, and apoptosis was expressed as a percentage in a total of 100 cells. For the TUNEL assay, results were scored as percentage of apoptotic cells (TUNEL-positive cells) of total of 100 cells.

Quantitative PCR. To document expression of mortalin and p53, 500 μL of hemolymph were removed from normal, leukemic, and 0.075 mmol/L etoposide-treated leukemic clams (with and without prior treatment with WGA). The hemolymph was centrifuged at 3,000 *g* for 10 min, RNA extracted using Trizol (Invitrogen), and cDNA synthesized using SuperScript First-Strand Synthesis System (Invitrogen). All samples were prepared for quantitative PCR (QPCR) using the Taqman Fast System (Applied Biosystems) and run in a 7500 Fast reverse transcription-PCR System (Applied Biosystems) at 45 cycles of 95°C with automatically set cycle threshold (Ct).

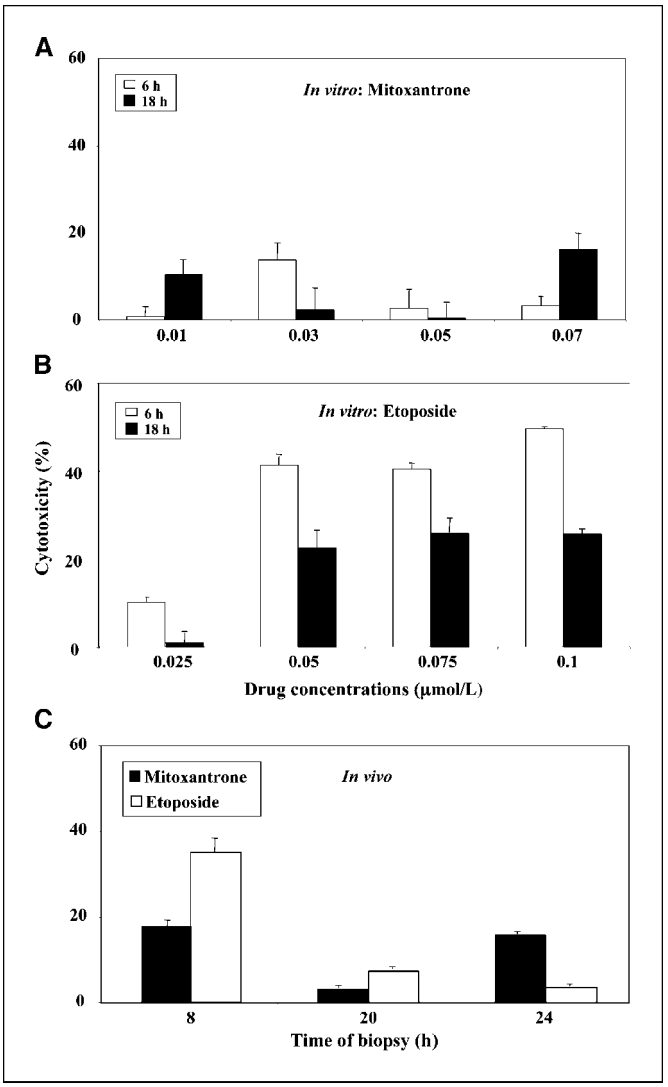

Figure 1. Cytotoxicity of leukemic clam hemocytes. LDH activity (% LDH) measured cell proliferation of leukemic clam hemocytes after treatment with mitoxantrone (0–0.07 mmol/L) *in vitro* (*A*), etoposide (0–0.1 mmol/L) *in vitro* (*B*) and mitoxantrone (0.07 mmol/L) and etoposide (0.075 mmol/L) *in vivo* (*C*). Cytotoxicity of leukemic clam hemocytes was expressed as percentage and determined using medium background absorbance, untreated cell absorbance, treated cell absorbance (experimental release), and maximal lysed cell absorbance (target maximum release).

