Apoptosis in LR73 Cells is Inhibited by Overexpression of the Multidrug Resistance (MDR) Protein

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Apoptosis, also known as programmed cell death, is one of two biological pathways for cell death. The induction of apoptosis is believed to be one important method of action of chemotherapeutic drugs used to treat cancer. Multidrug resistant (MDR) tumor cells, which exhibit increased tolerance to chemotherapeutic drugs, are a major problem in the effective treatment of cancer. MDR cells have been shown to exhibit a resistance to apoptosis induced by chemotherapeutic agents. It is possible that MDR cells are resistant to the induction of the "apoptotic cascade." Thus, apoptosis was induced by withdrawal of fetal calf serum (FCS), and by treatment with colchicine at different dosages. The experiments were performed with three different cell lines: LR73 Chinese hamster ovary fibroblasts; LR73#27 (a hu MDR1 transfectant); and 1-1, a mu MDR1 transfectant further selected with 50 ng/mL vinblastine. Apoptosis was quantified by densitometric analysis of the percentage of total cellular DNA in "ladder" form. When apoptosis was induced by serum withdrawal, after three days, 53% of the DNA isolated from LR73 cells was in ladder form; while only 40% of the DNA from the LR73#27 cells, and 19% of the DNA isolated from the 1-1 cells were in ladder form. This timecourse was altered in the presence of verapamil, which reverses the effects of the MDR protein. These data are consistent with the hypothesis that the MDR protein increases resistance to the induction of the apoptosis.

Introduction[1]

Apoptosis. Apoptosis is a process whereby cells die in a controlled, programmed manner in response to specific stimuli. It is characterized by distinct changes in cell volume, nuclear organization and the topology of the cell membrane. In contrast, the other common pathway for cell death, necrosis, is characterized by a gradual dissolution of cell structure and eventual rupture of the cell membrane. Necrosis generally occurs after major toxic insult, when cells are too damaged to survive. Apoptosis, on the other hand, is frequently induced by less dramatic events. Apoptosis, sometimes referred to as "programmed cell death" or "cellular suicide," was first described as a series of cell morphological changes (Kerr et al., 1972).

Apoptosis occurs throughout human life. It plays an important role during embryogenesis, when the cells that make up the tail and the webbing between the digits of the hands and feet are programmed to die (Hinchliffe, 1981). It is also an important mechanism in the regulation of the immune system (Smith et al., 1989). When those cells that recognize proteins in the body as a foreign substance are eliminated, they generally die in a programmed manner, i.e., by apoptosis. In addition, cytotoxic T-cells and natural killer T-cells are believed to eliminate virally infected cells and tumor cells by inducing them to undergo apoptosis (Shi et al., 1989).

There are three stages of apoptosis that are best described by morphological criteria (Kerr et al., 1972). First, nuclear chromatin, which is a complex of nucleic acids and proteins in the cell nucleus, condenses. The nucleus decreases in size, and cell volume decreases by about one-third. The second stage of apoptosis involves characteristic membrane "blebbing," which means the cell membrane forms folds and becomes invaginated. In this phase, both the nucleus and the cytoplasm begin to break apart into small, membrane-bound apoptotic bodies. In the third stage, the apoptotic bodies bud off of the cell and are degraded as they are "ingested" by phagocytes and neighboring epithelial cells. At about this time, an endonuclease (or endonucleases) begins to cleave DNA. The cleaved DNA forms a characteristic "ladder" pattern of fragments of 100 + n(100) base pairs in size ($1 < n < 8$) that can be readily visualized by agarose gel electrophoresis (Wyllie, 1980). Thus, many researchers now rely on DNA fragmentation, rather than on morphological criteria, to detect apoptosis (Corcoran et al., 1994).

In contrast to necrosis, which typically occurs for many cells simultaneously, apoptosis can occur one cell at a time and typically takes several hours to complete. Apoptosis in organisms is not accompanied by inflammation, and surrounding cells ingest the resulting apoptotic bodies. Apoptosis occurs in many physiological conditions, in response to some viral infections, and can occur in response to mild hypoxia. The immune system may induce apoptosis in potentially cancerous cells. Apoptosis can also occur as a result of exposure to low doses of certain toxic compounds, such as chemotherapeutic drugs (Corcoran et al., 1994).

In contrast to apoptosis, necrosis begins with an initial phase of generalized cell swelling. Over a period of hours to days, there is gradual dissolution of organelles and rupture of the plasma membrane. DNA is not fragmented in a patterned way, but some DNA degradation may occur. Necrosis typically occurs in groups of cells, and takes many hours or days to complete. If degradation of DNA occurs, occasional small fragments of DNA are produced, resulting in a "smeary" ladder pattern when the DNA is electrophoresed on agarose gels. Necrosis in organisms is typically accompanied by acute inflammation and secondary scarring. Necrosis is never physiological, it is always pathological, meaning it is caused by an external insult. It can be complement-mediated, caused by severe hypoxia, or caused by high levels of various toxic compounds (Corcoran et al., 1994).

Apoptosis can be induced in a variety of apparently unrelated ways. However, all methods of induction likely enter a "common final pathway," which then initiates the morphological changes and DNA fragmentation that investigators use to define and quantitate apoptosis. Apoptosis can be induced by cytochalasin B (a fungal metabolite), by a reduction in intracellular pH (Barry and Eastman, 1993), by topoisomerase II inhibitors such as etoposide (Barry et al.,

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1993), and by a variety of chemotherapeutic drugs such as doxorubicin and annamycin (Ling et al., 1993). Apoptosis can also be induced by γ radiation, and by elevations in intracellular Ca^{2+} (Zhivotovosky et al., 1993; Furuya et al., 1994). Apoptosis induced by anthracycline antibiotics can be inhibited by cellular resistance to these drugs (Ling et al., 1993). Apoptosis can also be inhibited by cyclosporin A (Shi et al., 1989), and, in some cell lines, by Ca^{2+} chelators (Zhivotovsky et al., 1993).

