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Subtelomeric regions in mammalian cells are deficient in DNA double-strand break repair

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ABSTRACT

We have previously demonstrated that double-strand breaks (DSBs) in regions near telomeres are much more likely to result in large deletions, gross chromosome rearrangements, and chromosome instability than DSBs at interstitial sites within chromosomes. In the present study, we investigated whether this response of subtelomeric regions to DSBs is a result of a deficiency in DSB repair by comparing the frequency of homologous recombination repair (HRR) and nonhomologous end joining (NHEJ) at interstitial and telomeric sites following the introduction of DSBs by I-SceI endonuclease. We also monitored the frequency of small deletions, which have been shown to be the most common mutation at I-SceI-induced DSBs at interstitial sites. We observed no difference in the frequency of small deletions or HRR at interstitial and subtelomeric DSBs. However, the frequency of NHEJ was significantly lower at DSBs near telomeres compared to interstitial sites. The frequency of NHEJ was also lower at DSBs occurring at interstitial sites containing telomeric repeat sequences. We propose that regions near telomeres are deficient in classical NHEJ as a result of the presence of cis-acting telomere-binding proteins that cause DSBs to be processed as though they were telomeres, resulting in excessive resection, telomere loss, and eventual chromosome rearrangements by alternative NHEJ.

1. Introduction

DNA double-strand breaks (DSBs) are a critical DNA lesion, responsible for both the toxic effects of ionizing radiation and radiation-induced chromosome rearrangements leading to cancer [1]. The repair of DSBs occurs through either homologous recombination repair (HRR) or nonhomologous end joining (NHEJ). HRR in mammalian cells primarily uses the sister chromatid as a template, and is therefore limited to DSBs that occur after DNA replication [2]. NHEJ involves the joining of broken ends, and therefore can occur at anytime during the cell cycle. There are two forms of NHEJ, classical (C-NHEJ) and alternative (A-NHEJ). C-NHEJ has been extensively studied and many of the proteins that are involved are known, whereas much less is known about A-NHEJ [3,4]. A-NHEJ has primarily been observed in cells deficient in C-NHEJ, and has therefore been proposed to serve as a backup mechanism for repair of DSBs. Both C-NHEJ and A-NHEJ produce mutations at the site of a DSB caused by ionizing radiation, however, A-NHEJ is commonly associated with large deletions [5,6] and chromosome rearrangements [5,7–10]. Another characteristic of A-NHEJ is that repair commonly occurs at sites with microhomology [6,8,11–13].

Not all DSBs are repaired equally well. Most DSBs generated by ionizing radiation are repaired within a few hours; however, some DSBs require many hours to be repaired [14,15]. One factor that can influence the efficiency of repair of DSBs is their location in the genome. Goodarzi et al. [16] found that DSBs within heterochromatin are repaired much more slowly than DSBs occurring in euchromatin. Moreover, they also found that unlike DSBs occurring in euchromatin, the repair of DSBs in heterochromatin is dependent on ATM.

The ends of chromosomes, called telomeres, are another structural feature that can influence DSB repair. Telomeres are composed of the TTAGGG repeat sequence and associated proteins that together form the T-loop structure that keep the ends of chromosomes from appearing as DSBs and prevent chromosome fusion [17,18]. Telomeres are maintained in human germ line cells by telomerase, but shorten during cell division in somatic cells due to insufficient telomerase expression [19]. The gradual telomere shortening that occurs during growth of somatic cells that do not express sufficient telomerase normally results in telomere-associated DSB repair foci and cell senescence [20,21]. However, in cells that are unable to senesce, telomeres continue to shorten, eventually resulting in extensive chromosome fusion [22].

