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Unscheduled Activation of Cyclin B1/Cdc2 Kinase in Human Promyelocytic Leukemia Cell Line HL60 Cells Undergoing Apoptosis Induced by DNA Damage

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Abstract

We have studied changes in cyclin A- and B1-dependent kinases during apoptosis induced in human promyelocytic leukemia (HL60) cells treated with the topoisomerase I inhibitor camptothecin. We found that cyclin B1/Cdc2 kinase activity transiently increases within 30 min after camptothecin treatment. This increase is followed by a rapid inactivation of the cyclin B1/Cdc2 kinase that is associated with Cdc2 tyrosine phosphorylation without any change in Cdc2 or cyclin B1 protein levels. The DNA polymerase inhibitor aphidicolin abrogates camptothecin-induced changes in cyclin B1/Cdc2 kinase activity, indicating that DNA replication-induced DNA damage is essential for both Cdc2 alterations and apoptosis activation. Apoptosis and the initial cyclin B1/Cdc2 kinase activation were amplified using synchronized S-phase cells, and cyclin A/cdk2 kinase did not change under these conditions. The same transient activation and subsequent inactivation of cyclin B1/Cdc2 kinase were observed after DNA damage by etoposide or bis-(2-chloroethyl)methylamine hydrochloride. These observations suggest that DNA damage promotes the transient and unscheduled stimulation of cyclin B1/Cdc2 kinase activity in HL60 cells prior to apoptosis.

Introduction

Apoptosis is a major form of cell death which is implicated in various processes such as embryogenesis, carcinogenesis, and immune and toxic cell killing. It is morphologically characterized by condensation and fragmentation of nuclear chromatin and cell shrinkage (1). HL60 human promyelocytic leukemia cells are particularly sensitive to apoptotic cell death following treatment with various chemotherapeutic agents (2, 3). The reasons behind this ultra sensitivity remain, however, poorly understood. Besides c-myc protooncogene overexpression, which is usually reported to be a potent inducer of apoptosis (4), HL60 also express Bcl-2, the well-established apoptosis-suppressor gene product (5). Furthermore, HL60 cells do not express p53 (6), which appears to be a key regulator of the induction of apoptosis following DNA damage (7).

The top1 inhibitor, CPT, is among the most promising anticancer drugs presently in clinical trials (8). CPT is a selective top1 inhibitor which traps top1-cleaveable complexes (9, 10). CPT causes rapid DNA synthesis inhibition and DNA damage after collision of DNA replication forks with CPT-stabilized top1-DNA cleavable complexes (11–13). HL60 cells subsequently die by apoptosis within 3 hours of CPT treatment. As described previously (14), CPT-induced apoptosis and DNA fragmentation, as measured by a filter elution assay, can be detected after 1 h of CPT treatment and increase rapidly within 3 h. This DNA fragmentation corresponds to internucleosomal DNA fragmentation as a common hallmark of apoptosis (1).

A number of cyclin-dependent protein kinases have been recently isolated and shown to regulate cell cycle events in mammalian cells (15, 16). Cdc2 (cdk1) kinase activation controls the G1/S transition by promoting the breakdown of the nuclear membrane, chromatin condensation, and microtubule spindle formation. This kinase is activated by threonine-161 phosphorylation and its association with other proteins, primarily cyclin B. During G2, Cdc25 phosphatase binds to and activates the cyclin B/Cdc2 complex by dephosphorylating Cdc2 phosphotyrosine 15 and phosphothreonine 14 residues, thus allowing catalysis of ATP within the ATP binding pocket (15, 16). During S-phase, phosphorylation of Cdc2 at these two amino acids keeps the kinase inactive, in spite of threonine-161 phosphorylation and cyclin B binding which together activate Cdc2. At the end of mitosis, cyclin B/Cdc2 complexes disassemble and lose activity. Another kinase, cyclin A/cdk2 kinase is primarily active during S and G2 and appears to be linked to DNA synthesis (15, 16). Alterations of cyclin-dependent protein kinases have been suggested to play a key role in the activity of chemotherapeutic agents (17).

