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Induction of the WAF1/CIP1 protein and apoptosis in human T-cell leukemia virus type I–transformed lymphocytes after treatment with Adriamycin by using a p53-independent pathway

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ABSTRACT **The WAF1/CIP1 protein has been identified as a downstream mediator of the tumor suppressor p53 in regulating cell cycle progression through a G_1-phase checkpoint. Recent work has implicated the functional status of p53 as a critical determinant in the apoptotic response of certain cell lines to DNA damaging agents. By using human T-cell leukemia virus type I-transformed lymphoid cell lines that differ in their level and function of wild-type p53, we investigated the induction of WAF1/CIP1 and apoptosis after exposure to Adriamycin, a genotoxic agent. We found that regardless of the p53 status in these cell lines, WAF1/CIP1 RNA was rapidly induced in response to Adriamycin treatment. An elevated level of WAF1/CIP1 protein was observed as well. Additionally, we demonstrated that apoptosis was induced in all cell lines analyzed despite some having functionally inactive p53 protein. Our data suggest that a p53-independent pathway may play a role in the apoptotic response observed in some cell lines after exposure to DNA damaging agents.**

Human T-cell leukemia virus type I (HTLV-I), the etiologic agent of adult T-cell leukemia transforms human T cells both *in vitro* and *in vivo* (1–3). We (24) and others (4–7) have demonstrated elevated steady-state levels in tumor suppressor p53 protein in several HTLV-I-transformed cell lines despite having a wild-type sequence in the p53 coding region usually associated with inactivating mutations. The mechanism responsible for the increased stabilization of p53 in these cells is presently unknown. Recently, our group has shown that levels of p53 protein were consistently higher in interleukin 2 (IL-2)-independent HTLV-I-transformed cell lines compared with IL-2-dependent ones and that the observed increase in p53 protein level correlated with its functional inactivation (24).

The gene for the WAF1/CIP1 protein has been identified as a downstream target of p53 in regulating cell cycle progression through a G_1-phase checkpoint (8, 9). Additionally, the presence of a functional p53 protein has been implicated as a critical determinant in the apoptotic response of certain cell lines to DNA damaging agents (10, 11). We wished to examine whether differences in the p53 status of HTLV-I-transformed lymphocytes, the *in vitro* correlate of the malignant cell of adult T-cell leukemia would translate into different responses to treatment with the genotoxic agent Adriamycin. By using several HTLV-I-transformed cell lines that differed in their IL-2 requirement and functional status of p53, we studied the induction of WAF1/CIP1 at the RNA and protein level after exposure to Adriamycin. We also measured cytotoxicity and apoptosis by using an MTT assay and DNA fragmentation assays. Our findings indicate that Adriamycin can induce WAF1/CIP1 and apoptosis in a p53-independent manner.

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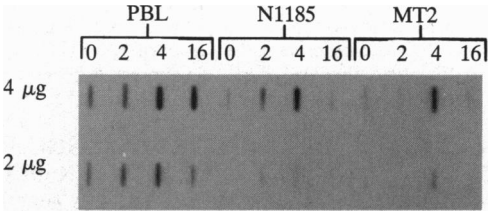

FIG. 1. p53-dependent and -independent induction of WAF1/CIP1 after exposure (0, 2, 4, and 16 h) to 1 μM Adriamycin. Peak levels of WAF1/CIP1 expression were 4 h after treatment as determined by slot-blot analysis at the indicated times. Equal amounts of RNA were loaded in each slot, as determined by hybridizing the same blot with a G3PDH probe (data not shown).

