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ACTIVATION OF PROGRAMMED CELL DEATH (APOPTOSIS) BY CISPLATIN, OTHER ANTICANCER DRUGS, TOXINS AND HYPERTHERMIA

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Abstract—Cell death induced by cisplatin was studied in Chinese hamster ovary cell lines, one proficient and the other deficient (100-fold sensitive) in DNA excision repair. Previous experiments demonstrated that cells progressed to and arrested in the G_2 phase of the cell cycle before dying. DNA double-strand breaks were detected following G_2 arrest and prior to loss of membrane integrity. These DNA breaks have been studied in more detail. DNA fragments were observed consisting of multimers of approximately 180 base pairs. These fragments are consistent with internucleosomal cleavage of chromatin by an endonuclease. At LC_{90} concentrations, DNA digestion began 48 hr after cisplatin treatment followed by loss of membrane integrity and cell shrinkage 24 hr later. High concentrations of cisplatin (170 logs of kill) induced DNA digestion 12 hr after drug treatment but loss of membrane integrity occurred 12 hr later. Both cell death and DNA fragmentation were inhibited by cycloheximide, suggesting the requirement for new protein synthesis. Cells incubated with many other agents demonstrated the same characteristic pattern of DNA degradation. At 90% lethal conditions, DNA digestion was induced within 30 min by hyperthermia, 18 hr by methotrexate, and 48–72 hr by all other agents tested. DNA digestion always preceded loss of membrane integrity and cell shrinkage. These observations are consistent with cell death occurring by the process of apoptosis, or prorammed cell death, and demonstrate the importance of DNA digestion as an early and presumably essential step in cell death. The results suggest that, irrespective of the primary site of action of a drug, cell death by most pharmacologic agents is mediated by activation of the signal transduction pathway for apoptosis. The results also suggest two signal pathways for apoptosis, one directly associated with drug action and a second that requires cell cycle-related events.

Cisplatin is an effective chemotherapeutic agent that elicits its antineoplastic activity by binding to DNA and disrupting template functions [1]. Inhibition of DNA synthesis has generally been considered critical for toxicity. However, recent work from this laboratory demonstrated that cells die at drug concentrations that do not inhibit DNA synthesis [2, 3]. These cells proceed through normal cell cycling, arrest in the G_2 phase, and die after a latency of a few days. During this time, protein and RNA synthesis as well as ATP and NAD pools appear normal [4]. DNA double-strand breaks are detected during this latency period and are considered the earliest indicator of cell death [2]. The current experiments were designed to investigate the origin of these DNA double-strand breaks.

Wyllie *et al.* [5] characterized two mechanisms of cell death: necrosis and apoptosis. These two processes differ both morphologically and biochemically. The morphological changes associated with necrosis are swelling, followed by rupture of membranes and dissolution of organized structure. By contrast, during apoptosis, cell shrinkage and chromatin condensation occur while normal organelle structure is maintained. Biochemically, necrosis results from loss of osmoregulation, with DNA digestion by lysosomal enzymes occurring as a late event. One of the earliest steps in apoptosis appears to be DNA digestion but, because the lysosomes remain intact, the endonuclease involved is believed to be non-lysosomal.

Apoptosis is an active process requiring new protein synthesis. It is observed in controlled deletion of cells during metamorphosis, differentiation and general cell turnover and appears normally to be regulated by receptor-coupled events [5]. For these reasons, apoptosis has been called "programmed cell death" or "cell suicide." It is important to realize that although every cell has this genetic program to commit suicide, it is usually suppressed. Under normal circumstances, only those cells no longer required by an organism activate this program.

Many studies of apoptosis have focused on mouse thymocytes because of their suicide response to glucocorticoids. Within a few hours of glucocorticoid addition, thymocyte DNA is digested by an endonuclease, giving rise to a nucleosome "ladder" of DNA fragments detected by electrophoresis. This endonucleolytic digestion is considered an essential step in apoptosis [5]. Recent studies have also demonstrated that radiation, dioxin and several anticancer drugs induce thymocytes to digest their DNA [6–8]. Most other studies have focused on immunologic systems such as T lymphocyte-mediated death of target cells or tumor necrosis factor-induced cell death [9, 10]. By contrast, cell death by anticancer agents and in other systems has been poorly studied, and apoptosis has been implicated on morphological grounds in only a few cases [5].

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In this paper, we demonstrate that cisplatin induced events analogous to those observed during apoptosis. A Chinese hamster ovary cell line, CHO/AA8, and a DNA excision repair-deficient cell line, CHO/UV41, were used [11]. We employed a modified gel electrophoresis procedure that facilitates direct-loading of cells into an agarose gel and provides rapid visualization of the characteristic nucleosome ladder of apoptosis [4]. We also investigated other elements of apoptosis: changes in membrane integrity, cell size and morphology, and the ability of cycloheximide to inhibit the process. Other drugs, as well as hyperthermia, were investigated to determine whether they induce the same mechanism of cell death.

MATERIALS AND METHODS

Materials. Cisplatin in the form of Platinol was purchased from Bristol-Meyers (Syracuse, NY). Etoposide was a gift from Bristol-Meyers. Ouabain was purchased from Boehringer Mannheim (Indianapolis, IN). All other drugs, chemicals and enzymes were purchased from the Sigma Chemical Co. (St. Louis, MO). Ultrapure agarose was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Molecular weight DNA standards were obtained from New England Biolabs (Beverly, MA). Cell culture materials were obtained from Gibco Laboratories (Grand Island, NY).

