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Cdc2 and Cdk2 play critical roles in low dose doxorubicin-induced cell death through mitotic catastrophe but not in high dose doxorubicin-induced apoptosis ☆

Seok Soon Park, Young-Woo Eom, Kyeong Sook Choi *

Institute for Medical Sciences, Ajou University School of Medicine, Suwon 442-749, Republic of Korea

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Abstract

In Huh-7 hepatoma cells, low dose (LD) doxorubicin treatment induces cell death through mitotic catastrophe accompanying the formation of large cells with multiple micronuclei, whereas high dose (HD) doxorubicin induces apoptosis. In this study, we investigated the role of Cdc2 and Cdk2 kinase in the regulation of the two modes of cell death induced by doxorubicin. During HD doxorubicin-induced apoptosis, the histone H1-associated activities of Cdc2 and Cdk2 both progressively declined in parallel with reductions in cyclin A and cyclin B protein levels. In contrast, during LD doxorubicin-induced cell death through mitotic catastrophe, the Cdc2 and Cdk2 kinases were transiently activated 1 day post-treatment, with similar changes seen in the protein levels of cyclin A, cyclin B, and Cdc2. Treatment with roscovitine, a specific inhibitor of Cdc2 and Cdk2, significantly blocked LD doxorubicin-induced mitotic catastrophe and cell death, but did not affect HD doxorubicin-induced apoptosis in Huh-7, SNU-398, and SNU-449 hepatoma cell lines. Our results demonstrate that differential regulation of Cdc2 and Cdk2 activity by different doses of doxorubicin may contribute to the induction of two distinct modes of cell death in hepatoma cells, either apoptosis or cell death through mitotic catastrophe.

Keywords: Cdc2; Cdk2; Doxorubicin; Mitotic catastrophe; Apoptosis; Hepatoma; Micronuclei; DNA fragmentation; Dose effect; Death mode

The anthracycline antibiotic, doxorubicin, is one of the most important anticancer agents for the treatment of solid tumors [1]. However, despite the widespread clinical use of doxorubicin, its anti-proliferative and death-inducing signal transduction pathways are far from well characterized. Doxorubicin is thought to act through DNA intercalation/binding, inhibition of topoisomerase II, free radical generation, or damage to cell membranes [2]. As with many other anticancer drugs, high doses (HDs) of doxorubicin induce apoptosis via activation of caspases and disruption of inner mitochondrial membrane potential [3]. However, several lines of evidence indicate that low doses (LDs) of various chemotherapeutic drugs are capable of inducing mitotic catastrophe [4–6]. Originally, mitotic catastrophe was defined as a type of cell death resulting from abnormal mitotic events that produce improper chromosomal segregation and cell division, leading to the formation of large, nonviable cells with multiple micronuclei and decondensed chromatin [4]. However, more recent studies have redefined mitotic catastrophe as an abnormal mitosis that leads to cell death (which can occur through necrosis or apoptosis) rather than an actual form of cell death [7,8]. We recently showed in human hepatocellular

☆ *Abbreviations:* PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; calcein-AM, calcein-acetoxymethyl ester; Etd-1; ethidium homodimer-1.

* Corresponding author. Fax: +82 31 219 4503.
E-mail address: kschoi@ajou.ac.kr (K.S. Choi).

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carcinoma (HCC) cells that LD doxorubicin induces cell death through mitotic catastrophe, and that this is a morphologically distinct death mode from the apoptosis induced by HD doxorubicin [9]. However, the molecular basis of this cell death through mitotic catastrophe is largely unknown, as are the underlying mechanisms by which two distinct modes of cell death are induced by different doses of the same anti-cancer drug. In this study, we investigated whether differential regulation of cell cycle regulators contributes to the induction of apoptosis or cell death through mitotic catastrophe in doxorubicin-treated cells. Our results reveal for the first time that the roles of Cdc2 and Cdk2 differ in cells undergoing LD doxorubicin-induced cell death through mitotic catastrophe versus those undergoing HD doxorubicin-induced apoptosis.

Materials and methods

Reagents. Doxorubicin and roscovitine were purchased from Sigma (St. Louis, MO). The following antibodies were purchased: anti-Cdc2, Cdk2, Cdk4, Cdk6, cyclin A, cyclin B, cyclin D, cyclin E, p15, p16, p19, p21, p27, BubR1, Chk1, and Chk2 (from Santa Cruz Biotechnology); CENP-A (from Upstate Biotechnology); and Plk1 (from BD Transduction Lab).

Induction of cell death through mitotic catastrophe or apoptosis by different doses of doxorubicin. Cell death through mitotic catastrophe was induced in Huh-7 cells by chronic exposure to 50 ng/ml doxorubicin. Briefly, Huh-7 cells were first plated in 10 cm dishes with 6 ml DMEM containing 10% FBS. After overnight culture, this medium was replaced with 6 ml of fresh DMEM containing 10% FBS and 50 ng/ml doxorubicin. After 3 days, 3 ml of fresh DMEM containing 10% FBS and 50 ng/ml doxorubicin was added to the pre-existing medium. Thereafter, 3 ml of doxorubicin-containing fresh medium was added every 3 days in the same manner, until day 12. SNU-398 and SNU-449 human HCC cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and grown in RPMI containing 10% FBS and antibiotics. Mitotic catastrophe was induced in SNU-398 cells by treatment with 50 ng/ml doxorubicin and in SNU-449 cells by treatment with 150 ng/ml doxorubicin. Doxorubicin-induced apoptosis in these cells was induced by treatment with 10 μg/ml doxorubicin for 48 h.