MATERIALS AND METHODS

Cell Culture and Drug Treatment. Peripheral blood lymphocytes (PBLs) were isolated from healthy donors as described (12). PBLs and the IL-2-dependent cell lines N1185 and N1186 (13) were grown in RPMI 1640 medium containing 20% (vol/vol) fetal calf serum, penicillin (50 μg/ml), streptomycin (50 μg/ml), neomycin (100 μg/ml), 2 mM L-glutamine, 10% (wt/vol) phytohemagglutinin, and recombinant IL-2 (40 units/ml). IL-2-independent cell lines C10MJ, MT2, HUT102, and DA202 (Advanced Bio-technologies) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50 μg/ml), streptomycin (50 μg/ml), neomycin (100 μg/ml), and 2 mM L-glutamine.

Cells (5–10 × 10^6 cells) were treated with Adriamycin at concentrations ranging from 0.1 μM to 10.0 μM. Exposure duration varied from 0 to 48 h.

Cytotoxicity. Cell death was assessed by the MTT assay carried out in 96-well microtiter plates as described by Cole (14). Briefly, cells were plated in quadruplicate wells with Adriamycin at concentrations of 0.1 μM–10.0 μM for 48 h. At 4 h prior to the end of drug exposure, MTT was added to each well for a final concentration of 0.5 μg/μl. Then at the end of drug exposure, the enzyme reaction was terminated with 100 μl of 1 M HCl/2-propanol, 1:24 (vol/vol), followed by thorough mixing. The plates were read at 550 nm on a Bio-Whittaker Microplate Reader 2001. Controls included cells with no drugs and medium plus drug but no cells. The assay was repeated in three experiments, with the standard deviation determined by standard methodology.

RNA Isolation and Slot Blot Analysis. Total cellular RNA was isolated from 5 × 10^6 cells by using RNA Stat-60 according to the manufacture's instructions (Tel Test, Friendswood, TX). A human WAF1/CIP1 DNA probe was amplified from human genomic DNA by using primers specific for exon 2 (sense,

Abbreviations: HTLV-I, human T-cell leukemia virus type I; IL-2, interleukin 2; PBL, peripheral blood lymphocyte; QIC, quantitative immunocytochemical.

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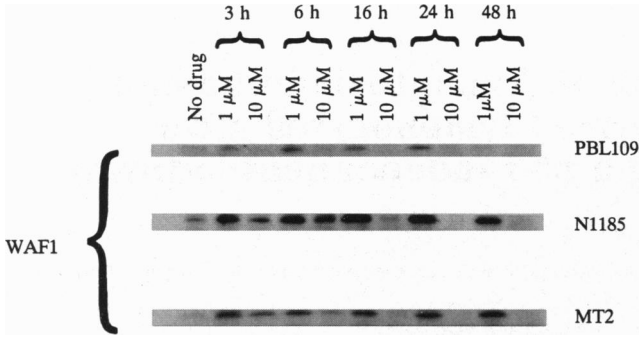

FIG. 2. Protein levels of WAF1/CIP1 after exposure to 1–10 μM Adriamycin. As early as 3 h after treatment, WAF1/CIP1 protein levels increased relative to the low basal levels observed. Adriamycin at 1 μM was effective in increasing the level of WAF1/CIP1 protein. At 10 μM Adriamycin, the level of protein expressed decreased due to the increasing cytotoxicity at this dose.

5′-CGGGATCCGGCGCCATGTCAGAACCGGC-3′; antisense, 5′-CGGAATTCCATGCTGGTCTGCCGCCGTTTTCG-3′). A control cDNA probe G3PDH was generated by using primers obtained from Clontech. The automated temperature cycling program was performed as below: Step-cycle file for 30 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min; time-delay file, 72°C for 7 min; soak cycle, 4°C. Slot-blot hybridization was carried out with three dilutions 1:2 (4 μg to 1 μg). Prehybridization, hybridization, and washing conditions were as described (15).

