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Suppression of DNA-dependent protein kinase sensitize cells to radiation without affecting DSB repair

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ABSTRACT

Efficient and correct repair of DNA double-strand break (DSB) is critical for cell survival. Defects in the DNA repair may lead to cell death, genomic instability and development of cancer. The catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) is an essential component of the non-homologous end joining (NHEJ) which is the major DSB repair pathway in mammalian cells. In the present study, by using siRNA against DNA-PKcs in four human cell lines, we examined how low levels of DNA-PKcs affected cellular response to ionizing radiation. Decrease of DNA-PKcs levels by 80–95%, induced by siRNA treatment, lead to extreme radiosensitivity, similar to that seen in cells completely lacking DNA-PKcs and low levels of DNA-PKcs promoted cell accumulation in G2/M phase after irradiation and blocked progression of mitosis. Surprisingly, low levels of DNA-PKcs did not affect the repair capacity and the removal of 53BP1 or γ-H2AX foci and rejoining of DSB appeared normal. This was in strong contrast to cells completely lacking DNA-PKcs and cells treated with the DNA-PKcs inhibitor NU7441, in which DSB repair were severely compromised. This suggests that there are different mechanisms by which loss of DNA-PKcs functions can sensitize cells to ionizing radiation. Further, foci of phosphorylated DNA-PKcs (T2609 and S2056) co-localized with DSB and this was independent of the amount of DNA-PKcs but foci of DNA-PKcs was only seen in siRNA-treated cells. Our study emphasizes on the critical role of DNA-PKcs for maintaining survival after radiation exposure which is uncoupled from its essential function in DSB repair. This could have implications for the development of therapeutic strategies aiming to radiosensitize tumors by affecting the DNA-PKcs function.

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1. Introduction

Ionizing radiation and endogenous processes, e.g. free radicals formed in normal cellular metabolism or collapse of replication forks during replication of DNA, may result in complex damage to the DNA. Among the different types of DNA damage, DNA double-strand break (DSB) is the most unfavorable type of DNA lesion, and unrepaired or misrepaired DSB can lead to gene mutations, chromosomal aberrations, permanent cell cycle arrest, apoptosis, mitotic cell death and cancer development [1–3].

In DSB repair two pathways have been implicated to process the break, non-homologous end joining (NHEJ) and homologous recombination (HR) [4]. Some of the key proteins involved in NHEJ are DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Ku70 and Ku80 that together form the DNA-dependent protein kinase (DNA-PK) complex [5]. DNA-PKcs is a member of the phosphatidylinositol 3 (PI-3)-like kinase family that includes ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) [6]. Besides functioning in NHEJ, DNA-PKcs is also required for V(D)J recombination of immune-globulin genes and T-cell receptor genes, and telomere length maintenance [7]. NHEJ is believed to play a more important role then HR in mammalian cells, and is present at all time during the cell cycle, HR is limited to late S, G2 and M phase [8–10].

Proteins involved in the repair and signaling of DSB play a vital role in cell survival and healthy human life [11–13]. Many tumors types characterized by genomic instability are caused by mutations in DSB-responsive genes. The expression of DNA-PKcs in tumor biopsies can be both up- and down-regulated in specific tumor types [14]. Several cancer types with decreased levels of both DNA-PKcs and Ku70/80 have been found [15–17] which could indicate that partial deficiency of proteins involved in DSB repair may contribute to increased risk of developing cancer [14,18]. In contrast, elevated levels of DNA-PKcs have been found in various tumor types and linked to poor survival [19].

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Besides its direct role in repair, it is becoming evident that DNA-PKcs may have other functions that promote cell survival. Arlander et al. (2008) suggested that DNA-PKcs contributes to the G2 checkpoints and cell cycle regulation [20]. Recently it was also shown that DNA-PKcs is involved in stabilization of the mitotic spindle formation in a way that reduced levels of DNA-PKcs may direct the cell toward mitotic catastrophe [21–23]. Further data indicates that DNA-PKcs is a central player in G2 progression and low levels of DNA-PKcs promotes a fast progression from S phase to G2, but interferes with progression through mitosis and leads to cell accumulation in G2 [22]. Human cells deficient in DNA-PK show a severe sensitivity to radiation and reduced capacity to repair DSBs [11,24,25] and it is possible that this inability to progress through the cell-cycle is affected by the presence of unrepaired DNA [26].

In the present investigation, we studied DNA repair in cells with reduced levels of DNA-PKcs. Human cells transfected with siRNA targeting DNA-PKcs, resulting in 5–20% of the original DNA-PKcs content, exhibited similar extreme radiosensitivity as cells completely lacking DNA-PKcs. Surprisingly, the low DNA-PKcs-levels had no significant effect on DSB repair capacity, but low levels of DNA-PKcs affected the cell cycle, resulting in siRNA treated cell accumulation in G2 after irradiation and inability to proceed through mitosis. These data support recent findings suggesting that DNA-PKcs is an important regulator of the cell-cycle while we here show that this function seems to be uncoupled from the cellular capacity to repair DSBs.