It was discovered in 1990 that the cleavage of double-stranded DNA characteristic of apoptosis generates two classes of chromatin fragments (Arends et al., 1990). It was further discovered that most of the cleavage products originate from DNA that is in a transcriptionally active configuration. The characteristic nuclear morphology of apoptosis may be explained by the cleavage of transcriptionally active genes, with less cleavage in the nucleolin-rich fibrillar center. That is, the morphological changes associated with apoptosis have been suggested to be the result of nuclease digestion of nuclear DNA that is primed for transcription into mRNA (Arends et al., 1990).

In 1993, it was postulated that the pertinent endonuclease involved in apoptosis is deoxyribonuclease II (Barry and Eastman, 1993). DNase II is an acidic endonuclease that is activated when intracellular pH falls below pH 7, with maximum endonuclease activity at pH 5.5. DNase II is not a Ca^{2+}/Mg^{2+} dependent endonuclease. This hypothesis is in contrast to other work which showed that Ca^{2+} is an important signaling mechanism for the induction of apoptosis (Zhivotovsky et al., 1993; Furuya et al., 1994).

Also in 1993, coinciding with the suggestion of DNase II as a possible endonuclease involved in apoptosis (Barry and Eastman, 1993), the changes in pH upon the induction of apoptosis by etoposide (a topoisomerase II inhibitor) were studied (Barry et al., 1993). This pH change for cells undergoing etoposide-induced apoptosis was measured in individual cells using flow cytometry. It was determined that in 15% of all the cells, an acidification of up to 1 pH unit occurred and correlated with the timecourse of the appearance of DNA digestion. When the cells were sorted on the basis of intracellular pH, only the acidic cells showed the morphology and DNA digestion characteristic of apoptosis (Barry et al., 1993).

Multidrug Resistance. Multidrug resistance (MDR) is a major obstacle to the effective treatment of cancer by chemotherapy, and has been a subject of much research in recent years. The MDR phenomenon was first described by Biedler and Riehm (1970), when they observed that cell lines grown in the presence of actinomycin D developed cross-resistance to many other drugs that are markedly different in structure and pharmacology (e.g., doxorubicin, vinblastine, colchicine). It was subsequently found that markers for the MDR phenotype frequently include the overexpression of the MDR protein (a polytopic integral plasma membrane protein also called p-Glycoprotein or p-GP) and the decreased retention of chemotherapeutic drugs in cells (Gottesman and Pastan, 1993; Hammond et al., 1989). The decreased retention of drugs by these cells makes these cells drug-resistant since, presumably, less toxic compound is available to bind to intracellular targets. Ling and colleagues established that the level of multidrug resistance is related in some fashion to the level of overexpression of MDR protein (Endicott and Ling, 1989). In recent years, other cellular consequences of MDR protein overexpression have been noted (reviewed in Roepe, 1995).

It is not currently known precisely how the MDR protein functions to lower intracellular levels of chemotherapeutic drugs. There are essentially two theories. One proposes that the MDR protein is a drug transporter (Hammond et al., 1989; Gottesman and Pastan, 1993), and the other suggests that MDR promotes decreased drug retention indirectly by elevating intracellular pH and decreasing electrical membrane potential (Roepe, 1995). Sequence analysis has revealed structural homology between the gene encoding human MDR1 and bacterial genes that encode various periplasmic transporters. This similarity has led to the proposal that the MDR1 protein lowers intracellular levels of chemotherapeutics via active drug transport. However, it is not understood how a single transport protein could recognize all of the compounds to which MDR cells are resistant, or those for which the cells exhibit decreased retention. Also, recent kinetic studies of drug transport (reviewed in Roepe, 1995) are inconsistent with the “drug pump” theory.

An alternative explanation (Roepe, 1995) is that MDR protein indirectly promotes decreased retention of lipophilic, weakly basic and/or cationic drugs, or drugs that bind to intracellular targets in a highly pH dependent manner. An altered pHi or altered plasma membrane electrical potential could also result in decreased drug retention, the definition of the MDR phenotype. This hypothesis is consistent with the observation of elevated intracellular pH and decreased plasma membrane electrical potential in many different cell lines overexpressing MDR1 (reviewed in Roepe, 1995).

When apoptosis was induced with the anthracycline antibiotics doxorubicin (an “MDR” drug whose cellular retention is usually altered by the MDR protein) and its analogue annamycin (a “non-MDR” drug) in P388 parental cells and P388 MDR derivatives created by selection with doxorubicin, it was discovered that the MDR cells were markedly resistant to apoptosis induced by doxorubicin. Conversely, they were not very resistant to apoptosis induced by annamycin. DNA fragmentation caused by doxorubicin and annamycin treatment was inhibited by the RNA synthesis inhibitor actinomycin D, the endonuclease inhibitor aurintricarboxylic acid, and the protein synthesis inhibitor cyclohexamide. These data indicate that DNA fragmentation is probably not directly caused by treatment with chemotherapeutic drugs, but instead requires gene expression and the synthesis of new proteins. It was also determined that drug-topoisomerase II-cleavable complexes occurred only in cells that had begun to undergo apoptosis. This suggests that DNA fragmentation and the induction of apoptosis by anthracycline antibiotics may be mediated through interaction between DNA and topoisomerase II and alteration of the supercoiled DNA in chromatin, allowing the endogenous nuclease easier access to the linker regions in the nucleosome, where they can then initiate DNA cleavage (Ling et al., 1993).