Protein Extraction and Western Blot Analysis. Treated cells (5–10 × 10^6 cells) were washed twice with PBS. Cell pellet was lysed with lysate buffer (10 mM Tris·HCl, pH 7.4/150 mM NaCl/1 mM EDTA/0.1% SDS/150 mM Phenylmethylsulfonyl fluoride). Total protein concentration in each sample was determined by using a micro BCA method (Pierce) according to the suppliers instructions. Protein at 25 μg per well was fractionated by electrophoresis in a Tris/glycine PAGE gel (Novex, San Diego) under denaturing reducing conditions. Transfer to supported nitrocellulose filter was carried out by using a Millipore electroblotting apparatus (Millipore). The filters were incubated first with WAF1 (Ab-1), a monoclonal antibody to WAF1/CIP1 (Oncogene Science), and then a secondary antibody (Goat anti-mouse IgG) conjugated with horseradish peroxidase was added. Chemiluminescence was performed with ECL (Amersham) according to the manufacturer's instruction. The filters were then exposed to x-ray film and the autoradiographs were analyzed by laser densitometry using a personal densitometer (Molecular Dynamics).

DNA Fragmentation Assays. Apoptosis was characterized by DNA fragmentation analyzed either by agarose gel electrophoresis of low molecular weight DNA (16) or immunoperoxidase detection of digoxigenin-labeled genomic DNA (17). Briefly, untreated or treated cells (24–48 h) were fixed in suspension with 70% ethanol for the agarose gel electrophoresis assay. The cells were then centrifuged at 800 × *g* for 5 min and the ethanol was thoroughly removed. The cell pellets (1–2 × 10^6 cells) were resuspended in 40 μl of phosphate/citrate (PC) buffer (192 parts of 0.2 M Na_2HPO_4/8 parts of 0.1 M citric acid, pH 7.8) at room temperature for 30 min. After centrifugation at 1000 × *g* for 5 min, the supernatant was transferred to new tubes and concentrated by vacuum. Three microliters of 0.25% Nonidet P-40 (Sigma) was then added, followed by 3 μl of RNase A (Sigma; 1 mg/ml). After a 30-min incubation at 37°C, 3 μl of proteinase K (1 mg/ml; Boehringer Manheim), was added and incubated for an additional 30 min at 37°C. Finally, 12 μl of loading buffer [0.25% bromophenol blue/0.25% xylene cyanol FF/30% (vol/vol) glycerol] was added and the mixture was analyzed on a 0.8% agarose gel at 2 V/cm for 16 h. After staining with ethidium bromide (5 μg/ml), the DNA in the gels was visualized under UV light and photographed. Measurement of DNA fragmentation by the immunoperoxidase assay was carried out using the Apop Tag detection kit (Oncor) according to the suppliers instructions with slight modification as described below. Cell suspension was fixed in 10% (vol/vol) formalin for 5 min then the slide preparation was counterstained with methyl green at pH 5.5 for 10 min. This was washed in three changes of distilled water, followed by three changes of absolute alcohol.

Apoptosis was relatively graded utilizing the quantitative immunocytochemical (QIC) score obtained from the CAS-200 Image Analysis System (Cell Analysis Systems, Elmhurst, IL). The zero point was established utilizing a sham preparation in which IL-2-deprived PBL-109 lymphocytes were stained for apoptosis in the absence of terminal deoxynucleotidyltransferase. All cell preparations were then microscopically examined at 400× magnification in real time and subject to random field image analysis of five microscopic fields for each preparation. This allowed between 500 and 1000 cells to be examined for each cell preparation. The QIC score was determined by taking the percentage of the summed total optical density for the positive apoptotic nuclear area divided by the summed total optical density of all the nuclei and multiplying the resulting apoptotic density expressed as a percentage times the percentage of positively stained nuclear area. The product was then divided by 10. Since percentages were utilized, the absolute numbers of cells examined were irrelevant to the final QIC score.

RESULTS

WAF1/CIP1 Expressions in HTLV-I-Transformed Lymphocytes. HTLV-I-transformed lymphocytes have been reported to have elevated levels of p53 protein when compared to normal PBLs (refs. 4–7, 24). The growth factor requirement of various HTLV-I-transformed lymphocytes differs with some requiring IL-2 supplementation whereas others may not. We have recently demonstrated that IL-2-independent cell lines have functionally inactive p53 protein associated with elevated steady-state levels (24). By using these phenotypically distinct cell lines, we investigated the ability of Adriamycin to induce WAFI/CIPI expression in cell lines deficient in functional p53 protein.