Cell culture. Chinese hamster ovary cells, AA8 and UV41, were obtained from Dr. Larry Thompson, Berkeley, CA, and maintained in α-Minimum Essential Medium supplemented with 2.5% fetal bovine serum, 2.5% horse serum, penicillin and streptomycin. Cells were allowed to attach to culture flasks for 24 hr, and then were incubated for 2 hr with appropriate concentration of cisplatin. Next the cells were washed with Hanks' Balanced Salt Solution and fresh medium was added. A short incubation time was chosen to ensure that the observed changes occurred after a lethal treatment, and therefore would not be affected by toxicity increasing with time of incubation. Other agents were incubated with cells for either 1 hr or continuously as noted in Table 1. The continuous incubations were required for cell cycle phase-specific drugs. Survival values were calculated from the number of colonies at 7 days and expressed as either LC_{50} or LC_{90}, that is, the drug concentration that reduced colony formation by 50 or 90% respectively. From 0–4 days, the cells were harvested for gel electrophoresis. In every experiment, floating cells in the culture medium were combined with attached cells harvested by trypsinization. The cells were counted with a Coulter Counter (Hialeah, FL) and sized with a Coulter Channelyzer. Aliquots containing 10^6 cells were rinsed in cold (4°) phosphate-buffered saline and centrifuged for 5 min at 1000 rpm. The supernatant was discarded and the cells were stored as a pellet on ice until electrophoresis (less than 10 min).

S49.1 mouse thymocytes were obtained from the Amercian Type Culture Collection (Rockville, MD) and maintained in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum, penicillin and streptomycin.

Electrophoresis. Cells were analyzed by a gel electrophoresis method adapted from that of Eckhardt [12] and detailed elsewhere [4]. Briefly, 10^6 cells were pipetted directly into the wells of a 2% agarose gel, lysed and digested with proteinase K; then the DNA was separated by electrophoresis. RNA was removed subsequently from the gel by incubation with ribonuclease A, and the DNA was visualized with ethidium bromide. This method obviates the need to purify DNA before electrophoresis. Hence, unlike previous methods, the majority of DNA is still high molecular weight and remains in, or close to, the loading well.

The approximate amount of DNA detected in these gels has been estimated previously [4]. Minimally detectable degradation represented about 50 ng while the maximum degradation detected in these experiments was about 500 ng. The total DNA applied to each well was 10–20 μg; the latter value reflects cells with twice the normal content of DNA as a result of arresting in G_2.

Trypan blue exclusion. Exclusion was assayed with aliquots of the cell preparation used for electrophoresis. Cells resuspended in culture medium were stained by addition of an equal volume of 0.4% trypan blue in 0.7% saline and counted on a hemocytometer. Alternatively, EDTA was added directly to cell cultures to give a final concentration of 10 mM. After 5 min, the cells were detached by slapping the flask and aliquots were stained with an equal volume of 0.4% trypan blue.

RESULTS

Toxicity. Survival of AA8 and UV41 cells after a 2-hr incubation with cisplatin was determined previously by colony formation, and survival curves have been published [3]. The repair-deficient UV41 cell line had an LC_{50} value of 0.1 μM. This compares to an LC_{50} of 12 μM in the AA8 cells. Hence, the UV41 cells are approximately 100-fold more sensitive to cisplatin. Toxicity with respect to the other agents was determined in AA8 cells and is expressed as LC_{90} values in Table 1.

DNA digestion. DNA digestion was assessed in the AA8 and UV41 cells after incubation with cisplatin. A characteristic pattern of DNA fragments was observed which consisted of multimers of approximately 180 base pairs (Fig. 1). These digestion products correspond to the nucleosome ladders characteristic of apoptosis [5]. The LC_{90} concentrations in the UV41 (0.4 μM) and AA8 cells (48 μM) produced distinct nucleosome ladders by 48 hr. At higher concentrations for UV41 (6 μM) and AA8 (96 μM), distinct nucleosome ladders were first detected at 24 hr. The most rapid DNA digestion was observed in UV41 cells 12 hr after incubation with 60 μM cisplatin (not shown). This concentration of drug extrapolates to 170 logs of kill in this cell line. These observations demonstrate that the occurrence of DNA degradation is always an indication of cell death, but that the timing of DNA digestion is related to drug concentration.

The amount of DNA detected as degraded in these experiments varied from about 0.5 to 5% of the total cellular DNA. For comparison, S49.1 mouse

