Human MDR 1 Protein Overexpression Delays the Apoptotic Cascade in Chinese Hamster Ovary Fibroblasts†

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ABSTRACT: Several laboratories have reported that overexpression of the multidrug resistance (MDR) protein is associated with intracellular alkalinization, and several investigators have reported that cells induced to undergo programmed cell death (apoptosis) acidify quite significantly. Because it is difficult to fully explain the resistance to apoptosis-inducing chemotherapeutic drugs that is exhibited by MDR tumor cells solely via altered drug transport alone [Hoffman *et al.* (1996) *J. Gen. Physiol. 108*, 295−313], we have investigated whether overexpression of the hu MDR 1 protein alters progression of the apoptotic cascade. LR73 fibroblasts induced to undergo apoptosis either via treatment with the chemotherapeutic drug colchicine or by serum withdrawal exhibit cellular volume changes, intracellular acidification, nuclear condensation, and chromosomal digestion ("ladder formation"), characteristic of apoptosis, in a temporally well-defined pattern. However, multidrug resistant LR73/20E or LR73/27 hu MDR 1 transfectants recently created in our laboratory without selection on chemotherapeutic drug are significantly delayed in the onset of apoptosis as defined by the above criteria, regardless of whether apoptosis is induced by colchicine treatment or by serum withdrawal. Thus, the delay cannot simply be due to the well-known ability of MDR protein overexpression to lower chemotherapeutic drug accumulation in MDR cells. LR73/27V500 "selectants", exhibiting similar levels of MDR protein overexpression but higher multidrug resistance due to selection with the chemotherapeutic drug vincristine, exhibit a slightly longer delay in the progression of apoptosis. Normal apoptotic cascade kinetics are partially restored by pre-treatment of the MDR cells with the MDR protein inhibitor verapamil. Untransfected LR73 cells not expressing MDR protein but elevated in pH_i via manipulation of CO_2/HCO_3^- as described [Hoffman *et al.* (1996) *J. Gen. Physiol. 108*, 295−313] are inhibited in DNA ladder formation, similar to LR73/hu MDR 1 transfectants. These results uncover an additional mechanism whereby MDR protein overexpression may promote the survival of tumor cells and further support the notion that in some systems intracellular acidification may be either causal or permissive for proper progression of the apoptotic cascade.

In recent years, molecular-level understanding of the regulation of programmed cell death (apoptosis) has expanded considerably. Nonetheless, it is still not understood how diverse extracellular signals, ranging from withdrawal of growth factors to administration of chemotherapeutic drugs, hormones, or elevated Ca^{2+}, lead to a similar sequence of well defined morphologic and biochemical changes that define apoptosis (Wyllie *et al.*, 1980; Steller, 1995). These changes appear to occur in a distinct sequence and include (in order) changes in cell volume, changes in cell pH_i,[1] nuclear condensation, activation of proteases, activation of endonucleases, membrane deformation, and vesicle-mediated exocytosis of digested cellular components. A central goal in apoptosis research is identifying the signal transduction pathway(s) that regulate these well-defined morphologic and biochemical changes but that are triggered by such a diverse collection of stimuli. Treatments or factors that alter the kinetics of the apoptotic cascade thus offer important clues about this signal transduction.

Since profound changes in cellular volume, pH_i, and Ca^{2+} appear to occur early on in apoptosis, one popular hypothesis is that changes in intracellular ionic equilibrium may be involved in some fashion in regulating apoptosis (Barry & Eastman, 1993; Barry *et al.*, 1993; Li & Eastman, 1995;

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[1] Abbreviations: pH_i, intracellular pH; MDR, multidrug resistance; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; G418, geneticin; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; EtOH, ethanol; EDTA, ethylenediaminetetraacetic acid; PMT, photon multiplier tube; HBSS, Hank's balanced salt solution; bp, base pairs; IL-2, interleukin 2; ICE, interleukin-converting enzyme; TUNEL, terminal dUDP nick end-labeling.

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Huang *et al.*, 1995; Pérez-Sala *et al.*, 1995; Gottlieb *et al.*, 1996; Gottlieb, 1996). This idea has added appeal in that many apoptosis-inducing treatments have important effects on the plasma membrane, within which key regulators of intracellular ion homeostasis function. These channels, pumps, exchangers, and co-transporters are capable of changing intracellular ionic composition quickly, without synthesis of new proteins. This is important since apoptosis can sometimes occur in the presence of protein synthesis inhibitors [e.g. Borner *et al.* (1995)].

Two other topics in modern biomedical research that might initially appear unrelated to the biochemistry of apoptosis are (1) the mechanism(s) whereby overexpression of MDR proteins confer cellular resistance to chemotherapeutic drugs and other toxic compounds and (2) our evolving appreciation that the efficacy of some chemotherapeutic drugs or drug treatments is not simply a consquence of their direct cytotoxic effects but is also linked to the facility with which the drugs induce apoptosis. That is, a paradigm of cancer pharmacology has been that chemotherapeutic drugs kill tumor cells via the direct damage they cause to nucleic acid, tubulin, and other targets. This paradigm has been modified in recent years to accommodate the fact that chemotherapeutic drugs are also very potent inducers of the apoptotic cascade [e.g. Eastman (1990), Lowe *et al.* (1993), Ling *et al.* (1993), and Sumantran *et al.* (1995)]. It is therefore currently argued that some well-understood actions of certain chemotherapeutic drugs may not completely define their cytotoxic potency.

Following this reasoning, we and others have hypothesized that perhaps some mechanisms that confer resistance to chemotherapeutic drugs might do so by circumventing their apoptosis-inducing ability and not necessarily by altering their access to or interaction with the principle (traditionally defined) intracellular target. If true, this would have important consequences for the further evolution of chemotherapy. One well-documented mechanism of resistance to chemotherapeutic drugs that is not yet completely understood (Roepe, 1995; Roepe *et al.*, 1996; Wadkins & Roepe, 1997) is the overexpression of the MDR 1 protein (Gottesman & Pastan, 1993; Endicott & Ling, 1989). Overexpression of MDR 1 protein is frequently (but not always) observed in drug-selected tumor cells, but overexpression can be unstable (Hoffman *et al.*, 1996). A recent study (Ling *et al.*, 1993) reported that upon treatment with doxorubicin, P388/Dox cells (a drug resistant P388 murine leukemia cell line selected with the chemotherapeutic drug doxorubicin) exhibited decreased DNA ladder formation, which is characteristic of apoptosis. However, it is not clear from this study whether resistance to ladder formation was a trivial consequence of the resistance to doxorubicin that was selected for in the P388/Dox cell line or to some inherent alteration in apoptosis. Also, because P388/Dox cells were selected with doxorubicin and exhibited very high levels of drug resistance that are not conferred by MDR protein overexpression alone (Hoffman *et al.*, 1996; Roepe *et al.*, 1996), it is not clear if MDR protein overexpression in these cells *per se* played any role in the phenomenon. Furthermore, although the presence of DNA ladder formation is frequently diagnostic of late-stage apoptosis, its absence does not necessarily indicate that apoptosis has been halted completely (Oberhammer *et al.*, 1993). It is thus worthwhile to further examine any possible relationship between MDR protein overexpression and the progression of apoptosis.

