DNA Repair 12 (2013) 936–946

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

The complexity of DNA double strand breaks is a critical factor enhancing end-resection

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ARTICLE INFO

Article history:
Received 22 March 2013
Received in revised form 9 August 2013
Accepted 17 August 2013
Available online 13 September 2013

Keywords:
DNA double strand breaks
homologous recombination
DNA end resection
CtIP
ATM
complex DNA damage structure

ABSTRACT

DNA double strand breaks (DSBs) induced by ionizing radiation (IR) are deleterious damages. Two major pathways repair DSBs in human cells, DNA non-homologous end-joining (NHEJ) and homologous recombination (HR). It has been suggested that the balance between the two repair pathways varies depending on the chromatin structure surrounding the damage site and/or the complexity of damage at the DNA break ends. Heavy ion radiation is known to induce complex-type DSBs, and the efficiency of NHEJ in repairing these DSBs was shown to be diminished. Taking advantage of the ability of high linear energy transfer (LET) radiation to produce complex DSBs effectively, we investigated how the complexity of DSB end structure influences DNA damage responses. An early step in HR is the generation of 3′-single strand DNA (SSD) via a process of DNA end resection that requires CtIP. To assess this process, we analyzed the level of phosphorylated CtIP, as well as RPA phosphorylation and focus formation, which occur on the exposed SSD. We show that complex DSBs efficiently activate DNA end resection. After heavy ion beam irradiation, resection signals appear both in the vicinity of heterochromatic areas, which is also observed after X-irradiation, and additionally in euchromatic areas. Consequently, ~85% of complex DSBs are subjected to resection in heavy ion particle tracks. Furthermore, around 20–40% of G1 cells exhibit resection signals. Taken together, our observations reveal that the complexity of DSB ends is a critical factor regulating the choice of DSB repair pathway and drastically alters the balance toward resection-mediated rejoining. As demonstrated here, studies on DNA damage responses induced by heavy ion radiation provide an important tool to shed light on mechanisms regulating DNA end resection.

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1. Introduction

DNA double strand breaks (DSBs) induced by ionizing radiation (IR) are deleterious damages leading to cell death and genomic instability if not properly repaired. It is well known that there are two major DSB repair pathways, DNA non-homologous end-joining (NHEJ) and homologous recombination (HR) [1,2], and recent work has suggested that NHEJ is the dominant pathway throughout the cell cycle in mammalian cells [3,4]. Although the actual mechanisms regulating repair pathway choice are still largely unknown, it has been proposed that the balance between the two repair pathways depends on the chromatin structure surrounding the damage site. In the case of X ray irradiation, around 20% of DSBs are repaired by HR in G2 cells. This frequency corresponds approximately to the magnitude of heterochromatin, and it is has been argued that heterochromatin-associated DSBs are subjected to end resection [4]. Another factor that was shown to have a significant influence on repair pathway choice is the complexity of the DSB end structure [4]. It is known that high linear energy transfer (LET) radiations, such as heavy ion beams, induce complex-type DSBs, since these radiations release their energy at high density along their trajectories. Although the actual chemical structures of such DSBs require further clarification, it has been reported that they often include clustered damages in the vicinity of break sites [5–8]. While the efficiency of NHEJ is diminished for complex DSBs, the interface with the usage of homologous recombination has not been fully explored [9–11]. In this work, we utilized heavy ion beams, carbon and iron

Abbreviations: DSBs, DNA double strand breaks; IR, ionizing radiation; HR, homologous recombination; LET, linear energy transfer; RPA, replication protein A; PIKK, phosphatidyl inositol 3′ kinase-related kinase; MEF, mouse embryonic fibroblast; MMEJ, micro homology-mediated end joining; CPT, camptothecin; CENP-F, centromere protein F; RBE, relative biological effectiveness; DAPI, 4′,6-diamidino-2-phenylindole.

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http://dx.doi.org/10.1016/j.dnarep.2013.08.009

ions, to produce complex DSBs, and aspired to elucidate the influence that the complexity of break ends has on the repair pathway choice. In the last decade, the micro laser irradiation system has become established as a powerful tool in the DNA damage response research field [12–15]. One advantage of the system is the ability to produce damages at any chosen position in a cell, which facilitates discrimination between spontaneous and induced damage signals. However, there are technical disadvantages to micro laser irradiation in that not all cells in a field are damaged precluding the use of Western blotting analysis and BrdU is frequently employed, which may influence the repair response. These factors restrict the utility of micro laser irradiation for analysis of the cellular responses to complex DSBs. In addition to the production of complex DSBs, particle beam radiation creates damage in the path traversed by the ion particles (DSB tracks). Consequently, when cells are irradiated in a horizontal direction, clear DSB signal tracks (such as γH2AX) can be observed in immunofluorescence experiments. Hence, radiation-induced damage signals are easily distinguished from background signals, which often appear in S phase cells [7,11,16].

An early step in HR is the generation of 3′-single strand DNA (SSD) via a process called DNA end resection. It has been elucidated that a critical player in this process is CtIP (CtBP-interacting protein, also known as RBBP8), which was reported to be a Brca1 binding partner with a function in transcription [17–25]. CtIP is phosphorylated by ATM [24], and initiates resection together with Mre11 nuclease. This activity is thought to be restricted to the vicinity of the DNA ends. Subsequently, the execution of a large-scale expansion of resection occurs by other nucleases and RecQ family helicases, such as Exo1 and BLM [17–19,21,23,26–29]. After exposure of SSD, RPA (a hetero-trimer consisting of RPA1, RPA2 and RPA3) accumulates and is phosphorylated by PIKK family kinases [30–34]. Recent findings suggest that the pathway choice is dictated by whether end joining or end resection occurs first. Hence we focused on examining if DNA end resection is efficiently activated in mammalian cells in response to complex DSBs induced by high LET heavy ion beams compared to simple DSBs induced by low LET radiation, such as X and γ rays.

