The Role of Cell Cycle Progression in Cisplatin-induced Apoptosis in Chinese Hamster Ovary Cells

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Abstract
Many anticancer drugs arrest cells in G2 of the cell cycle and subsequently induce cell death by apoptosis. The current experiments establish a detailed sequence of events that occur in Chinese hamster ovary CHO/UV41 cells following incubation with cisplatin. Synchronized CHO/UV41 cells were damaged with cisplatin in early S. The cells progressed at a normal rate through S but arrested in G2. The arrested cells exhibited normal levels of the mitosis-promoting kinase p34cdcc in its fully phosphorylated, inactive form. After a protracted arrest, the cells dephosphorylated p34cdcc and underwent an aberrant mitosis and cytokinesis in which the chromosomes segregated unequally due to the formation of multipolar mitotic spindles. These cells subsequently lost contact with the extracellular matrix, and only then digested their DNA in a manner characteristic of apoptosis. This sequence of events could be dramatically accelerated by the addition of caffeine to G2-arrested cells, which induced dephosphorylation of p34cdcc and passage through an aberrant mitosis. It has previously been suggested that protein synthesis is required for both caffeine-induced premature mitosis and apoptosis. However, when added in G2, cycloheximide could inhibit neither the caffeine-induced mitosis nor apoptosis. Inhibition was only seen if cycloheximide was added before complete synthesis of the proteins required for mitosis. These results demonstrate that, in this model, the proteins thought to be involved in apoptosis are those required for normal cell cycle progression. It is hypothesized that the DNA digestion results from loss of signal transduction originating from the extracellular matrix but that earlier events leading to loss of cell adhesion are critical for the induction of apoptosis.

Introduction
Cisplatin is an effective chemotherapeutic drug for a variety of tumors. It kills cells as a consequence of its covalent interaction with DNA to produce both intrastrand and interstrand cross-links (1, 2). This binding to DNA is not in itself sufficient to cause cell death. Cisplatin is much more toxic to replicating cells than quiescent cells, and many observations have implicated inhibition of DNA synthesis as the critical step. However, recent experiments have demonstrated a poor correlation between inhibition of DNA synthesis and cell death. In particular, the DNA repair-deficient CHO/UV41 cells have been shown to progress at an unaltered rate through S of the cell cycle, even after incubation with cisplatin concentrations that kill 99% of the cells (3). In contrast, the DNA repair-competent CHO/AAP cells demonstrated inhibition of DNA synthesis, even at concentrations of cisplatin that they survive. At toxic concentrations of cisplatin, both cell lines arrested in G2 of the cell cycle before dying. However, those experiments were performed in asynchronous cell populations, which did not facilitate clear resolution of the relationship between G2 arrest and cell death.

Recent results have demonstrated that cisplatin, as well as many anticancer drugs, causes cell death by apoptosis (4). Apoptosis is defined by morphological criteria including chromatin condensation, cell shrinkage, and cell surface blebbing and is frequently associated with digestion of chromatin-associated DNA between the nucleosomes to give a characteristic pattern observed upon electrophoresis of the DNA (5). Apoptosis is a late event in cell death resulting from a number of distinct pathways (6). In addition to the induction of apoptosis by cytotoxins, other pathways of apoptosis include activation-induction such as can occur through positive stimulus at the T-cell receptor, inactivation-induction such as following removal of growth factors, and during cell-mediated immunity. An important question to be resolved is which events are involved in the final common steps of apoptosis and which events are specific to individual pathways.

Considering the role of G2 arrest in the response of cells to cisplatin, the current experiments were designed to investigate the relationship between cell cycle progression and apoptosis. Arrest of cells in G2 is a common response of cells to many cytotoxic agents. Analysis of the yeast Saccharomyces cerevisiae has identified RAD9 mutants that fail to arrest in G2 after induction of damage by γ-radiation and UV light (7, 8). These mutants show enhanced killing, presumably because they lack the time in G2 required to adequately repair the DNA damage. Likewise, caffeine has been shown to bypass drug-induced G2 arrest in mammalian cells and thereby enhance toxicity (9, 10). The G2/M transition is regulated by the p34cdcc/cyclin B complex (11). During S and G2, cyclin B is synthesized and associates with the constitutively expressed p34cdcc. This targets p34cdcc for phosphorylation. Entry into mitosis is caused by dephosphorylation of p34cdcc, thereby activating the complex as "histone H1 kinase," which phosphorylates many proteins in addition to histone H1. Exit from mitosis occurs upon degradation of cyclin B.

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3 The abbreviations used are: CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.
The present experiments were performed in synchronized CHO/UV41 cells, selected because of their lack of DNA repair for cisplatin lesions. This facilitated killing of the cells with very low concentrations of cisplatin that did not slow the rate of passage through S and therefore led to synchronous arrest and onset of apoptosis. We have established a detailed sequence of events that occur as the damaged cells progress through the cell cycle, arrest in G2, then pass through an aberrant mitosis, and subsequently undergo apoptosis.