The role of the telomere in protecting chromosome ends and preventing chromosome fusion can influence the response to DNA damage within subtelomeric regioins. Ricchetti et al. [23] found

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that I-SceI-induced DSBs near telomeres in yeast are much more likely to result in gross chromosome rearrangements (GCRs) than DSBs occurring at interstitial locations, and concluded that this difference in response to DSBs near telomeres was due to differences in DSB repair. We have also reported that I-SceI-induced DSBs occurring near telomeres in a human tumor cell line are much more likely to result in large deletions, GCRs, and chromosome instability than DSBs occurring at interstitial locations within a chromosome [24]. This increased likelihood of telomere loss and GCRs as a result of DSBs within subtelomeric regions is not limited to human cancer cells, because I-SceI-induced DSBs near telomeres in mouse ES cells results in similar types of rearrangements and chromosome instability [25,26]. The likelihood of telomere loss and GCRs as a result of DSBs within subtelomeric regions has important implications for the chromosome instability associated with cancer. We have previously proposed that the sensitivity of subtelomeric regions to DSBs plays an important role in the high rate of spontaneous telomere loss commonly observed human cancer cells [24,27,28]. The chromosome fusions resulting from telomere loss initiate chromosome instability through breakage/fusion/bridge (B/F/B) cycles [29], which can generate many of the chromosome rearrangements leading to cancer [30]. The importance of B/F/B cycles resulting from telomere loss in cancer was demonstrated by the high rate of carcinomas in mice that are deficient in both telomerase and p53 [31]. The sensitivity of subtelomeric regions to DSBs could also be important in ionizing radiation-induced carcinogenesis, because we have demonstrated that the sensitive region extends at least 100 kb from a telomere and therefore poses a large target for DSB-induced chromosome instability [32].

In the current study, we have investigated whether the increased frequency of large deletions and GCRs that result from DSBs within subtelomeric regions in mammalian cells is due to a deficiency in DSB repair. These studies involve the analysis of the frequency of DSB repair by HRR and NHEJ through the activation of the gene for green fluorescent protein (GFP) following the introduction of DSBs with the I-SceI endonuclease. The repair of I-SceI-induced-DSBs through activation of GFP has been used extensively in mammalian cells to study the mechanisms of DSB repair and DSB-induced chromosome rearrangements [12,33–37]. We compared the frequency of repair of I-SceI-induced DSBs in a GFP gene integrated at telomeric sites, interstitial sites, and interstitial sites containing telomeric repeat sequences, to determine whether telomeres or telomeric repeat sequences influence the efficiency DSB repair.

2. Materials and methods

2.1. Plasmids

The pNCT-tel plasmid has been previously described [38]. pNCT-tel contains a neomycin-resistance (Neo) gene for positive selection with G418, a herpes simplex virus thymidine kinase (HSV-tk) gene for negative selection with ganciclovir, an I-SceI recognition site for introducing DSBs with the I-SceI endonuclease, and 0.8 kb of telomeric repeat sequences for seeding the formation of new telomeres (Fig. 1). The pQCXIH-ISceI retroviral vector that was used for expression of the I-SceI gene was constructed as previously described [24].

The pDR-GFP-tel plasmid was generated from the pDR-GFP plasmid [12,39] by the insertion of telomeric repeat sequences (Fig. 1). The first step in construction of pDR-GFP-ISceI was to remove the unique NotI restriction site in pDR-GFP. This was accomplished by first digesting the plasmid with NotI, and using the Klenow fragment of *E. coli* DNA polymerase (Invitrogen) to fill in the single-stranded NotI overhang prior to ligation. The next step was to insert a linker containing the NotI and XhoI restriction sites into the unique SspI restriction site located between the β-lactamase gene and chicken β-actin promoter for the GFP gene. A NotI/XhoI restriction fragment containing 0.8 kb of telomeric repeat sequences from the pNTP-tel plasmid [40] was then inserted into the NotI/XhoI sites in the pDR-GFP plasmid.

