



Novel DNA damage checkpoint in mitosis: Mitotic DNA damage induces re-replication without cell division in various cancer cells

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ABSTRACT

DNA damage induces multiple checkpoint pathways to arrest cell cycle progression until damage is repaired. In our previous reports, when DNA damage occurred in prometaphase, cells were accumulated in 4 N-DNA G1 phase, and mitosis-specific kinases were inactivated in dependent on ATM/Chk1 after a short incubation for repair. We investigated whether or not mitotic DNA damage causes cells to skip-over late mitotic periods under prolonged incubation in a time-lapse study. 4 N-DNA-damaged cells re-replicated without cell division and accumulated in 8 N-DNA content, and the activities of apoptotic factors were increased. The inhibition of DNA replication reduced the 8 N-DNA cell population dramatically. Induction of replication without cell division was not observed upon depletion of Chk1 or ATM. Finally, mitotic DNA damage induces mitotic slippage and that cells enter G1 phase with 4 N-DNA content and then DNA replication is occurred to 8 N-DNA content before completion of mitosis in the ATM/Chk1-dependent manner, followed by caspase-dependent apoptosis during long-term repair.

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1. Introduction

The cells possess a defense system called checkpoints to resist from DNA damage and genotoxic insults, which are present at the G1/S boundary, intra-S, and G2/M transitions [1,2]. In the event of DNA damage, sensor proteins and signal transducer proteins associated with each checkpoint detect DNA damage and transmit signals to their appropriate effectors, which initiate cell cycle arrest, DNA repair, or apoptosis. When repair of DNA damage is completed, the cells restart the cell cycle. If cells do not successfully complete DNA repair, the cells continue cell cycle arrest and then must be removed by apoptosis or cellular senescence or oncogenesis [3,4]. The ataxia-telangiectasia mutated (ATM) activates checkpoints by transmitting a DNA damage transduction signal when cells are exposed to ionizing radiation or drugs that trigger DNA double strand breaks [5]. The ataxia-telangiectasia and Rad3-related (ATR) is known to be activated by stalled replication forks and UV-radiation-induced DNA damage [6,7]. ATM/ATR transmits actual signals to major downstream targets, such as Chk1 and Chk2 [3]. These checkpoint kinases negatively regulate Cdc25 phosphatase family proteins that dephosphorylate Cdks involved in the cell cycle transition [8]. Cdk1 activity and its destruction are required for regulation of the mitotic exit, proper chromosome segregation, cytokinesis, and progression to G1 phase in the normal cell cycle. Although the activation status of Cdk1 is dependent upon cyclin B1 binding, the tyro-

sine-15 phosphorylation site of Cdk1 is regulated by the Cdc25 phosphatase family through the ATM-Chk1/2-Cdc25C pathway [9,10]. Plk1 is associated with phosphorylation of Cdc25 and mitotic cyclin, centrosome maturation [11], establishment of bipolar spindle [12], and activity of the anaphase-promoting complex (APC/C) [13]. Plk1 is also a target of DNA damage checkpoint in an ATM-dependent manner [14,15]. Plk1 inhibition in this situation leads to cell cycle arrest [16,17]. In addition, mitotic DNA damage delays mitotic exit in association with inhibition of Cdk1 and Plk1, cytokinesis failure, and abnormal G1 phase with 4N DNA content, even cyclin B1 level is still high [16–18]. These cells have decreased phosphorylation of Cdc25C compared with mitotic phase cells [19]. DNA damage may generate an abnormal DNA phenotype in mitosis called multiploidy. Multiploidy induces chromosomal instability and is a common feature of tumor cells. It may promote tumor formation with a combination of additional factors in tissue or act to protect organisms from tumor formation by cell death [18,20]. In this report, we showed that mitotic DNA damage induced abnormal cell cycle arrest in G1 phase with 4N DNA content without cell division in the ATM/Chk1-dependent manner, and multiploidy followed to DNA re-replication without completion of mitosis was observed.

2. Method and materials

2.1. Cell culture and treatments

Cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS (Hyclone). To synchronize in prometaphase,

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cells were treated with nocodazole (100 ng/ml, Sigma) for 16 h and collected by shake-off. For mitotic DNA damage, mitotic cells were treated with doxorubicin (5 μ M, Sigma) for 1 h. Cells were incubated in fresh media for the indicated time. For inhibition of DNA replication, mitotic cells with doxorubicin shock were treated with hydroxyurea (2 mM, Sigma) for 24 h. To detect BrdU incorporation, cells were treated with bromodeoxyuridine (BrdU) (10 μ M, Sigma). To address caspase dependency, mitotic cells were treated with Z-VAD-FMK (100 μ M, R&D system) for 2 h prior to doxorubicin shock, and continuously incubated with Z-VAD-FMK (10 μ M) during releasing.

2.2. Cell transfection, gene silencing, and overexpression

Cells were transfected with plasmid DNAs by the calcium chloride technique. Plasmid construct of short hairpin RNA (shRNA) for gene silencing were provided by SABiosciences (Frederick, MD, USA). SureSilencing™ shRNA plasmid provided by SABiosciences (Frederick, MD, USA) expressed was used for gene-specific knock-down. Target sequences for knockdown of human Chk1 and ATM genes was as follow: 5'-1TTG GTT GAC TTC CGG CTT TCT-3' and 5'-CCA GAA TGT GAA CAC CAC CAA-3', respectively.