Summary. Apoptosis is an important mechanism for cell death that is distinct from necrosis. Apoptosis can be induced in cells in many ways, including via treatment with chemotherapeutic drugs. Multidrug-resistant tumor cells are a serious obstacle to the effective treatment of cancer for several reasons. These include the fact that it is more difficult to induce programmed tumor cell death (apoptosis) in these MDR cells. The work of Ling et al. (1993) that examined apoptosis induced by different anthracycline antibiotics suggests that MDR cells may be resistant to the induction of apoptosis. However, from the data presented in their

paper it is unclear if the MDR cells are resistant to apoptosis because less drug is available to induce apoptosis or because the cells are densensitized to events downstream of those directly mediated by drugs. In other words, if MDR protein acts to exclude these drugs from cells, it is not possible, based on present data, to determine if the MDR protein alters the biochemistry of the "apoptotic cascade."

It is possible to induce apoptosis in cells without treating them with chemotherapeutic drugs. One way is to induce apoptosis by withdrawal of fetal calf serum (FCS) from the media in which cells are typically grown. By quantifying apoptosis induced via FCS withdrawal in cell lines expressing varying amounts of MDR1, we sought to determine if the MDR protein directly inhibits the induction of apoptosis or if the inhibition of apoptosis induced by chemotherapeutic drugs in cells overexpressing MDR results from the exclusion of these drugs from the cells.

Methods

Materials. Vinblastine was obtained in powdered form from Eli Lilly and Co (Indianapolis, In.). Verapamil and colchicine were obtained from Sigma Chemical Co. (St. Louis, Mo.) in powdered form. Doxorubicin was obtained as a 1 mg/mL solution from Adria Laboratories (Milan, Italy). The Chinese hamster ovary fibroblast cell line LR73 and the murine MDR1 transfectant cell line 1-1 were generously provided by Dr. Phillipe Gros, McGill University. The LR73#27 cell line, a hu MDR1 transfectant, was obtained from Dr. L.Y. Wei, Memorial Sloan-Kettering Institute. G-418 sulfate was obtained from Gibco BRL (Gaithsburg, Md.). Proteinase K and RNase A were obtained from Gibco BRL (Gaithsburg, Md.) in powdered form. Photographic grade sodium sulfite was obtained from Sigma Chemical Co. (St. Louis, Mo.). Dulbecco's modified eagle medium (DME) with high glucose (with or without 10% fetal calf serum (FCS)) and 200 units/L each of penicillin and streptomycin were obtained from the media core facility of Memorial Sloan-Kettering Cancer Center. Trypsin (0.05% trypsin, and 0.53 mM EDTA in saline), was obtained from Gibco BRL (Gaithsburg, Md.). All other chemicals were reagent grade or better and were purchased from commercial sources.

Procedures. *Tissue Culture.* All cell lines were grown in a Nuaire programmable incubator at 37°C in an atmosphere of 5% CO_2. 1-1 cells were grown in the presence of 50 ng/mL vinblastine. LR73#27 cells were grown in the presence of 200 μg/mL G418 to maintain hu MDR1 expression.

Growth Assays. To determine the effect that FCS withdrawal, doxorubicin, and colchicine had on the growth rate of the three cell lines, it was necessary to determine how many cells survived after varied times under different conditions (see *Results*). Cells were harvested by trypsinization, and counted using a hemacytometer. Then, 1 x 10^4 cells were plated in each well of a six-well plate. Each well contained either normal media, FCS-free media, or normal media with 100 nM colchicine. After different periods of time, the cells were again harvested by trypsinization and were counted using a hemacytometer.

Induction of apoptosis. When inducing apoptosis by withdrawal of FCS, each plate of cells was first washed with 2 mL of phosphate buffered saline (PBS; without Mg^+ or Ca^+), 10 mL of serum-free media was added, and the plates then grown in the absence of FCS for different periods of time (see *Results*). When apoptosis was induced by means of doxorubicin and colchicine, plates were similarly washed with PBS, but 10 mL of media containing FCS was then added along with varied concentrations of the drug (see *Results*), and the plates of cells were then grown for different amounts of time. When apoptosis was induced in the presence of 10μM verapamil, the verapamil was added at the same time as the drug or just after FCS-free media was added.

Extraction of DNA. The media from each plate was first collected and placed in a 15 mL conical tube, and each tube was placed on ice. This was necessary in order to prevent DNA degradation and to stop the action of any enzymes then cleaving the DNA. Each plate was then washed with 2 mL of cold (approximately 4°C) PBS. Cells attached to the plate were then harvested with 1 mL of trypsin (0.05% trypsin, and 0.53 mM EDTA in saline), and pooled with any cells in the growth media. Cells were then centrifuged for 10 minutes at 2,000 RPM at 4°C in a Sorvall refrigerated centrifuge. The supernatant was then aspirated, and the cell pellet was resuspended in 5 mL of PBS and centrifuged again as above. The supernatant was then aspirated, and the pellet was resuspended in 1 mL of lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.5% sodium dodecyl sulfate (SDS), 0.1 mg/mL Proteinase K).