Results

Cell Cycle Progression. Many methods to arrest cells in the G1 phase lead to a heterogeneous population of cells progressing through the cell cycle upon subsequent release. Aphidicolin is an excellent agent to arrest cells because, upon release, they progress immediately and synchronously through S. However, it is essential that no cells are in S at the time of addition of aphidicolin because these cells can subsequently progress to and arrest in G2 (12) with delayed apoptosis (4), thereby complicating the experiments. We have tested many methods for reducing the S population before the addition of aphidicolin but have found them to give incomplete synchronization, to be unreliable, or to be toxic to some of the cells. Mitotic shake yields an excellent population of synchronized cells in mitosis but a limited number of cells. Furthermore, these cells still enter S somewhat heterogeneously. These two problems were overcome by performing repeated mitotic shakes, followed by arrest with aphidicolin. Upon release from aphidicolin, these cells progressed immediately into S and then into the next mitosis at 10 h (Fig. 1A). By 14 h after release, cells began to enter a second S phase. There was also approximately 14 h between the first mitosis at 10 h and the second mitosis at 24 h. These results are consistent with a normal cell cycle time of about 14 h and demonstrate that the aphidicolin treatment did not alter cell cycle traverse. This protocol was completely nontoxic to the cells.

Synchronized CHO/UV41 cells were damaged with cisplatin for 1 h immediately upon release from aphidicolin arrest. At the drug concentration used (0.5 μg/ml), 99% of the cells were killed (3). This cell line was specifically chosen because of its deficiency in DNA excision repair. Accordingly, the level of cisplatin-induced DNA damage did not vary during the subsequent incubations. This facilitated the use of a relatively low cisplatin concentration that, although killing all of the cells, was inadequate to cause inhibition of DNA synthesis. This was confirmed by a similar rate of progression through S after cisplatin treatment as in the undamaged cells (Fig. 1B). The damaged cells entered G2 synchronously, but then arrested there. The duration of arrest was about 10 h. At 20 h after release from aphidicolin and cisplatin treatment, new daughter cells began to appear on the flow cytometry profiles, and the G2 population began to decline. The width of the G2 peak following this mitosis was much broader than in the control cells due to unequal chromosome segregation as discussed below.

p34cdc2 Protein Levels and Phosphorylation Status. Activation of the p34cdc2/cyclin B complex is required for entry of cells into mitosis (11). Cyclin B is newly synthesized in each cell cycle and then binds to p34cdc2, targeting it for phosphorylation and inactivation. At the G2/M transition, dephosphorylation of p34cdc2 activates the kinase. We used Western blot analysis to investigate the phosphorylation status of p34cdc2 kinase as a monitor of the passage of cells though the G2 phase and mitosis. Immunoblots were probed with a polyclonal anti-p34cdc2 antibody raised against the carboxyl terminus of the protein and therefore specific for this particular kinase (Fig. 2). The immunoblots showed the three forms of p34cdc2, the character of which has been well established (11, 13–15). The fastest migrating band is the hypophosphorylated form of the protein, which may contain phosphorylation at threonine-161. The other two bands represent additional phosphorylation on threonine-14, tyrosine-15, or both. Using antiphosphotyrosine antibodies, we confirmed that the slowest migrating band contained the majority of the phosphotyrosine (data not shown). The latter two phosphorylations are responsible for the inactivity of the complex.

At the time of release of control cells from aphidicolin arrest, the three phosphorylated forms of p34cdc2 were present at about the same intensity, with a slight predominance of the slowest migrating, hyperphosphorylated band (Fig. 2). During the 8 h following release from the aphidicolin block, there was both an increase in the total level of protein and also an accumulation of the hyperphosphorylated form. Most of the protein was hyperphosphorylated at the time the cells were preparing to undergo mitosis. At 10 h, a shift in the migration of the protein was observed, as is characteristic of its dephosphorylation (from the slowest to the fastest migrating band). The timing of this dephosphorylation was consistent with progression through mitosis and reemergence of G1 daughter cells in the flow cytometric profiles.

Cells damaged with cisplatin in early S showed exactly the same pattern of p34cdc2 synthesis and phosphorylation during the first 8 h following damage (Fig. 2). However, unlike the undamaged cells, no dephosphorylation occurred at 10 h, and the cells stayed arrested in G2. Therefore, this arrest is associated with an inhibition of the dephosphorylation of p34cdc2. At later time points, p34cdc2 was dephosphorylated, and the cells underwent mitosis and cell death (see below).