Results

Treatment with 115 nmol/L of the nuclear export blocker leptomycin B for 8 and 24 h did not result in localization of p53 in the nucleus, suggesting that overactive nuclear export of p53 mediated by CRM1 is not responsible for cytoplasmic sequestration in leukemic clam hemocytes (data not shown).

LDH activity decreased for leukemic clam hemocytes treated with etoposide at 6 h *in vitro* (0–0.1 mmol/L etoposide) and 8 h *in vivo* (0.075 mmol/L etoposide only; $P < 0.001$), indicating increased cytotoxicity (ANOVA on arcsine transformed data; Fig. 1). LDH activity increased at 18 h of treatment *in vitro* (0–0.1 mmol/L etoposide) and 20 to 24 h treatment *in vivo* (0.075 mmol/L etoposide only) using etoposide and indicating decreased cytotoxicity. Treatment of leukemic clam hemocytes for 8 h with mitoxantrone *in vivo* (0.07 mmol/L mitoxantrone) resulted in increased cytotoxicity. After treatment for 20 h, cytotoxicity decreased, followed by an increase at 24 h of treatment. *In vitro* treatment with mitoxantrone (0–0.07 mmol/L mitoxantrone) resulted no changes in these variables ($P = 0.087$).

Nuclear clam p53 increased after treatment with 0.07 mmol/L mitoxantrone *in vivo* ($P = 0.047$), whereas *in vitro* treatment with this drug had no effect (Kruskal-Wallis followed by Bonferroni test; Fig. 2). Both *in vitro* and *in vivo* treatments with etoposide (0.075 mmol/L) resulted in increased nuclear clam p53. This result was most pronounced during *in vivo* treatment.

After treatment of leukemic clam hemocytes with etoposide *in vitro* and *in vivo*, the Comet assay indicated increased DNA fragmentation, whereas treatment with mitoxantrone yielded DNA fragmentation *in vivo* only (Mann-Whitney test; Fig. 3*A* and *B*). DNA fragmentation was notably higher in leukemic clam hemocytes treated with etoposide *in vitro*. After treatment with mitoxantrone and etoposide, apoptotic leukemic clam hemocytes increased both *in vitro* and *in vivo*. However, apoptosis occurred less frequently after treatment with mitoxantrone than after treatment with etoposide (Mann-Whitney test; Fig. 3*C* and *D*). Percentages of apoptotic leukemic clam hemocytes were significantly lower when assessed by observations of Romanovsky-stained cells than by the TUNEL assay. Analysis by Romanovsky staining detects apoptosis at a later stage when leukemic clam hemocytes are forming internal vesicles; the more sensitive TUNEL assay provides a quantitative estimate of apoptotic DNA damage at an earlier stage and yields a significantly higher estimate for apoptotic leukemic clam hemocytes.

After transfection of leukemic clam hemocytes with the nuclear pore blocker WGA followed by etoposide treatment, p53 was found only in the cytoplasm of leukemic clam hemocytes and was not transported into the nucleus, and no increase in apoptotic cells was noted (data not shown). Frequencies of apoptotic cells were lower in leukemic clam hemocytes pretreated with WGA (15.3%) than in hemocytes treated directly with 0.075 mmol/L etoposide (74.4%).

Expression of p53 did not differ between untreated normal and leukemic clam hemocytes. In leukemic clam hemocytes treated