Staining of nuclei. Cells were washed twice with PBS and fixed in 4% formaldehyde for 10 min at room temperature, and then washed three times with PBS. Fixed cells were permeabilized in 0.1% Triton X-100/2% BSA, and stained with 1 μg/ml Hoechst 33258 (Sigma). Stained cells were examined by fluorescence microscope (Axiovert 200M, Carl Zeiss).

Measurement of cellular viability. After treatments, cell viability was assessed by double labeling of cells with 2 μmol/L calcein-acetoxymethyl ester (calcein-AM) and 4 μmol/L ethidium homodimer-1 (Etd-1). Calcein-positive live cells were counted under a fluorescence microscope, since Etd-1-positive dead cells were floated from the culture plates following doxorubicin treatment.

DNA fragmentation analysis by agarose gel electrophoresis. To isolate the fragmented DNA from Huh-7 cells treated with doxorubicin, cells were lysed using mild detergent buffer containing 0.5% Triton X-100, 5 mM Tris (pH 7.4), and 20 mM EDTA. At this step, small-sized cellular membrane pores, through which only apoptotic fragmented DNAs but not intact chromosomal DNAs can pass, are made. Lysates were collected, vortexed briefly, and microcentrifuged at 16,000*g* for 15 min at 4 °C to remove intact chromosomal DNAs. Supernatants were extracted with phenol–chloroform–isoamylalcohol (25:24:1), precipitated in ethanol containing 300 mM sodium acetate, and resuspended in Tris–EDTA buffer (pH 7.4). The soluble DNA samples were subjected to electrophoresis on 1.5% agarose gel. To isolate total chromosomal DNAs from cells treated with 50 ng/ml doxorubicin, genomic DNA samples were purified following the manufacturer's protocol (Promega, WI) and subjected to electrophoresis on 1.0% agarose gel.

TUNEL assay. Cells cultured on coverslips were treated with 50 ng/ml or 10 μg/ml doxorubicin for the indicated time points and fixed with 4% paraformaldehyde (pH 7.4) for 10 min. TUNEL assay of the fragmented DNAs was performed as recommended by the manufacturer (Boehringer–Mannheim).

Cell cycle analysis. Trypsinized and floating cells were pooled, washed with PBS–EDTA, and fixed in 70% (v/v) ethanol. DNA contents were assessed by staining cells with propidium iodide and monitoring by FACScan (Becton–Dickinson). Cell distribution was determined with a ModFit LT program (Verity Software House).

Cdc2 and Cdk2 immune complex kinase assay. After treatments, cells were lysed in buffer A (1% Nonidet P-40, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 20 mM NaF, 1 mM Na_3VO_4, and 10 μg/ml PMSF) at 4 °C for 15 min. Cell lysates were cleared by centrifugation at 13,000 rpm for 15 min. A total of 500 μg of protein was used for each immunoprecipitation. Cdc2 and Cdk2 in cell extracts were incubated with the specific antibody (1 μg/reaction) for 3 h at 4 °C. 15 μl of protein A/G–agarose (Oncogene Research Products, MA) was added into the mixture, which was then further incubated for 1 h. Immune complexes were centrifuged at 2500 rpm for 5 min and the precipitates were washed three times with buffer A and twice with kinase buffer [50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT]. Cdk kinase assays on histone H1 were performed by mixing the respective immune complexes with 5 μg of histone H1 and 10 μCi of [γ-^{32}P]ATP in 30 μl of kinase buffer. The kinase reaction was performed at 30 °C for 30 min and then terminated with 2× SDS–PAGE sample buffer. The reaction mixtures were resolved by SDS–polyacrylamide gel electrophoresis analysis. Gels were stained with Coomassie blue staining solution and dried. The extent of phosphorylation was measured by liquid scintillation counting of the gel slices of each substrate.

Results

Different does of doxorubicin induce two distinct modes of cell death, cell death through mitotic catastrophe and apoptosis

When we compared the effects of various doses of doxorubicin in Huh-7 human hepatoma cells, we found that treatment with 10 μg/ml doxorubicin induced apoptosis, which was characterized by cellular shrinkage, apoptotic blebbing, chromatin condensation, and fragmentation (Figs. 1A and B). In contrast, chronic treatment of Huh-7 cells with 50 ng/ml doxorubicin induced cell death through mitotic catastrophe, which was characterized by the formation of large cells with multiple micronuclei and decondensed chromatin (Figs. 1A and B). Cell viability was examined using calcein-AM and Etd-1 to detect live and dead cells, respectively. The results revealed that apoptosis was rapidly induced by 10 μg/ml doxorubicin, leading to loss of 50% cellular viability at 24 h. In contrast, cell death through mitotic