Mitosis Induced by Caffeine. Caffeine and related methylxanthines are known to cause premature mitosis in S-arrested cells and also to circumvent G2 arrest after DNA damage (9, 10, 16). Accordingly, we used caffeine to determine what effect accelerated mitosis would have on the onset of apoptosis. The CHO/UV41 cells damaged with cisplatin and arrested in G2 were incubated with 5 mM caffeine. Flow cytometry revealed that G1 daughter cells began to appear about 3 h after the addition of caffeine (Fig. 1C), therefore demonstrating that caffeine was able to overcome the G2 arrest. At 14 h (4 h after the addition of caffeine), almost all cells were in Gi. Similar to the results observed following incubation of cells with cisplatin alone, the width of the G1 peak is clearly much broader than in control cells. Incubation with 2 mM caffeine also overcame the G2 arrest, but it took twice as long to occur (data not shown).

Western blot analysis of p34cdc2 showed that most of the protein was dephosphorylated 2 h after the addition of caffeine (Fig. 2). The dephosphorylation of p34cdc2 1 h before the appearance of new G1 daughter cells was in concordance with the traverse of mitosis after its induction. Moreover, this is consistent with dephosphorylation of p34cdc2, leading to activation of the kinase which drives the cells into mitosis.
Cell Growth and Toxicity. The cisplatin-induced G2 arrest was transient, with cells undergoing mitosis around 20 h after incubation with cisplatin (Fig. 1 B). As the cells leave the G2 arrest, we observed that an increasing percentage of the population was rounding up and floating off the surface of the flasks. We also observed that incubation of cells with caffeine increased the number and rate that cells detached from the flask. Therefore, we investigated the relationship between cell growth, detachment, and membrane integrity.

The increase in cell number with time was determined for the various experimental groups (Fig. 3). The control cells doubled their number 12 h after the release from aphidicolin and doubled it again by 36 h. Cisplatin-damaged cells started to increase their cell number about 24 h after the treatment and had doubled completely by about 36 h. This was consistent with the appearance of G1 cells by flow cytometry. However, the cells never doubled again throughout the time course of these experiments. G2-arrested cells incubated with caffeine at 10 h doubled their cell number rapidly between 4 and 6 h after the addition of caffeine, also consistent with the results of flow cytometry. These cells also did not undergo any further doubling up to 72 h.

At each time point, we determined the number of attached and detached cells (Fig. 3). For control cells, most remained firmly attached to the surface throughout the duration of the experiment. In contrast, about 24 h following cisplatin treatment, cells started to detach from the surface until by 72 h, 60% of the cells had detached. The addition of caffeine to G2-arrested cells forced the cells to rapidly undergo mitosis; many cells detached from the surface at 16 h but then reattached again. These cells then detached continuously, so that by 72 h, 80% of the cells
had detached. Hence, the majority of cells had clearly detached while in the subsequent G₁ phase.

Since addition of caffeine to G₂-arrested cells accelerated the detachment from the surface, we determined whether caffeine also accelerated the rate of cell death. Trypan blue exclusion was used to determine membrane integrity as an indicator of cell viability (Fig. 3). The majority of undamaged cells excluded trypan blue except at 72 h, when the cultures had begun to overgrow. In cisplatin-treated cells, membrane integrity remained above 80% for the first 24 h but subsequently decreased rapidly in the detached cells. At 48 h, almost all the detached cells had lost membrane integrity, while the attached cells were still excluding dye until at least 60 h. Caffeine treatment led to an even earlier loss of membrane integrity, which was clearly detectable by 24 h in the detached cells. At later time periods, even the few cells remaining attached to the flask began to lose membrane integrity. The results clearly show that detached cells initially exclude trypan blue, demonstrating that detachment occurs before cells lose membrane integrity.

**DNA Digestion Induced by Cisplatin.** The results presented so far have established a sequence of events in which dephosphorylation of p34<sup>cdc2</sup> precedes mitosis, followed by detachment of the cells from the surface, and finally loss of membrane integrity. Previous studies have shown that CHO/UV41 cells undergo DNA digestion and apoptosis when treated with cisplatin (4). Therefore, we determined the point in this sequence at which the DNA digestion occurred. Control cells exhibited no DNA digestion (Fig. 4). At 20 h after cisplatin treatment, no DNA digestion was detected in the total cell population. Similarly, no DNA digestion was observed in 10⁶ attached cells. However, when 5 × 10⁵ detached cells were analyzed separately, a faint nucleosome ladder was observed. It should be recalled that at this time only about 10% of the cells are detached, so this represents 5-fold more detached
cells than would be present in $10^6$ cells from the total population. At 24 h, more DNA digestion was detected in the detached population but none in the attached cells.

