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Investigation of switch from ATM to ATR signaling at the sites of DNA damage induced by low and high LET radiation

Janapriya Saha[a], Minli Wang[a], Francis A. Cucinotta[b,c,*]

[a] Division of Space Life Sciences, Universities Space Research Association, 3600 Bay Area Blvd., Houston, TX 77058, USA
[b] NASA, Lyndon B. Johnson Space Center, Houston, TX 77058, USA
[c] University of Nevada Las Vegas, Department of Health Physics and Diagnostic Sciences, 4505 Maryland Parkway, Las Vegas, NV 89154-3037, USA

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ABSTRACT

Upon induction of DNA damage by ionizing radiation (IR), members of the phosphatidylinositol 3-kinase-like kinase family of proteins namely ataxia-telangiectasia mutated (ATM), DNA-PKcs, and ATM- and Rad3-related (ATR) maintain genomic integrity by mounting DNA damage response (DDR). Recent reports suggest that activation of ATM and ATR are oppositely regulated by the length of single stranded overhangs generated during end processing by nucleases at the break sites. These stretches of single stranded overhangs hold the clue for the transition from ATM to ATR signaling at broken DNA ends. We investigated whether differential processing of breaks induced by low and high LET radiation augments the phenomenon of switching from ATM to ATR kinase and hence a concomitant NHEJ to HR transition at the sites of DNA damage. 82-6 human fibroblasts were irradiated with 1 or 2 Gy of γ-rays and particle radiation of increasing LET in order to increase the complexity and variability of DNA double strand breaks (DSB) structures. The activation kinetics of ATM and ATR kinases along with their downstream substrates were determined utilizing Western blotting and immunofluorescence techniques. Our data provide evidence of a potential switch from ATM to ATR kinase signaling in cells treated with γ-rays at approximately 2 h post irradiation, with induction and completion of resection denoted by Rad51 foci resolution kinetics and observed with a significant decline of phosphorylated ATR kinase 8 h after IR. On the other hand, irradiation with high LET 600 MeV/u ^{56}Fe (180 keV/μm) and 170 MeV/u ^{28}Si (99 keV/μm) particles show a similar Rad51 foci decay kinetics, however, exhibiting prolonged resection, evident by the persistent phosphorylated ATM and ATR kinase until 24 h post irradiation. This residual effect, however, was significantly reduced for 250 MeV/u ^{16}O particles of moderate LET (25 keV/μm) and absent for γ-rays. Hence, our results support the hypothesis that the transition from ATM to ATR signaling at DNA break sites is extended for longer periods of time, indicated by sustained resection due to the complex type of damage induced, a hallmark of high LET radiation, which may contribute to its increased biological effectiveness.

1. Introduction

IR-induced DNA double strand breaks (DSBs) induce important signal transduction processes and may lead to cell death, mutation, or genomic instability. Two DNA-damage-activated protein kinases - ATM and ATR, along with DNA-PKcs, members of the phosphatidylinositol kinase-like kinase family are key regulatory proteins that protect eukaryotic cells from DNA damage and the resultant genomic instability. These two kinases respond to DNA damage by phosphorylating downstream effectors that coordinate the cell cycle through checkpoints, permitting DNA repair, or may initiate apoptosis in the event of extensive damage and overwhelmed repair systems. Although the ATM and ATR kinases phosphorylate some common substrates, they are differentially activated by distinct types of DNA damage.

Exposure of cells to IR triggers rapid autophosphorylation of serine-1981 that causes dimer dissociation and initiates monomer formation of ATM [1]. It is recruited to sites of damage by the Mre11-Rad50-Nbs1 (MRN) complex [2,3]. The ATM kinase phosphorylates a large number of downstream molecules such as γ-H2AX, pATF2, NBS1, Chk2, p53, SMC1 and BRCA1, the deficiency of some of which leads to defective G1/S, G2/M and intra-S checkpoints [4–10]. In the

Abbreviations: (IR), Ionizing radiation; (HZE), High charge and energy; (LET), Linear energy transfer; (DDR), DNA damage response; (DSB), DNA double strand breaks; (ATM), Ataxia-telangiectasia mutated; (ATR), ATM- and Rad3-related; (NSRL), NASA space radiation laboratory; (BNL), Brookhaven national laboratory.

* Corresponding author. Tel.: +1 7028950977; fax: +1 7028954819.
E-mail address: francis.cucinotta@unlv.edu (F.A. Cucinotta).

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case of ATR, RPA-coated single-stranded DNA (ssDNA) generated as a result of stalled DNA replication forks or during processing of chromosomal lesions is crucial for the localization of ATR to sites of DNA damage in association with ATR-interacting protein (ATRIP) [11–14].

Although unlike ATR, ATM is not an essential gene, in humans, ATM deficiencies result in the disease ataxia-telangiectasia (AT) [15]. Cells from AT patients are sensitive to IR but not to UV exposure. These observations suggested that ATM and ATR function in parallel pathways. However, several reports indicate that ATR also responds to DSBs including those produced at stalled replication forks, although the response may be delayed as compared to ATM [16]. The ATR-ATRIP complex is required to maintain the G2/M checkpoint in response to IR and form intranuclear foci at IR induced damage sites. It has also been shown that in AT cells, phosphorylation of p53 in response to IR is delayed but not completely abrogated. It has also been shown that ATM activation triggers subsequent ATR activation upon DSB induction [17,18].

Recent successes in designing biochemical assays has allowed for the study of the substrate preference of ATM and provided insights of how blunt DNA ends or ends with short overhangs at resected DSBs activate ATM and how the single-strand/double-strand junctions of their substrates are crucial for ATM activation. The processing of these ends by various protein complexes belonging to family of nucleases or helicases like MRN [19,20], Exo1 [21–23], CtIP [24,25] and BLM [26] leads to increased overhang length, which suppresses ATM and leads to subsequent ATR activation [27].