The sample was then incubated for 18 hours at 50°C. The sample was split evenly into two 1.5 mL eppendorf tubes, resulting in 500 μL of sample in each tube. Then, 500 μL of phenol(pH 8)/chloroform/isoamyl alcohol (25:25:1) was added to the sample. The sample was then mixed using a Vortex Genie 2. The sample was then spun for 15 minutes at 14K RPM in an eppendorf microcentrifuge located inside a 4°C cold room. The supernatant was then removed and placed in a new eppendorf tube; the pH 8 phenol was discarded. Then, 1 mL of cold 100% EtOH and 50 μL of 3M sodium acetate were added to the sample in order to precipitate the DNA. The samples were placed in a -20°C freezer overnight.

The next day, the samples were removed from the freezer and spun at 14K RPM for 15 minutes in an eppendorf microcentrifuge in a 4°C cold room. The supernatant was aspirated, and 500 μL of 70% EtOH was added to each tube in order to remove excess salt from the precipitated DNA. The samples were then centrifuged for another 15 minutes at 14K RPM at 4°C. The supernatant was then aspirated, and the samples were dried in a speed vac until all of the 70% ethanol had evaporated. The samples were then resuspended in 1x TE (10 mM Tris-HCL pH 8.0, 1 mM EDTA pH 8.0). Samples were then RNased using 40 mg/mL of RNase A, in the presence of 0.1% SDS, and incubated in a 37°C water bath for 1 hour. Samples were then re-extracted and precipitated as above, and resuspended in 1x TE. An aliquot of the sample was then diluted, and the absorbance relative to water was determined at 260 nm using a Beckman spectrophotometer. The amount of DNA present was then calculated using the relationship: 1 optical density $(O.D.)_{260}$ = 50 μg of double-stranded DNA.

Agarose gel electrophoresis. DNA was electrophoresed on agarose gels in a mini-gel electrophoresis cell (Bio-Rad, Hercules, Ca.). Agarose gels were composed of 1.6% agarose with 40 mM Tris-acetate, 1 mM EDTA pH 8, and 0.5 μg/mL ethidium bromide. Typically, 10 μg of DNA, mixed with 4 μL of 6x DNA loading buffer (0.25% bromophenol blue,

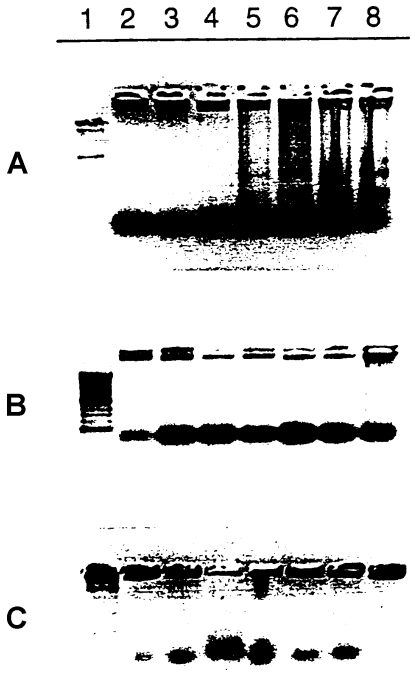

Figure 1. DNA extracted from LR73 cells (A), LR73#27 cells (B), and 1-1 cells (C) induced to apoptose by colchicine. Each lane contains 10 μg of DNA that has been electrophoresed on a 1.6% agarose gel. Lane 1: 100 base-pair ladder; lane 2: cells grown for three days without colchicine; lane 3: cells induced with 25 nM colchicine for 1 day; lane 4: cells induced with 100 nM colchicine for 1 day; lane 5: cells induced with 25 nM colchicine for 2 days; lane 6: cells induced with 100 nM colchicine for 2 days; lane 7: cells induced with 25 nM colchicine for 3 days; lane 8: cells induced with 100 nM colchicine for 3 days.

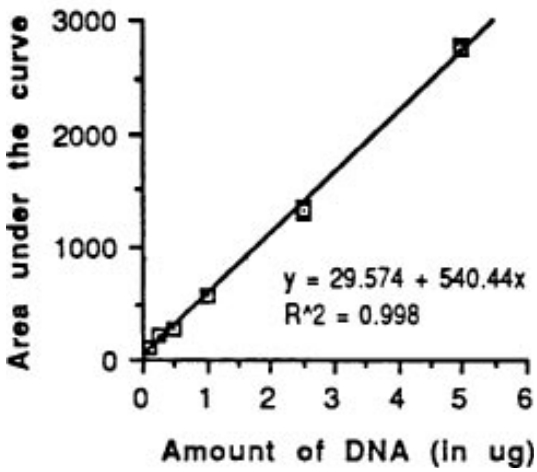

Figure 2. Standard curve generated using the average of five 0.8% agarose gels containing YH1 plasmid that were electrophoresed and quantitated with a densitometer.

0.25% xylene cyanol FF, 30% glycerol in water) was applied to each well of an 8-well agarose gel. The agarose gel was then electrophoresed for one to two hours at 40 volts. A ladder of 100 base pairs (Gibco BRL, Gaithsburg, Md.) was used as a standard. After one to two hours, the gel was removed from the electrophoresis cell, and the ethidium bromide-stained DNA on the gel was visualized under UV light.