Caffeine treatment of $G_2$-arrested cells caused a marked increase in the amount of DNA digestion. At 20 h, which was 10 h after the addition of caffeine to $G_2$-arrested cells, DNA degradation was detected in the total cell population. However, like cells damaged only with cisplatin, the nucleosome ladders were detected only in the detached cells, which represented about 40% of the total population at this time. A distinct nucleosome ladder in the detached cells was detected as early as 16 h, a time at which no DNA digestion was detected in cells incubated with only cisplatin (data not shown). These results demonstrate that DNA digestion occurred 6 h after the addition of caffeine, 4 h after dephosphorylation of $p34^{cdk2}$, and 2 h after the majority of cells had entered $G_1$.

The above results show that all of the DNA digestion occurs in the detached cells, but it does not establish a clear sequence for these events. Therefore, we used a technique that assays breaks in individual cells, the increased sensitivity of which should detect any breaks occurring initially in the attached cells which lead to rapid detachment. This assay involves labeling the broken DNA ends with biotinylated dUTP/fluorescein and analyzing the cells by flow cytometry (17). The cells were concurrently stained with propidium iodide to confirm the phase of the cell cycle. Representative results are shown in Fig. 5. At 20 h after release from aphidicolin, cells damaged with cisplatin were still predominantly in $G_2$ as expected, with about 5% of the cells having increased fluorescence indicative of DNA breaks. In contrast, at 16 h following release with caffeine added at 10 h, the majority of cells were in $G_1$, and 12% exhibited DNA breaks. This population was separated into attached and detached cells; only about 1% of the attached cells had DNA breaks, whereas 35% of the detached cells exhibited breaks. The remainder of the detached cells at this time point were equally distributed between $G_1$ and $G_2$ and probably reflect cells still undergoing mitosis rather than cells initiating apoptosis. By 20 h after release with caffeine added at 10 h, >93% of the attached cells still exhibited no DNA breaks, whereas 90% of the detached cells exhibited breaks. The results show that there is a very close temporal relationship between cell detachment and DNA fragmentation, and it is not possible in these experiments to determine which occurred first.
When cells observed napolan spindle, I time was from cells figures showed caffeine cells produce around multiple chromosomes. However, remaining caffeine added to the remaining chromosomes led to cell. In contrast, addition of caffeine, the expected bipolar mitotic spindle, greater than 80% of the mitoses in damaged cells had multipolar mitotic spindles; these were frequently tetrapolar spindles, but occasionally tripolar spindles were observed (Fig. 7).

**Inhibition of Protein Synthesis.** Apoptosis has often been shown to be inhibited by cycloheximide, suggesting the requirement for new or ongoing protein synthesis for toxicity (5). Cycloheximide has also been shown to inhibit caffeine-induced mitosis (18). Therefore, we questioned whether the prevention of apoptosis by cycloheximide might be due to the synthesis of proteins required for passage through the cell cycle. In the following experiments, all cells were harvested 24 h after release from aphidicolin and analyzed for the phosphorylation status of p34<sup>cdc2</sup>.

When cycloheximide was added to control cells at 8 or 10 h after release from aphidicolin, the cells still dephosphorylated p34<sup>cdc2</sup> by 24 h; dephosphorylation was only inhibited if cycloheximide was added at or before 6 h, i.e., while the cells were still in S (Fig. 8). This suggests that by the time these cells reach G<sub>2</sub>, they have synthesized all of the proteins required for mitosis. Similar results were obtained if caffeine was added to control cells at 10 h; cycloheximide had to be added 4 h before the caffeine to prevent dephosphorylation.

When cisplatin-treated cells were incubated with cycloheximide, little dephosphorylation occurred by 24 h, even when cycloheximide was added at 10 h (Fig. 8). In the absence of cycloheximide, these cells would have dephosphorylated p34<sup>cdc2</sup> by the time of harvest (Fig. 2). This loss in capacity to dephosphorylate p34<sup>cdc2</sup> is presumed to result from the decay of a critical protein following addition of cycloheximide. That all of the necessary proteins had been synthesized by 10 h is shown by the treatment with caffeine, which still caused dephosphorylation, even in the presence of cycloheximide. Cycloheximide was only able to prevent the caffeine-mediated dephosphorylation if it was added about 4 h before the addition of caffeine. Therefore, this result was identical to that observed in the absence of cisplatin.