Compared to x-rays or γ-rays, high linear energy transfer (LET) radiation leads to increased clustering and complexity of DSBs [28], providing an important tool to study altered DNA damage processing. In this study, we investigated the ATM to ATR switch in hTERT-immortalized human skin fibroblasts (82-6) after 1 or 2 Gy γ-rays, high LET 600 MeV/u ^{56}Fe and 170 MeV/u ^{28}Si and moderate LET 250 MeV/u ^{16}O particles with LETs of approximately 180, 99 and 25 keV/μm, respectively, utilizing Western blotting and immunofluorescence techniques. Live cell imaging experiments were performed with HT22-mouse hippocampal neuronal cell line stably expressing mCherry-53BP1.

2. Materials and methods

2.1. Cell culture

Human hTERT-immortalized skin fibroblast (82-6 hTERT [29], referred here as 82-6) were cultured in DMEM (GIBCO) medium supplemented with 1× Antibiotic-Antimycotic and 10% FBS. Mouse hippocampal neuronal cell line-HT22 cells stably expressing mCherry-53BP1 (a kind gift from Dr. Huichen Wang, Emory University, Atlanta, Georgia) were maintained in DMEM medium supplemented with 10% FBS containing 1 μg/ml Puromycin. All cells were grown as monolayers at 37 °C under 5% CO_2.

2.2. Irradiation of cells and chemical treatments

HZE particle experiments were performed at NASA Space Radiation Laboratory (NSRL), Brookhaven National Laboratories (BNL), Long Island, New York. A 20 × 20 cm^2 beam with a dose uniformity of +2%, and a dose-rate of ~0.5 Gy/min was used. ^{137}Cs gamma radiation (γ-rays) experiments were completed at NASA Lyndon B. Johnson Space Center (Houston, Texas) with a dose-rate of 0.3 Gy/min. T25 flasks containing exponentially growing cells were exposed vertically with the cell surface perpendicular to the beam. Eight-well chamber slides were exposed either vertically or horizontally as indicated. The tracks of high LET particle exposures can be visualized by the accumulation of DDR proteins in the form of foci when cells are irradiated horizontally to the beam.

2.3. Western blotting/antibodies

Cells were lysed in NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific), supplemented with Halt protease inhibitor cocktail. The kit enables stepwise lysis of cells, separation of the cytoplasm from the intact nucleus and then extraction of nuclear proteins away from genomic DNA and mRNA and both active nuclear and cytoplasmic proteins can be recovered from the same cell culture. Western blotting was used to detect the phosphorylated and native forms of ATM, ATR, and Chk2 kinases along with loading controls TATA box binding protein (TBP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins in the nuclear and cytoplasmic extracts. Mouse anti-pATM(Ser1981) (#600-401-400, Rockland Immunochemicals), Rabbit anti-ATM (#2873S, Cell Signaling), Rabbit anti-pATR(Ser428) (#2853, Cell Signaling), Rabbit anti-ATR (#2790S, Cell Signaling), Rabbit anti-ATRIP (#2737, Cell Signaling), Rabbit anti-pChk2(Thr68) (#2661, Cell Signaling), Rabbit anti-pChk1(Ser345) (#2348, Cell Signaling), Mouse anti-γH2AX(Ser139) (#05-636, Millipore), Rabbit anti-Rad51 (#PC130, Calbiochem), Mouse anti-Cyclin A (#611269, BD Biosciences), Mouse anti-Cyclin B1 (#05-373, Millipore), Mouse anti-TBP (#05-1531, Millipore) and Mouse anti-GAPDH (#AB2302, Millipore) were used.

2.4. Immunofluorescence

Cells were grown on LabTek 8-well chamber slides and fixed with 4% paraformaldehyde for 15 min. After permeabilization with 0.3% Triton X-100 in PBS for 5 min at RT, the slides were washed three times in PBS for 5 min and subsequently blocked with PBS containing 10% normal goat serum (Sigma Aldrich) for 1 h. The cells were then incubated with indicated primary antibodies in 2% goat serum at 4 °C overnight followed by secondary antibody conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen) and nuclei were counterstained with DAPI. Immunofluorescence was evaluated with a fluorescence microscope Axioplan 2 (Zeiss, Germany).

2.5. Cell cycle analysis by flow cytometry

Exponentially growing 82-6 cells were irradiated and collected at different time points and fixed with 70% ethanol along with proper controls. The cells were further processed for staining utilizing the Guava Cell Cycle Reagent (Millipore) according to the manufacturer's protocol. Data from the stained cells were acquired with Guava PCA system (Millipore). The histograms were analyzed using ModFit LT software (Verity Software House).

2.6. Live cell imaging

HT22 cells stably expressing mCherry-53BP1 were plated on 35-mm glass-bottomed tissue culture dish (MatTek Corporation, MA) 24 h prior to irradiation. The cells were then exposed to 1 Gy of 600 MeV/u ^{56}Fe irradiation in a horizontal orientation to the beam for visualization of particle tracks and immediately shifted to the live cell imaging setup at NSRL for time lapse imaging. To accommodate a number of cells per view, up to 10 fields were imaged simultaneously for duration of 36 h post IR. Images were captured with Zeiss Axiovert 200 Fluorescence/Live Cell imaging microscope (Zeiss AG, Germany) utilizing a 63 × 1.4-NA oil immersion objective. Images were analyzed utilizing the Zeiss AxioVision image processing software.