Possible Role for p34<sup>cdc2</sup> Kinase in Etoposide-induced Cell Death of Chinese Hamster Ovary Cells

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ABSTRACT

In an effort to shed light upon the processes of antitumor drug-induced cell death, we have carried out a systematic study of the effects of the anti-topoisomerase II agent, etoposide, on Chinese hamster ovary cells. Treatment of Chinese hamster ovary cells for 1 h with a 2-log cell-killing concentration of etoposide induces a high incidence of DNA single-strand breaks which are rapidly repaired upon drug removal. p34<sup>cdc2</sup> kinase activity is inhibited within 1 h of addition of etoposide. Following removal of drug, cells accumulate transiently in G<sub>2</sub>. Upon recovery of p34<sup>cdc2</sup> kinase activity (between 12 and 24 h posttreatment), approximately 50% of cells progress through mitosis which results in micronucleation. Examination of mitotic figures at various posttreatment incubation times indicates that micronucleation of daughter cells could be attributed to abnormal segregation of chromosomes during mitosis. Unexpectedly, p34<sup>cdc2</sup> kinase activity remains elevated relative to untreated controls until 36 h post-etoposide treatment, a point where no further cell division takes place. This activity decreases by 48 h posttreatment, concomitant with a decrease in cell viability as estimated by the ability to exclude trypan blue. These results indicate that etoposide may induce cytotoxicity via gross chromosomal fragmentation, and that p34<sup>cdc2</sup> kinase may be involved in this process.

INTRODUCTION

The antitumor epipodophyllotoxin, etoposide, appears to exert cytotoxicity by induction of DNA strand breakage via interaction with the enzyme DNA topoisomerase II (for recent reviews, see Refs. 1 and 2). The extent of DNA strand breakage shows a direct correlation with cytotoxicity using a variety of mammalian tumor cell lines (3, 4). However, etoposide remains weakly bound intracellularly and rapidly effluxes into drug-free medium (5). This is paralleled by rapid resealing of drug-induced DNA SSB (3). It is apparent, therefore, that etoposide cytotoxicity is not a direct consequence of DNA strand breakage with a decrease in cell viability as estimated by the ability to exclude trypan blue. These results indicate that etoposide may induce cytotoxicity via gross chromosomal fragmentation, and that p34<sup>cdc2</sup> kinase may be involved in this process.

We have carried out a systematic study of events distal to formation and resealing of etoposide-induced DNA SSB. The purpose of this was to establish the chronological sequence of events in order to form a useful hypothesis regarding the mechanism of cell death. The results of this study include the observations that etoposide-induced G<sub>2</sub> arrest is transient and that the subsequent mitosis results in micronucleated daughter cells via irregular chromosome segregation. Abnormally elevated p34<sup>cdc2</sup> kinase activity was detected in drug-treated cells, indicating that perturbations in the activities of cell cycle-regulated proteins may be a cause/effect of mammalian cell death.

MATERIALS AND METHODS

Cell Culture, Drug Treatments, and Cytological Techniques. Wild-type CHO cells were maintained on monolayer at 37°C in α-minimal essential medium supplemented with 5% fetal calf serum in a 5% CO<sub>2</sub> atmosphere. Drug treatments were carried out using 25 μM etoposide for 1 h (1.5% survival compared with solvent-treated controls, as determined by colony-forming assay). Control cultures received the equivalent solvent exposure (0.04% dimethyl sulfoxide). Following drug treatment monolayers were rinsed twice with drug-free medium (37°C) and incubated in drug-free medium at 37°C. Cell counts were determined using a hemacytometer and a Model ZM Coulter Counter (Coulter Electronics, Inc.) following trypsinization of cells. Estimations of cell viability by trypan blue exclusion were achieved by light trypsinization of cells followed by resuspension in medium plus 5% fetal calf serum (overtrypsinization resulted in loss of viability). Trypan blue was then added to 0.04%, and viability was estimated by counting at least 500 cells. For morphological examination of etoposide-treated cells, approximately 10,000 phosphate-buffered saline-washed cells were spun onto a clean microscope slide (Cytospin 2; Shandon Southern Products, Ltd., Runcorn, England), fixed for 15 min sequentially in 50%, 75%, and 95% (v/v) ethanol, and then air dried. They were then stained for 15 min with propidium iodide (50 μg/ml in 50 mM sodium citrate, pH 7.6) containing 500 μg/ml of RNase A and observed under a Nikon Optiphot fluorescence microscope with an HFX-II camera attachment.