It can be concluded that once the cells reach G<sub>2</sub>, they already have synthesized all of the proteins required for mitosis. Accordingly, caffeine was able to induce mitosis without the requirement for additional protein synthesis. To prevent cells from entering mitosis, it was necessary to add the cycloheximide during S. We have previously demonstrated that cycloheximide can delay DNA digestion and apoptosis in UV41 cells for up to 2 days (4), and in the current experiments, cycloheximide was only able to prevent the fragmentation of DNA characteristic of apoptosis under conditions in which it was able to block mitosis (data not shown). These results are consistent with the hypothesis that the requirement for protein synthesis in apoptosis fol-

**Cell Morphology.** Since only the detached cells were undergoing DNA digestion characteristic of apoptosis, it was of interest to compare the morphology of these cells with the remaining attached cells. Control cells 16 h after the release from aphidicolin showed a mixed population of mitotic cells and interphase cells; the majority of mitotic cells were in the detached population (Fig. 6). These mitotic cells showed well-organized segregation of their chromosomes.

At 16 h following cisplatin treatment, the attached cells were considerably larger than the control cells and showed no evidence of mitosis (Fig. 6). In contrast, the addition of caffeine led to chromosome condensation and segregation observed at 16 h. The mitotic figures were clearly different from undamaged cells in that the chromosomes were often scattered throughout the cytoplasm. A number of the cells showed chromosomes apparently segregating to more than two locations in the cell. Some of the cells also showed multiple nuclei and micronuclei. By 20 h, all of the mitotic figures had disappeared, and nuclear membranes had re-formed. However, the membranes presumably reformed around isolated chromosomes or a few chromosomes to produce micronuclei or several larger nuclei. Similar events were observed in cells damaged only with cisplatin, but the events occurred at later time points (data not shown). These events appear consistent with the results of flow cytometry in which the G<sub>1</sub> population exhibited a heterogeneous content of DNA, presumably because of unequal segregation of the chromosomes. One further observation at late time points was the occasional appearance of highly condensed cells typical of shrunk apoptotic cells seen in tissue sections (5).

To investigate the cause of the aberrant chromosome segregation, we analyzed the mitotic cells by immunofluorescence with an anti-tubulin antibody. In contrast to undamaged cells which exhibited the expected bipolar mitotic spindle, greater than 80% of the mitoses in damaged cells had multipolar mitotic spindles; these were frequently tetrapolar spindles, but occasionally tripolar spindles were observed (Fig. 7).
Fig. 6. Cell morphology assayed by Giemsa staining. A, undamaged cells attached to the flask 16 h after release from aphidicolin. B, undamaged cells detached from the flask at 16 h; note two pairs of cells neatly segregating chromosomes. C, cisplatin-damaged cells attached to the flask at 16 h; note swollen but otherwise normal morphology. D, cells damaged with cisplatin followed by caffeine and attached to the flask at 16 h; aberrant nuclear morphology is becoming apparent. E and F, cells damaged with cisplatin followed by caffeine and detached from the flask at 16 h; note many cells with scattered chromosomes. G, cells damaged with cisplatin followed by caffeine and attached to the flask at 20 h; many cells contain multiple nuclei and micronuclei. H and I, cells damaged with cisplatin followed by caffeine and detached from the flask at 20 h; most cells contain multiple nuclei and micronuclei; highly condensed cells are occasionally observed.
Cisplatin-induced $G_2$ Arrest and Apoptosis

Cisplatin-induced G2 arrest is due to loss of proteins required for cell cycle passage.

Discussion

DNA damage induced by agents such as cisplatin has been shown to cause $G_2$ arrest prior to inducing apoptosis (3, 19). However, the biochemical mechanisms involved in cell cycle arrest and apoptosis are still poorly understood. To investigate this, we synchronized CHO/UV41 cells at the $G_1/S$ transition using mitotic shake and aphidicolin block. This provided a population of cells which, upon release from aphidicolin, progressed synchronously through S, $G_2$, and M phases, and even retained some synchrony through a second cell cycle.

Cisplatin treatment in early S caused these cells to progress at a normal rate through S and then to arrest at $G_2$. If these experiments had been performed with the DNA repair-proficient CHO/AA8 cell line, a higher drug concentration would have been required to obtain a $G_2$ arrest, and this concentration would have caused slowed progression through S (3).

During S and $G_2$, cells synthesize cyclin B, which complexes with the p34$^{cdc2}$ kinase as an essential step in regulating passage into mitosis. This complex becomes phosphorylated and inactive (20–22). At the $G_2$/M transition, dephosphorylation of p34$^{cdc2}$ activates its kinase activity, which drives the cell into mitosis (13, 23, 24). We demonstrated that cisplatin-treated CHO/UV41 cells behaved exactly as control cells in that they successfully produced the hyperphosphorylated form of p34$^{cdc2}$. This demonstrates that there is no block to the synthesis of p34$^{cdc2}$ or cyclin B, or to the phosphorylation of the complex. However, the arrested cells failed to dephosphorylate p34$^{cdc2}$, thereby causing the $G_2$ arrest. Accumulation of the hyperphosphorylated form is consistent with results reported for $G_2$ arrest following nitrogen mustard treatment of human lymphoma cells (25), camptothecin treatment of HeLa cells (26), and etoposide treatment of CHO cells (27).