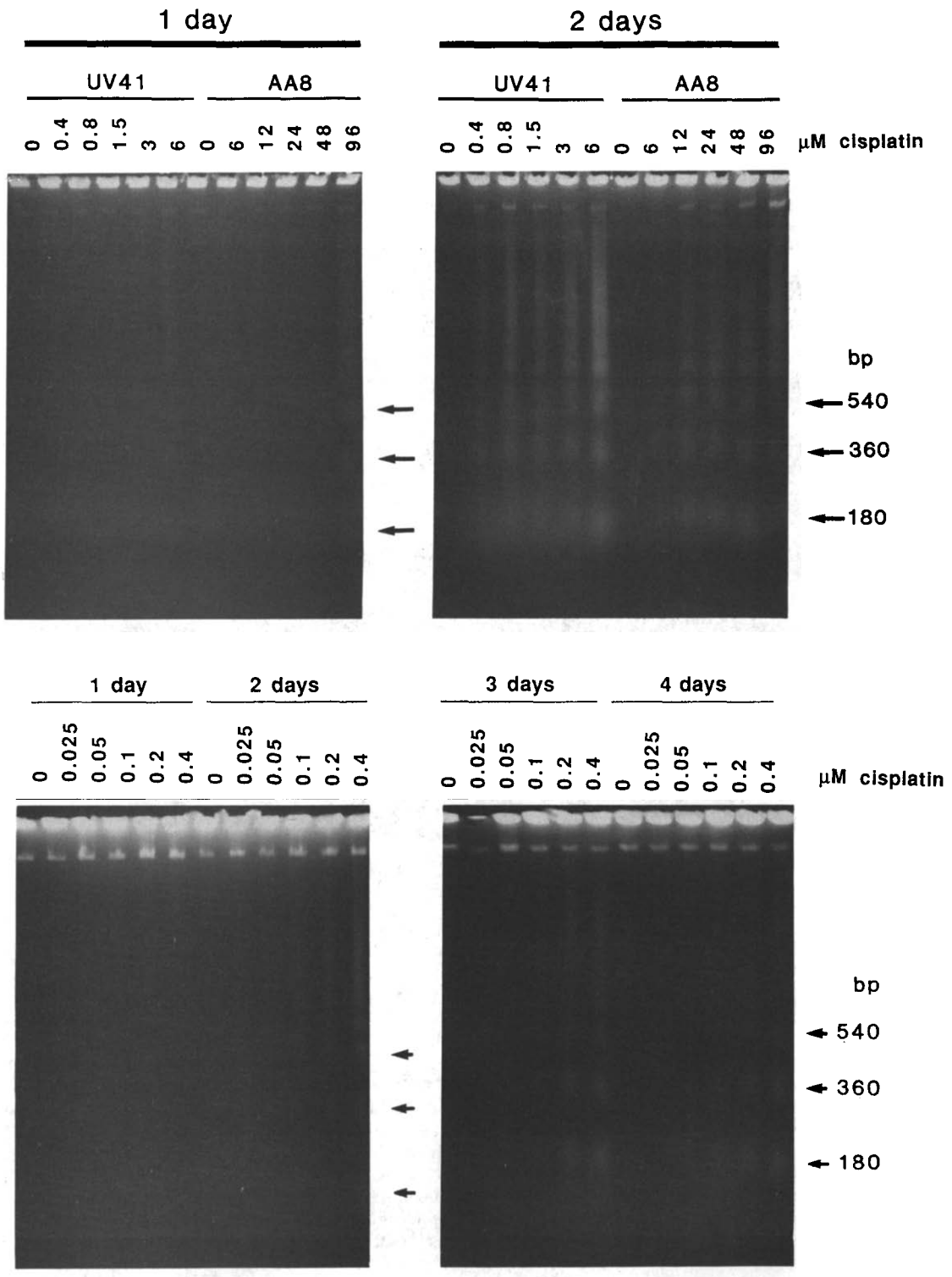

Fig. 1. DNA degradation in CHO/AA8 and CHO/UV41 cells following incubation with cisplatin. Cells were incubated with the indicated concentrations of cisplatin for 2 hr and harvested 1–4 days later; then DNA degradation was analyzed by gel electrophoresis. Top panels: AA8 and UV41 cells 1 and 2 days after incubation with cisplatin. Bottom panels: UV41 cells 1–4 days after incubation with cisplatin at lower concentrations than in the top panels. The molecular weights indicated were obtained from adjacent lanes containing MspI-digested pBR322 DNA standards.