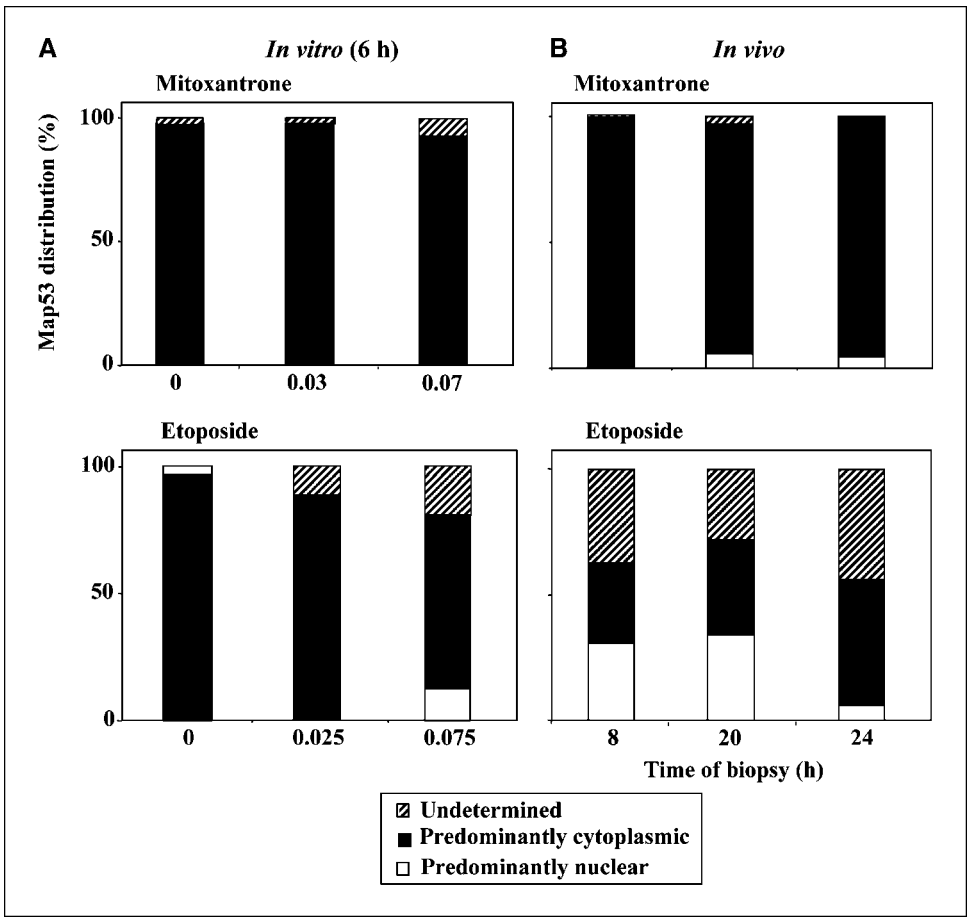

Figure 2. Localization of clam p53 within leukemic clam hemocytes using immunocytochemistry. Distribution (%) of nuclear and cytoplasmic p53 in leukemic clam hemocytes: with mitoxantrone (0–0.07 mmol/L) and etoposide (0–0.75 mmol/L) *in vitro* (*A*) and with mitoxantrone (0.07 mmol/L) and etoposide (0.075 mmol/L) *in vivo* (*B*). Results for clam p53 distribution for a total of 200 leukemic clam hemocytes are subdivided into three different scoring categories: leukemic clam hemocytes with predominantly nuclear clam p53, leukemic clam hemocytes with predominantly cytoplasmic clam 53, and leukemic clam hemocytes where the intracellular distribution of clam p53 was undetermined. Map53 = clam p53 (48).

with etoposide, p53 was overexpressed 602-fold compared with untreated leukemic hemocytes, which showed higher expression than normal clam hemocytes by 30% (Table 1). When transfected with the nuclear pore blocker WGA, followed by treatment with etoposide, p53 was overexpressed 893-fold compared with untreated normal clam hemocytes. Expression of mortalin differed between normal and leukemic clam hemocytes by 1,634 and 619 times for the long and short versions respectively. Additional increase of mortalin expression was observed in leukemic clam hemocytes treated with etoposide by 204-fold for the long variant and 159-fold for the short variant.