The activation of p34$^{cdc2}$ is the result of an interplay between many proteins. The phosphorylation state of p34$^{cdc2}$ is directly dependent upon the relative activities of several other kinases, which inactivate the complex by phosphorylating it on both threonine-14 and tyrosine-15, andcdc25 phosphatase, which dephosphorylates these sites to activate the p34$^{cdc2}$ kinase (28–31). Proteins have

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In the absence of caffeine, the control cells remained in the $G_1/S$ phase, whereas the transfected cells entered the $G_2/M$ phase. After 10 h of caffeine, the control cells arrested in the $G_1/S$ phase, whereas the cisplatin-treated cells entered the $G_2/M$ phase.

Fig. 7. Immunofluorescence of mitotic spindles in cisplatin-damaged UV41 cells. At 16 h after release from aphidicolin with caffeine added at 10 h, mitotic cells were collected and stained with anti-β-tubulin antibody, followed by fluorescein-conjugated goat anti-mouse antibody. All pictures are from the same treatment. A few cells exhibit apparently normal bipolar mitotic spindles, but about 80% exhibited at least three spindle poles.
been identified that delay the onset of mitosis in damaged cells. For example, radiation damage to the yeast Saccharomyces cerevisiae leads to a G2 arrest except in RAD9-deficient mutants (7, 8). The RAD9 protein usually inhibits passage into mitosis until adequate DNA repair has occurred, although eventually even in the absence of repair, cells adapt and undergo mitosis (32). It is assumed that a similar control mechanism operates in human cells, but it has not yet been characterized.

The G2 arrest was not irreversible, and eventually, like damaged yeast cells (32), the cisplatin-damaged CHO cells entered mitosis; they then digested their DNA in a manner characteristic of apoptosis. However, the internucleosomal DNA digestion was only detected in the detached cells. The sequence of events that occurs is significant. By 24 h after cisplatin, many of the cells passed through mitosis and into G1, as observed by flow cytometry. This occurred concurrently with dephosphorylation of most of the p34<sup>cdc2</sup>, and mitotic figures were observed in the detached cells. The cell numbers had doubled completely by 36 h, but 80% of the cells remained firmly attached to the flask; these cells must have reattached to the flask after passing through mitosis. Cells continued to detach from the surface throughout the time course of the experiment, suggesting that many of the cells detached during the G1 phase. Microscopic observation demonstrated that the cells had reformed nuclear membranes, but many of the cells exhibited multiple nuclei and micronuclei which presumably arose from the formation of nuclear membrane around the chromosomes that were seen to segregate abnormally.

The sequence of events is even clearer following addition of caffeine. Caffeine forced the cisplatin-damaged cells to enter mitosis within 3–4 h; by flow cytometry, they had almost all entered G1. This clearly followed the caffeine-induced dephosphorylation of p34<sup>cdc2</sup>. The delay observed between addition of caffeine and the dephosphorylation of p34<sup>cdc2</sup> (about 2 h) did not appear to be due to the requirement for any translational event because it was not prevented by cycloheximide. Also within 4 h of the addition of caffeine, more than 90% of the cells detached from the surface, consistent with passage through mitosis. At least some of these cells reattached as they entered G1 and detached slowly over the remainder of the experiment. Again, the internucleosomal DNA digestion was only detected in the detached cells. Furthermore, fluorescent labeling of the DNA breaks in individual cells showed no breaks in the attached cells but extensive DNA breaks in each detached cell (excluding those in mitosis). Therefore, detachment and DNA digestion are very closely related events.

Microscopic observation following caffeine also showed aberrant chromosome segregation and subsequent formation of micronuclei. Flow cytometry showed that the G1 cells appearing after mitosis had a heterogeneous DNA content. The cause of the aberrant mitosis was the formation of multipolar spindles. Presumably cells died as a result of unequal segregation of critical chromosomes during this mitosis. Suppression of mitosis by cisplatin delays cell death, whereas caffeine was able to overcome this suppression, leading to an earlier mitosis, enhanced rate of cell detachment, and an earlier cell death. Therefore, cell death is a consequence of unequal chromosome segregation resulting from the formation of aberrant mitotic spindles, but how a cisplatin lesion in the DNA can cause this is currently unknown.