After exposure to Adriamycin, total RNA was extracted from IL-2-dependent and IL-2-independent cell lines at various times and analyzed for WAF1/CIP1 expression by slot blot and Northern blot analysis. As demonstrated in Fig. 1, the level of message peaked at 4 h with comparable levels in N1185 and MT2 cells. Similar results were observed in other IL-2-dependent and IL-2-independent cell lines examined (data not shown).

A parallel set of experiments was carried out to assess the level of WAF1/CIP1 protein induced by Adriamycin treat-

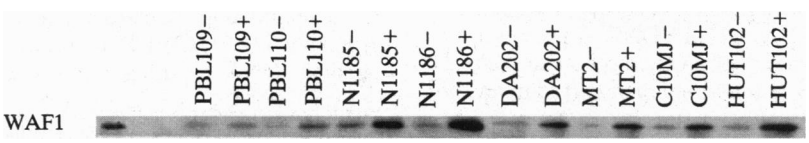

FIG. 3. Survey of IL-2-dependent and IL-2-independent cell lines revealing induction of WAF1/CIP1 protein after exposure to 1 μM Adriamycin for 48 h.

Table 1. Cytotoxicity assay

Cell line	IL-2 requirement	Adriamycin 0.1 μM	Adriamycin 1.0 μM	Adriamycin 10.0 μM
PBL109	+	65.4 ± 7.4	13.6 ± 3.4	6.6 ± 1.8
PBL110	+	72.1 ± 32.9	26.3 ± 13.7	13.2 ± 6.7
N1185	+	86.1 ± 8.1	41.9 ± 24.7	14.0 ± 4.8
N1186	+	68.1 ± 14.6	21.3 ± 3.5	9.3 ± 2.5
DA202	−	72.7 ± 3.1	40.9 ± 4.08	9.5 ± 0.9
MT2	−	63.7 ± 6.6	41.3 ± 9.6	13.8 ± 1.9
C10MJ	−	67.9 ± 7.9	8.3 ± 0.4	6.0 ± 0.3
HUT102	−	56.7 ± 8.8	13.0 ± 0.9	7.9 ± 3.6

As demonstrated in the MTT assay and expressed as percent viable cells, chemosensitivity to Adriamycin across three orders of magnitude of drug concentration did not correlate with the p53 status of the respective cell lines. Normal donor lymphocytes (PBL109 and PBL110) served as controls.

ment. Total cellular protein was prepared and analyzed by Western blot revealing low basal levels of WAF1/CIP1 protein but increasing as early as 3 h after drug exposure (Fig. 2). These results were obtained in both IL-2-dependent and IL-2-independent cell lines (Fig. 3). Interestingly, both IL-2-dependent cell lines demonstrated a greater relative increase in WAF1/CIP1 expression after drug treatment than the IL-2-independent cell lines. These findings support the presence of a p53-independent pathway involved in the induction of WAF1/CIP1 after Adriamycin treatment of HTLV-I-transformed lymphocytes.

Cytotoxicity and Apoptosis. Previous work has suggested that the presence of functional p53 is a strong determinant of response to chemotherapy in transformed cell lines (10, 11). We wished to examine whether this was a universal prerequisite; therefore, by using HTLV-I-transformed lymphocytes differing in their functional p53 status, we examined the cytotoxic effect of Adriamycin. We analyzed the chemosensitivity of these cell lines by using the MTT assay with normal donor lymphocytes serving as controls. Since prior studies reported that higher doses of DNA damaging agents could kill chemoresistant cell lines despite having inactive p53 (18–21), we carried out our studies across three orders of magnitude of drug concentration. As displayed in Table 1, there is no correlation with cell viability and p53 status.

