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Marked contribution of alternative end-joining to chromosome-translocation-formation by stochastically induced DNA double-strand-breaks in G_2-phase human cells

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ABSTRACT

Ionizing radiation (IR) induces double strand breaks (DSBs) in cellular DNA, which if not repaired correctly can cause chromosome translocations leading to cell death or cancer. Incorrect joining of DNA ends generating chromosome translocations can be catalyzed either by the dominant DNA-PKcs-dependent, classical non-homologous end-joining (c-NHEJ), or by an alternative end-joining (alt-EJ) process, functioning as backup to abrogated c-NHEJ, or homologous recombination repair. Alt-EJ operates with slower kinetics as compared to c-NHEJ and generates larger alterations at the junctions; it is also considered crucial to chromosome translocation-formation. A recent report posits that this view only holds for rodent cells and that in human cells c-NHEJ is the main mechanism of chromosome translocation formation. Since this report uses designer nucleases that induce DSBs with unique characteristics in specific genomic locations and PCR to detect translocations, we revisit the issue using stochastically distributed DSBs induced in the human genome by IR during the G_2-phase of the cell cycle. For visualization and analysis of chromosome translocations, which manifest as chromatid translocations in cells irradiated in G_2, we employ classical cytogenetics. In wild-type cells, we observe a significant contribution of alt-EJ to translocation formation, as demonstrated by a yield-reduction after treatment with inhibitors of Parp, or of DNA ligases 1 and 3 (Lig1, Lig3). Notably, a nearly fourfold increase in translocation formation is seen in c-NHEJ mutants with defects in DNA ligase 4 (Lig4) that remain largely sensitive to inhibitors of Parp, and of Lig1/Lig3. We conclude that similar to rodent cells, chromosome translocation formation from randomly induced DSBs in human cells largely relies on alt-EJ. We discuss DSB localization in the genome, characteristics of the DSB and the cell cycle as potential causes of the divergent results generated with IR and designer nucleases.

1. Introduction

It is widely accepted that DNA double strand breaks (DSBs) are precursor lesions for chromosomal aberrations, including a wide spectrum of chromosomal translocations [1–3]. Ionizing radiation (IR) is a potent inducer of DSBs, which if miss-repaired can lead to translocations [4] that cause cell death, and which, if not lethal, can feed genomic instability and carcinogenesis [5–8]. Indeed, chromosomal translocations are a hallmark of many human cancers [9,10]. Typically, in a cell exposed to IR, only a very small fraction of induced DSBs manifest as chromosomal translocations [11] and therefore mechanisms and circumstances that lead to this form of genomic "accident" are the subject of intensive research.

Chromosomal translocations are products of DSB processing that has failed its aim in restoring the original structure of the genome. In higher eukaryotes DSBs are processed by three pathways with widely different functional fidelities and activity-fluctuations throughout the cell cycle. As a result of fundamental differences in the principles of operation, their contribution to chromosome translocation formation is widely different.

Error-free processing of DSBs is only ensured by Rad51-dependent homologous recombination repair (HRR) [12]. As the term implies, HRR requires a homologous template for function and is therefore restricted to the late S- and G_2 phases of the cell cycle, where a sister chromatid becomes available [13,14]. HRR restores the original DNA sequence at the break-site and prevents translocations [15,16]. Indeed, junction analysis at translocations fails to identify signatures of HRR.

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DNA-PKcs-dependent classical non homologous end joining (c-NHEJ) is a dominant DSB repair pathway in higher eukaryotes [17,18]. During this process, DNA ends are instantly captured by the Ku heterodimer (Ku70/80) that recruits DNA-PKcs to the DSB [19]. DNA-PKcs changes its conformation and dimerises to hold the two DNA ends together and to generate a scaffold for subsequent processing and rejoining. Ultimately, processed ends are ligated by the Lig4/XRCC4 complex in a process stimulated by XLF/Cernunnos and PAXX. DNA polymerases μ and λ, polynucleotide kinase and the Artemis nuclease are responsible for end processing and the occasionally required DNA polymerization.

c-NHEJ proceeds with very fast kinetics (half times of about 15 min) and is nearly equally active throughout the cell cycle [20–22]. By its nature it can ensure neither the restoration of the original DNA sequence at the generated junction, nor the rejoining of the correct DNA ends. As a result c-NHEJ can, in principle, cause chromosomal translocations. Yet, extensive data suggests that c-NHEJ actually suppresses the formation of chromosomal translocations [23–26], a result most likely reflecting its high speed and the fact that the ends of a DSB are initially directly proximal and therefore topologically privileged for direct rejoining.

A slow, alternative form of end joining (alt-EJ) comes to the fore under certain conditions and is thought to provide backup maintenance of genome stability [27] whenever c-NHEJ is compromised — either globally by mutations or locally on individual DSBs by various factors including DNA replication stress [18,28]. Parp1 [29,30], Lig3 and possibly its interacting partner Xrcc1 [29,31], Histone H1 [32], as well as Lig1 [33–35] are shown to be crucial for functional alt-EJ. Therefore, sensitivity to Parp1 or Lig1/Lig3 inhibitors is considered direct evidence for DSB processing by alt-EJ. Additionally, proteins involved in DSB end-resection i.e., Mre11 [36–38], NBS1 [39], and CtIP [40] are also implicated in the function of alt-EJ.