Discussion

Nongenotoxic and genotoxic strategies for reactivating p53 function are of high interest because they may lead to promising cancer therapies (23, 26–28). Based on an understanding of the normal p53-mediated pathway for suppressing cancer induction, strategies for reactivating p53 have been proposed. Among others, these include stabilizing p53 using small molecule Mdm2 antagonists (e.g., nutlins; refs. 29–32) and inducing *de novo* expression of p53 after DNA damage resulting from genotoxic stress or gene therapy (33–35).

Previous studies in humans and clams have shown that the cationic inhibitor of mortalin, MKT-077, competes with mortalin for p53 binding and results in translocation of p53 to the nucleus followed by rapid apoptosis (8, 36, 37). Use of this small molecule does not induce DNA damage or elevate transcription of p53.

In the present study, we use genotoxic stress to promote transcription of *de novo* wild-type p53, translocation of p53 to the nucleus, and apoptosis of leukemic clam hemocytes. The apoptotic outcome can be prevented after transfection with the nuclear pore blocker WGA. Although cytoplasmic sequestration of p53 occurs in a variety of human cancers, no one has previously linked treatment with these topoisomerase II poisons to reversal of mortalin-based cytoplasmic sequestration and apoptotic death of cancer cells in any organism.

Mortalin is a member of the Hsp70 family of proteins that is not up-regulated by heat. In mammals, it functions in mitochondrial import, energy generation, chaperoning of misfolded proteins, as a stress sensor, and is involved in carcinogenesis and old age disorders (e.g., Parkinson's, Alzheimer's, and Huntington's diseases; ref. 9). We have identified two variants of mortalin in the clam, a full-length and a truncated variant. Both contain an NH_2-terminal mitochondrial localization signal and a p53 binding site, but the truncated variant is missing amino acids represented by exon 3, which contain ATP binding and ATPase functions necessary for releasing p53 from binding. Both variants are complexed in the cytoplasm of leukemic clam hemocytes with p53. At this point, it is unclear if either overexpressed variant is exclusively responsible for sequestering p53 in the cytoplasm of leukemic clam hemocytes. Although both variants of mortalin are also minimally up-regulated after treatment with etoposide, mortalin-based cytoplasmic tethering mechanism is apparently still overwhelmed, allowing nuclear translocation of untethered clam p53.