An earlier report (11) demonstrated that the induction of wild-type p53 and WAFI/CIPI correlated with G_1 arrest and susceptibility of cells to apoptosis after exposure to ionizing radiation and etoposide. We observed the induction of WAF1/CIP1 in cells with functionally inactive p53 after exposure to the genotoxic agent Adriamycin. This finding prompted us to examine whether apoptosis would occur in these cells by a p53-independent pathway. By using an immunohistochemistry assay that labels 3′-OH DNA ends generated by DNA fragmentation and an agarose DNA ladder assay, we measured the induction of apoptosis in cell lines with functional and inactive p53 proteins.

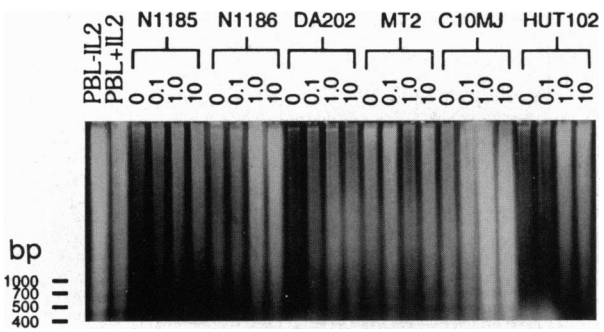

FIG. 4. DNA oligomer formation observed on a 0.8% agarose gel. The cell lines were treated for 24 h with 0.1 μM–10 μM Adriamycin and then low molecular weight DNA was isolated for agarose gel electrophoresis. Note the dose–response increase in DNA ladder formation. PBL with (+) or without (−) IL-2 was used as a positive control for DNA ladder formation.

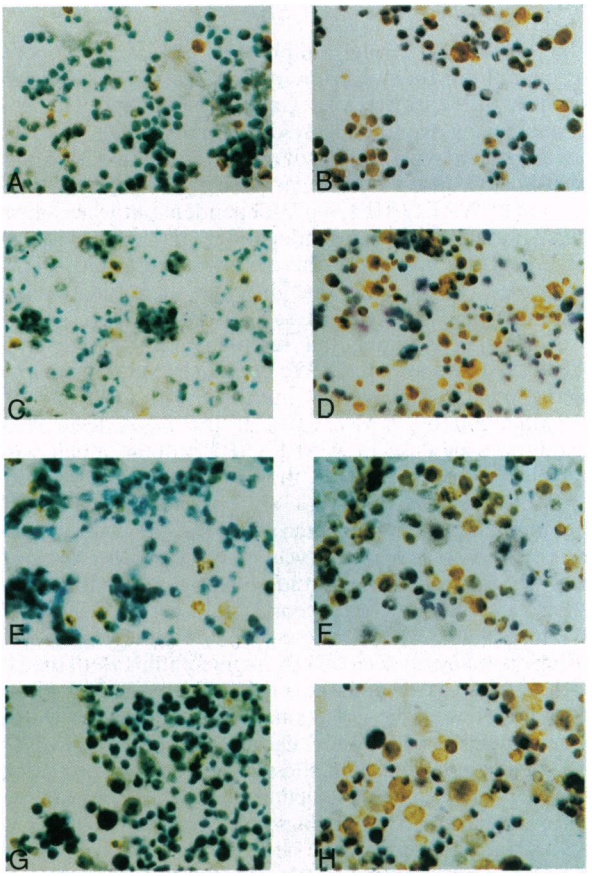

FIG. 5. Immunoperoxidase detection of digoxigenin-labeled genomic DNA staining positive for the apoptotic nuclear area. (×400.) Untreated cell lines showing only occasional positively staining nuclei: *A*, N1185; *C*, N1186; *E*, MT2; *G*, Hut102. Cell lines treated with 1 μM Adriamycin demonstrating significant numbers of positively staining nuclei: *B*, N1185; *D*, N1186; *F*, MT2; *H*, Hut102. (See Table 2 for quantitation.)