We and others have documented rather significant changes in steady state pH_i and pH_i regulation in drug-selected MDR cell lines, as well as "pure" MDR transfectants never exposed to chemotherapeutic drugs (Hoffman *et al.*, 1996; Roepe *et al.*, 1996; Hoffman & Roepe, 1997). In general, overexpression of the MDR protein appears to elevate pH_i and alter the efficiency of Cl^-/HCO_3^- exchange (Luz *et al.*, 1994; Hoffman & Roepe, 1997). Others [e.g., Barry and Eastman (1993), Li and Eastman (1995), Huang *et al.* (1995), and Gottlieb *et al.* (1996)] have recently shown that rather significant intracellular acidification appears to accompany apoptosis. The molecular-level mechanism of this change in pH_i is not yet known. It is unclear whether the acidification is causal, permissive, or incidental with regard to progression of the apoptotic cascade in many cell types, although some important data bearing on these issues have recently been reported (Li & Eastman, 1995; Pérez-Sala *et al.*, 1995; Gottlieb *et al.*, 1996). We wondered whether intracellular alkalinization induced by MDR protein overexpression might influence the acidification that occurs during apoptosis, and whether this might alter other events that characterize the apoptotic cascade. The answers to these questions could drastically modify our understanding of how MDR protein overexpression confers resistance to chemotherapeutic drugs and other compounds with potent apoptosis-inducing ability.

Thus, we have measured pH_i changes and other biochemical events in LR73 cells, LR73/hu MDR 1 transfectants, and drug-selected LR73/hu MDR 1 transfectant derivatives induced to undergo apoptosis via either colchicine treatment or serum withdrawal. We compared chemotherapeutic drug-based and serum withdrawal-based methods for induction of apoptosis in order to test whether any alterations in the apoptotic cascade were simply a trivial consequence of reduced intracellular drug accumulation conferred by MDR protein overexpression or whether they illustrate perturbations in underlying signal transduction that is relevant for normal progression of the apoptotic cascade. We examined recently created "true" hu MDR 1 transfectants that have not ever been exposed to chemotherapeutic drugs in order to distinguish between effects of MDR 1 protein overexpression versus the plethora of other drug-selection effects present in most other model MDR cell lines. The results have interesting implications for our understanding of chemotherapeutic drug resistance as well as of the biochemistry of the apoptotic cascade.

MATERIALS AND METHODS

Materials. 2′,7′-Bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) and nigericin were purchased from Molecular Probes (Eugene, OR) and used without further purification. Colchicine and G418 in powder form were from Sigma. The integrity of commercial 5% CO_2 (balance air) mixtures used in this work was checked using standard HCO_3^- solutions and application of the Henderson−Hasselbalch relation. DME media was from the Memorial Sloan−Kettering Cancer Center media laboratory. All other chemicals were reagent grade or better, purchased from commercial sources, and used without further purification or analysis.

Tissue Culture. Construction of the LR73-derived hu MDR 1 transfectant cell lines used in this work has been described (Hoffman *et al.*, 1996). In brief, a vector harboring hu MDR 1 cDNA under control of the CMV promoter was

co-transfected along with pSVDNAneo (Invitrogen), and clones selected with G418 only were analyzed further. Several isolated clones (including no. 27 used in the present work) were found to express high levels of hu MDR 1 protein (Hoffman *et al.,* 1996) that are comparable to the levels observed for chemotherapeutic drug-selected cells.

To select a LR73/hu MDR 1 transfectant derivative exhibiting even higher levels of drug resistance, a single clone of LR73/27 was grown in the presence of 500 nM vincristine to produce the cell line LR73/27V500. Selection was deemed complete when 27V500 grew in the presence of drug at a similar rate, relative to parental cells grown in the absence of drug. This was essentially the case after five to six passages [see Hoffman *et al.* (1996)].

All cells were grown at 37 °C in a 5% CO_2 atmosphere in DME medium supplemented with 10% fetal calf serum, 100 units of penicillin/mL and 100 μg of streptomycin/mL. The vincristine-selected cells and the hu MDR 1 transfectants were maintained in selective medium (harboring either 500 nM vincristine or 250 μg of active G418/mL, respectively) until the last passage prior to analysis. Stock suspensions of the stable cell lines were frozen in liquid nitrogen and resuscitated as needed.

For single-cell photometry analysis of pH_i, cells were grown as above on glass cover slips (Corning Glassworks, 18 mm^2/0.11 mm thick) that were immobilized in standard tissue culture plates with a dab of autoclaved silicon vacuum grease (Dow-Corning) as described previously (Hoffman *et al.,* 1996; Luz *et al.,* 1994; Wei *et al.,* 1995).

Induction of Apoptosis, Isolation of DNA, and Quantitation of Ladder Patterns. To produce mass populations of cells at different stages of apoptosis, a 100 cm^2 plate of cells grown to confluency as above was harvested by trypsinization and split 1:4. Cells were allowed to attach, grown to 70%−80% confluency, and then induced to undergo apoptosis either via addition of colchicine (from a 100 mM stock solution made in H_2O) or by withdrawal of serum (see Results). To serum-starve the cells, DME containing FCS was removed, the plate was washed with PBS, and 10 mL of serum-free DME was then added to the plate. The plate was then incubated as above for variable time (3 or 6 h, etc.; see Results). When apoptosis was induced by serum withdrawal in the presence of verapamil, 10 μM verapamil was added to the dish of cells under normal culture conditions for 1 h prior to induction and then also included in the serum-free medium during induction.

For analysis of apoptotic cells at the single-cell level (see single-cell photometry below), cells were treated as above but grown on glass cover slips immobilized in the 100 cm^2 dish with a dab of autoclaved vacuum grease.

To isolate DNA from mass populations of the fibroblasts, all cells (including any floating in media) were collected and centrifuged at 2000 rpm at 4 °C for 10 min in a Sorvall RT6000B centrifuge. The pellet was washed with PBS and resuspended in 900 μL of extraction buffer (100 mM NaCl/10 mM Tris-HCl/25 mM EDTA/0.5% SDS, pH 8.0). 100 μL of a 1 mg/mL solution of proteinase K was then added, and the lysate was digested overnight in a 50 °C water bath. After digestion, the mixture was extracted with an equal volume of water-saturated, buffered (pH = 7.80) phenol/$CHCl_3$ (1:1), and nucleic acid precipitated for 2 h at −20 °C by addition of sodium acetate (to 300 mM) and 2 vol of 100% EtOH. The nucleic acid was collected by centrifugation, washed with 70% EtOH, dried in a speed-vac, resuspended in 100 μL of TE (10 mM Tris HCl/1 mM EDTA, pH 8.0) and treated with RNAse (40 μg/mL final concentration) at 37 °C for 1 h. Phenol/$CHCl_3$ extraction, EtOH precipitation, lyophilization, and rehydration were carried out again as above, and DNA was quantified by absorbance spectroscopy with a Beckman DU65 spectrophotometer. To visualize laddered DNA, 10 μg was applied to lanes of a 1.6% agarose gel containing 0.5 μg/mL EtBr, and the samples were electrophoresed for approximately 90 min at 40 mV constant voltage. Gels under UV illumination were photographed with a Polaroid camera, and the images were scanned with an Acturus 600 dpi scanner. To quantify ladder DNA, the negative photographic image was digitized with a Stratascan densitometer interfaced to an AST pc and band intensities were analyzed using Stratascan software (Roepe *et al.,* 1993).

Colony Formation Assays. Cells grown in normal DME media containing 10% FCS were trypsinized, resuspended in normal DME media containing 10% FCS, counted, and plated at a density of 500 cells/well of a standard six-well plate. The cells were allowed to attach for 12 h. Normal DME media was then aspirated, the wells were washed twice with PBS, and DME media lacking serum was added. The cells were deprived of serum for variable time (0, 6, 12, or 24 h, see Results). Serum-free media was then aspirated, normal DME media containing 10% FCS was added back, and colonies were allowed to form for 3 days. Medium was aspirated once again, the wells were washed with PBS, and the plates were stained with methylene blue. Colonies were then counted by eye, and data were expressed as % colonies formed in the absence of serum deprivation (i.e., 0 h minus serum, see Figure 2 in Results). Relative plating efficiency of the different cell lines in the presence of serum was similar. In each assay, each time point was analyzed in triplicate. The results from three such assays (nine determinations in all) were averaged and expressed as the mean ± SD (see Figure 2 in Results).

