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Inhibition of homologous recombination by hyperthermia shunts early double strand break repair to non-homologous end-joining

Judith W.J. Bergs[a,1], Przemek M. Krawczyk[b,1], Tijana Borovski[a], Rosemarie ten Cate[a], Hans M. Rodermond[a], Jan Stap[b], Jan Paul Medema[a], Jaap Haveman[a], Jeroen Essers[c], Chris van Bree[a], Lukas J.A. Stalpers[a], Roland Kanaar[c], Jacob A. Aten[b], Nicolaas A.P. Franken[a,*]

[a] Laboratory for Experimental Oncology and Radiobiology (LEXOR), Center for Experimental Molecular Medicine, Department of Radiation Oncology, Academic Medical Center, University of Amsterdam, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands
[b] van Leeuwenhoek Centre for Advanced Microscopy – AMC, Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, The Netherlands
[c] Department of Cell Biology & Genetics, Cancer Genomics Center, Department of Radiation Oncology, Erasmus Medical Center, 3000 CA Rotterdam, The Netherlands

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ABSTRACT

In S and G2 phase mammalian cells DNA double strand breaks (DSBs) can potentially be repaired by homologous recombination (HR) or non-homologous end-joining (NHEJ). Results of several studies suggest that these two mechanistically distinct repair pathways can compete for DNA ends. Because HR and NHEJ differ with respect to error susceptibility, generation of chromosome rearrangements, which are potentially carcinogenic products of DSB repair, may depend on the pathway choice. To investigate this hypothesis, the influence of HR and NHEJ inhibition on the frequencies of chromosome aberrations in G2 phase cells was investigated. SW-1573 and RKO cells were treated with mild (41 °C) hyperthermia in order to disable HR and/or NU7441/cisplatin to inactivate NHEJ and frequencies of chromosomal fragments (resulting from unrepaired DSBs) and translocations (products of erroneous DSB rejoining) were studied using premature chromosome condensation (PCC) combined with fluorescence in situ hybridization (FISH).

It is shown here that temporary inhibition of HR by hyperthermia results in increased frequency of ionizing-radiation (IR)-induced chromosomal translocations and that this effect is abrogated by NU7441- or cisplatin-mediated inhibition of NHEJ. The results suggest that in the absence of HR, DSB repair is shifted to the error-prone NHEJ pathway resulting in increased frequencies of chromosomal rearrangements. These results might be of consequence for clinical cancer treatment approaches that aim at inhibition of one or more DSB repair pathways.

1. Introduction

DNA double strand breaks (DSBs) are potentially lethal lesions that can be induced by endogenous DNA-related processes as well as by exogenous agents, including ionizing radiation (IR) and chemicals. In mammalian cells, DSBs trigger a complicated cascade of reactions initiated by recognition of the breaks by the ATM kinase and the Mre11/Rad50/NBS1 (MRN) complex and subsequent phosphorylation of the histone protein H2AX at chromatin domains surrounding DSB sites [1]. Phosphorylated H2AX (γ-H2AX) is considered to be one of the earliest markers of DSBs [1–4]. Damaged chromatin attracts other repair- and checkpoint activation-related proteins, including MDC1 and 53BP1 [5,6] which form so-called ionizing radiation induced foci (IRIF). IRIF are considered to be markers of ongoing repair activities and can be visualized by (immuno)fluorescence microscopy [7]. Failure of repair proteins to form IRIF has been linked to damage response deficiencies [8,9]. After initial recognition, repair of the DSBs is performed by homologous recombination (HR) or non-homologous end-joining (NHEJ) [10].

HR requires a DNA sequence homologous to the broken strand and thus acts predominantly during the S and G2 phases of the cell cycle when a DNA template is available in the form of a sister chromatid [11]. Major HR factors include Rad51, Rad51 paralogs, Rad52, Rad54, BRCA2 and RPA [12,13].

NHEJ mediates rejoining of DSBs without the requirement of a homologous sequence. After induction of a DSB, the KU heterodimer, consisting of the KU70 and KU80 proteins, binds DNA ends and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) leading to formation of the DNA-PK holo-enzyme [11]. DNA-PK then forms a functional complex with Artemis, which

* Corresponding author. Tel.: +31 20 5663641.
E-mail address: n.a.franken@amc.uva.nl (N.A.P. Franken).
[1] These authors have contributed equally.

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provides nucleolytic processing activity required to prepare DNA ends for ligation [14]. Ligation of open DNA ends is accomplished by the Ligase IV/XRCC4 complex. Recently, another factor involved in the ligation process, called Cernunnos (XLF), has been described [15,16]. The NHEJ process is active during all cell cycle phases but it plays a major role in the elimination of DSBs during G0/G1 when the chromosomes consist of only one chromatid [17].

In S and G2, both HR and NHEJ can potentially repair DSBs and it has been suggested that the two pathways might compete for DNA ends [18,19]. In support of this theory, absence of DNA-PKcs or KU80 was reported to stimulate HR [20–24], whereas inactivation or inhibition of DNA-PKcs had an opposite effect [22,23,25]. NHEJ has been shown to precede HR [20,26,27]. In contrast, other studies have suggested cooperation, rather than competition of HR and NHEJ in DSB repair [23,28,29]. Wu et al. [30] suggested an alternative repair pathway, called back-up NHEJ, in G2-phase of cells deficient for the classical NHEJ.