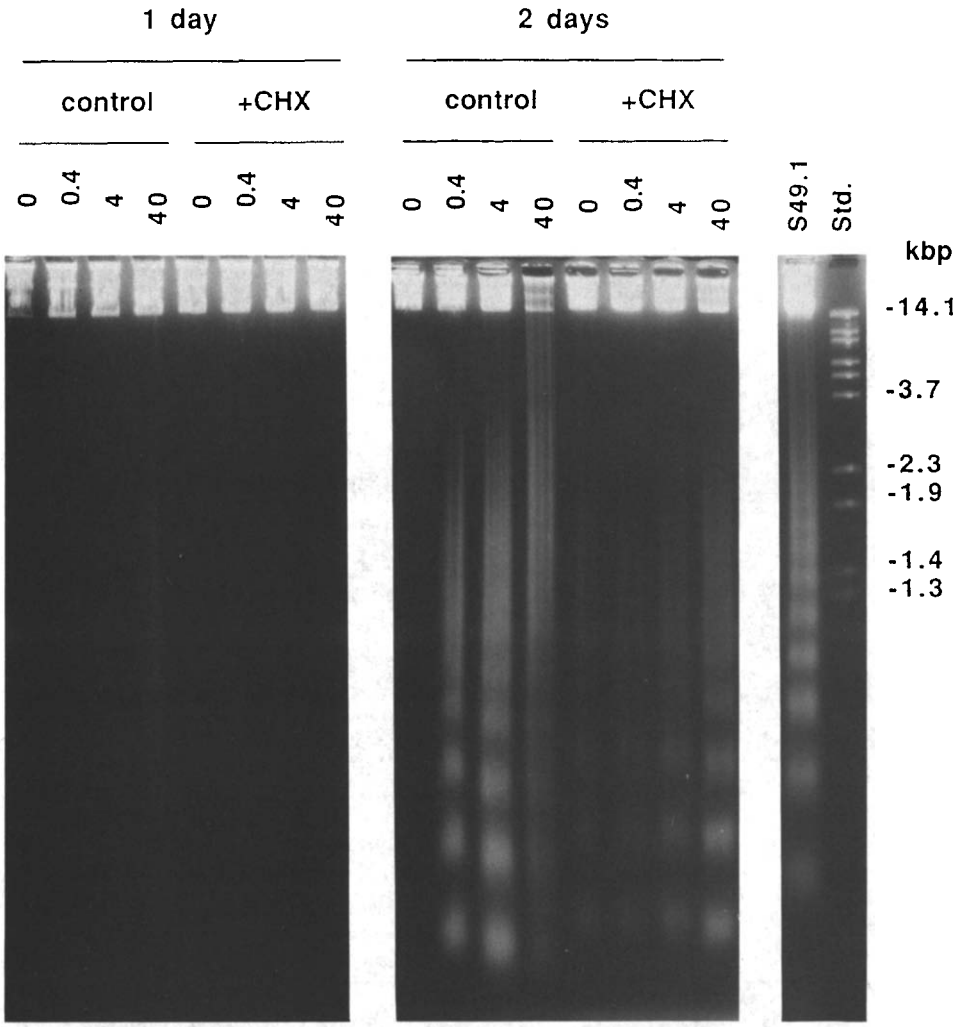

Fig. 2. Inhibition by cycloheximide of cisplatin-induced DNA degradation. CHO/UV41 cells were incubated with 0–40 μM cisplatin for 2 hr, followed by a 1- or 2-day incubation in the presence or absence of 1 μg/mL cycloheximide. Cells were harvested and DNA degradation was assessed. The right panel is a comparison with the degradation induced in S49.1 mouse thymocytes after a 24-hr incubation in 10 μg/mL dexamethasone. The smallest DNA fragments visible are dinucleosome length; the mononucleosomes have just eluted from the gel. The molecular weight standards are BstEII-digested λ DNA.

thymocytes were also analyzed for DNA digestion after a 24-hr incubation with dexamethasone (Fig. 2). Under these conditions, it is usually assumed that all of the cells are undergoing apoptosis [13, 14]. The nucleosome ladder was readily detected, and the DNA in the ladder represented about 5% of the total cellular DNA.

In subsequent studies with cisplatin, we focused on the UV41 cell line to avoid observing any DNA breaks formed by excision repair events. In addition, these cells exhibit a very pronounced G_2 arrest prior to dying [3].

Membrane integrity. To determine whether the observed DNA digestion was an early event in cell death, we assessed the timing of trypan blue uptake as a measure of loss of membrane integrity. In UV41 cells, trypan blue uptake was first observed 96 hr after incubation with an LC_{50} concentration and 72 hr after incubation with an LC_{90} concentration of cisplatin (Fig. 3A). In each case this occurred 24 hr after distinct nucleosome ladders were observed. The most rapid loss of membrane integrity occurred following treatment with 40 μM cisplatin. Trypan blue uptake was observed after 24 hr with almost total loss of membrane integrity by 48 hr. Even in this case, DNA degradation preceded loss of membrane integrity by at least 12 hr.

Inhibition of protein synthesis. UV41 cells were incubated with 1 μg/mL cycloheximide following a 2-hr incubation with cisplatin. The toxicity of continuous treatment with cycloheximide does not facilitate 7-day colony-forming assays, so toxicity was monitored at shorter time points by trypan blue exclusion (Fig. 3B). At very toxic concentrations of cisplatin (40 μM), cycloheximide delayed toxicity by about 1 day. However, at lower cisplatin con-

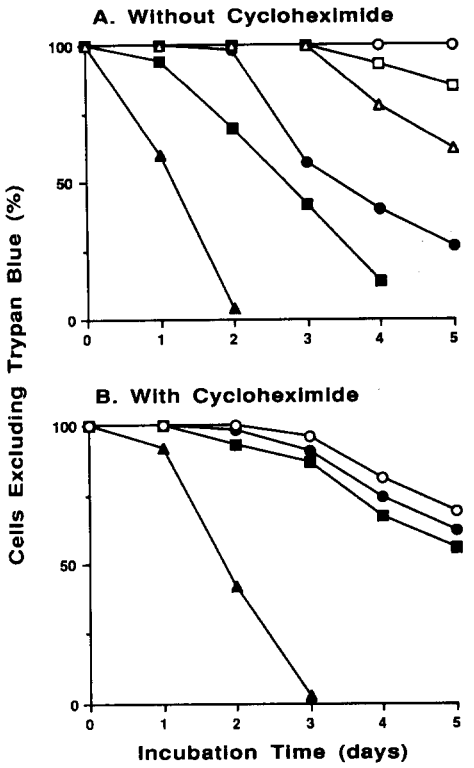

Fig. 3. Exclusion of trypan blue by CHO/UV41 cells at various times after a 2-hr incubation with the following concentrations of cisplatin: control (○), 0.1 μM (□), 0.2 μM (△), 0.4 μM (●), 4 μM and (■) and 40 μM (▲). In panel B, the cells were incubated in the presence of 1 μg/mL cycloheximide continuously after the cisplatin incubation.