Quantitation of DNA Fragmentation. In order to quantitate the percent of DNA in fragmented form, it was necessary to obtain a photographic negative of the gel. In order to obtain this negative, Polaroid 665 positive/negative film was exposed for 10 seconds while the gel was illuminated with 320 nm light from a UV lightbox, and the film was then allowed to develop for 30 seconds. The negative was then separated from the positive in complete darkness to ensure that the negative maintained the maximum possible resolution. The negative was then immersed in an 18% sodium sulfite bath for 1 minute in order to clear the residual developer layer and the opaque backcoat. The residual sodium sulfite was then washed off the negative by holding it under running water for three to four minutes. The negative was then air-dried, with care taken to avoid drying marks.

The negative was then scanned using a Stratagene Stratascan 7000 densitometer interfaced to an AST personal computer, running Stratascan 1D densitometry software. To determine if the densitometer was able to accurately quantitate the amount of DNA, a standard curve was produced using known quantities of DNA. It was determined that the amount of DNA vs. the density of the film was 99.8% consistent with a linear curve (see *Results*). In order to determine the amount of DNA in fragmented form, the amount of DNA smaller than 900 base pairs was ratioed vs. the total amount of DNA in each lane. The results were then graphed using Cricket Graph 1.3.2 software and a Macintosh IIcx computer.

Control. To ensure that only the variables under consideration effected the results, multiple controls were used. When apoptosis was induced by FCS withdrawal, or by treatment with colchicine, the same experiment was performed on all three cell lines at once, and one plate of cells from each cell line was grown in normal media without any drugs. DNA was also isolated from these untreated plates to be used as a control. When apoptosis was induced by FCS withdrawal or colchicine in the presence of verapamil, one plate of cells from each cell line was grown in normal media without any drugs, and one plate of cells from each cell line was grown in the presence of verapamil.

When plates of cells were induced to apoptose, each set of plates had previously (one to two days prior to the experiment) been split from the same “parental” plate. All experiments were arranged so that at the end of the testing interval (usually three days), all of the experimental plates and the control were at approximately the same degree of confluency (i.e., cell density/100 cm^2). When the DNA was electrophoresed on an agarose gel, it was always run alongside a 100 base pair ladder that served as a standard against which any apoptotic ladder in the samples was compared.

Results

Apoptosis was first induced in LR73 cells, LR73#27 cells, and 1-1 cells by treatment with 25 nM or 100 nM colchicine. DNA was extracted (see *Methods*), and electrophoresed on a 1.6% agarose gel (see Fig. 1). Note that “apoptotic DNA ladders” are observed in LR73 cells (Fig. 1A) only two days after apoptosis was induced by the addition of 25 nM or 100 nM colchicine, but DNA ladders are not seen in LR73#27 cells (Fig. 1B) until three days following treatment with 100 nM colchicine. DNA ladders were not observed at all for 1-1 cells (Fig. 1C) treated with either 25 nM or 100 nM colchicine even three days following treatment.

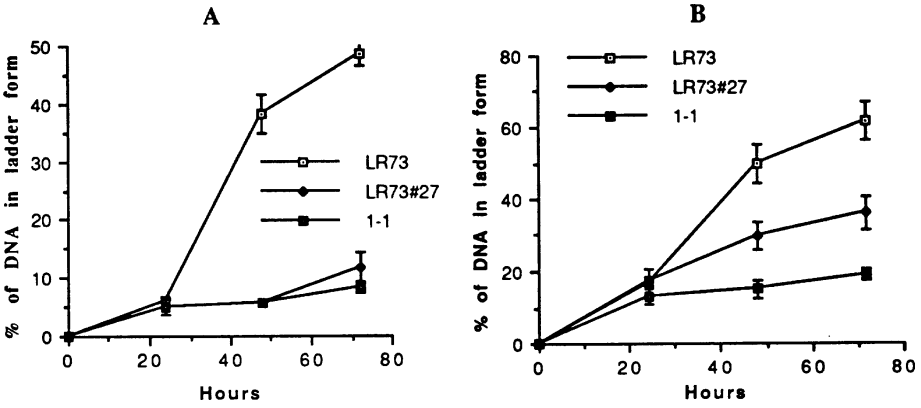

Figure 3. Timecourse of apoptosis induced by 25 nM (A) and 100 nM (B) colchicine. Each point is the average of three determinations of fragmented DNA electrophoresed on a 1.6% agarose gel and quantitated using a densitometer (see *Methods*). Error bars represent standard deviation.

The "percent apoptosis" (percent DNA in ladder form) for these cells was measured by using a densitometer to quantitate the percent of DNA in each lane that was in ladder (i.e., apoptotic) form (see *Methods*). The accuracy of this method was determined by electrophoresing small amounts of YH1 plasmid DNA that had been independently quantitated using a spectrophotometer (see *Methods*). As shown in Fig. 2, the densitometer provided an accurate measure of DNA content. The experiment shown in Fig. 3 was repeated three times, and the time of induction was plotted vs. the percent of DNA in fragmented form (Fig. 3).

It was possible that the apparent reduced efficiency of induction of apoptosis in LR73#27 cells and 1-1 cells was solely related to the resistance that these cells exhibit to colchicine. In order to determine if this was true, or if the apparent reduction of apoptosis was due to other actions of the MDR protein, cells were induced at the same effective dose of colchicine. As seen in Fig. 4, when apoptosis was induced by addition of the same effective dose of colchicine, the kinetics of apoptosis in all three cell lines are similar to that induced by the addition of 100 nM colchicine (Fig. 3).