Alkaline Elution of Drug-Treated Cells. Exponentially growing CHO cells were labeled with [<sup>3</sup>H]thymidine (50 nCl/ml) for 24 h, after which they were drug treated, rinsed twice with drug-free medium (37°C), and incubated at 37°C in drug-free medium. Cells were harvested at various posttreatment times, and DNA SSB were estimated by alkaline elution, as previously described (10). Internal standard cells were included in each experiment. These were murine leukemia L1210 cells, which had been labeled for 24 h with 100 nCi/ml of [<sup>3</sup>H]thymidine and irradiated with 300 cGy using a 60Co source at a dose rate of 196 cGy/min. cGy-equivalent values of drug-induced SSB were calculated based on the elution of untreated control cells and cells which had received 300 cGy, according to established protocols (10). One cGy-equivalent approximates to 1 strand break per 9 × 10<sup>6</sup> base pairs (11).

p34<sup>cdc2</sup> Kinase Assays. CHO cells were lysed in 50 mM Tris-Cl, pH 7.4, 250 mM sodium chloride, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM sodium fluoride containing the protease inhibitors antipain, aprotinin, chymostatin, leupeptin, and pepstatin A each at a concentration of 20 μg/ml, and 2 mM phenylmethylsulfonyl fluoride. Lysates were incubated on ice for 30 min, followed by centrifugation at 13,000 × g for 5 min at 4°C. Supernatants were recovered, and their protein concentration was estimated (12). For each kinase assay, p34<sup>cdc2</sup> was...
immunoprecipitated from 100 µg of total cellular protein using affinity-purified polyclonal antiserum (kindly provided by Dr. Giulio Draetta, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), and histone H1 kinase activity was assayed, as previously described (13).

RESULTS

Cell Division, Viability, and Nuclear Integrity. Treatment of exponentially growing CHO cells with 25 µM etoposide for 1 h (1.5% survival by colony-forming assay) resulted in immediate cessation of cell division (Fig. 1A). At 12 h posttreatment approximately 80% of cells were arrested in G2, consistent with previously published data regarding the effects of etoposide on the cell cycle distribution of mammalian tumor cell lines (6, 14–16). However, the G2 arrest was transient, as between 12 and 24 h post-etoposide treatment the cell number increased by approximately 1.5-fold (Fig. 1A). After this point, no further cell division took place, and cells began to lift from the surface of the culture vessel at 36 h posttreatment. Between 48 and 96 h posttreatment the number of cells attached to the culture dish significantly decreased (Fig. 1A).

The trypan blue exclusion test, a determination of the integrity of the plasma membrane, has been considered a marker for cell viability. Thus, in order to systematically characterize the process of etoposide-induced cell death in CHO cells, it was necessary to determine at which point this ability was lost. Etoposide-treated CHO cells retained the ability to exclude trypan blue until 48 h post-treatment (Fig. 1B). However, by 48 h post-etoposide treatment only approximately 50% of cells excluded trypan blue, and this figure had dropped to roughly 30% by 72 h posttreatment (Fig. 1B). As noted, at this point cells were beginning to lift from the surface of the culture vessel, and none of these cells retained the ability to exclude trypan blue (data not shown).

Thus, it appears that loss of plasma membrane integrity is the conclusion of a chain of events which ultimately determines the death of a cell. In order to gain further insights into these processes, etoposide-treated cells were studied by fluorescence microscopy. At 12 h post-etoposide treatment each cell contained one intact nucleus (Fig. 2A). However, micronucleation of cells exposed to etoposide was apparent as early as 18 h posttreatment (Fig. 2B), concomitant with the passage of cells through mitosis after overcoming G2 arrest. By 36 h post-etoposide treatment less than 30% of cells contained an intact single nucleus (Fig. 2D). Wright/Giemsa staining of etoposide-treated cells followed by light microscopy revealed that micronucleation was associated with a light staining, vacuolated cytoplasm (data not shown). Observation of mitotic figures at 18 and 24 h posttreatment indicated that chromosome fragmentation was a likely explanation for the micronucleation phenomenon (Fig. 2, B and C). At 48 h posttreatment, cells exhibited a high degree of fragility, consistent with their inability to exclude trypan blue. These results were confirmed by electron microscopy of etoposide-treated cells (data not shown). The time course of nuclear fragmentation is represented graphically in Fig. 3.