As depicted in Fig. 4, there is a strong dose–response relationship in the formation of DNA oligomers consistent with internucleosomal cleavage. While we did observe some background levels of DNA ladder formation in untreated cells, this was far less than that observed in treated cell lines. We used an additional assay to validate our findings of DNA fragmentation considered to be a hallmark of apoptosis. Fig. 5 and Table 2 show a marked increase in DNA fragments in cell nuclei. Both of the above assays independently confirm the presence of apoptosis in Adriamycin-treated cells.

Table 2. Apoptotic score

Cell line	Apoptotic score (QIC) No drug	Apoptotic score (QIC) Adriamycin (1.0 μM)	Fold increase after drug treatment
N1185	143	687	4.8
N1186	45	531	11.8
MT2	20	433	21.6
HUT102	84	589	7.0

By utilizing the QIC score, we were able to relatively grade the induction of apoptosis in cell lines after a 24-h exposure to 1 μM Adriamycin. A minimum of 500 cells were evaluated per treatment group (see text for details).

DISCUSSION

Earlier work has strongly implicated p53 as an important determinant of chemosensitivity (10, 11). A recent report examining the sensitivity of several human lymphoma cells to DNA damaging agents suggested that p53 gene mutations are often associated with evasion of apoptotic death (11). Michieli *et al.* (21) have described the existence of two pathways for the induction of WAF1/CIP1, a p53-dependent pathway activated by DNA damage and a p53-independent pathway activated by mitogens at the entry into the cell cycle. By using HTLV-I-transformed lymphocytes that differ in their functional p53 status we studied the ability of Adriamycin, a major component of several active lymphoma regimens (22, 23) to induce WAF1/CIP1 and apoptosis. We demonstrated that treatment of active p53 containing cells and those with inactive protein have induction of WAF1/CIP1 at the RNA level with a concomitant increase in WAF1/CIP1 protein, albeit with a greater increase observed in those cell lines with functional p53. As has been described (8, 11, 21), basal levels of both RNA and protein are low in untreated cells. The chemosensitivity of these cell lines to Adriamycin was shown to be dose responsive across three orders of magnitude of drug concentration with chemosensitivity showing no correlation with p53 status.

We investigated whether these cell lines underwent apoptosis by using two assays to detect DNA fragmentation. Both the DNA ladder agarose assay and the immunohistochemistry method consistently showed the induction of apoptosis in cells with and without functional p53 protein. Our data are consistent with p53 not being the only determinant essential for the upregulation of the WAF1/CIP1 gene or induction of apoptosis in response to genotoxic agents. These findings would appear to differ with previous studies describing the necessity for functional p53 protein to effect an apoptotic death upon exposure to DNA damaging agents. However, there are two prior observations that support our findings. In one study (11), a lymphoblastoid cell line, Ramos, containing only mutant p53 protein displayed chemosensitivity to cisplatinum and nitrogen mustard as well as DNA fragmentation comparable to cell lines with wild-type p53 protein. Another group reported (21) that at higher doses Adriamycin induced WAF1/CIP1 expression in embryonic fibroblasts derived from p53 knock-out mice. They suggested that Adriamycin might act through both a p53-dependent and -independent pathway albeit at different drug concentrations. Our work confirms and extends these observations with the documentation of WAF1/CIP1 induction and apoptosis occurring in response to Adriamycin exposure of cell lines deficient in functional p53. We did observe greater increases in WAF1/CIP1 induction in those cell lines with active p53, suggesting that both mechanisms may be working concurrently. While WAF1/CIP1 functions to arrest cells in G_1 by associating with cyclin–Cdk complexes, the determinant(s) critical for undergoing apoptosis are not clear at present. Further studies in cell lines that have WAF1/CIP1 induction in a p53 independent manner may identify targets for rational drug development.

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