Histologic Staining for Compacted Nuclei. Cells were grown as above, but on sterile, glass microscope slides fixed to the bottom of a large tissue culture plate with sterile vacuum grease. At the appropriate time after induction of apoptosis (see above), the slides were removed, fixed in 3:1 methanol:glacial acetic acid, and stored overnight in 70% EtOH at 20 °C if they were to be stained with hematoxylin/eosin (see below). If they were to be stained via the TUNEL protocol (see below), they were fixed in 10% formalin for 10 min at room temperature and then washed several times in distilled water.

For hematoxylin/eosin staining, the slides were first coded, rinsed twice in distilled H_2O, and stained with 0.5% hematoxylin (Fisher) for 7 min. They were rinsed with running tap water, dipped 15−20 times in bluing agent (250 mL of distilled water with 10 drops of ammonium hydroxide), and rinsed again with running water and then with 95% EtOH. Cells were then stained with 0.25% eosin (eosin-Y-alcoholic, Shandon) for 30 min and then rinsed once with 95% EtOH, twice with 100% EtOH, and three times with 100% xylene. A cover slip was added using mounting medium (Micromount from Surgipath), and cells were viewed under a microscope. We first counted the total number of cells in a field (>100 in each case) and then counted the number of cells with condensed nuclei (see Results). Three fields were counted on each slide, and the average "% condensed nuclei" was calculated. The identity

of the sample on a given slide was unknown during counting. For each time point after serum starvation, three such experiments using three separately prepared slides were performed and the data averaged (see Results). Data are expressed as the mean ±SD for the nine determinations; differences between the control and MDR cell lines are significant (student's *T*-test, $P < 0.05$).

For TUNEL staining (visualization of DNA nick end-labeling), cells were first rinsed with 10 mM Tris HCl, pH 8.0, for 5 min. They were then treated with 20 μg of proteinase K/mL in 10 mM Tris HCl, pH 8.0, in a moist chamber for 15 min at room temperature. After being washed four times in distilled water (2 min each wash) cells were incubated in 3% H_2O_2/PBS for 5 min, washed three times more in distilled water, and then incubated in preincubation buffer (4 mM Tris base/17.5 mM sodium cacodylate/125 μM $CoCl_2$, pH 7.20) for 15 min in a moist chamber at room temperature. Preincubation buffer was aspirated, and cells were treated with 75 μL of incubation buffer (100 mM sodium cacodylate/2.5 mM $CoCl_2$/0.005% BSA/0.1 mM DTT/0.35 nM biotin-16-dUTP/3.75 units of TDTA, pH 7.0) for 60 min in a moist chamber at 37 °C. The reaction was terminated by treatment with 300 mM NaCl/30 mM sodium citrate for 15 min at room temperature, and the cells were then washed 3× in PBS and blocked with 2% human albumin for 10 min. After 3 more washes with PBS, cells were treated with avidin/biotin−HRP (1.4 μg of avidin/0.3% biotinylated HRP) for 30 min in a moist chamber, washed with PBS again, and stained in 10% diaminobenzidine. They were then counterstained with 0.5% hematoxylin and mounted using Micromount.

Single-Cell Photometry and Measurement of Steady State pH_i. We have constructed a single-cell photometry apparatus by interfacing a Nikon Diaphot epifluorescence microscope and associated optics to a Photon Technologies Inc. Alphascan fluorometer (Hoffman *et al.*, 1996; Wei *et al.*, 1995; Luz *et al.*, 1994). Signals from PMT s connected in T-format to the side port of the microscope were transferred to a Dell 433/L computer and analyzed with PTI software [see Wei *et al.* (1995) for more detail].

Cells were grown on sterile glass cover slips as described above and analyzed after 1.5 but before 4 days after plating, i.e., before confluency but after several cell divisions. Cover slips were incubated with 5 μM BCECF−AM for 30 min before mounting on the microscope stage, and they were then continuously perfused at a constant rate (approximately 4 mL/min) with HBSS buffer (118 mM NaCl/24.2 mM $NaHCO_3$/1.3 mM $CaCl_2$/0.5 mM $MgCl_2$/0.6 mM Na_2HPO_4/0.5 mM KH_2PO_4/10 mM glucose) that had been equilibrated with 5% CO_2 and to 37 °C. We limited exposure to excitation light to the time of data collection in order to limit photobleaching. Uniform BCECF staining was verified [see Hoffman *et al.* (1996), Wei *et al.* (1995), and Luz *et al.* (1994)], and buffers harboring HCO_3^- were continuously purged with 5% CO_2. Buffer pH was monitored with a microelectrode. We also followed previously described procedures (Hoffman *et al.*, 1996; Wei *et al.*, 1995; Luz *et al.*, 1994) to verify that leaking of the esterified BCECF−AM was minimal in the time required to make a measurement, and not any different for the different cell lines. The K^+/nigericin BCECF calibration curves obtained as described (Hoffman *et al.*, 1996; Wei *et al.*, 1995; Luz *et al.*, 1994) for these cell lines verify that, although absolute ratiometric readings differ from cover slip to cover slip, pH_i-dependent behavior of BCECF (shape and slope of the 439/490 nm ratios vs pH_i curve) is nearly identical for the different cell lines [data not shown; see Hoffman *et al.* (1996)].

Steady state pH_i were calculated as described (Hoffman *et al.*, 1996; Wei *et al.*, 1995; Luz *et al.*, 1994), and K^+/nigericin standard curves were generated for each cover slip used to measure steady state pH_i. Steady state pH_i data were obtained for 25−50 cells for a given cover slip perfused with HBSS before perfusing with K^+/nigericin solutions. These steady state data were then plotted as histograms (see Results), and we also averaged these data to obtain a mean pH_i for the cell population. Since induction of apoptosis is not uniform, the calculated mean pH_i underestimates the true extent of cellular acidification that occurs during apoptosis for individual cells.

From the same steady state pH_i data, we were also able to obtain information pertaining to relative cell volume following the 439 nm isosbestic method of Muallem *et al.* (1992). To confirm that the 439 nm data collected in the pH_i experiments reveal significant changes in cell volume, we also perfused these cover slips with HBSS of various tonicity to generate empirical calibration curves [described in Muallem *et al.* (1992)].

RESULTS

DNA Ladder Pattern Kinetics and Colony Formation after Serum Starvation

Apoptosis is defined by changes in cell morphology (see below) as well as activation of protease and endonuclease activities that ultimately lead to a distinct DNA "ladder" pattern in most cases (e.g. Figure 1). Upon withdrawal of serum from the growth medium for LR73 fibroblasts or control LR73/neo-transfectants, laddered DNA is clearly apparent within 12 h (Figure 1A, lane 5) and even faintly visible at 3−6 h (Figure 1A, lanes 3 and 4). Via densitometry, we estimate that about 5% of the DNA isolated from LR73 cells 6 h post serum withdrawal is in ladder form. In early work (D. Lamming and P. D. Roepe, unpublished) we noticed slightly slower kinetics of ladder formation upon treatment of these LR73 cells with 100 nM levels of the chemotherapeutic drug colchicine, with 5−10% DNA observed in ladder form within about 6−12 h (not shown). We also noticed a significant decrease in colchicine-induced ladder formation for the cell line V500, a multidrug resistant derivative of LR73 cells recently derived in our laboratory (see Materials and Methods). That is, V500 cells did not harbor any laddered DNA after treatment for 24 h with 100 nM or even 400 nM colchicine (not shown). The defect did not appear to be due to a significant difference in growth rate between the two cell lines (not shown). Since V500 is mildly resistant to colchicine and exhibits decreased intracellular retention of colchicine (Hoffman *et al.*, 1996), it was not surprising to find that treatment with 100 nM colchicine was a less efficient inducer of apoptosis for V500, relative to LR73. However, since V500 is about 4-fold resistant to colchicine relative to LR73 (Hoffman *et al.*, 1996), 400 nM should have been a similar "effective dose" for V500 compared to 100 nM for LR73. Because V500 treated at a similar effective dose still did not yield laddered DNA within 24 h, it suggested that a defect in the apoptotic pathway might exist for these cells.