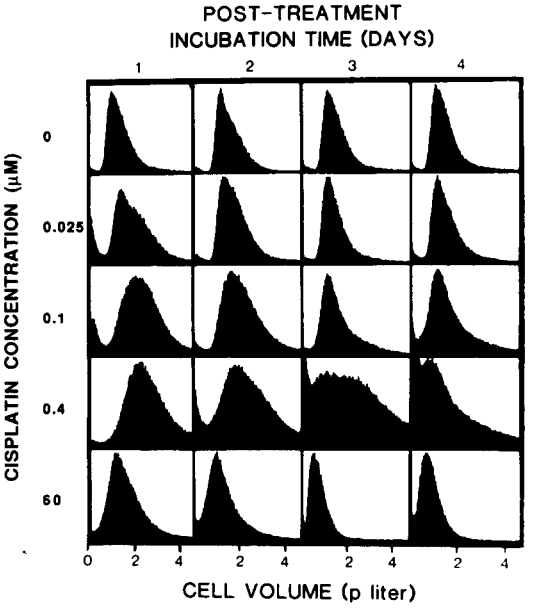

Fig. 4. Changes in size of CHO/UV41 cells following a 2-hr incubation with the indicated concentrations of cisplatin.

centrations, cycloheximide delayed the loss of membrane integrity by almost 2 days. This delay resulted in "survival" close to that for cells incubated only with cycloheximide and suggests that cycloheximide provided total protection from cisplatin-induced toxicity within the limits of its own toxicity.

In parallel experiments, the effect of cycloheximide on DNA digestion was studied (Fig. 2). Digestion was faintly visible within 1 day after 40 μM cisplatin, but this was not observed if the cells were incubated with cycloheximide during this period. After 2 days, DNA digestion was evident at all cisplatin concentrations. In cells incubated with 40 μM cisplatin, much of the high molecular weight DNA had also disappeared from the well. The amount of DNA degradation was markedly less in cells incubated with cycloheximide. Faint DNA degradation was also visible in cells incubated with only cycloheximide for 2 days. This level of DNA degradation was comparable to that in cells incubated with 0.4 μM cisplatin plus cycloheximide. Therefore, under these conditions, cycloheximide appears to provide complete protection from cisplatin-induced DNA degradation.

Cell size. While measuring trypan blue exclusion, it was evident that the platinated cells excluding dye were larger than control cells, whereas the blue cells were much smaller and often fragmented. To better assess the cisplatin-induced changes in cell size, the cells were harvested and analyzed on a Coulter channelyzer. At most cisplatin concentrations, the first detectable change was an increase in heterogeneity and median cell size (Fig. 4). At the LC_{50} concentration (0.1 μM), conditions under which many cells survive, this increased heterogeneity was transient with return to a normal size distribution by 3 days. This increase in size correlated with the time that these concentrations cause G_2 arrest and release [3]. At the LC_{90} concentration (0.4 μM), the cell size increased initially but shrank by day 4 to a median size of less than 1 pL; a size considerably less than that of undamaged cells. The highest concentration led to a marked shrinkage visible at 72 hr without an initial increase in size. At this concentration, these cells do not undergo G_2 arrest and seem to die from all phases of the cell cycle. Therefore, under the toxic conditions investigated, all the dying cells shrank. This occurred after DNA degradation and perhaps simultaneously with loss of membrane integrity.

Other agents. Cell death induced by other agents was investigated in the AA8 cell line. First, a survival curve was generated for each drug. Based upon these values, drug concentrations were selected that represented an equivalent range of toxicities. Cells were harvested and the integrity of their DNA was assessed. The results obtained with methotrexate are presented here as a further example (Fig. 5). Cells were incubated with methotrexate for up to 24 hr. At 6-hr intervals, cells were harvested and their DNA was analyzed by gel electrophoresis. Internucleosomal digestion of DNA was readily apparent and was both time and concentration dependent. At the higher drug concentrations, digestion was visible at 12 hr, while at all toxic concentrations it was detectable by 18 hr.