It has been reported that both caffeine-induced premature mitosis and apoptosis are inhibited by cycloheximide (5, 18). In the case of apoptosis, it has consistently been thought that cycloheximide prevents the synthesis of lethal proteins, although not necessarily the endonuclease. We have previously suggested that these lethal proteins might be normal proteins involved in cell cycle passage (33). The results presented here support this hypothesis. Once the cells have reached G2, they appear to have synthesized all of the proteins necessary for mitosis such that the addition of cycloheximide cannot prevent mitosis. This is true in both undamaged cells as well as during caffeine-induced mitosis in cisplatin-damaged cells. To prevent mitosis in both situations, it was necessary to add cycloheximide during S. This is in fact consistent with similar studies on caffeine-enhanced premature chromatid condensation because those experiments were performed with agents that arrested the cells in S (9). S-arrested cells continue to synthesize proteins required for mitosis; thus, cycloheximide would be able to block caffeine-induced premature mitosis by preventing synthesis of these essential proteins. Similarly, cycloheximide only blocked apoptosis under conditions that inhibited mitosis. Hence, the proteins required for both caffeine-induced premature mitosis and apoptosis appear to be normal cell cycle regulatory proteins.

What then is the cause of DNA digestion and apoptosis? In parallel studies, we have investigated endonucleases in CHO cells and have demonstrated the presence of deoxyribonuclease II (34). This endonuclease is activated by acidic shifts in intracellular pH. Others have suggested the involvement of a Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonuclease in apoptotic DNA digestion which occurs as a result of sustained increases in intracellular Ca<sup>2+</sup> (reviewed in Ref. 35). We have demonstrated in several systems that acidification occurs without any increase in Ca<sup>2+</sup> and in cells that contain deoxyribonuclease II but no detectable Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonuclease (36, 37). Considering that DNA digestion occurred only in detached cells, it could result from loss of contact with the extracellular matrix (i.e., the surface of the flask). Extracellular matrix proteins such as fibronectin, which communicate through integrin-type receptors, have been shown to activate a signal transduction pathway through a focal adhesion kinase (38). The extracellular matrix can also activate the Na<sup>+</sup>/H<sup>+</sup> antiporter, leading to intracellular alkalization (39). Therefore, loss of attachment is likely to inactivate the Na<sup>+</sup>/H<sup>+</sup> antiporter, leading to intracellular acidification and activation of deoxyribonuclease II. This is similar to the morphology seen when a cell undergoes apoptosis in vivo; a cell is seen to shrink away from its neighbors (5), and this loss of contact could lead in a similar manner to apoptotic DNA digestion.

In summary, these experiments have established a sequence of events that occurs during the induction of apoptosis by cisplatin. Damaged cells progress to arrest in G2. Upon subsequent dephosphorylation of p34<sup>cdc2</sup>, the cells undergo an aberrant mitosis with multiple mitotic spindles leading to unequal chromosome segregation. In the subsequent G1 phase, the cells detach from the surface of the flask. Only at this time do the cells show the characteristic DNA digestion of apoptosis. Subsequently, the cells loses membrane integrity. It is suggested that the DNA digestion results from loss of a signal transduction pathway originating from the extracellular matrix, which leads to intracellular acidification and subsequent activation of an endonuclease.
Materials and Methods

Cell Culture. CHO/UV41 cells (a gift from Dr. Larry H. Thompson, Berkeley, CA) were maintained as exponentially growing monolayer cultures in α-minimum essential medium supplemented with 2.5% fetal bovine serum, 2.5% horse serum, penicillin, and streptomycin in a humidified incubator at 37°C with 5% CO₂.

Cell Synchronization. Cells were synchronized at the G₁/S transition by mitotic shake followed by incubation in aphidicolin as follows. In order to obtain large numbers of cells, CHO/UV41 cells were grown in T150 flasks for 2–3 days until they reached 50–70% confluence. The flasks were gently slapped, and the medium was removed and discarded. Fresh medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid was added; this was used during all steps of the mitotic shakes to help maintain the pH of the medium. Mitotic cells were collected every 30 min for up to 6 h. This was achieved by gently removing the medium, adding 5 ml of fresh medium, gently slapping the flask, removing the medium containing mitotic cells, replacing the original medium, and returning the flasks to an incubator. The mitotic cells were centrifuged, resuspended in 2 μg/ml aphidicolin, and placed in T25 or T75 flasks. Cells from each repeat shake were aliquoted into each flask. Thirty min after adding cells from the final shake, the medium, together with any cells that had not adhered to the flask, was removed. The adherent cells were then incubated for 16 h with 2 μg/ml aphidicolin.

Treatment of Synchronized Cells. Cells were released from the G₁/S block by incubating in aphidicolin-free medium. As required, the cells were incubated with 0.5 μg/ml cisplatin (Bristol-Myers Squibb) for 1 h immediately after release from aphidicolin. After this incubation, the cells were rinsed and incubated in fresh medium. In some experiments, cells were exposed to 5 mM caffeine (from a 50 mM stock in medium) or 5 μg/ml cycloheximide (from a 1 mg/ml stock in water).