The present study represents an attempt to correlate apoptosis and cyclin-dependent kinase alterations in HL60 cells. The rapidity of apoptosis in HL60 cells treated by DNA-damaging agents, the lack of inhibitory effect of the protein synthesis inhibitor, cycloheximide (14), and the facile induction of apoptosis by the protein kinase inhibitor, staurosporine (18), suggest that intracellular signaling and thus, protein phosphorylation might play a key role in apoptosis. Our results show the unscheduled activation of cyclin B1/Cdc2 kinase activity during HL60 apoptosis induced by DNA-damaging agents.

Materials and Methods

Drugs, Antibodies, and Chemicals

CPT was a generous gift from Drs. M. E. Wall and M. C. Wani (Research Triangle Park, NC) and VP-16 was generously provided by Bristol-Myers-Squibb Laboratories, Syracuse, NY. They were freshly dissolved in DMSO at 10 mM and further diluted in water prior to each experiment. Nitrogen mustard was obtained from Sigma Chemical Co. and was prepared as a 10 mM stock solution in 0.1 M hydrochloric acid.

Anti-cyclin B1 and anti-cyclin A monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Cdc2 monoclonal antibody was obtained from 1Cn Biomedicals (Costa Mesa, CA) for Western blotting and anti-Cdc2 polyclonal antibodies from Gibco-BRL (Grand Island, NY) for immunoprecipitation. Anti-phosphotyrosine monoclonal antibody was from UBI (Lake Placid, NY).

Radiolabeled precursor [3H]thymidine (53.6 mCi/mmol) was purchased from New England Nuclear (Boston, MA). All other chemicals were of reagent grade and were purchased from either Sigma or other local sources.

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2 The abbreviations used are: top1, DNA topoisomerase I; CPT, camptothecin; VP-16, 4',6-diamino-2-phenylindole; etoposide; nitrogen mustard, bis-(2-chloroethyl)methylamine hydrochloride.

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Cell Culture and DNA Labeling

HL6O cells were grown in RPMI 1640 supplemented with 10% FCS (GIBCO-BRL), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 95% of air and 5% CO₂. For filter elution assays, HL6O cells were incubated with 0.01 μCi/ml [³H]thymidine for one doubling time, washed with fresh medium twice, and chased in isotope-free medium overnight before drug treatment.

Cell Synchronization and Cell Cycle Analysis

Cell synchronization was performed by double thymidine block. Exponentially growing cells were incubated with 2 mM thymidine for 12 h, followed by washing and incubation in fresh medium for 16 h, and treatment with 2 mM thymidine for 14 h. Cells were then washed by centrifugation and released in fresh medium. At the indicated times, cells were harvested and samples taken for cell cycle analysis and kinase assays in the absence or presence of CPT. Cells for cell cycle analysis were washed in PBS, fixed in 70% ethanol, treated with RNase, stained with propidium iodide, and subjected to flow cytometry (Becton Dickinson) to measure DNA content.

Filter Elution Assays for Measurement of DNA Fragmentation

DNA fragmentation related to apoptosis was measured by filter elution assay as described previously (18). Briefly, cell samples were deposited on protein-adsorbing filter (Metriceel; Gelman Science, Ann Harbor, MI), washed with 5 ml 0.02 M Na₂EDTA, and lysed with 5 ml LS10 [2 M NaCl, 0.04 M Na₂EDTA, and 0.2% sarkosyl (pH 10)]. The cell deposition (medium, M), Na₂EDTA, and lysis (L) samples, and filters (F) were collected and counted by liquid scintillation (18). DNA fragmentation was calculated as the percentage of DNA eluting from the filter as:

\[ \% \text{ DNA fragmentation} = 100 \times \frac{(M + E + L)}{(M + E + L + F)} \]

Cyclin, cdk5, and Kinase Assays

Cells were pelleted, washed once in cold PBS, and lysed on ice as described previously (19). Protein determination was performed using a protein assay kit according to the manufacturer’s instructions (Pierce, Rockford, IL). Eighty μg of total cell protein was loaded per lane on a standard SDS-protein gel for Western blotting analysis. Seven hundred μg of cell protein per sample was used for immunoprecipitation, which was performed as described (19).