In this report, we provide evidence that complex DSBs efficiently provoke DNA end resection. We show that massive phosphorylation of CtIP and RPA2 arises within 20 min and at a later time point, respectively, after heavy ion irradiation. Further, immunofluorescence experiments reveal that 85 percent of heavy ion-induced DSBs in tracks are processed by resection in G2 cells. In addition, we show that 20–40 percent of human G1 cells exhibit resection signals, demonstrating that they are equipped with the biochemical potential to trigger resection responses. Collectively, we conclude that the complexity of DSB end structure is a critical factor that can drastically change the processing of DSB ends.

2. Materials and methods

2.1. Cell culture

U2OS, HeLa, U251 and mouse embryonic fibroblast (MEF) cells, 1BR-hTERT, a human normal fibroblast immortalized with hTERT [35], and 48BR, a human primary fibroblast [35], were grown in MEM Eagle medium supplemented with 10% fetal bovine serum and Antibiotic–Antimycotic (Gibco, #15240). All cells were maintained in a humidified incubator with 5% CO_2.

2.2. IR irradiation and drug treatment

Exponentially grown cells were irradiated with X rays at a dose rate of 1.0–1.3 Gy/min, or with γ rays at 7.4 Gy/min. Heavy ion beam irradiation experiments were performed at the Heavy-Ion Medical Accelerator in Chiba (HIMAC) at the National Institute of Radiological Sciences (NIRS, Japan). LET values of 70 keV/μm and 200 keV/μm were used for carbon ion (290 MeV/nucleon) and iron ion (500 MeV/nucleon), respectively. Particle fluences of the beams are 0.089/μm^2/Gy and 0.031/μm^2/Gy, respectively, at the LET values. The dose rates of heavy ion beams varied depending on the irradiation conditions. When cells were treated with KU55933, an ATM inhibitor (Calbiochem, #118500), they were pre-incubated with the drug (+ATMi) at 20 μM or with DMSO (-ATMi) for 30 min before irradiation. The treatments were continued until the harvest of cells after irradiation.

2.3. Western blotting and immunofluorescence staining

Cells were lysed with RIPA buffer containing a protease inhibitor (Complete, Roche #11 697 498 001) and a phosphatase inhibitor cocktail (PhosSTOP, Roche #04 906 837 001), and centrifuged to prepare the supernatant as whole cell lysate (WCL). WCLs were used for all Western blotting except for detection of γH2AX. After centrifugation, pellets were suspended in RIPA buffer containing the inhibitors, and sonicated to prepare samples for γH2AX blotting, since the majority of histones are present in the pellets. After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane, and then detected by a conventional Western blotting method using appropriate antibodies as indicated in the figures. For immunofluorescence staining, cells were grown on chamber slides (Nunc Lab-Tek). After IR irradiation or mock irradiation, they were fixed with cold methanol for 20 min, permeabilized with 0.5% Triton X-100/PBS for 10 min on ice. After blocking with 3% bovine serum albumin/PBS, cells were sequentially incubated with primary antibodies and fluorescence-labeled secondary antibodies for one hour each at 37 °C. ProLong Gold antifade reagent with DAPI (Invitrogen) was used to mount cells.

2.4. Antibodies and siRNAs

Anti-CENP-F (#ab5), pS2056-DNA-PKcs (#ab18192), H3K9me (#ab71604) and RPA2 (#ab2175) antibodies were purchased from Abcam. Anti-BrdU (Bromodeoxyuridine) (#347580) antibody was purchased from Becton, Dickinson (BD). Anti-pS4/8-RPA2 (#A300-245A) antibody was purchased from Bethyl Laboratories. Anti-Chk1 (#2345), pS345-Chk1 (#2348), Chk2 (#2662), pT68-Chk2 (#2661), CtIP (#9201), Mre11 (#4847) and pS676-Mre11 (#4859) antibodies were purchased from Cell Signaling Technology. Anti-ATM (#1549), pS1981ATM (#2152), Cyclin A2 (#1547), γH2AX (#2212) and pT21-RPA2 (#3237) antibodies were purchased from Epitomics. Anti-actin (#MAB1501), Cyclin B1 (#05-373) and γH2AX (#05-636) antibodies were purchased from Millipore. Anti-Ku80 (#MS-285) antibody was purchased from Thermo Scientific. Negative control (non-targeting) siRNA (34390843) and CtIP-targeting siRNA (#s11850) were purchased from Life Technologies. U2OS cells were transfected with the siRNA twice using Lipofectamine RNAiMAX (Invitrogen).

3. Results and discussion

3.1. Heavy ion beam irradiation induces massive phosphorylation of CtIP and RPA

To estimate the level of DNA end resection after IR, we analyzed the phosphorylation of CtIP and RPA2 in HeLa cells following heavy ion and X ray exposure. The phosphorylated form of CtIP can be easily distinguished from the unphosphorylated form due to its retardation in gel electrophoresis [24]. Iron ion induced substantially more CtIP and RPA2 phosphorylation compared with X rays after both 3 Gy and 10 Gy (Fig. 1A). CtIP showed a more rapid

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