DNA Strand Breakage and Inhibition of p34cdc2 Kinase Activity. Drug-induced G2 arrest appears to be followed by a mitosis resulting in micronucleation, the consequences of which do not allow further cell division. In order to investigate whether residual DNA strand breakage could contribute to these processes, alkaline elution was carried out on CHO cells at various times after exposure to etoposide. Fig. 4 demonstrates that a 1-h exposure of CHO cells to etoposide (25 µM) caused a high incidence of DNA SSB (0-h posttreatment incubation). However, upon removal of drug and incubation of cells in drug-free medium, DNA SSB rapidly resealed, with less than 20% of original SSB present at 1 h posttreatment (Fig. 4). cGy-equivalent values of SSB were negligible at 12, 18, and 24 h post-etoposide treatment. These results were confirmed by agarose gel electrophoresis of genomic DNA extracted from etoposide-treated CHO cells, which showed no significant degradation of DNA up to 24 h posttreatment (data not shown).

Inactivation of the cell cycle-regulated protein kinase p34cdc2 may contribute to G2 arrest (see Ref. 9). The following experiment indicates that regulation of p34cdc2 kinase activity, after G2 arrest, is abnormal and may contribute to the cell death process. Immunoprecipitated p34cdc2 kinase activity was initially inhibited after etoposide exposure (compare Lanes 1 and 2 with Lanes 3 and 4 in Fig. 5). Subsequently the kinase activity recovered and remained elevated (approximately 2-fold) compared with asynchronously growing control cultures until at least 36 h posttreatment (Fig. 5, Lanes 3 to 9). By this time, however, no further cell division took place (Fig. 1A). This differed from the anticipated reduction in p34cdc2 kinase activity to at least control levels following mitosis (compare Lanes 1 and 9 in Fig. 5). However, elevated kinase activity was less than that of an enriched G2-M population at 5 h (Lanes 11) and 6 h (Lanes 12) postrelease of a double thymidine block (see Ref. 9). p34cdc2 kinase activity of CHO cells decreased significantly at 48 h posttreatment (Fig. 5, Lane 10), concomitant with their loss of viability (Fig. 1B).

DISCUSSION

This study demonstrates that the sequence of events leading to cell death in CHO cells treated with etoposide occurs in the following manner. Drug treatment induces DNA strand break-
ETOPOSIDE-INDUCED CYTOTOXICITY

Fig. 2. Postmitotic micronucleation of etoposide-treated CHO cells. Following drug treatment (25 μM etoposide for 1 h), cells were incubated in drug-free medium for 12 h (A), 18 h (B), 24 h (C), and 36 h (D), after which they were harvested by trypsinization and washed twice with phosphate-buffered saline. Approximately 10,000 cells were spun onto a glass microscope slide, stained with propidium iodide, and photographed under fluorescent illumination.

Fig. 3. Time course of nuclear fragmentation in CHO cells following etoposide treatment (25 μM, 1 h). Exponentially growing CHO cells were treated with etoposide and incubated in drug-free medium for the indicated times, after which they were harvested and prepared for staining (see Fig. 2). Approximately 500 cells were examined for each time point, and the proportion of those with a single intact nucleus calculated.

Fig. 4. Formation and repair of etoposide-induced DNA SSB in CHO cells. Following etoposide treatment (25 μM, 1 h), cells were either harvested immediately or incubated in drug-free medium. At the indicated times cells were harvested and processed for estimation of DNA SSB by alkaline elution. Each point represents the mean ± SEM of two determinations.

Vacuolated, and the ability to regulate homeostasis is lost along with the integrity of the plasma membrane, allowing uptake of trypan blue.

Morphologically, cell death has been classified as either necrotic or apoptotic (17). Kerr et al. (18) described a particular cell death process, termed apoptosis, which follows a chain of deliberate events culminating in cell death. Other workers have identified an early event in apoptosis as being internucleosomal DNA cleavage brought about by activation of a putative endonuclease (19). Apoptosis appears readily inducible in lymphoid
tissue exposed to glucocorticoids (20). Recently it was reported that anti-hormone-induced cell death of human tumor cells followed the apoptotic pathway (21), and that monoclonal antibodies raised against a human B-lymphoblast cell line could induce tumor regression via apoptosis (22). Other workers have reported apoptotic death induced by etoposide in CHO cells (23); however, the time of appearance of the internucleosomal pattern of DNA cleavage (25 h posttreatment) occurs considerably later than the appearance of micronucleated cells reported in this study (within 18 h posttreatment). We have found no evidence for an internucleosomal pattern of DNA cleavage in CHO cells treated with etoposide until at least 24 h posttreatment. However, in agreement with Sorensen and Eastman (24) we find that loss of trypan blue exclusivity occurs after a host of other cell death-associated changes have appeared. We conclude, therefore, that the trypan blue viability test is crude, at best, and that its relationship to cytotoxicity greatly depends upon the time at which drug-treated cells are assayed.