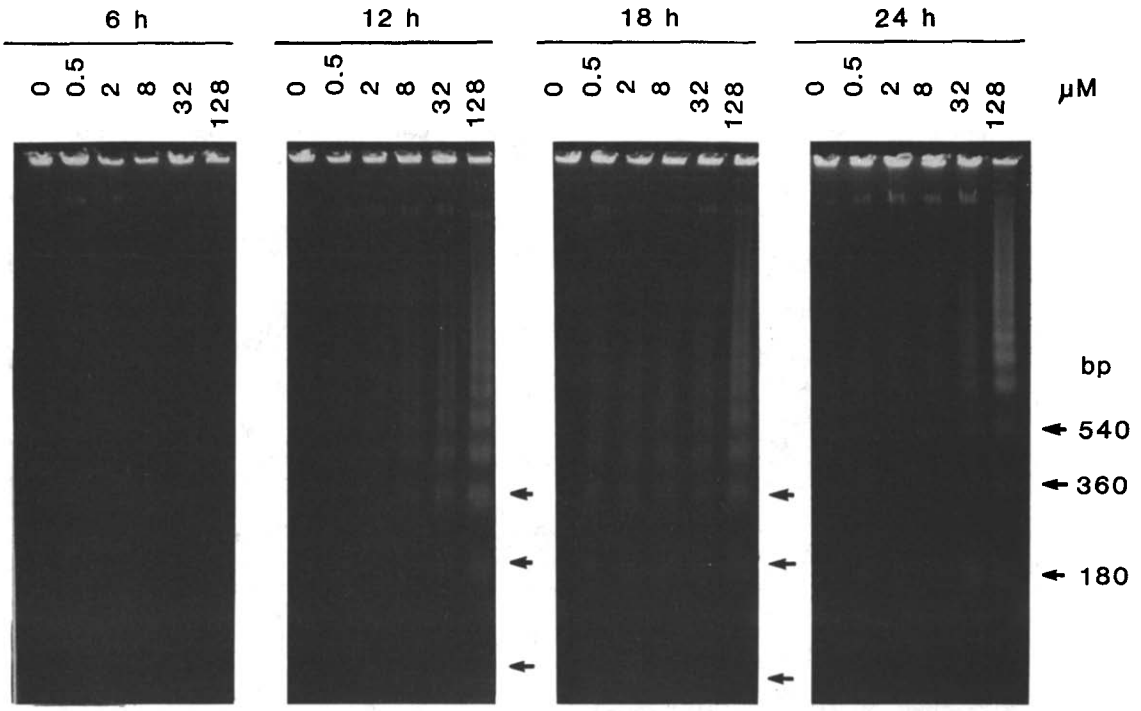

Fig. 5. DNA degradation produced in CHO/AA8 cells during incubation with methotrexate. Cells were incubated with 0–128 μM methotrexate for 6–24 hr. The cells were harvested and DNA degradation was analyzed by gel electrophoresis.

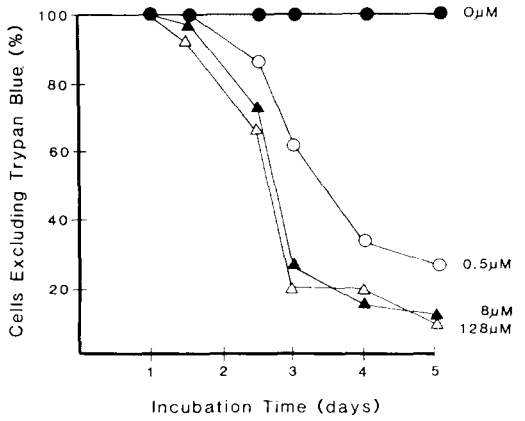

Fig. 6. Exclusion of trypan blue by CHO/AA8 cells during continuous incubation with the indicated concentrations of methotrexate.

For these DNA breaks to be indicative of apoptosis, they should occur as an early event in cell death, in particular, prior to loss of membrane integrity. Trypan blue exclusion clearly demonstrated no loss in membrane integrity until 36 hr of methotrexate treatment and not until 3 days did about 50% of the cells take up dye (Fig. 6). This was long after DNA degradation was observed.

Cells incubated with methotrexate were investigated for changes in cell size (Fig. 7). As with cisplatin, the earliest change was an increase in cell size. By 60 hr, a subpopulation of cells demonstrated marked shrinkage. At longer time periods, all the dying cells shrank. The timing of this shrinkage closely correlated with the loss of membrane integrity. It is important to emphasize that cell shrinkage and trypan blue uptake occurred 1–2 days after DNA degradation was detected.

With every other agent investigated, we detected internucleosomal digestion of the DNA, but the timing appeared characteristic for the agent (Table 1). At equitoxic conditions, methotrexate and hyperthermia induced DNA degradation within 18 hr and 90 min (60 min at 43° followed by 30 min at 37°) respectively. With all the other agents, DNA digestion was not observed until 2–3 days after treatment. Hyperthermic treatment was studied in more detail; cell shrinkage and trypan blue uptake were not observed before 24 hr (not shown). The toxicity resulting from hyperthermia could be eliminated completely by incubation with 1 μg/mL cycloheximide from 2 hr before to 2 hr after hyperthermia (tested up to 90 min at 43°).

DISCUSSION

Our previous observation that DNA double-strand breaks occur in dying cells, and appear to be an early indicator of cell death [2], led to the present

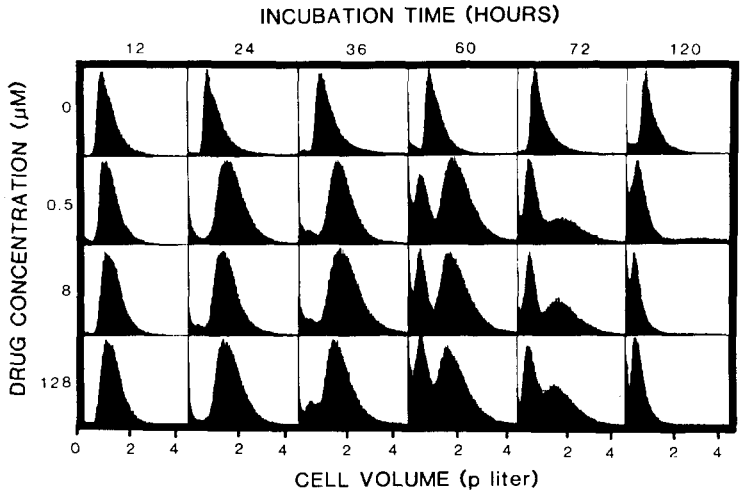

Fig. 7. Change in cell size during incubation with methotrexate. CHO/AA8 cells were incubated with 0–128 μM methotrexate for 6–24 hr and then sized on a Coulter counter with channelyzer.