To test this, we measured appearance of laddered DNA in V500 induced to undergo apoptosis by a method that did

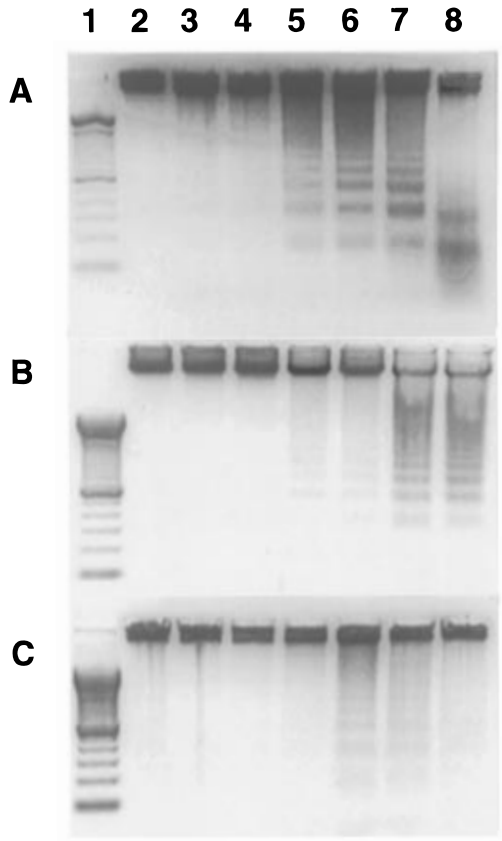

FIGURE 1: Kinetics of the appearance of laddered DNA for (A) LR73 fibroblasts, (B) LR73/hu MDR 1 transfectant clone no. 27 [see Hoffman *et al.* (1996)], and (C) LR73/27V500 cells (see Materials and Methods) induced to undergo apoptosis via withdrawal of serum from the culture medium (see Materials and Methods). Lane 1 is 100 bp DNA marker (100 bp at bottom, 2072 bp at top, approximately 100 bp increments in the well-separated bands); lane 2 is control DNA isolated from exponentially growing cells at 5% CO_2; lanes 3−8 are DNA isolated from cells 3, 6, 12, 24, 48, and 72 h after withdrawal of serum from the DME media, respectively. Note the more rapid appearance of laddered DNA, as well as the more rapid progression to low molecular weight ($\leq$550 bp) DNA fragments for LR73, relative to the MDR derivatives upon induction of the apoptotic cascade. Virtually identical results are seen for LR73/neo control transfectants (Hoffman *et al.*, 1996) relative to LR73 cells (not shown), indicating that G418 selection does not affect the kinetics of the apoptotic cascade in these cells. A significant portion of the delay in ladder formation for V500 must be due to overexpression of hu MR 1 protein, since LR73/hu MDR 1 clone no. 27 (panel B) was not previously exposed to chemotherapeutic drugs.

not rely upon treatment with chemotherapeutic drugs (e.g., via serum withdrawal, cf. Figure 1C). Indeed, laddered DNA could not be isolated from these cells even 12 h after serum withdrawal (Figure 1C, lane 5) but does begin to appear at about 24 h. However, in several experiments, it appeared as if laddered DNA for V500 did not increase in intensity upon further incubation in serum-free medium. In fact, it often appeared (e.g., Figure 1C) as if the laddered DNA *decreased* in intensity, suggesting that only a subpopulation of V500 was effectively induced. Growth of both V500 and LR73 arrests after serum withdrawal but does not start to detach before about 48 h. Regardless, any floating cells that might have appeared in the medium were included in our DNA isolations (see Materials and Methods). The data suggest that a multidrug resistance phenotype for these cells effects progression of the apoptotic cascade via a mechanism that is independent of chemotherapeutic drugs.

Recently, we have determined that the MDR phenotype exhibited by chemotherapeutic drug-selected cells is due to the sum of several events caused by drug selection (Hoffman *et al.*, 1996), only one of which is overexpression of the MDR protein. To determine whether the delay in ladder formation for V500 is due to the overexpression of hu MDR 1 protein *per se* (Hoffman *et al.*, 1996) similar experiments were performed for LR73/hu MDR 1 transfectants [LR73/hu MDR 1 clone no. 27, see Hoffman *et al.* (1996)]. These transfectants express similar levels of hu MDR 1 protein, relative to V500 (Hoffman *et al.*, 1996), but have never been exposed to any chemotherapeutic drugs. Therefore, any altered phenotype for these cells is due to hu MDR 1 protein overexpression alone. Similar to the case for V500, we do not observe any laddered DNA for the hu MDR 1 transfectants after 6 h post serum withdrawal (Figure 1B, lane 4). Faint laddered DNA is seen at 12 h post withdrawal for LR73/hu MDR 1 no. 27 (Figure 1B, lane 5). Note, however, that the laddered DNA for clone no. 27 at 12 h post withdrawal does not contain a significant number of DNA fragments of size $<$550 bp, whereas laddered DNA fragments of 550, 350, and 150 bp are clearly isolated for LR73 (the bottom band for the molecular weight markers in lane 1 is 100 bp, and the bands above are 200, 300, 400 bp, etc.). That is, the ladder DNA that is present for clone no. 27 at 12 h is not digested to the same extent relative to the ladder DNA for control LR73 cells. Bands $\leq$550 bp are easily seen for DNA isolated from LR73 at 12, 24, and 48 h after serum withdrawal (Figure 1A, lanes 5−7, respectively). At later time (72 h postwithdrawal) almost all laddered DNA isolated from LR73 is in either 150 or 350 bp form. In contrast, 350 bp DNA is not seen for clone no. 27 at 12 or 24 h, and no 150 bp DNA is seen even at 72 h postwithdrawal. Thus, appearance of laddered DNA and progression to lower molecular weight DNA ladder fragments occur with slower kinetics for MDR cells, and this effect is specifically related to overexpression of MDR protein.

The above results suggest that hu MDR 1 transfectants not previously selected with chemotherapeutic drug exhibit a defect in the apoptotic cascade initiated by serum withdrawl. If this is true, cell survivability (e.g., as assayed by colony formation assays) should differ for the MDR 1 transfectants relative to mock transfectant controls. Although measurement of relative survivability or relative colony formation does not distinguish between apoptotic and necrotic cell death, a clear difference in survivability would be expected. Figure 2 shows that survivability of the true hu MDR 1 transfectants upon serum starvation (as assayed by colony formation assays, see Materials and Methods) is dramatically increased relative to the controls. Although there is some apparent loss of viability at 12−24 h, survivability is substantially increased in the MDR 1 transfectants relative to the control and mirrors the apparent decrease in formation of laddered DNA. Thus, it is reasonable to suggest that a significant "delay" in the apoptotic cascade accompanies overexpression of the hu MDR 1 protein. Importantly, this behavior is independent of any effect of chemotherapeutic drugs, since in this case apoptosis is being induced by serum withdrawl.