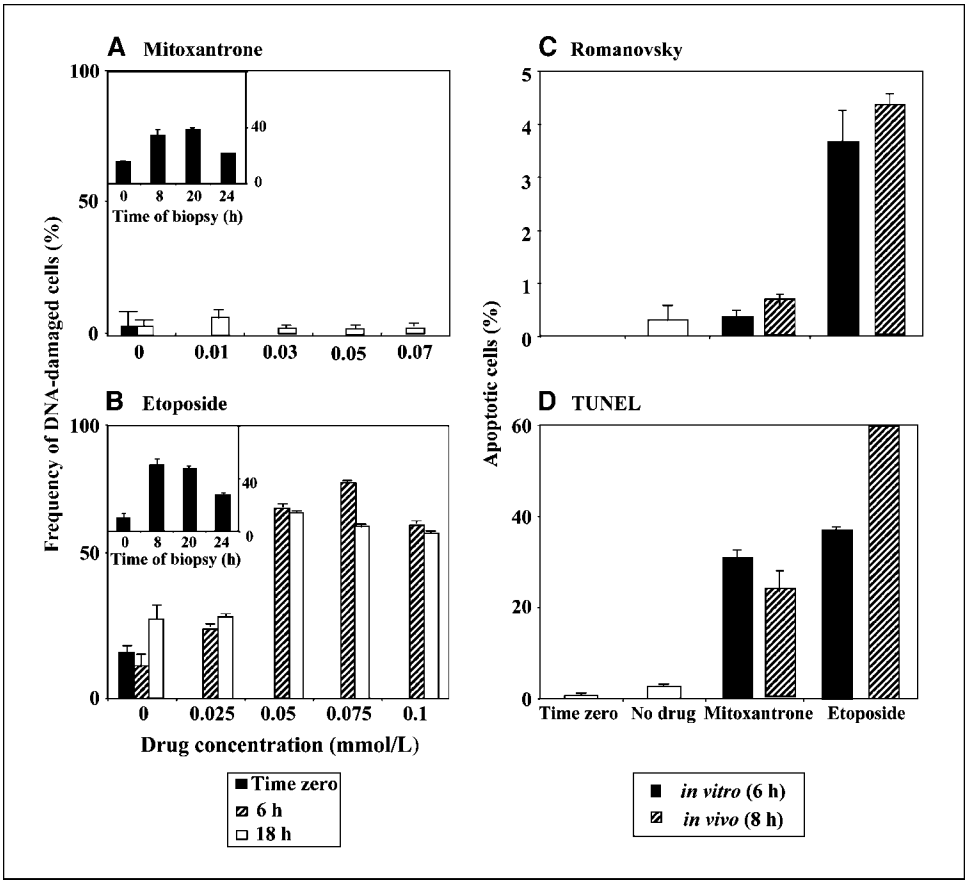

Figure 3. DNA fragmentation and apoptosis assays of leukemic clam hemocytes. Frequency of DNA-damaged cells (Comet assay; %) of leukemic clam hemocytes after treatment with mitoxantrone (*A*) and etoposide (*B*). Results were determined by migration of denatured and cleaved DNA fragments in 100 cells ("comet tails") indicative of DNA damage. *Large graphs,* results *in vitro*; *insets, in vivo* experimental data and frequency of apoptotic cells in leukemic clam hemocytes treated with mitoxantrone and etoposide using Romanovsky stain (*C*) and TUNEL assay (*D*) as detection methods; apoptosis was determined by the development of intracellular vacuoles and the degree of nuclear fluorescence, respectively. Analysis by Romanovsky staining detects apoptosis at a much later stage when leukemic clam hemocytes are forming internal vesicles and is qualitative; the more sensitive TUNEL assay provides a quantitative estimate of DNA damage at an earlier stage of apoptosis and, thus, detects a greater number of apoptotic leukemic clam hemocytes. Both results were expressed as a percentage of 100 cells counted.

Etoposide forms a complex between DNA and DNA topoisomerase II, resulting in decreased DNA religation and strand breaks (38) and secondary cancers can result (39, 40). Overexpression of wild-type p53 facilitates etoposide-induced death of cancer cells in human colon carcinoma cell lines (HCT116 and RKO; ref. 3). In the current study, treatment of leukemic clam hemocytes with etoposide resulted in DNA damage and increased expression of clam p53 followed by nuclear translocation of some p53 and apoptosis.

Mitoxantrone causes crosslinking and DNA strand breaks and interferes with the function of DNA toposiomerase II and with DNA repair. Mitoxantrone kills both proliferating and nonproliferating cells (41, 42). In the current study, mitoxantrone was minimally effective at reversing cytoplasmic sequestration of clam p53 *in vivo* and had no obvious effects *in vitro*. Discrepancies between data acquired *in vivo* and *in vitro* for mammalian cells derived from the same source tissues are common in preclinical pharmacokinetic studies (22).

The most attractive interpretation of our data is that treatment with etoposide and mitoxantrone poisons elevates p53 levels in the cytoplasm of leukemic clam hemocytes, and that *de novo* p53 protein overwhelms mortalin tethering, resulting in nuclear translocation of some p53 followed by cytotoxicity, DNA damage, and apoptosis of leukemic clam hemocytes. Upon entering the nucleus after etoposide or mitoxantrone treatment, *de novo* clam p53 may be phosphorylated and stabilized by ATM that senses DNA damage induced by these drugs (43). Such stabilization would effectively increase the intranuclear concentration of p53 because Mdm2 could not bind p53 at its TAD and p53 would not be subject to Mdm2-induced ubiquination and digestion by 26S proteosomes (44). Mdm2 levels may be low or nonexistent anyway because cytoplasmically sequestered p53 was not originally present in the nucleus to induce transcription of this gene. After treatment with topoisomerase II poisons, other proteins up-regulated by p53-mediated transcription would be active in evaluating and repairing DNA or, failing that, in inducing apoptosis of leukemic clam hemocytes with severely damaged DNA. This condition would simplify the p53-mediated response, leading to the rapid apoptosis of leukemic clam hemocytes that we have observed. We saw limited evidence (15%) for transcription-independent initiation of apoptosis