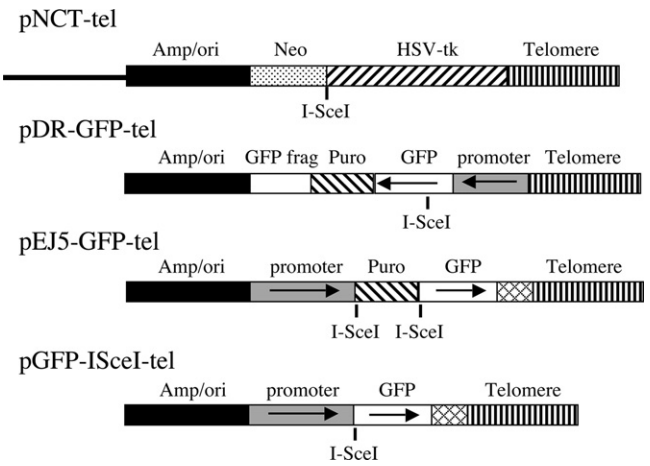

Fig. 1. The structure of the plasmids used in this study. The pNCT-tel plasmid is located adjacent to the telomere on chromosome 16p in clone B3 of the EJ-30 tumor cell line, and was previously used to demonstrate the sensitivity of subtelomeric regions to DSBs. The pNCT-tel plasmid contains a β-lactamase gene for resistance to ampicillin and bacterial origin of replication (Amp/ori), a Neo gene for resistance to G418, and a gene for Herpes simplex virus thymidine kinase (HSV-tk). The pEJ5-GFP-tel and pDR-GFP-tel plasmids were generated from the pEJ5-GFP and pDR-GFP plasmids by insertion of telomeric repeat sequences. The telomeric repeat sequences were added to seed the formation of a new telomere following targeted integration through the shared homology in the Amp/ori sequences. The pDR-GFP-tel plasmid was used to monitor the frequency of HRR and contains a GFP gene that is defective due to an I-SceI site in the coding sequence, a puro gene for selection with puromycin, and a complimentary fragment of the GFP gene for repair of the I-SceI-induced DSB. The pEJ5-GFP-tel plasmid was used to monitor the frequency of NHEJ and contains a puro gene flanked by I-SceI sites, which is located between the GFP coding sequence and promoter. NHEJ between the two I-SceI-induced DSBs results in activation of the GFP gene. The pGFP-ISceI-tel plasmid was used to determine the frequency of large deletions (>50 bp) and GCRs. The cell clones containing the pGFP-ISceI-tel plasmid were generated from the clones containing the pEJ5-GFP-tel plasmid by I-SceI-mediated deletion of the puro gene, and contain an active GFP gene with an I-SceI site located between the coding sequence and promoter.

The pEJ5-GFP-tel plasmid was generated from the pEJ5-GFP plasmid [12] by the insertion of telomeric repeat sequences (Fig. 1). The first step was to eliminate two NotI restriction sites flanking a 300 bp fragment containing the polyA addition sequences on the GFP gene. This was accomplished by first digesting the plasmid with NotI, then using the Klenow fragment of *E. coli* DNA polymerase (Invitrogen) to fill in the single-stranded NotI overhang prior to ligation. We next eliminated a unique SacI restriction site, by digesting the plasmid with SacI, then using the T4 DNA polymerase (Invitrogen) to remove the single-stranded SacI overhang prior to ligation. We then inserted a linker containing the NotI and SacI restriction sites between two PvuII restriction sites located between the beta lactamase gene and the GFP gene. The 0.8 kb NotI/SacI fragment containing telomeric repeat sequences in the pNTP-tel plasmid [40] was then inserted into the NotI/SacI sites in the pEJ5-GFP plasmid. We then inserted a SmaI/XbaI restriction fragment containing the PolyA addition sequences from the pPGKpuro plasmid into complementary FseI/NheI restriction sites at the end of the GFP gene.

2.2. Cell lines

All of the cell lines used in this study were derived from clone B3 of the EJ-30 human bladder cell carcinoma cell line. EJ-30 is a