Table 1. Earliest time that DNA degradation was detected in CHO/AA8 cells incubated with equitoxic concentrations of different agents

Drug	Target	LC_{90} (exposure time)	Time to DNA degradation (hr)
Methotrexate	Dihydrofolate reductase	0.3 μM (continuous)	18
5-Fluorodeoxyuridine	Thymidylate synthetase	1.0 μM (continuous)	48
5-Fluorouracil	Thymidylate synthetase and DNA/RNA precursor	10 μM (continuous)	72
Aphidicolin	DNA polymerase α and δ	0.2 μM (continuous)	48
Etoposide	DNA topoisomerase II	40 μM (1 hr)	48
Cisplatin	DNA (bifunctional)	48 μM (2 hr)	48
N-Methyl-*N'*-nitro *N*-nitrosoguanidine	DNA (monofunctional)	2 μM (1 hr)	72
Ouabain	Na^+,K^+-ATPase	1.3 mM (continuous)	48
Hyperthermia	Unknown	43° (1 hr)	0.5

experiments designed to better characterize this DNA degradation. Electrophoresis of DNA from cells damaged with cisplatin clearly demonstrated the multimeric pattern characteristic of internucleosomal cleavage of the genomic DNA. In both AA8 and UV41 cells, the appearance of this DNA digestion 48 hr after incubation with cisplatin correlated with toxicity. At non-toxic concentrations of cisplatin, no DNA degradation was detected. At higher concentrations, DNA degradation could be induced to occur much more rapidly. Our results show a comparable extent of DNA digestion in cells dying from either cisplatin or dexamethasone (Fig. 2). In the latter case, it is usually considered that dexamethasone is killing all the cells [13, 14]. Accordingly, when 5% of the DNA is detected as nucleosome fragments, it is consistent with all the cells dying. The nucleosome-length DNA only represents the end product of excessive digestion; much of the DNA is digested yet still electrophoreses as relatively high molecular weight DNA. The presence of this high molecular weight DNA is also observed morphologically in dying cells as condensed chromatin just inside the nuclear envelope [5]. Furthermore, the presence of a given nucleosome fragment is probably transient, as demonstrated by the UV41 cells after incubation with 40 μM cisplatin (Fig. 2). More than 50% of the high molecular weight DNA had been digested within 2 days, while less than 5% of the DNA was present in the nucleosome ladder. Presumably, nucleosome fragments can be either lost from the cells or digested further so that they are no longer detected. The appearance and disappearance of nucleosome fragments are therefore dynamic, and only some of the digestion products will be detected at any specific time point. The concentrations of cisplatin used in the current experiments produced greater than 90% kill and an amount of DNA digestion that is comparable to cells dying from dexamethasone treatment. It can be concluded, therefore, that these results reflect a toxic response to cisplatin of all of the cells, rather than just a small fraction of cells dying and digesting all of their DNA to nucleosome-length fragments.

The final indicator of cell death used in these studies was the loss of membrane integrity as assessed by trypan blue uptake. This assay has often been regarded as an inaccurate measure of cell death. Generally, this has resulted from assessing trypan blue exclusion too soon after drug treatment. We have demonstrated recently that lethally damaged cells retain apparently normal metabolic activities for several days [4]. Specifically, their RNA and protein synthetic machinery continue to function, while NAD and ATP levels remain normal. The current experiments further demonstrate that cell death represents a series of changes with loss of membrane integrity being a late event. The important point for these experiments is the timing of loss of cell membrane integrity. In all cases, trypan blue uptake occurred after DNA degradation had been detected. In most cases, this was at least 24 hr after distinct internucleosomal digestion had occurred. Only at the highest concentration did cells start to take up trypan blue more rapidly, but this was also after DNA degradation was detected. This confirmed our earlier report that DNA breakage was an early event in cell death [2].

This type of DNA digestion has been considered characteristic of the process termed programmed cell death or apoptosis. Wyllie [15] proposed four cardinal elements to characterize apoptosis: first, volume reduction accompanied by an increase in cell density, convolution and blebbing of the cell surface; second, chromatin condensation associated with activation of an endogenous endonuclease; third, recognition by phagocytic cells; and finally a dependence upon active protein synthesis. In recent studies of cisplatin action in murine leukemia L1210 cells, we observed cell shrinkage, cell surface blebbing and the characteristic internucleosomal digestion of chromatin [4]. We have now extended these studies to CHO cells and to other characteristics of apoptosis.