Table 1. QPCR results (%) for cDNA from leukemic clam hemocytes and leukemic clam hemocytes transfected with the nuclear pore blocker WGA followed by 10 h treatment with 0.075 mmol/L etoposide

	p53 expression	Mortalin LV expression	Mortalin SV expression
LCH	47.0	1,633.6	619.0
LCH + Etopo	602.1	1,837.9	778.1
LCH + WGA + Etopo	893.8		

NOTE: All results were normalized against Ct values from normal clam hemocytes, assuming that Ct values from normal clam hemocytes were the baseline; LCH, leukemic clam hemocytes; Etopo, etoposide; mortalin LV, long variant of mortalin; mortalin SV, short variant of mortalin.

via transport to the mitochondria when cells were treated with WGA followed by etoposide (45). Under these conditions, it is likely that, *de novo* p53 mRNA is retained in the nucleus and that additional p53 protein is not produced (46). Finally, when mortalin is overexpressed and tethers p53 in the cytoplasm of leukemic clam hemocytes, the interaction of p53 with BCL2 or other antiapoptotic molecules at the mitochondria may be restricted or absent, curtailing, or preventing this alternative apoptotic induction pathway (9, 47).

Acknowledgments

Received 3/14/2006; revised 7/17/2007; accepted 9/18/2007.

Grant support: National Cancer Institute grants (CA71008-01 and CA104112-01) and Hatch (353; C. Walker) and a Pfizer SURF award (E. Jerszyk).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Initial studies treating leukemic clam hemocytes with etoposide were accomplished by Ben Low, Jennifer Currier, and Allison Kelleher who were undergraduate or graduate students in the Walker laboratory.