2.3. Analysis of cell cycle and BrdU incorporation by flow cytometry

Cells were trypsinized, fixed in ice-cold 80% ethanol for 16 h or longer, and incubated with RNaseA (100 μ g/ml) at 37 °C for 2 h. Cells labeled by propidium iodide (40 μ g/ml) were analyzed by flow cytometry of 25,000 events (FACSCaliber, Becton Dickinson). For quantification of BrdU incorporation, cells labeled with BrdU were harvested and fixed in ice-cold 70% ethanol for 16 h or longer. Cells were treated with 1 ml of HCl (1.5 M) at RT for 20 min, and incubated for 1 h in PBS containing 0.5% Tween-20, 0.5% BSA, and anti-BrdU antibody (1:200 dilution, SantaCruz). Cells were incubated with FITC-conjugated secondary anti-mouse antibody (1:100 dilution, SantaCruz) and propidium iodide (40 μ g/ml).

2.4. Microscopic analysis

For confocal microscopic analysis of BrdU incorporation, cells were cultured on glass coverslips and fixed in 4% paraformaldehyde for 15 min at RT. After washing, cells were incubated in Triton X-100 buffer (0.3% Triton X-100 in PBS) for 15 min at RT. For DNA denaturation, cells were incubated with 2 M HCl for 20 min, and were neutralized using 0.1 M sodium borate for 2 min. Cells were blocked in blocking buffer (10% Tween 20 and 2% horse serum in PBS) for 30 min, and were stained with BrdU conjugated FITC antibodies. The fluorescence signals were detected and captured by confocal microscopy (LSM510). For time-lapse microscopic analysis, mitotic cells were grown in glass bottom dish and detected on a microscope (Zeiss Axiovert S100) equipped with a LCI long-term microincubator chamber system with CO₂.

2.5. Western Blotting and antibodies

For Western Blot analysis, cells were lysed in NP-40 cell lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 8, 2 mM EDTA, pH 8.0, 2 mM EGTA, pH 8.0, and protease inhibitors). Cell lysates were separated on SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore). Following incubation with antibodies in TBS containing 0.05% Tween-20 and 5% skim-milk, protein signals were visualized using the ECL™ system (Amersham Biosciences). Antibodies for Chk1, ATM and PARP-1 were obtained from Santa Cruz Biotechnology. Anti-Caspase-3 antibodies were obtained from Cell Signaling.

3. Results

3.1. Mitotic HeLa cells with DNA damage do not undergo cytokinesis and enter G1 with 4 N-DNA content, and re-replication of DNA occurs without cell division

We previously reported that prometaphasic cells accumulate in G2-like interphase and not in mitosis as a result of DNA damage by doxorubicin shock [17]. Under this condition, activated Cdk1 in prometaphase became inactivated, and mitotic Plk1 was also dephosphorylated and inhibited by protein phosphatase 2A, which might function as a downstream target of the ATM/Chk1 pathway. Upon inactivation of two major mitotic kinases, cells cannot undergo the remaining steps of mitosis; instead, they enter G1 phase with 4N DNA content. These phenotypes of the mitotic DNA damage response were detected from 3 h after release into fresh media for repair [16].

To investigate the fate of mitotic cells with DNA damage after prolonged culture through this novel mitotic DNA damage response, cells were incubated for 24 h or longer. Cells synchronized in prometaphase were treated with doxorubicin for 1 h and then cultured continuously after washing for damage repair (Fig. 1A). During culture, we followed the cell cycle progression, and phenotypic changes in cells were detected by time-lapse imaging (Fig. 1B, C). Most mitotic cells without DNA damage by doxorubicin shock progressed into cytokinesis for further division within 30 min and became two daughter cells in G1 phase (Fig. 1B). After incubation for 13–15 h, cells entered into a new mitotic period and started a second round of cell division. On the other hand, when cells were treated with doxorubicin, they did not enter cytokinesis and remained as a single cell even during prolonged culture. Under this condition, cells with DNA damage turned into enlarged cells with multi-nuclei within 20 h of incubation (Fig. 1C). To distinguish between a defect in chromosomal segregation or nuclear division and a defect in cytokinesis, we performed time-lapse microscopic analysis using fluorescent label on chromosomes by expressing histone H2B-GFP. During releasing for 6 h, mitotic chromosomes were not segregated properly through normal anaphase, and cellular phenotypes of late mitosis were not observed (Fig. 1D, upper panels). As with Fig. 1C, multi-nuclei were observed in mitotic cells with DNA damage within 24 h after release (Fig. 1D, lower panels).

Normal mitotic cells without doxorubicin shock contained 4 N-DNA content, and most of the cells entered a new round of cell division, finally accumulating in interphase with 2 N-DNA content (Fig. 1, 20 h in a), as in normal asynchronous culture (Fig. 1E, g). When the doxorubicin concentration was increased from 0.2 μ M to 5 μ M, accumulation of cells in interphase containing 4 N-DNA content within 8 h after release increased (Fig. 1E, 8 h in b–f). After incubation for 20 h or longer, cells with 4 N-DNA content were characterized by multiploidy (Fig. 1E, 20 h in c–f). These data suggest that if mitotic DNA damage can be recovered from, multiploid cells will divide normally, but serious damage causes cellular defects.

Accumulation of cells in G1 phase with 4 N-DNA contents within 8 h of release was not changed by treatment with hydroxyurea in comparison with that without treatment. However, multiploid cells containing 8 N-DNA content totally disappeared after release for 20 h with hydroxyurea treatment (Fig. 2B, 20 h in d–f). Bromodeoxyuridine (BrdU) incorporation was starting in 5-hour releasing time point in cells with mitotic DNA damage (Fig. 2, b in C and BrdU-FITC in b, D). After 20 h-incubation, nuclear BrdU were diffused into the whole cytoplasm under the damage condition (Fig. 2D, 20 h in b). These data suggested that suggesting that mitotic cells containing DNA damage do not progress into cytokinesis and enter new interphase with 4 N-DNA content, whereas re-replication of 4 N-DNA occurs once during continuous culture without further cell division.

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