In Vitro H1 Kinase Assays. Immune complexes were resuspended in kinase buffer as described (19) and then incubated at 37°C for 20 min. Reactions were stopped by boiling samples in 3X SDS-gel loading buffer for 5 min.

Gel Electrophoresis and Western Blotting. Samples were loaded onto precast SDS-polyacrylamide gels (NOVEX, San Diego, CA) and electrophoresed at 125 V for 2 h. For quantification of kinase activity of immunoprecipitated, gels were dried, and the extent of histone H1 phosphorylation was measured by autoradiography and Phosphorimager (Molecular Dynamics, Sunnyvale, CA). For immunoblotting, proteins were transferred to Immobilon membranes (Millipore, Bedford, MA), and blots were detected as described (19).

Results and Discussion

Cyclin B1/Cdc2 Immune Complex Kinase Activity Increases Transiently and Then Rapidly Decreases Following CPT Treatment. To investigate relationships between cyclin-dependent kinases and CPT-induced apoptosis, immunoprecipitates were prepared using anti-cyclin B1 antibody, and histone H1 kinase activity was measured at various times after CPT treatment. Fig. 1A shows a representative cyclin B1/Cdc2 kinase activity profile from immunoprecipitates of HL60 cells treated for up to 4 h with 1 μM CPT. As quantitated in Fig. 1B, kinase activity increased transiently with a peak after a 30-min CPT treatment and then decreased quickly below control levels. In case of 0.15 μM CPT treatment, similar changes in kinase activity were observed, but their kinetics was slower than in the case of 1 μM CPT treatment (Fig. 2B, inset). The magnitude of this initial increase was relatively small but reproducible in at least five independent experiments for each time point. Inset: cyclin B1/Cdc2 kinase activity after treatment with 0.15 μM CPT. The means (bars, SD) were from at least three independent experiments. C. autoradiography of cyclin B1/Cdc2 kinase activity of immunoprecipitates from HL60 cells treated with 1 μM CPT in the absence or presence of 1 μM aphidicolin (APH).

Neither Cdc2 Nor Cyclin B1 Protein Levels Change during the Cyclin B1/Cdc2 Kinase Activity Alterations Induced by CPT. To see whether the changes in cyclin B1/Cdc2 H1 kinase activity were due to changes in protein levels, we performed immunoblotting of whole cell lysates from CPT-treated cells. As shown in Fig. 2, A and B, Cdc2 or cyclin B1 protein levels did not change during CPT treatment.

Since Cdc2 kinase is inactivated when threonine-14 and tyrosine-15 of Cdc2 are phosphorylated, we performed immunoblotting analyses of Cdc2 immunoprecipitates with an antiphosphotyrosine antibody. As shown in Fig. 2C, tyrosine-phosphorylated Cdc2 bands were observed only 2 and 4 h after CPT treatment without being seen in the
exponentially growing HL60 cells. The mechanism of transient Cdc2 kinase activation remains to be determined. Activation of the Cdc2-activating kinase, which was recently discovered and phosphorylates threonine-161 of the Cdc2 protein, can be invoked for the activation of Cdc2 kinase (20). However, reagents to test this possibility were not available at the time of the study. It is also possible that the structure of the cyclin B1/Cdc2 kinase complex could be modified by the binding of a yet unknown activating factor.

Previous studies in synchronized HeLa cells treated briefly with CPT did not reveal similar increases in Cdc2 kinase activity (21). However, HeLa cells did not appear to undergo apoptosis following CPT treatment. Suppression of Cdc2 kinase activity was observed in HeLa cells, although the mechanism of this suppression was complex. Cyclin B protein was maintained at high levels in spite of suppression of cyclin B protein synthesis, presumably due to cyclin B stabilization. Cdc2-tyrosine dephosphorylation was also blocked in CPT-treated HeLa cells. Moreover, in Chinese hamster ovary cells, treatment with the topoisomerase II inhibitor, VP-16, was shown to block Cdc2 kinase activation, due to inhibition of Cdc2 tyrosine dephosphorylation, and to cause G2 arrest without apoptosis (22–24). Nitrogen mustard produced similar effects on Cdc2 in CA46 cells, a human myeloma cell line, and induced the rapid but transient cyclin B1/Cdc2 kinase activation observed for CPT (Fig. 4). This was again followed by a subsequent decrease in kinase activity and apoptosis (data not shown) during the next 2 h of treatment.