Apoptosis was then induced by a method not based on chemotherapeutic drugs in order to test whether the MDR protein itself, not necessarily decreased drug retention caused by the MDR protein, was the reason that LR73#27 cells and 1-1 cells which overexpress the MDR protein exhibit resistance to the induction of apoptosis. Apoptosis was induced by the withdrawal of fetal calf serum (FCS) from the media in which the cell lines are typically grown. As seen in Fig. 5, LR73 cells exhibit an apoptotic ladder one day after the induction of apoptosis by this method, LR73#27 cells exhibit an apoptotic ladder three days after the induction of apoptosis, and 1-1 cells do not exhibit an apoptotic ladder. As shown in Fig. 6, the timecourse of apoptosis induced by FCS withdrawal is similar to that when apoptosis was induced by colchicine.

The effects of verapamil, a drug which can partially inhibit the function of the MDR protein (Ford and Hait, 1990) on apoptosis induced by colchicine and by fetal calf serum

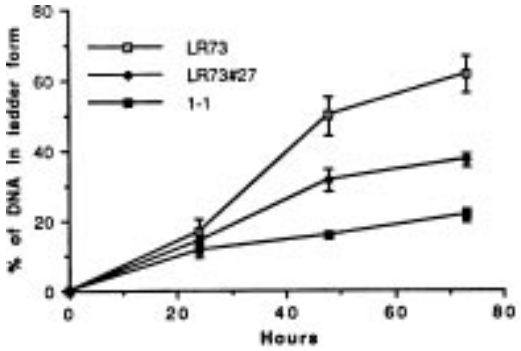

Figure 4. Timecourse of apoptosis induced by the IC_{50} of colchicine for each cell line: LR73 induced with 100 nM colchicine; LR73#27 induced with 400 nM colchicine; 1-1 induced with 1200 nM colchicine. Each point is the average of three determinations of fragmented DNA electrophoresed on a 1.6% agarose gel and quantitated using a densitometer (see *Methods*). Error bars represent standard deviation.

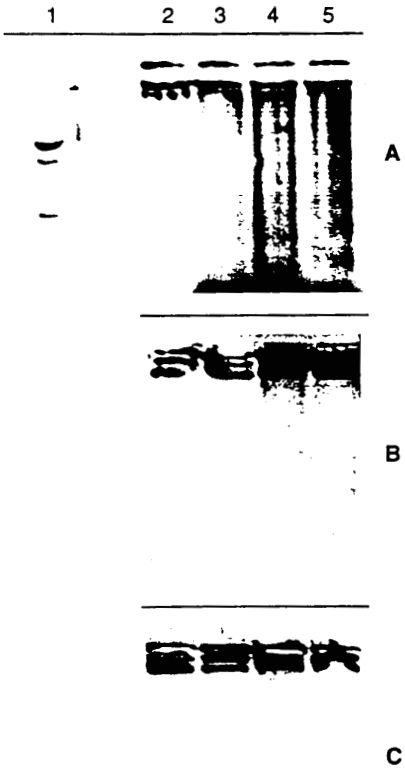

Figure 5. DNA extracted from LR73 (A), LR73#27 (B), and 1-1 (C) cells induced to apoptose by fetal calf serum (FCS) withdrawal. Each lane contains 10 µg of DNA that has been electrophoresed on a 1.6% agarose gel. Lane 1: 100 base-pair ladder; lane 2: cells grown for three days with FCS; lane 3: cells induced by FCS withdrawal for 1 day; lane 4: cells induced by FCS withdrawal for 2 days; lane 5: cells induced by FCS withdrawal for 3 days.

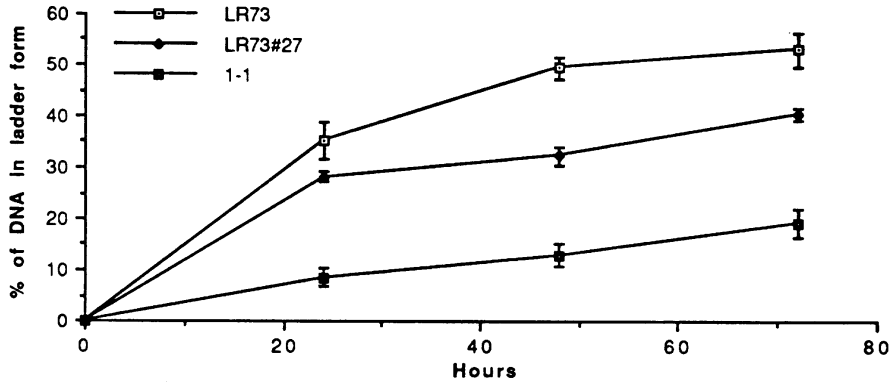

Figure 6. Timecourse of apoptosis induced by fetal calf serum (FCS) withdrawal. Each point is the average of five determinations of apoptotic DNA electrophoresed on a 1.6% agarose gel and quantitated using a densitometer (see *Methods*). Error bars represent standard deviation.

withdrawal were studied. As seen in Fig. 7, the percent of DNA in ladder form in LR73#27 cells and in 1-1 cells increases when apoptosis is induced by 100 nM colchicine (A) or by FCS withdrawal (B) in the presence of verapamil. The percent of LR73 cells undergoing apoptosis is not effected by the presence of 10 mM verapamil (data not shown).

Finally, it was necessary to measure the growth rate of all three cell lines to insure that the MDR cells did not have decreased growth rates in the presence of 100 nM colchicine or in the absence of FCS, since an increase in cell density may be associated with an increased rate of apoptosis. As seen in Fig. 8, 1-1 cells have the highest growth rate under all conditions. If the cells were undergoing apoptosis due to higher cell density, 1-1 cells, not LR73 cells, would apoptose more efficiently.