Undamaged cells had a median volume of 1.2 pL. Dying cells demonstrated shrinkage to about 0.5 pL. The timing of this shrinkage was dependent upon the concentration of cisplatin. At the highest concentration studied, the UV41 cells shrank on day 2, whereas at the LC_{90} concentration, shrinkage was not observed until day 4. The timing of this shrinkage was similar to the timing of trypan blue uptake. Under light microscopy, it was evident that the cells taking up trypan blue were indeed the shrunken cells. However, we observed a previously unreported increase in size heterogeneity and median cell size occurring before shrinkage. This increased size correlates with the time at which these cells undergo G_2 arrest [3]. This increase may not have been observed before for several reasons. First, most of the observations of apoptotic cells have been made in whole tissues in which apoptotic cells are typically quite isolated and infrequent [5]. Because of this relative infrequence, it would be difficult to observe cells undergoing early apoptosis, particularly as such swollen cells may not have been recognized as abnormal. Second, work in thymocytes shows a much more rapid induction of apoptosis in response to dexamethasone [13, 14]. The only condition under which we saw no increased size and heterogeneity was at the highest cisplatin concentration, a condition that led to rapid death. These observations suggest two distinct signal pathways for apoptosis, one in which death is slow and probably dependent upon some event that occurs at the G_2/M phase of the cell cycle and, second, a rapid form of cell death that is independent of cell cycle stage.

Perhaps the most significant element of apoptosis is the active participation of the cell in death. Not only is expression of an endonuclease required but the synthesis of some protein is also required (though not necessarily synthesis of the endonuclease). It is well recognized that cycloheximide protects thymocytes from glucocorticoid-induced death [14]. There have also been a number of reports that cycloheximide can protect against a variety of other agents, for example, vincristine and colchicine [16], methotrexate [17], and hyperthermia [18]. We have demonstrated here that cycloheximide can also delay the onset of toxicity induced by cisplatin in Chinese hamster ovary cells. The duration of these experiments was limited because cycloheximide was toxic to the cells after continued incubation. It has been reported that cycloheximide can induce apoptosis by itself [8], which may question the absolute need for new protein synthesis in apoptosis.

The elements of apoptosis that were measured here suggest that cisplatin kills by the mechanism of apoptosis. The major criteria is that internucleosomal degradation of genomic DNA occurred before any other indicator of cell death. As has been suggested for dexamethasone-induced death of thymocytes [5], this DNA digestion appears to play an integral role in causing cell death rather than just being a response to cell death.

Other agents were tested to determine whether apoptosis could be induced by disruption of a wide range of molecular targets. Several of these agents were already known to induce DNA breaks. In the case of etoposide, DNA breaks occur as a direct result of the interaction of the drug with DNA and topoisomerase II [19]. After removal of the drug, these DNA breaks are reversible yet the cells still die. We observed that, at later times, DNA breaks are again detectable, but at this time they appear as the characteristic nucleosome ladder of apoptosis. DNA breaks induced by both methotrexate and 5-fluorodeoxyuridine were previously thought to result from inhibition of repair of spontaneous damage to DNA [20, 21]. The current results suggest that these breaks arise by activation of the endonuclease involved in apoptosis.

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), although not used as an anticancer drug, has been used in previous studies of cell death [22]. It is a monofunctional alkylating agent which, at lethal concentrations, rapidly produces DNA strand breaks that stimulate poly(ADP-ribose) synthetase activity. The DNA breaks are thought to arise as an intermediate in DNA repair. Death was hypothesized to result from depletion of NAD, the precursor for poly(ADP-ribose), followed by depletion of ATP. These events occurred within a few minutes of incubation of cells with high concentrations of MNNG. An inconsistency with this model is that depletion of NAD can be prevented by 3-aminobenzamide, an

inhibitor of poly(ADP-ribose) synthetase, yet 3-aminobenzamide potentiates toxicity. An alternate hypothesis is that 3-aminobenzamide activates an endonuclease normally inhibited by poly(ADP-ribosyl)ation [23]. This is consistent with the pathway for cell death suggested here. Cells incubated with an LC_{90} concentration of MNNG produced a nucleosome ladder of DNA after 2 days.

Ouabain was tested because we anticipated its primary action would be disruption of osmoregulation and loss of membrane integrity inducing necrotic death. Surprisingly, even this drug induced internucleosomal degradation of DNA along with apoptotic cell shrinkage. It is possible that some agents can induce both apoptosis and necrosis. In this regard, it has been reported recently that minimally toxic conditions of methotrexate, ethanol, H_2O_2 and heat induce apoptosis, while more drastic conditions can induce necrosis [24]. Non-pharmacologic situations are unlikely to kill by apoptosis.

DNA breaks also occur under a variety of other circumstances, for example, after incubation with hydroxyurea, an inhibitor of ribonucleotide reductase [25], and mitomycin C, a bifunctional DNA alkylating agent [26]. With X-irradiation and bleomycin, DNA double-strand breaks are considered a primary effect of the agent. The phenomenon of sister chromatid exchange is also generally attributed to such breaks. The results presented here suggest that some of these breaks may be attributable to induction of an endonuclease. It is perhaps a semantic argument as to whether the endonuclease is expressed erroneously during cytotoxic treatment or represents designed deletion of damaged cells. The importance of this pathway is that it represents a common mechanism of cell death and that the endonuclease is probably an essential component. These results also suggest two variations of apoptosis: a rapid, and presumably more direct, activation of the endonuclease, and a delayed activation that requires passage to a specific cell cycle phase, the G_2/M phase, and the activation of events normally associated with the G_2/M phase transition. Although cells may die faster at higher concentrations of drug, the outcome is the same; the DNA is digested and the cells die. The slow process may be more pertinent to a clinical situation in which high concentrations of drug cannot be administered. Future experiments will need to determine how agents with such diverse targets induce a common mechanism of cell death.

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