References

1. Clifford B, Beljin M, Stark GR, Taylor WR. G_2 arrest in response to topoisomerase II inhibitors: the role of p53. Cancer Res 2003;63:4074–81.
2. Lutzker SG, Levine AJ. Apoptosis and cancer chemotherapy. Cancer Treat Res 1996;87:345–56.
3. Blagosklonny MV, El-Deiry WS. Acute overexpression of wt p53 facilitates anticancer drug-induced death of cancer and normal cells. Int J Cancer 1998;75:933–40.
4. Karpinich NO, Tafani M, Rothman RJ, Russo MA, Farber JL. The course of etoposide-induced apoptosis from damage to DNA and p53 activation to mitochondrial release of cytochrome c. J Biol Chem 2002;277:16547–52.
5. Moll UM, Wolff S, Speidel D, Deppert W. Transcription-independent pro-apoptotic functions of p53. Curr Opin Cell Biol 2005;17:631–6.
6. Arima Y, Nitta M, Kuninaka S, et al. Transcriptional blockade induces p53-dependent apoptosis associated with translocation of p53 to mitochondria. J Biol Chem 2005;280:19166–76.
7. Kelley ML, Winge P, Heaney JD, et al. Expression of homologues for p53 and p73 in the softshell clam (Mya arenaria), a naturally-occurring model for human cancer. Oncogene 2001;20:748–58.
8. Walker C, Böttger S, Low B. Mortalin-based cytoplasmic sequestration of p53 in a non-mammalian cancer model. Am J Pathol 2006;168:1526–30.
9. Kaul SC, Deocaris CC, Wadhwa R. Three faces of mortalin: A housekeeper, guardian and killer. Exp Gerontol 2007;42:263–74.
10. Moll UM, Riou G, Levine AJ. Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. Proc Natl Acad Sci U S A 1992;89:7262–6.
11. Moll UM, LaQuaglia M, Benard J, Riou G. Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proc Natl Acad Sci U S A 1995;92:4407–11.
12. Nagpal J, Jamoona A, Gulati ND, et al. Revisiting the role of p53 in primary and secondary glioblastomas. Anticancer Res 2006;26:4633–9.
13. Wadhwa R, Taira K, Kaul SC. An Hsp70 family chaperone, mortalin/mthsp70/PBP74/Grp75: what, when, and where? Cell Stress Chaperones 2002;7:309–16.
14. Kaul SC, Duncan E, Sugihara T, Reddel RR, Mitsui Y, Wadhwa R. Structurally and functionally distinct mouse hsp70 family members Mot-1 and Mot-2 proteins are encoded by two alleles. DNA Res 2000;7:229–31.
15. Nikolaev AY, Li M, Puskas N, Qin J, Gu W. Parc: a cytoplasmic anchor for p53. Cell 2003;112:29–40.
16. Kuperwasser C, Pinkas J, Hurlbut GD, Naber SP, Jerry DJ. Cytoplasmic sequestration and functional repression of p53 in the mammary epithelium is reversed by hormonal treatment. Cancer Res 2000;60:2723–9.
17. Kim IS, Kim DH, Han SM, et al. Truncated form of importin α identified in breast cancer cell inhibits nuclear import of p53. J Biol Chem 2000;275:23139–45.
18. Rodriguez-Lopez AM, Xenaki D, Eden TO, Hickman JA, Chresta CM. MDM2 mediated nuclear exclusion of p53 attenuates etoposide-induced apoptosis in neuroblastoma cells. Mol Pharmacol 2001;59:135–43.
19. Zhao LY, Liao D. Sequestration of p53 in the cytoplasm by adenovirus type 12 E1B 55-kilodalton oncoprotein is required for inhibition of p53-mediated apoptosis. J Virol 2003;77:13171–81.
20. Wadhwa R, Takano S, Robert M, et al. Inactivation of tumor suppressor p53 by mot-2, a hsp70 family member. J Biol Chem 1998;273:29586–91.
21. Dundas SR, Lawrie LC, Rooney PH, Murray GI. Mortalin is over-expressed by colorectal adenocarcinomas and correlates with poor survival. J Pathol 2005;205:74–81.
22. O'Connor PM, Jackman J, Bae I, et al. Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer Res 1997;57:4285–300.
23. Martins CP, Brown-Swigart L, Evan GI. Modeling the therapeutic efficacy of p53 restoration in tumors. Cell 2006;127:1323–34.
24. Sible J, Marsh AG, Walker CW. Effect of extrinsic polyamines on post-spawning testes of the sea star, (*Asterias vulgaris*) (Echinodermata): implications for seasonal regulation of spermatogenesis. Invertebr Reprod Dev 1992;19:257–65.