Discussion

In order to test the hypothesis that overexpression of the MDR protein inhibits the induction of apoptosis, it was necessary to induce apoptosis both by chemotherapeutic drugs and by a means that did not involve the use of chemotherapeutic drugs, in both sensitive cell lines and in cell lines that overexpress the MDR protein. Three cell lines were used in this study: LR73 Chinese hamster ovary fibroblasts which do not overexpress the MDR protein; LR73#27 transfectants, which overexpress the MDR protein but which were not previously selected with any chemotherapeutic drugs; and 1-1 transfectants further selected on vinblastine, which also overexpress the MDR protein. Two different cells lines that overexpressed the MDR protein by approximately the same amount were used to distinguish the effects of chemotherapeutic drug selection from the specific effects of MDR protein itself on the inhibition of apoptosis.

Apoptosis can be induced in several ways. First, to validate the techniques used, apoptosis was induced with colchicine, a chemotherapeutic drug known to efficiently induce apoptosis in many cell types. As shown in Fig. 1, apoptotic ladders can be observed after only two days of treatment with 25 nM or 100 nM colchicine in LR73 cells, but are not observed in LR73#27 cells until three days of treatment with 100 nM colchicine, and no DNA ladders are observed with these dosages in 1-1 cells. As can be seen in Fig. 3, when time of induction was plotted vs. percent of DNA in ladder form, much more of the DNA from LR73 is in ladder form than is the DNA from LR73#27 cells, which in turn harbored more DNA in ladder form than did the 1-1 cells. In Fig. 3B, when apoptosis was induced with 100 nM colchicine for three days, 62% of the DNA from LR73 cells was in ladder form, while only 36% of the DNA from the LR73#27 cells and 18.5% of the DNA from 1-1 cells was in ladder form.

However, these results could be the effect of a different "effective drug dose" for each cell line caused by the overexpression of the MDR protein. Therefore, the different cell lines were induced to apoptose at the same effective dose of colchicine, that is, their relative $IC_{50.}$ As shown in Fig. 4, when apoptosis was induced at the same growth inhibitory concentration, the timecourse of apoptosis still differed dramatically.

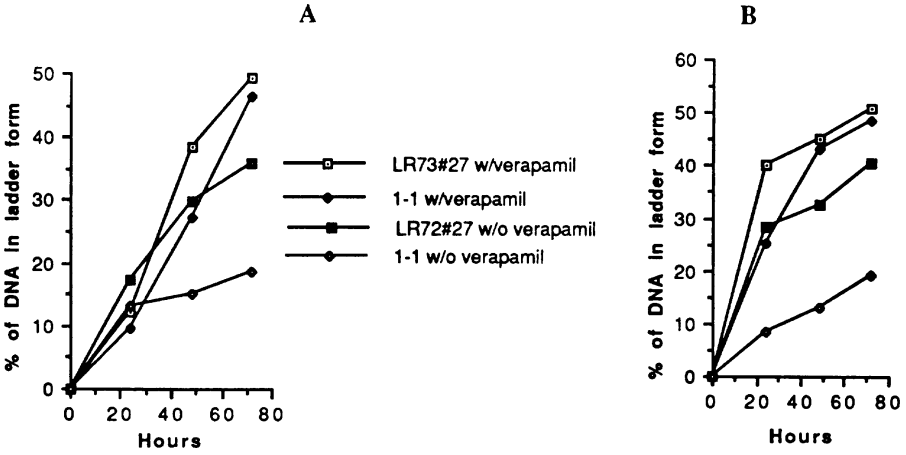

Figure 7. Timecourse of apoptosis induced by 100 nM colchicine (A) and by FCS withdrawal (B) in the presence or absence of 10 µM verapamil. Each point is the average of two experiments.

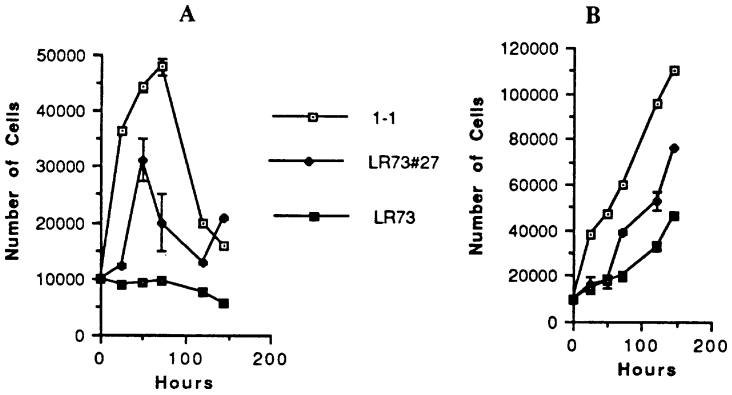

Figure 8. Growth of LR73 cells, LR73#27 cells, and 1-1 cells without fetal calf serum (A) and in the presence of 100 nM colchicine (B). Each data point represents the average of three sets of data. Error bars represent standard deviation.

Apoptosis was then induced by the withdrawal of fetal calf serum (FCS) from the media in which the cell lines are typically grown. This method of inducing apoptosis was selected due to its inability to be directly effected by the MDR protein if, in fact, the MDR protein functions as a "drug pump." The "drug pump" model, although widely debated, is still the prevailing model used to explain the decreased drug retention that defines the MDR phenotype. If the MDR protein also functions by inhibiting the induction of apoptosis, apoptosis induced by withdrawal of FCS should be inhibited in LR73#27 cells and 1-1 cells. If the inhibition of apoptosis observed when apoptosis was induced by chemotherapeutics was caused by decreased drug retention, or by a "drug pump," apoptosis induced by FCS withdrawal should not be inhibited in cells overexpressing the MDR protein.