HR has been proposed as the major mechanism for error-free repair of DSBs [31,32], whereas NHEJ is considered to be error-prone [33]. One endpoint that could be therefore used to dissect interactions between HR and NHEJ is formation of chromosome translocations, which result from erroneous DSB repair. Inhibition of HR in G2 phase cells, when both pathways are active, might stimulate the erroneous NHEJ or back-up NHEJ [20–24,30] and thereby increase the frequencies of chromosomal aberrations. Multiple NHEJ inhibitors have been described, but repression of HR is difficult to obtain. However, it was recently demonstrated that mild hyperthermia efficiently induces degradation of BRCA2 and inhibits HR [34]. Results presented here indeed confirm that hyperthermia induces HR defects in SW-1573 and RKO cells. Furthermore, the results demonstrate that inhibition of HR by hyperthermia increases frequencies of ionizing-radiation induced chromosome aberrations, whereas additional inhibition of NHEJ counteracts this effect. To further investigate whether absence of HR induces the induction chromosomal translocations, aberration studies were also carried out in WT and mutant HR mouse embryonic fibroblast (MEF) cells. The results presented here support the hypothesis that inhibition or absence of HR stimulates the fast and error-prone NHEJ.

2. Materials and methods

2.1. Cell cultures

The human colon cancer cell line RKO was grown in Mc Coy's 5A medium with 25 mM Hepes (Gibco-brl life technologies, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS) and glutamine, penicillin and streptomycin. The cells were maintained at 37 °C in an incubator with humidified air supplemented with 5% CO_2. The doubling time of these cells during exponential growth is approximately 24 h.

The human squamous lung carcinoma cell line SW-1573 was grown in Leibowitz-15 (Gibco-brl life technologies, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS) and glutamine, penicillin and streptomycin. The cells were maintained at 37 °C in an incubator with humidified air without additional CO_2. The doubling time of these cells during exponential growth is approximately 24 h.

Mouse embryonic fibroblast cells: $P53^{-/-}$ $LIG4^{+/+}$ $Rad54^{+/+}$ (WT) and $P53^{-/-}$ $LIG4^{+/+}$ $Rad54^{-/-}$ [30,35] (kindly provided by the laboratory of Dr. G. Iliakis with permission from Dr. F. Alt) were grown in DMEM (Gibco-brl Life Technologies, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS) and glutamine, penicillin and streptomycin. The cells were maintained at 37 °C in an incubator with humidified air supplemented with 10% CO_2 The doubling time of these cells during exponential growth is approximately 12–14 h.

2.2. Treatments

2.2.1. Irradiation

For induction of chromosomal aberrations, cells were treated with 4 Gy of γ-rays. The γ-irradiation was applied using a ^{137}Cs source at a dose rate of about 0.7 Gy/min. For induction of DSB repair protein foci, irradiation was performed using the ^{137}Cs γ-source as described above or an Am-241 α-particle source [34,36–38].

2.2.2. Hyperthermia 41 °C incubation

Incubation of the cells at 41 °C was performed by submerging the Petri dishes in a thermostatically controlled waterbath for 1 h. RKO cells were heated in a 5%CO_2/95% air atmosphere with an air inflow of 2 L/min. SW-1573 cells did not receive additional CO_2.

2.2.3. NHEJ inhibition

NHEJ was inhibited by using a specific DNA-PK inhibitor NU7441 or cisplatin. The specific DNA-PK inhibitor (NU7441 also known as KU57788 or AZ 12623380) was kindly provided by Dr. Graeme Smith (KuDOS Pharmaceuticals, AstraZeneca UK Ltd.) [39]. NU7441 was dissolved in DMSO as 10 mM stock and stored at −20 °C. For experiments NU7441 was further diluted in DMSO and added directly to the culture medium at a final concentration of 1 μM. To check whether NU7441 indeed inhibited DNA-PK, the activity was measured using the promega SignaTECT® DNA dependent protein kinase assay system, according to manufacturer's protocol, on whole-cell extracts from SW-1573 cells. Cisplatin (Platosin®, Pharmachemie, Haarlem, The Netherlands) was diluted just before use in culture medium from a freshly prepared 3.3 mM stock. Cells were incubated with 1 μM, 50 μM, and 100 μM cisplatin (cDDP) for 1 h at 37 °C.

2.2.4. Combination treatments

Incubation of the cells at 41 °C, NU7441 or cDDP (at 37 °C) was performed for 1 h immediately prior to irradiation. The NU7441 or cDDP-containing medium was replaced with fresh medium just before irradiation. Simultaneous NU7441 or cDDP and 41 °C incubation was performed for 1 h immediately prior to irradiation.

2.2.5. Detection of DSB repair protein foci co-localizing with γ-H2AX on DSB sites

Detection of γ-H2AX, Mre11, Rad51 or Mdc1 foci was performed as described earlier [34–37]. For RKO cells, only Rad51 foci could be analyzed since the stringent washing procedure necessary for Mre11 detection resulted in loss of the cells from the mylar bottom of the Petri dishes. These mylar bottoms of dishes are required for the short range α-particles to reach the nuclei.

2.2.6. Analysis of chromosomal aberrations

For studying chromosomal aberrations in SW 1573 and RKO cells the premature chromosome condensation (PCC) FISH technique was carried out. For this cultures containing approximately 15% (SW-1573) or 20% (RKO) G2 phase cells were used. To induce chromosome condensation, 80 nM of calyculin A was added for 1 h either immediately after treatment or 24 h after treatment. Preparations were obtained by standard cytogenetic techniques. In MEF cells chromosomal aberrations were studied in colcemid-induced metaphases. For this irradiated and unirradiated cells were treated for 16 h with 0.1 μg/ml colcemid (Demecolcine solution, Sigma) and then metaphases were harvested.

Visualization of chromosomes was accomplished by FISH. In RKO cells, the chromosomal spreads were hybridized to whole-chromosome FISH probes (Metasystems, Germany) for