Although alt-EJ functions throughout the cell cycle, its activity is markedly enhanced in G_2-phase [41,42]. The increased activity of alt-EJ in G_2-phase, as well as its dependence on proteins involved in DNA end-resection can be explained by our recent observation that alt-EJ efficiently backs up abrogated HRR in S- and G_2-phase of the cell cycle. Collectively, these observations raise alt-EJ to a universal backup for the two primary modes of DSB processing, HRR and c-NHEJ, as well as of abrogated DNA replication forks, in higher eukaryotes [26,28,67].

The function of alt-EJ can also be demonstrated during class switch and V(D)J recombination [43,44], as well as in reporter assay systems based on I-Sce-I and newly developed designer-nucleases [45], and there is evidence for distinct alt-EJ sub-pathways [46–48]. Although alt-EJ operates on similar fundamental principles as c-NHEJ, it is frequently slower in its function, causes larger sequence alterations at the generated junction, and is considered a major contributor to chromosome translocation formation [26].

Junctions generated by alt-EJ show more frequent and more extensive use of microhomologies than junctions generated by c-NHEJ [25,49–53]. Indeed, extensive deletions and microhomology at the junction are frequently invoked as “signatures” of alt-EJ events, and notably, are frequently observed in recurrent translocations of cancer cells [54].

The above outline of DSB processing pathways implies that translocation formation is in principle only possible through c-NHEJ or alt-EJ, and that among these two modes of DSB processing, alt-EJ will have a larger contribution and the responsibility for large deletions and microhomologies occasionally present. This is indeed frequently observed in several studies and represents the most widely accepted view [23–26]. It is also supported by the observation that mutation in components of c-NHEJ uniformly increase the formation of chromosome translocations in cells exposed to IR [20,22,55,56].

Surprisingly, a recent report presents evidence that the preponderance of alt-EJ in chromosome translocation formation is species-specific and valid as discussed above only for rodent cells [57]. The investigators transiently express designer nucleases generating DSB pairs at specific locations in the human genome and analyze by PCR, formation of the “expected”, reciprocal chromosome translocations 48 h later. Under these conditions chromosome translocations are robustly induced in repair proficient cells, and the generated junctions fail to display the above described “signatures” of alt-EJ. Notably, in c-NHEJ mutants of the same genetic background, the incidence of chromosomal translocations is uniformly reduced, albeit to a variable degree, and the junctions generated under these conditions do exhibit the typical features of alt-EJ. The authors conclude that in human cells, c-NHEJ mainly sustains chromosome translocation formation, in stark contrast to rodent cells, where alt-EJ plays a dominant role [57].

While species-specific quantitative shifts in the relative contributions of c-NHEJ and alt-EJ in chromosome translocation formation can be partly rationalized by the long-known 50-fold higher levels of DNA-PKcs in human as compared to rodent cells [58], qualitative shift of the reported magnitude could not be anticipated from other well-studied manifestations of c-NHEJ and alt-EJ in human and rodent cells, and indeed it appears incompatible with some of the results discussed above.

Since mechanistic understanding of chromosome translocation formation is essential for our fundamental understanding of the process of carcinogenesis itself, as well as of IR-induced cell killing and cancer, we revisited this question using stochastically induced DSBs and classical cytogenetics for the detection of chromosome translocations in the same human cell lines used in the above report — wild-type or c-NHEJ mutants. We focus specifically on cells irradiated during the G_2-phase of the cell cycle and analyze chromosome translocations, which in this case manifest as chromatid translocations, in the first metaphase.

Our results confirm for human cells observations reported in rodent cells, including markedly increased incidence of translocations in c-NHEJ mutants and a contribution of alt-EJ to translocation formation in c-NHEJ proficient cells.

2. Materials and methods

2.1. Cell culture

All cell lines were maintained at 37 °C in an atmosphere with 5% CO_2 and 95% air. HCT116 wt and *Lig4*$^{-/-}$ cells (a generous gift from Dr. E.A. Hendrickson) were grown in McCoy’s 5A medium (Sigma M-4892) supplemented with 10% fetal bovine serum (FBS-Sigma F0804) and antibiotics [Penicillin G (Pan-Pharma P06-08100P), Streptomycin (Calbiochem-5711)]. NALM-6 wt and *Lig4*$^{-/-}$ cells (a generous gift from Dr. N. Adachi) were grown in RPMI (Gibco-51800) supplemented with 10% FBS, 100 μM 2-mercaptoethanol and antibiotics.

2.2. Chemicals and inhibitors

Colcemid (L-6221, Biochrom AG) was used at 0.1 μg/ml to accumulate cells at metaphase (Stock: 10 μg/ml in PBS w/o Ca^{2+} and Mg^{2+}). Carnoy’s fixative was prepared by mixing 3 parts methanol (Sigma–Aldrich) and 1 part glacial acetic acid (Carl Roth GmbH & Co.) just before use. Routinely, 3 ml of ready to use Giemsa stain (Carl Roth GmbH & Co.) was diluted in 50 ml Sorenson’s buffer (10582-013, Gibco, Invitrogen) to stain metaphase chromosomes. Entellan (Merck) was used as mounting medium.

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