The micronucleation phenomenon may represent the manifestation of chromosomal instability during mitosis, which may arise from acentric chromosome fragments or lagging whole chromosomes. This has been observed to occur spontaneously in fibroblasts from those individuals expressing chromosome instability and/or mutagen hypersensitivity (25). It is induced by X-irradiation (26) and by a wide variety of genotoxic agents (27–30). Consistent with the studies reported here, we propose that micronucleation results from a prolonged mitosis, during which the activities of specific cell cycle-regulated proteins are altered. Lau and Pardee (31) induced micronucleation of nitrogen mustard-treated baby hamster kidney cells by circumventing G2 arrest with caffeine. In their study the degree of micronucleation induced by nitrogen mustard correlated with its cytotoxicity. However, DNA damage alone may not be a prerequisite for micronucleation. The p34\(^{\text{cdc2}}\) kinase complex interacts directly with p13 in human cells, in an as yet undefined manner (32). Microinjection of antibodies to p13, or of p13 itself, causes postmitotic micronucleation in rat fibroblasts (33). These findings are likely to have significance in the drug-induced micronucleation phenomenon, as our results suggest that, in etoposide-treated CHO cells, p34\(^{\text{cdc2}}\) kinase is not fully activated compared with synchronized G2 cells, which could account for a prolonged mitosis (33). Residual DNA strand breakage, which might result in delayed mitosis, did not appear to account for the observed morphological effects, as DNA SSB had resealed within 12 h of drug removal, and no strand breaks were detected between 12 and 24 h posttreatment. Thus, the appearance of fragmented chromosomes probably results from gross alterations of chromosome structure rather than random DNA SSB.

It is possible, however, that residual DNA damage remained undetectable by the techniques used, or that DNA recombination events resulting from cleavable complex formation may ultimately be responsible for chromosome breaks and cytotoxicity.

Currently we are studying whether abnormal spindle formation may contribute to chromosome fragmentation in drug-treated cells. Time-lapse microscopy of drug-treated cells may define the relationship between extended mitoses and micronucleation. Our observations may be only a part of a cellular response to cleavable complex formation, although they form the basis for further characterization of the etoposide-induced cell death pathway.

Gaulden (34) proposed that DNA-damaging agents may alter the activity of specific nuclear proteins, including topoisomerase II, thereby affecting chromosomal structure resulting in fragmentation. Our observation, that p34\(^{\text{cdc2}}\) kinase activity remains elevated until 36 h post-etoposide treatment, may contribute to this hypothesis. The putative intracellular target for etoposide, topoisomerase II, must be active in order for separation of chromosomes during mitosis (35, 36). However, while we have not measured actual catalytic activity of topoisomerase II, etoposide-treated CHO cells retain an active drug target until at least 18 h posttreatment, which was assayed by retreated cells with drug and quantitating DNA SSB by alkaline elution (data not shown). Therefore, it is unlikely that inactivation of the drug target leads to the noted effects on nuclear integrity.

Spontaneous premature chromosome condensation occurs when the completion of DNA synthesis is uncoupled from mitosis (37) and has been associated with micronucleation of CHO cells (38). The factors which control this response have yet to be identified. p34\(^{\text{cdc2}}\) kinase activity may be one of these factors, however, as it is a component of maturation promoting factor, an inducer of chromosomal condensation (39). We found no evidence of spontaneous premature chromosome condensation in etoposide-treated CHO cells. Teniposide, an analogue of etoposide, induces extensive chromosome damage in CHO cells, as revealed by forcing the chromosomes to condense by fusion with a mitotic cell (8). Unfortunately, the drug effects were assayed at 30 h posttreatment (8), a point at which micronucleation is apparent under our experimental conditions. It would be prudent to assay for these lesions at, for example, 12 h posttreatment in G2-arrested cells in order to determine whether gross chromosomal aberrations may have an affect on mitosis in etoposide-treated cells.

In summary, we have demonstrated that the procession of etoposide-induced cell death in CHO cells, at cytotoxicity relevant concentrations of drug, involves a transient G2 arrest followed by abnormal chromosomal segregation which results in micronucleation of daughter cells. Internucleosomal DNA cleavage presumably occurs subsequent to micronucleation. Associated with these events is an inability to regulate p34\(^{\text{cdc2}}\) kinase activity following mitosis. These observations may contribute to our understanding of the pathways of chemotherapeutic drug-induced tumor cell death.

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