These results suggest that several different types of DNA damage can induce the unscheduled activation of cyclin B1/Cdc2 kinase activity in HL60 cells, which is prior to or commensurate with the induction of apoptosis. The importance of Cdc2 activation in the process of apoptosis remains to be explored, but it is conceivable that

Fig. 2. Immunoblotting analyses of whole cell lysates or immunoprecipitates from HL60 cells treated with 1 μM CPT. A, immunoblotting of whole cell lysates with anti-Cdc2 antibody; B, with anti-cyclin B1 antibody; C, immunoblotting of Cdc2-immunoprecipitates with anti-phosphotyrosine antibody. CPT treatment times (h) are indicated under pictures.

The Initial Increase in Cyclin B1/Cdc2 Kinase Activity Is Amplified during CPT Treatment of S-phase Cells. Since cyclin B1/Cdc2 kinase activity is primarily activated during the G2-M transition, we sought to determine whether the kinase activation could occur in S-phase, as HL60 cells in S-phase are selectively sensitive to apoptosis (26). Cell cycle synchronization was achieved with double thymidine block. Under these conditions, the fraction of cells in S-phase was 70–80% compared with 30–40% in unsynchronized exponentially growing HL60 cells (data not shown). CPT was added to the synchronized HL60 cells 1 h after the release from the second thymidine block in order to let DNA synthesis recover from the double thymidine block. The cells were subsequently incubated with CPT for up to 2 h. As shown in Fig. 3, cyclin B1/Cdc2 kinase activity again peaked after 15 min CPT treatment and decreased very rapidly to control level. Under these conditions, the cyclin B1/Cdc2 kinase activation was greater than in the exponentially growing cells (2.5-fold versus 1.3-fold). These data indicate that the cyclin B1/Cdc2 activation can occur in S-phase cells and is, therefore, unscheduled. By contrast, the cyclin A/cdk2 kinase activity did not show any increase but rather a slight decrease during CPT treatment (Fig. 3B). Accordingly, we conclude that unscheduled stimulation of cyclin B1/Cdc2 kinase activity probably takes place in the cells that are committed to apoptosis.

The Same Cyclin B1/Cdc2 Kinase Activation Is Observed after VP-16 or Nitrogen Mustard Treatment. We also studied the effects of the topoisomerase II inhibitor, VP-16, and an alkylating agent, nitrogen mustard, on cyclin B1/Cdc2 kinase activity. Both drugs induced the rapid but transient cyclin B1/Cdc2 activation observed for CPT (Fig. 4). This was again followed by a subsequent decrease in kinase activity and apoptosis (data not shown) during the next 2 h of treatment.

These results suggest that several different types of DNA damage can induce the unscheduled activation of cyclin B1/Cdc2 kinase activity in HL60 cells, which is prior to or commensurate with the induction of apoptosis. The importance of Cdc2 activation in the process of apoptosis remains to be explored, but it is conceivable that
this or other kinases could be involved in some of the mechanistic (morphological) changes of apoptosis. In fact, recent reports indicate that premature activation of cyclin-dependent kinases occurs very early in the apoptotic process (27, 28). Taken together, these results indicate that unscheduled and transient cyclin B1/Cdc2 activation take place in response to DNA damage in HL60 cells that are committed to apoptosis.

Significance. Apoptosis has recently emerged as a key response of cells to cancer chemotherapy. Its occurrence may account for the common response of leukemias and the side effects (bone marrow and gastrointestinal toxicity) of chemotherapy. However, the biochemical and signal transduction pathways of apoptosis are still poorly known. The possibility of a functional link between cell cycle regulatory pathways and apoptosis may give a promising lead. The significance of unscheduled cyclin B1/Cdc2 kinase activation as an early event of apoptosis remains hypothetical. Since Cdc2 kinase activation could cause the breakdown of nuclear membrane and chromatin condensation, cyclin B1/Cdc2 might be involved in the nuclear changes (permeabilization to cytoplasmic nucleases, activation of nucleases, chromatin disruption, and condensation) that are observed during apoptosis.

References

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