25. Geyer RK, Yu ZK, Maki CG. The MDM2 RING-finger domain is required to promote p53 nuclear export. Nat Cell Biol 2000;2:569–73.
26. Fuster JJ, Sanz-Gonzalez SM, Moll UM, Andres V. Classic and novel roles of p53: prospects for anticancer therapy. Trends Mol Med 2007;13:192–9.
27. Marx J. Oncology. Recruiting the cell's own guardian for cancer therapy. Science 2007;315:1211–3.
28. Yu Q. Restoring p53-mediated apoptosis in cancer cells: new opportunities for cancer therapy. Drug Resist Updat 2006;9:19–25.
29. Tovar C, Rosinski J, Filipovic Z, et al. Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. Proc Natl Acad Sci U S A 2006;103:1888–93.
30. Vousden KH, Lane DP. p53 in health and disease. Nat Rev 2007;8:275–83.
31. Buolamwini JK, Addo J, Kamath S, Patil S, Mason D, Ores M. Small molecule antagonists of the MDM2 oncoprotein as anticancer agents. Curr Cancer Drug Targets 2005;5:57–68.
32. Chene P. Inhibition of the p53-2 interaction with low molecular weight compounds. Cell Cycle 2004;3:460–1.
33. Mistry AR, Felix CA, Whitmarsh RJ, et al. DNA topoisomerase II in therapy-related acute promyelocytic leukemia. N Engl J Med 2005;352:1529–38.
34. Appella E, Anderson CW. Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem 2001;268:2764–72.
35. Bossi G, Sacchi A. Restoration of wild-type p53 function in human cancer: relevance for tumor therapy. Head Neck 2007;29:272–84.
36. Wadhwa R, Sugihara T, Yoshida A, et al. Selective toxicity of MKT-077 to cancer cells is mediated by its binding to the hsp70 family protein mot-2 and reactivation of p53 function. Cancer Res 2000;60:6818–21.
37. Wadhwa R, Yaguchi T, Hasan MK, Mitsui Y, Reddel RR, Kaul SC. Hsp70 family member, mot-2/mthsp70/GRP75, binds to the cytoplasmic sequestration domain of the p53 protein. Exp Cell Res 2002;274:246–53.
38. Felix CA. Secondary leukemias induced by topoisomerase-targeted drugs. Biochim Biophys Acta 1998;1400:233–55.
39. Tishler RB, Calderwood SK, Coleman CN, Price BD. Increases in sequence specific DNA binding by p53 following treatment with chemotherapeutic and DNA damaging agents. Cancer Res 1993;53:2212–6.
40. Knippschild U, Milne DM, Campbell LE, et al. p53 is phosphorylated *in vitro* and *in vivo* by the δ and ε isoforms of casein kinase 1 and enhances the level of casein kinase 1 δ in response to topoisomerase-directed drugs. Oncogene 1997;15:1727–36.
41. Fox EJ. Mechanism of action of mitoxantrone. Neurology 2004;63:15–8.
42. De Isabella P, Palumbo M, Sissi C, et al. Topoisomerase II DNA cleavage stimulation, DNA binding activity, cytotoxicity, and physico-chemical properties of 2-aza- and 2-aza-oxide-anthracenedione derivatives. Mol Pharmacol 1995;48:30–8.
43. Xie G, Habbersett RC, Jia Y, et al. Requirements for p53 and the ATM gene product in the regulation of G_1/S and S phase checkpoints. Oncogene 1998;16:721–36.
44. Zaika A, Marchenko N, Moll UM. Cytoplasmically "sequestered" wild type p53 protein is resistant to Mdm2-mediated degradation. J Biol Chem 1999;274:27474–80.
45. Zhao Y, Chaiswing L, Velez JM, et al. p53 translocation to mitochondria precedes its nuclear translocation and targets mitochondrial oxidative defense protein-manganese superoxide dismutase. Cancer Res 2005;65:3745–50.
46. Kehlenbach RH. *In vitro* analysis of nuclear mRNA export using molecular beacons for target detection. Nucleic Acids Res 2003;31:e64.
47. Wadhwa R, Taira K, Kaul SC. Can mortalin be a candidate target for cancer therapy? Cancer Ther 2003;1:173–8.
48. Geyer RK, Nagasawa H, Little JB, Maki CG. Role and regulation of p53 during an ultraviolet radiation-induced G_1 cell cycle arrest. Cell Growth Differ 2000;11:149–56.

Genotoxic Stress–Induced Expression of p53 and Apoptosis in Leukemic Clam Hemocytes with Cytoplasmically Sequestered p53

Stefanie Böttger, Emily Jerszyk, Ben Low, et al.

Cancer Res 2008;68:777-782.