Nuclear Morphology

Although an altered pattern of DNA digestion for the MDR cells is intriguing, we wished to further test progression of apoptosis in a detailed way by independent measures. By

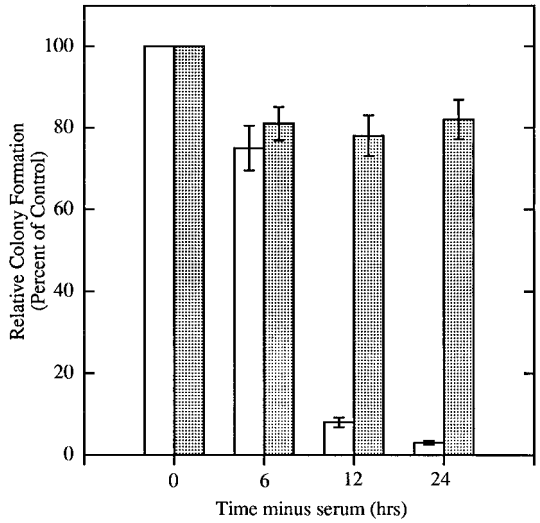

FIGURE 2: Results of colony formation assays for LR73/neo control cells (open bars) and pure hu MDR 1 transfectants (speckled bars) exposed to serum-free media for various times. Cells were plated and exposed to serum-free media as described in Materials and Methods, allowed to grow in normal media, and then stained with methylene blue, and surviving colonies >50 cells scored visually. Note the dramatically increased survivability of the MDR 1 transfecants relative to the control at 12 and 24 h postserum withdrawl, consistent with a significant delay in initiation of the apoptotic cascade.

staining cells with hemotoxylin/eosin at various time after serum withdrawal (see Figure 3A,B), one can easily distinguish normal nuclei from the nuclei of apoptotic cells that exhibit condensed or "collapsed" chromatin.[2] The latter appear as dark, dense nuclear staining (Figure 3B), whereas the former have a more speckled and diffuse appearance. Nick end-labeling methods can also be used to visualize collapsed nuclei for these cells (see footnote 2), but we found it was more difficult to precisely quantify collapsed nuclei with this method. By quantifying the percent collapsed nuclei for the different cell lines as a function of time after serum withdrawal (Figure 4), we again conclude that progression of the apoptotic cascade is delayed in these MDR cell lines and that the delay is most likely due to overexpression of the MDR protein [compare data for clone no. 27 (Figure 4 closed circles) vs data for V500 (Figure 4 open triangles)].

Intracellular pH Changes

As another detailed measure of the progression of apoptosis, we measured intracellular pH (pH_i) for single cells via single-cell photometry. Figure 5 presents a series of histograms showing pH_i for populations of LR73 cells grown on cover slips and analyzed for pH_i one cell at a time as described (Hoffman *et al.*, 1996; Wei *et al.*, 1995; Luz *et al.*, 1994). We find that serum withdrawal leads to fairly

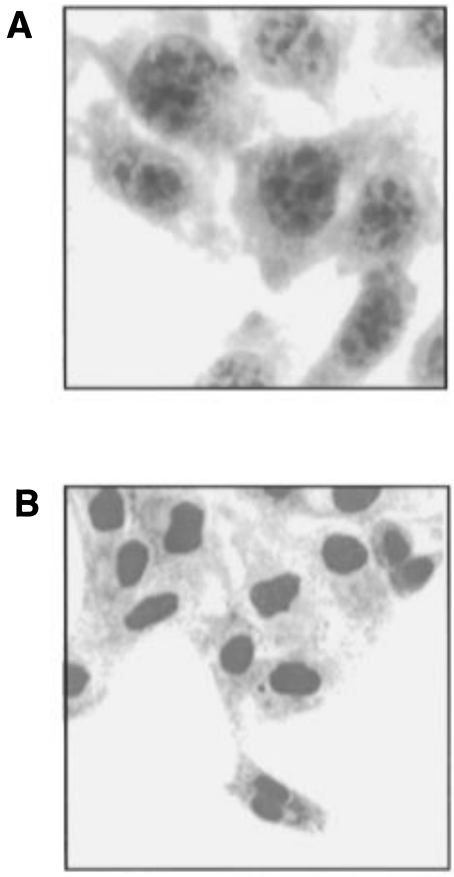

FIGURE 3: Nuclear morphology of control (A) and apoptotic (B) LR73 cells. Apoptosis was induced by serum starvation for 12 h. Cells were fixed and stained with hemotoxylin/eosin as described in Materials and Methods. Note the more compact dense staining of the apoptotic nuclei, which also corresponds to dense staining revealed in the TUNEL protocol (not shown; see Materials and Methods and footnote 2).

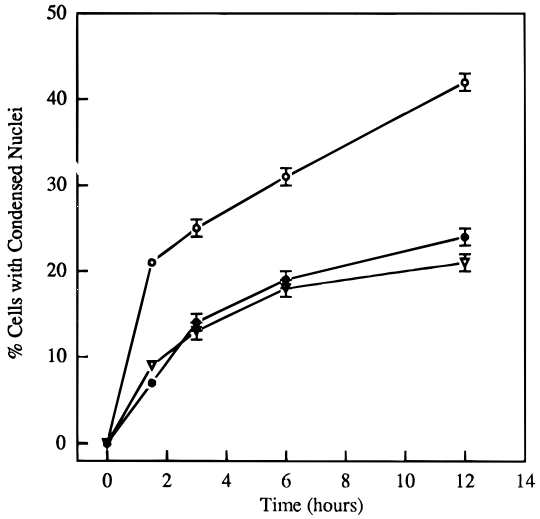

FIGURE 4: Quantitation of nuclear morphology data for LR73 (open circles), LR73/hu MDR 1 clone no. 27 (closed circles), and LR73/27V500 (open triangles) at various times after serum withdrawal. Similar to the case for ladder formation, nuclear condensation is delayed in the MDR derivatives. Data shown are the average of three experiments ±SD. Each experiment is the average of three counts from three separate microscopic fields.

[2] Many "classical" morphologic features of apoptotic cells were first described for cells in tissue. However, these features may not be exactly analogous to those exhibited by cells grown in culture. In culture, for the most part, the most common nuclear change that can be easily identified is condensation and with it some nuclear fragmentation. While fragmentation in apoptotic LR73 cells can be easily identified with nick end-labeling (i.e., TUNEL staining; data not shown but see Methods), *counting* cells in such a medium is difficult because the fixation and staining that accompany the TUNEL protocol frequently obscure the unlabeled cells, making precise quantitation (i.e., calculation of % nuclei in condensed form) extremely difficult if not impossible. The characteristic features of the apoptotic cells are discrete enough and are also easily recognizable in routinely fixed, hematoxylin- and eosin-stained cover slips. This method clarifies all the tissue culture cells grown on thin glass cover slips and allows for more precise counting of apoptotic nuclei. The method is also substantially faster and less expensive.