As is shown in Fig. 5, apoptosis induced by FCS withdrawal results in a clear apoptotic ladder after only one day in LR73 cells, but does not appear until three days after FCS withdrawal in LR73#27 cells, and a clear ladder does not appear at all in 1-1 cells. As shown in Fig. 6, when the percent of DNA in ladder form is plotted vs. the time of FCS withdrawal, the timecourse of apoptosis induced by FCS is seen to be similar to the timecourse of apoptosis induced by colchicine (Fig. 3, Fig. 4). Three days after fetal calf serum withdrawal, 53% of the DNA from LR73 was in ladder form. However, after three days of FCS withdrawal, only 40% of the DNA from LR73#27 cells and only 19% of the DNA from 1-1 cells was in ladder form.

After showing that apoptosis was apparently inhibited in cells overexpressing the MDR protein, the kinetics of apoptosis induced in the presence of 10 μM verapamil was studied. Verapamil is a Ca^{2+} channel blocker that has been shown to be effective in partially reversing the MDR phenotype. As shown in Fig. 7, apoptosis in LR73#27 cells and 1-1 cells induced by either 100 nM colchicine or by FCS withdrawal increases in the presence of verapamil. The percent of DNA in ladder form harbored by 1-1 cells more than doubles in the presence of verapamil.

It remained possible that the large percent of apoptosis observed in LR73 cells when apoptosis was induced by colchicine or by FCS withdrawal was due to a higher growth rate of LR73 cells under these conditions. It has been observed that cells spontaneously undergo apoptosis at a very high cell density. As shown in Fig. 8, under all conditions, 1-1 cells exhibit the highest growth rate. If high cell density was the reason that the cells apoptosed, 1-1 cells should have exhibited the most apoptosis. That they do not suggests that the above results were not unduly skewed by apoptosis caused by high cell density.

Conclusions. The results of this study can be summarized as follows:

(1) Apoptosis induced without chemotherapeutics (by the withdrawal of FCS) follows the same kinetic pattern as does apoptosis induced by some chemotherapeutic drugs (e.g., colchicine). A similar pattern is seen during the first 24 hours of apoptosis induced by doxorubicin (data not shown).

(2) When apoptosis is induced by colchicine at the same effective concentration, the MDR cells continue to be more resistant to the induction of apoptosis.

(3) When apoptosis is induced by FCS withdrawal or by colchicine in the presence of verapamil (which can partially reverse the effects of the MDR protein), the percent of DNA in ladder form increases.

This study demonstrates that the overexpression of MDR protein is directly related to apparent inhibition of induction of the apoptotic cascade. When apoptosis is induced by several different means, MDR cells consistently apoptose less efficiently than do sensitive cells. Prior to this study, it could be argued that the inhibition of apoptosis induced by chemotherapeutic drugs for MDR cells was a result of decreased drug retention (i.e., an indirect result of MDR protein function). However, the inhibition of apoptosis in MDR cells when apoptosis is induced by FCS withdrawal implies that the overexpression of MDR protein is directly involved in the inhibition of the apoptotic cascade. Furthermore, since verapamil (a specific inhibitor of MDR protein function) can reverse this inhibition, it is likely that the function of MDR protein is responsible for this effect.

It is possible that the MDR protein inhibits the induction of apoptosis by altering the intracellular pH of MDR cells. Studies have shown that several MDR cell lines exhibit an elevated intracellular pH (reviewed in Roepe, 1995). It has been suggested that the action of DNase II, an acidic endonuclease, is responsible for some of the effects of apoptosis (Barry and Eastman, 1993). If this is true, the MDR protein could inhibit the induction of apoptosis by elevating intracellular pH and inhibiting the action of DNase II.

Future Research. Although this work suggests that the MDR protein may inhibit the induction of apoptosis, the mechanism by which it accomplishes this is still unclear. Although this work shows that verapamil can reverse this effect, it is

also unknown how verapamil inhibits the function of the MDR protein. One hypothesis for verapamil function proposes that it acts as a competitive inhibitor for the MDR protein "drug pumping." Future research should include studies of apoptosis in other cell lines that overexpress the MDR protein; changes in pHi in apoptosing cells; and, finally, an eventual determination of the molecular mechanism of MDR protein function.

Applications. The MDR phenotype is a serious problem in the effective chemotherapeutic treatment of cancer. Understanding how the MDR protein interferes with the action of chemotherapeutic drugs and the role of the MDR protein in the inhibition of apoptosis may provide the information needed to develop more effective chemotherapeutic drug protocols for clinical applications.

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Endnotes

(1) Abbreviations used in this paper: MDR, multidrug resistance; FCS, fetal calf serum; DNA, deoxyribonucleic acid; pHi, intracellular pH; RNA, ribonucleic acid; DME, Dulbecco's modified eagle medium; EDTA, ethylenediaminetetraacedic acid; G418, antibiotic Geneticin-418 sulfate; PBS, phosphate buffered saline; RPM, revolutions per minute; SDS, sodium dodecyl sulfate; TE, 10 mM Tris-HCl, 1 mM EDTA pH 8.0; UV, ultraviolet light; IC_{50}, concentration of drug that inhibits growth by 50%.

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