Harvest of Different Cell Populations. For harvest of total cells (attached and detached), flasks were placed on ice, and cells were scraped directly into the medium. In some experiments, the cells were separated into those either attached or detached to the flask. The detached population included those loosely attached to the flask. These cells were dislodged by gently slapping the flask before removal of the medium. After removal of the medium containing the detached cells, the flasks were gently rinsed with cold medium to prevent cross-contamination of the various populations. The attached cells were then harvested using a scraper. Cells were centrifuged and rinsed with cold PBS containing phosphatase inhibitors (100 mM sodium fluoride and 10 μM sodium orthovanadate). The cells were counted with a Coulter counter, and appropriate aliquots were centrifuged for 5 min at 1000 rpm. These cells were analyzed for their morphology, cell cycle distribution, DNA degradation, trypan blue exclusion, and p34cdc2 phosphorylation states.

Cell Cycle Analysis. Cell cycle analysis was performed by a modification of a previously described procedure (40). Briefly, the total cell population was harvested by scraping the cells directly into the medium. Cells were fixed in 70% cold ethanol at least overnight. The fixed cells were incubated for 30 min at 37°C in PBS containing 1 mg/ml ribonuclease A and 100 μg/ml propidium iodide and subsequently analyzed for their DNA content on a Becton-Dickinson FACScan flow cytometer.

Analysis of p34cdc2. Cells to be analyzed for p34cdc2 were stored in liquid nitrogen. After thawing on ice, the cells were lysed in 50 mM Tris, pH 8.0, 250 mM NaCl, 1% Triton X-100, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml sodium pyrophosphate (the latter two were added immediately before use). The cells were incubated on ice for 30 min and then centrifuged for 10 min at 10,000 × g; then supernatants were transferred to clean tubes. Protein concentration was measured using the bicinchoninic acid protein assay reagent (Pierce). Standard curves were generated using known concentrations of bovine serum albumin.

Protein extracts were separated by electrophoresis on a 10% polyacrylamide/sodium dodecyl sulfate minigel (Bio-Rad Mini Protein II). To fully resolve the various phosphorylated forms of p34cdc2, the gels were electrophoresed until these proteins were close to the bottom of the gel. After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane (Immobilon; Millipore). The membranes were blocked by a 30-min incubation at room temperature in TBST (50 mM Tris, pH 7.3, 200 mM NaCl, and 0.1% Tween-20) containing 5% dry milk. Subsequent incubations with antibodies and washes were performed in TBST containing 1% dry milk. The blocked membranes were incubated in the presence of 1.3 μg/ml polyclonal rabbit anti-p34cdc2 antibody (carboxyl terminus; Gibco-BRL) for 1 hour at room temperature and then thoroughly washed. The membranes were subsequently incubated with a polyclonal goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad). Detection of the conjugate was performed using the enhanced chemiluminescence detection kit (Amersham Corp.) according to the manufacturer’s instructions.

DNA Degradation. DNA was analyzed by a gel electrophoresis method (19) with modifications described elsewhere (34). Briefly, 5 × 10⁵ or 10⁶ cells were directly added to the wells of a 2% agarose gel where they were lysed and digested with ribonuclease A and proteinase K. The gel was electrophoresed at room temperature for 16 h at 45 volts, and the DNA was visualized with 2 μg/ml ethidium bromide.

DNA breaks were also measured in individual cells according to the method of Gorczyca et al. (17). Briefly, cells were fixed in 1% formaldehyde for 15 min, resuspended and stored in 70% ethanol, rehydrated, and incubated with terminal deoxynucleotidyl transferase and biotin-16-DUTP for 30 min. After rinsing, the cells with DNA breaks were stained with fluoresceinated avidin, and total DNA was stained with propidium iodide. Cells were then analyzed on a Becton-Dickinson FACscan flow cytometer for red (propidium iodide) and green (fluorescein) emissions. Unstained cells were gated from the analysis.

Trypan Blue Exclusion. Aliquots of cells resuspended in PBS were mixed with an equal volume of 0.4% trypan blue stain in 0.85% saline and counted on a hemocytometer.

Cell Morphology Studies. Cells were centrifuged onto glass slides by a cytopsin (300 × g for 5 min) and stained with Giemsa with the Diff-Quik kit (Baxter).

Immunofluorescence. Cells in mitosis were harvested by gently shaking the flask, collected by centrifugation, and resuspended in PBS. The cells were attached to polylysine-coated cover slips and fixed in methanol at −20°C for 5 min. The cells were stained with a monoclonal anti-
β-tubulin antibody (Sigma; diluted 1:20 in PBS with 3% bovine serum albumin). The cells were washed with PBS and incubated with a fluorescein-conjugated goat anti-mouse secondary antibody (Sigma). Cells were photographed in the Robert Day Allen Laboratory for Light Microscopy on a Zeiss Axioshot at ×100.

References