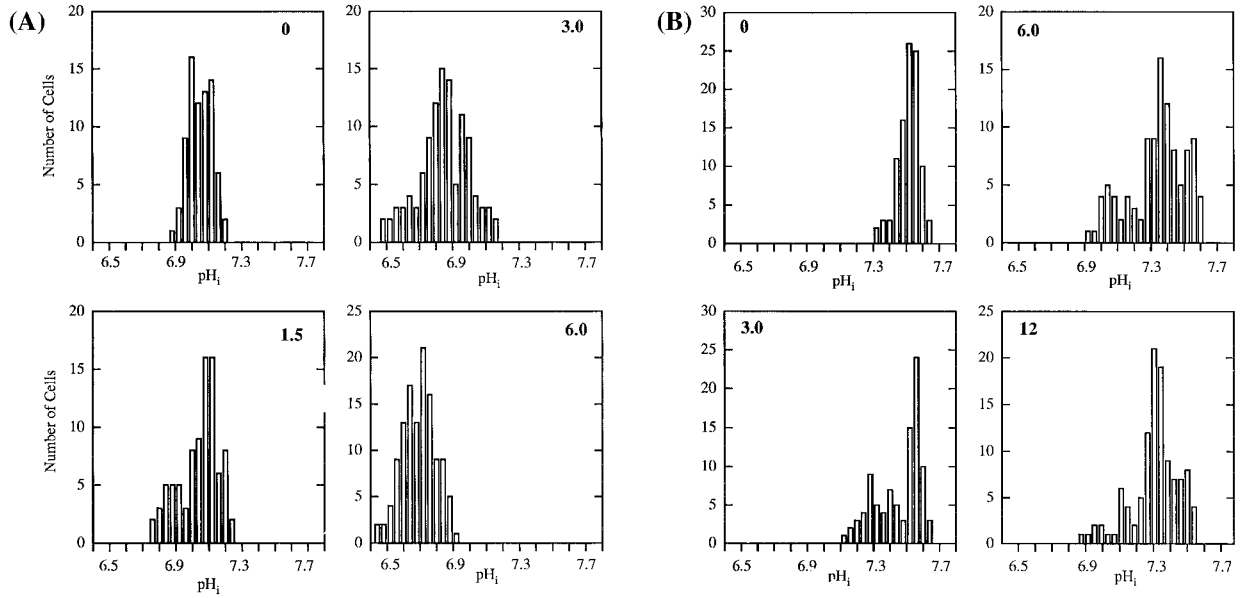

FIGURE 5: Histograms of steady state pH_i for LR73 (A) or LR73/hu mDR 1 no. 27 (B) at various times after serum withdrawal (for LR73, 0, 1.5, 3, 6 h; for clone no. 27, 0, 3, 6, 12 h). The number in the upper right-hand corner of each panel indicates hours after serum withdrawal. Steady state pH_i was measured for individual cells under constant perfusion by single-cell photometry (Hoffman *et al.*, 1996; Wei *et al.*, 1995; Luz *et al.*, 1994) as described in Materials and Methods. Clearly, overexpression of the hu MDR protein in these cells leads to significant intracellular alkalinization under control conditions as previously described (Hoffman *et al.*, 1996) and also a significant delay in the rate and extent of acidification for cells induced to undergo apoptosis via serum withdrawal. Similar results are obtained upon treatment with similar effective dose of colchicine (not shown).

rapid intracellular acidification for LR73 cells as they proceed into the apoptotic cascade. A few cells with acid pH_i (6.70−6.75) are seen even within 0.5−1.0 h (not shown). Importantly, our single-cell method allows us to inspect each individual cell for morphologic features as well as pH_i, allowing us to conclude that acidified cells in the population (which initially appear as a minor shoulder on the left of the overall histogram) correspond to cells with condensed nuclei (W. Roberts and P. D. Roepe, unpublished). A more detailed single-cell analysis of the kinetics of this acidification relative to distinct morphologic changes will be published elsewhere (W. Roberts, Mary M. Hoffman, and P. D. Roepe, in preparation).

In contrast to data for LR73 cells, histogram data for MDR clone no. 27 (Figure 5B) shows acidification that is not as pronounced and that does not occur with similar kinetics (note that we observe very little change in pH_i at 1.5 h post serum withdrawal for these cells; data not shown). Upon averaging the data in these histograms and plotting mean steady state pH_i for the cell populations vs time after serum withdrawal (Figure 6), we note that the extent of acidification begins early and plateaus within 6−12 h for LR73 cells. Conspicuously, acidification of mean pH_i does not occur as quickly or to the same extent for clone no. 27, although some acidified cells are apparent as a shoulder to the histogram at 6 h (Figure 5B at 6 h, top right). Thus, overexpression of MDR protein appears to decrease the extent and delay the kinetics of intracellular acidification that occurs upon induction of apoptosis.

This observation could provide a relatively simple explanation for the delay in the apoptotic cascade for the MDR cells *if* intracellular acidification that occurs early in apoptosis (Figure 5A) were either causal or permissive for subsequent events in the cascade. There is currently some debate with regard to this point (see Discussion) related to differences in the cascade for various cell types. To test whether

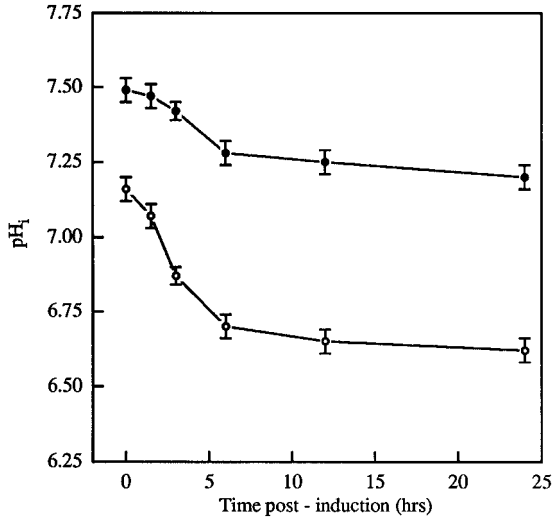

FIGURE 6: Plot of the mean cell pH_i for LR73 (open circles) and LR73/hu MDR 1 no. 27 (closed circles) cells vs time after induction of apoptosis via serum withdrawal. Data shown are the average of at least 50 cells at each time point ±SD.

intracellular alkalinization directly delayed subsequent events in the apoptotic cascade for these LR73 cells, we induced control cells to undergo apoptosis via serum withdrawal at 5% CO_2 and then allowed DNA digestion to progress at normal (Figure 7, lane 3) vs elevated pH_i (Figure 7, lane 2) for 1.5 h. The pH_i was elevated via manipulation of % CO_2 and $[HCO_3^-]_{ex}$ as recently described (Hoffman *et al.*, 1996). We observe that when pH_i is raised artificially in LR73 cells *after* committment to apoptosis (see Discussion), DNA laddering is delayed (compare lane 2, Figure 7 to lane 3). These experiments are somewhat different than other recent work wherein apoptosis was *induced* under either acid or alkaline extracellular conditions (Li & Eastman, 1995). The data suggest that intracellular alkalinization delays later-stage events in apoptosis (e.g., DNA laddering) in these cells and

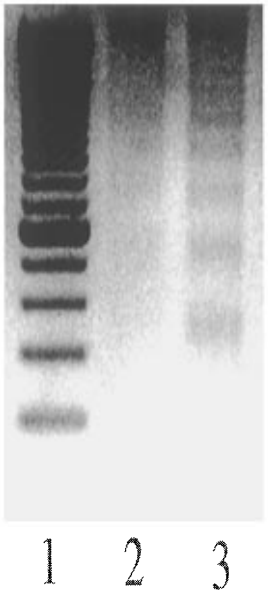

FIGURE 7: Ladder DNA formation for LR73 cells pulsed to high pH_i. Lane 1 is 100 bp molecular weight standards, lane 2 is LR73 induced to undergo apoptosis by 3 h of serum withdrawal as described in Materials and Methods, followed by incubation at 5% CO_2 in the presence of medium harboring serum and 75 mM HCO_3 for an additional 1.5 h. Lane 3 is LR73 similarly induced to undergo apoptosis by 3 h after serum withdrawal at 5% CO_2 but was then incubated for an additional 1.5 h in DME media plus serum at 5% CO_2 with no added HCO_3^-. Single-cell photometry experiments verified that LR73 cells incubated at 75 mM HCO_3 after serum starvation for 3 h exhibited elevated pH_i (between 6.85 and 7.15) compared to similar apoptotic cells at normal $[HCO_3]_{ex}$ (see Figure 5A). The results shown are representative of several experiments and indicate that alkalinization of the cytosol protects against late events in the apoptotic cascade (e.g., DNA laddering).

offers a reasonable framework for interpreting the delay caused by MDR protein overexpression.