2. Materials and methods

2.1. Cell lines and culture conditions

Human epithelial cancer cell line A431 (ATCC, Manassas, VA, USA) was grown in Ham's F-10. Human oral squamous carcinoma cell line H314 (ATCC) was grown in DMEM/Ham's F-12. Human colorectal carcinoma cell line HCT116 (ATCC) was grown in McCoy's. Normal human skin fibroblast GM5758 cell line (Human Genetic Mutant Cell Repository, Camden, NJ, USA) was grown in MEM, supplemented with 2× concentration of MEM vitamins, essential and non-essential amino acids. The human glioma cell lines M059J deficient in DNA-PKcs (ATCC) and M059K (ATCC) with wild-type DNA-PKcs were cultured in DMEM/Ham's F12 supplemented with non-essential amino acids. Culture medium for all the cells was supplemented with 10% FCS (Sigma, St. Louis, MO), 2 mM L-glutamine, 100 IU/ml penicillin and 10 μg/ml streptomycin (Biochrom Kg, Berlin Germany). All cells were cultured in a humidified incubator with 5% CO_2 at 37 °C and trypsinized with trypsin–EDTA (0.25% trypsin, 0.02% EDTA, Biochrom Kg).

2.2. Irradiation and inhibitor treatment

Cells were irradiated with ^{137}Cs γ-ray photons (Gammacell 40 Exactor, MDS Nordion, Kanata, Canada) at a dose rate of ∼1 Gy/min. NU7441 was obtained from Selleckchem (Houston, TX). NU7441 was dissolved in dimethyl sulfoxide. For treatment, cells were pretreated in 37 °C for 1 h with 2–10 μM NU7441 before irradiation. Clonogenic survival assay NU7441 was removed 24 h after irradiation with fresh medium. Dimethyl sulfoxide was used in mock treated cells.

2.3. Antibodies

Primary mouse-monoclonal antibodies used were, DNA-PKcs (ab1832, Abcam), *p*-DNA-PKcs (Thr2609) (ab18356, Abcam), β-actin (Sigma) and γ-H2AX (Millipore). Primary rabbit-polyclonal antibody used were 53BP1 (Bethyl Lab, Montgomery, TX), *p*-DNA-PKcs (Ser2056) (ab18192, Abcam), *p*-DNA-PKcs (Thr2609) (Santa Cruz), *p*-AKT (Ser437) (Cell Signaling) and *p*-Histone H3 (Millipore). Nucleus was stained with DAPI (Invitrogen). Secondary antibodies Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 donkey anti-rabbit (Invitrogen).

2.4. siRNA transfection

For the siRNA transfection of DNA-PKcs a SMART pool provided by Dharmacon (Thermo Scientific) was used with four siRNA specific for *PRKDC* (catalogue no. L-005030-00-0020) and a SMART pool as non-specific control (mock) (catalogue no. D-001810-10-20). Transfections were performed with DharmaFECT 1 Transfection Reagent (catalogue no T-2001-01) according to the instructions of the supplier. The concentration of siRNA was 100 nM and was added to the cells together with DharmaFECT I. Cells were seeded one day prior transfection, and cells were analyzed three days after transfection.

2.5. SDS-PAGE and Western blot analysis

Cells were lysed in ice cold lysis buffer containing 1% Tween-20, 20 mM Tris (pH 8.0) 137 mM NaCl, 10% glycerol, 2 nM EDTA, 1 mM activated sodium orthovanadate (Sigma) and protease inhibitor cocktail (P8340, Sigma). Cell extracts were loaded and electrophoresed on NuPAGE Novex 3–8% Tris–acetate gels (Invitrogen). Wet transfer to nitrocellulose membranes was done overnight and then the membranes were blocked for 1 h in 5% BSA, PBS and then incubated with the primary antibody overnight at 4 °C. Secondary antibodies (626520 and 656120) (Invitrogen) labeled with Horse Radish Peroxidase were incubated for 1 h at room temperature. Immunoreactive bands were visualized in a CCD camera (SuperCCD HR, Fujifilm, Japan) after treatment with electrochemiluminescent solution (Immobilon). The intensity of the bands was analyzed using Image J software (NIH, US).

2.6. Cell survival

Cells were counted and an appropriate number was seeded into T-25 cell flask in triplicate for each dose. Cells were irradiated with 1–4 Gy, 4 h after seeding. After 1–2 weeks cells were fixed in 95% EtOH and stained with Mayer's hematoxylin and colonies with >50 cells were scored.

2.7. Mitotic analysis

Three days after transfection with siRNA cells were irradiated with 5 Gy, and let to repair for 24 h in fresh medium before fixation and immunostaining with *p*-Histone H3 (*p*-H3) antibody. At the microscope *p*-H3 positive cells were scored, at least >250 cells per data point.

For nocodazole treatment, cells were treated with 1 μl/mg nocodazole (Sigma), 3 days after transfection with siRNA, and incubated for 6 h. After 6 h, nocodazole was removed and cells were irradiated with 2 Gy and let to repair for 18 h in fresh medium before fixation and immunostaining with *p*-H3 antibody. At the microscope *p*-H3 positive cells were scored, at least >400 cells per data point.

2.8. Immunofluorescence staining and quantification of foci

Cells were grown on microscope slides for three days after transfection and were irradiated with 1–5 Gy. After repair of DNA for various times at 37 °C, the cells were fixed in methanol for 20 min at −20 °C followed by permeabilization in ice cold acetone for 10 s. Blocking was performed in 10% FCS-PBS for 1 h at room temperature and then incubation overnight at 4 °C with primary antibodies. Secondary antibodies were incubated for 1 h at 37 °C (dilution 1:400).

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