Reversal with Verapamil

Finally, to further test whether MDR 1 overexpression delays the kinetics of apoptosis, similar experiments were performed in the presence of 10 μM verapamil, a concentration of the channel blocker that partially reverses the MDR phenotype in our true hu MDR 1 transfectants (Hoffman *et al.*, 1996) but that does not induce apoptosis in and of itself. As shown in Figure 8, when apoptosis is induced for clone no. 27 or for V500 via serum withdrawal in the presence of 10 μM verapamil, the kinetics of DNA ladder formation are partially restored (compare top two panels [A and C, without verapamil] to bottom two panels [B and D, with verapamil]). Because verapamil only partially reverses the MDR phenotype mediated solely by MDR protein (Hoffman *et al.*, 1996), we do not expect kinetics of ladder formation etc. that are *identical* to those found for control LR73 cells, but the partial restorative effect is consistent with partial reversal of MDR protein effects at this nontoxic concentration of verapamil [see Hoffman *et al.* (1996)]. As shown in Figure 9, the percent of cells exhibiting condensed nuclei is also increased for clone no. 27 and for V500 upon induction in the presence of verapamil.

Considering all the data together, we suggest that MDR 1 overexpression delays the onset of apoptosis in these cells via an increase in pH_i, thereby delaying or altering the extent of the acidification that normally must occur for proper execution of the cascade.

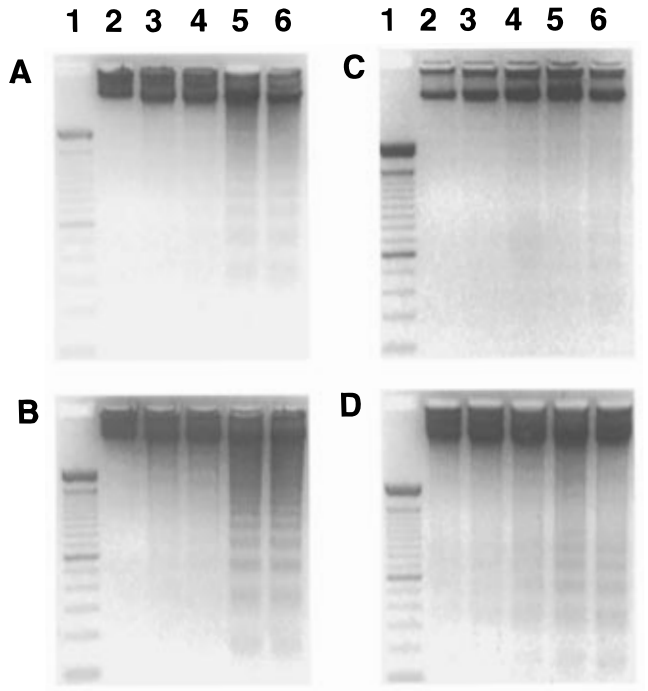

FIGURE 8: Reversal of the delay in the apoptotic cascade caused by MDR protein overexpression via incubation with 10 μM verapamil. DNA ladder formation kinetics for clone no. 27 and V500 − verapamil (A and C, respectively) are compared to kinetics for clone no. 27 and V500 + verapamil (B and D, respectively). Lane 1 is molecular weight markers, lane 2 is control DNA, and lanes 3−6 are DNA extracted 3, 6, 12, and 24 h after withdrawal of serum. Note this concentration of verapamil is not cytotoxic in and of itself to either the control or MDR cells [see Hoffman *et al.* (1996)] and does not affect kinetics of the apoptotic cascade for the control LR73 or LR73/neo cells (not shown) as measured by nuclear morphology, laddering, or changes in pH_i.

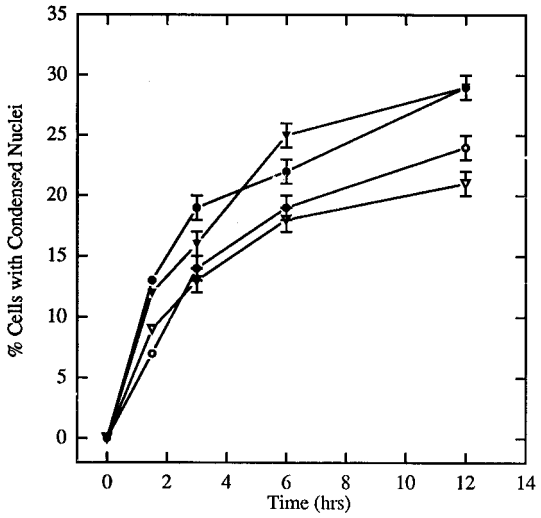

FIGURE 9: Comparison of nuclear morphology for clone no. 27 and V500 induced to undergo apoptosis by serum withdrawal in the absence (open circles and open triangles, respectively) or the presence (closed circles and triangles, respectively) of 10 μM verapamil (see also caption to Figure 8 for description of verapamil effects on the control cells).

DISCUSSION

By a variety of independent measures, we find that overexpression of the hu MDR 1 protein in LR73 CHO fibroblasts delays the onset of apoptosis that is induced by serum withdrawal. Similar to others (Barry & Eastman, 1993; Li & Eastman, 1995; Pérez-Sala *et al.*, 1995; Gottlieb *et al.*, 1996), we find that acidification is an early event in

the apoptotic cascade. Based on our initial data, it is conceivable that small (0.05−0.10) changes in pH_i occur for individual cells within minutes of induction of the cascade. Additional single-cell photometry work will test this idea (W. Roberts, Mary M. Hoffman, and P. D. Roepe, in preparation). As reported previously (Hoffman *et al.*, 1996) hu MDR 1/LR73 transfectants are alkaline, and they exhibit an altered extent of acidification as well as a delay in the acidification that accompanies apoptosis for LR73 cells under similar conditions. Thus, the delay in morphologic changes and DNA laddering (both of which occur *after* acidification) that we find in the MDR transfectants may be due to the altered pH_i homeostasis for these cells. Indeed, artificial elevation of pH_i by manipulation of $[HCO_3^-]_{ex}$ is also found to delay DNA laddering in these cells. It is interesting to note that mutations in another member of the ABC protein family, the CFTR, has also been found to alter apoptosis in C127 cells (Gottlieb & Dosanjh, 1996), presumably also through affecting acidification that occurs early on in the cascade. These results with the true hu MDR 1 transfectants are significant because they uncover another facet to the mechanism whereby MDR 1 protein overexpression increases the survivability of tumor cells. In contrast to other theories of MDR 1 protein function, this facet does not necessarily require any altered interaction between drug and drug target [e.g., "removing" drug from the cell, lowering availability of the target, and/or titrating drug−target interactions; see Gottesman and Pastan (1993) and Wadkins and Roepe (1997)] but rather envisions that altered signal transduction is responsible for the resistance.

Cellular acidification follows soon after volume changes in these cells but precedes other key events including nuclear condensation and DNA laddering. The relative sequence of these events is independent of the method of induction of apoptosis. That is, significant acidification for LR73 cells clearly begins within 1.5 h post serum withdrawal, before the appearance of laddered DNA (at 6−12 h), whereas a similar acidification occurs 3−4 h after 100 nM colchicine treatment, but also just before the appearance of laddered DNA (at 12−24 h; data not shown). Thus, similar to other reports (Barry & Eastman, 1993; Li & Eastman, 1995; Pérez-Sala *et al.*, 1995; Gottlieb *et al.*, 1996) the kinetics and extent of acidification are temporally linked to subsequent events in the apoptotic cascade regardless of the method of induction. This supports the possibility that intracellular acidification may be either causal or permissive with regard to proper progression through the apoptotic cascade. As suggested previously (Barry & Eastman, 1993) this idea has significant appeal because a key endonuclease (DNase II) that likely plays a role in DNA ladder formation is acid activated [with a pH optimum below 7.0; see Barry and Eastman (1993)]. Also, other events that occur during the cascade [such as ICE protease-mediated cleavage of a variety of protein substrates; see Patel *et al.* (1996) and references within] are likely pH dependent. Significant pH_i changes likely reflect significant changes in volume and perhaps changes in intracellular concentrations of other ions (e.g., Ca^{2+}) that may be more obvious for some cell types depending upon how pH_i and $[Ca^{2+}]_i$ are regulated for the cell type in question. Ca^{2+} and/or volume changes could be more important direct influences on the cascade for those cells [see Furuya *et al.* (1994)]. This idea also has appeal, since regulation of pH_i, volume, and $Ca^{2+}{}_i$ is tightly linked for many cell types (McCarty & O'Neil, 1992). It is believed that phosphatases and/or kinases are involved in regulating key early events in the apoptotic cascade and that these activities may thus also influence changes in intracellular ionic equilibrium (Morana *et al.*, 1996). Single-cell photometry approaches like those used in the present study may provide an avenue for deciphering (on a cell-by-cell basis) the precise temporal arrangement of activation of phosphatases and alterations in the activities of key regulators of pH_i. This approach will also likely shed light on putative connections between pH_i, volume, and $[Ca^{2+}]_i$ regulation in apoptotic cells.

Although our data mostly agree with other studies that have examined pH_i phenomena linked to apoptosis, in contrast to our interpretation that the MDR 1 protein-induced delay in the apoptotic cascade is linked to the protein's effects on pH_i, data from at least one elegant recent study argue that acidification of pH_i may not be absolutely obligate for the cascade in CTLL cells (Li & Eastman, 1995). This conclusion was reached via experiments wherein apoptosis was induced by IL-2 withdrawal for 16 h. at high extracellular pH (8.0). Since pH_i is raised above 7.0 when induced CTLL cells are suddenly placed at $pH_o = 8.0$, and since cells incubated at $pH_o = 8.0$ still exhibited laddered DNA, it was quite logically argued (Li & Eastman, 1995) that low pH_i was unecessary for DNA laddering. However, although a pH_o of 8.0 clearly elevates CTLL pH_i for some period of time (Li & Eastman, 1995), it is unknown whether such conditions would maintain elevated pH_i for 16 h. Thus, perhaps cells induced to undergo apoptosis at $pH_o = 8.0$ for 16 h do *eventually* reach a lower pH_i that allows for DNA digestion, or perhaps $pH_o = 8.0$ alters the efficiency of induction of apoptosis in this example, "masking" some effect on ladder formation. Indeed, we find that although induced LR73 cells shifted to $pH_o = 8.0$ similarly exhibit $pH_i > 7.0$ for at least 30 min (not shown), after that time it appears that some members of the population begin to drift in the acid direction (W. Roberts and P. D. Roepe, unpublished). Therefore, although for the most part our results are in agreement with those of Li and Eastman (1995) and others who have examined pH_i for apoptotic cells, the question of whether acidification of pH_i during apoptosis is causal, permissive, or merely incidental with regard to subsequent events in the cascade should perhaps be examined further in more model systems using a variety of approaches for manipulating pH_i.

Unfortunately, it is well-known that inducing stable, long-term perturbations in pH_i is very difficult (Hoffman *et al.*, 1996), and in most cases the apoptotic cascade requires several hours−days to run its course. Thus, it is challenging to manipulate pH_i and pH_i regulation on a time scale commensurate with other long-term biochemical events that occur during apoptosis (e.g., DNA laddering). We have initially approached this dilemma in the following way: we first noted that LR73 cells induced by serum withdrawal for 3 h and then placed back in serum-containing medium at 5% CO_2 for another 3 h exhibited production of laddered DNA that was similar to cells incubated in serum free medium for 6 h (not shown), as did cells incubated without serum for 4 h and then with serum for 2 h, etc. This suggested to us that the efficiency of DNA laddering was dependent both on induction of apoptosis (i.e., "committment" to the cascade) as well as the time allowed for DNA "digestion". Presumably, LR73 fibroblasts are efficiently

induced by 3 h of serum withdrawal, but not all cells that have entered the apoptotic cascade have yet fully digested their DNA. Therefore, we induced plates of cells under normal (5% CO_2) conditions and *then* placed them in environments of different % CO_2 and different $[HCO_3]_{ex}$, but only for short (1 h) periods of time, wherein the perturbations in pH_i caused by these manipulations are relatively stable. The data from experiments performed in this way argue that alkaline pH_i protects against production of laddered DNA (Figure 7). This conclusion is in agreement with another recent study (Pérez-Sala *et al.*, 1995) that used treatment with lovastatin to elevate pH_i. However, since lovastatin has a number of effects, results with the drug could formally be interpreted several ways. In the present work, pH_i was elevated solely via manipulation of $[HCO_3^-]_{ex}$.

Aside from what these data help illustrate with regard to apoptotic acidification, delay of the apoptotic cascade by overexpression of MDR protein has important implications for understanding how the protein (and possibly other resistance mechanisms) also confer resistance to chemotherapeutic drugs and other toxic insults. It is widely known that MDR protein overexpression confers reduced intracellular accumulation of a variety of chemotherapeutic drugs. Therefore, it has generally been assumed that the protection provided to tumor cells overexpressing MDR proteins is through the protein decreasing the efficiency of interaction between drug and principle target (e.g. DNA, tubulin, etc.), and therefore the direct cytotoxic damage that is a consequence of this interaction. However, it is also appreciated at this point that most chemotherapeutic drugs also act via inducing apoptosis, and that this inducing ability may or may not require that the drug bind to its principle intracellular target [see, for example, Sumantran *et al.* (1995), Eastman (1990), and Lowe *et al.* (1993)]. Also, it is difficult for us to quantitatively explain MDR protein-mediated drug resistance via altered intracellular drug retention alone (Hoffman *et al.*, 1996). Therefore, we interpret results from the serum starvation experiments to indicate that some component of the chemoprotective function of MDR protein overexpression is also via inhibition of the apoptotic cascade. If acidification is indeed an important event in the apoptotic cascade for many cell types, and if MDR protein delays the cascade by perturbing that acidification, then the present data also predict that MDR protein overexpression might confer "resistance" to other apoptotic inducers (e.g., withdrawal of serum). If true, this idea might (in part) explain several recent curious but paradoxical findings, such as overexpression of MDR protein in some cells that are resistant to radiation (Su *et al.*, 1994), which is also a potent inducer of apoptosis. It may also help explain the ability of MDR protein overexpression to confer certain forms of immunologic resistance (Weisburg *et al.*, 1996). However, in at least one case [complement-mediated cytotoxicity, see Weisburg *et al.* (1996)] cell death likely occurs too quickly to be directly linked to the apoptotic cascade, and other "necrotic-like" events also occur. Nonetheless, along with further describing acidification phenomena that occur during apoptosis, the observations in this paper suggest that additional, "nonclassical" protective functions of the MDR protein should be investigated and that the connection between apoptotic cascade signal transduction and altered intracellular ionic equilibria should be explored further.

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