Lytic infection of Kaposi’s sarcoma-associated herpesvirus induces DNA double-strand breaks and impairs non-homologous end joining

Yi Xiao, Jungang Chen, Qingjiao Liao, Yang Wu, Can Peng and Xulin Chen

Kaposi’s sarcoma-associated herpesvirus (KSHV) has been associated with the development of Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman’s disease (MCD) (Ablashi et al., 2002). Several reports have shown that chromosomal instability occurs in KSHV-infected cells or tissues. KSHV infection of primary human umbilical vein endothelial cells induces chromosome instability (Pan et al., 2004). Chromosomal imbalances have also been observed in KSHV-positive PEL cell lines (Nair et al., 2006). Furthermore, the loss of copies of chromosomes 14 and 21 and non-random translocations and deletions in the short arm of chromosome 3 were identified in two KS cell lines (Popescu et al., 1996; Thompson & Compton, 2011). Comparative genomic hybridization of microdissected KS lesions revealed the recurrent loss of chromosome Y in early tumour stages and additional chromosomal changes in late tumour stages (Nair et al., 2006; Pyakurel et al., 2006).

It has been reported that herpes simplex virus, human cytomegalovirus and Epstein–Barr virus infection can cause DNA double-strand breaks (DSBs) in host cells (Gaspar & Shenk, 2006; Kudoh et al., 2005; Shirata et al., 2005). Following activation of the DNA DSB repair pathway, the cell must repair the broken DNA using homologous recombination or non-homologous end joining (NHEJ) (Kao et al., 2005). NHEJ is the primary pathway used to repair DSBs and is an important mechanism for preserving genomic integrity (Burma et al., 2006). However, NHEJ mediates repair by directly ligating the ends of a DSB together and can often be mutagenic, which is especially true when the NHEJ pathway is impaired (Lord & Ashworth, 2012). Because chromosomal instability has been found in all three KSHV-associated diseases, KS, PEL and MCD, the production and the improper repair of DSBs could be the mechanism behind the tumorigenesis. We hypothesized that KSHV infection can induce DNA DSBs and impair NHEJ.

To understand whether the latent or the lytic infection of KSHV can cause DNA DSBs, KSHV latently infected human B-cell lymphoma BCBL-1 cells were treated with tetradecanoyl phorbol acetate (TPA) to induce lytic replication, and non-infected B-cell lymphoma BJAB cells were also treated with TPA as a control. The level of phosphorylated histone H2AX (γH2AX), which becomes phosphorylated on serine-139 in response to DSBs (Rogakou et al., 1998), was used as a biomarker of the cellular response to DSBs. As shown in Fig. 1(a), the level of γH2AX in BCBL-1 cells was very low in the viral latent infection but increased significantly following TPA treatment. However, the level of γH2AX in BJAB cells remained very low and was unchanged in both TPA-treated and untreated cells. These results suggested that KSHV lytic replication may contribute to tumorigenesis by causing DNA DSBs and interfering with the repair of DSBs.
during lytic replication (Jenner et al., 2001). At these time points, we examined the levels of γH2AX during TPA treatment in BCBL-1 cells. As expected, there was no PF-8 expression up to 10 h after TPA treatment, whereas the level of γH2AX increased to a high level at 10 h that was similar to the level at 24 and 48 h after treatment (Fig. 1b). The copy number of the viral genome determined by quantitative PCR showed that there was no viral DNA replication up to 10 h after TPA treatment (Fig. 1d). It appeared that the DNA DSBs caused by KSHV lytic infection were in the host DNA, as confirmed in a neutral comet assay (Fig. 1c) and the DSBs were induced before the viral DNA replication began in the viral lytic infection. To verify whether the lytic infection of KSHV interferes with the NHEJ of DNA with DSBs within the host cells, a NHEJ reporter plasmid, pEGFP-Pem1-Ad2 (Seluanov et al., 2004), was digested with I-SceI to produce a DSB within the ORF of GFP and transfected to BCBL-1 cells treated with TPA or sodium butyrate (NaB) or left untreated. At the dose used, TPA or NaB showed no toxicity to BCBL-1 cells. As shown in Fig. 1(e) and (f), compared with BCBL-1 cells in latent virus replication, the repair of DSBs in the reporter plasmids were impaired when BCBL-1 entered lytic virus replication. Previously, we discovered that KSHV PF-8 is the processivity factor of the KSHV DNA polymerase Pol-8, and binds to Pol-8 and stabilizes Pol-8 on the primer

![Fig. 1. Lytic replication of KSHV induces DNA DSBs in host cells. (a) BCBL-1 cells and BJAB cells were treated with TPA (20 ng ml⁻¹) for 48 h or left untreated. The levels of γH2AX and H2AX in cells were detected by Western blotting. (b) Expression of KSHV PF-8 and phosphorylation of H2AX in BCBL-1 cells were detected at different time points after the cells were treated with TPA. (c) The production of DSBs in cells was determined by a neutral comet assay using BCBL-1 cells treated or untreated with TPA (upper panel). Olive tail moment, defined as the product of the tail length and the fraction of total DNA in the tail, was measured using CASP software (lower panel). The y axis represents the abundance of DNA double strand breaks in arbitrary (Olive tail moment) units. (d) The copy number of the viral genome was quantified by real-time PCR using primers specific for KSHV orf73 in BCBL-1 cells treated with TPA for different periods of time. (e) BCBL-1 cells were treated with TPA (20 ng ml⁻¹) or NaB (0.3 mM) or left untreated (NT) for 12 h in the presence of 0.1 % DMSO, followed by electroporation with the linearized NHEJ reporter plasmid pEGFP-Pem1-Ad2 digested with I-SceI. At 14 h post-transfection, the BCBL-1 cells were fixed and expression of GFP was observed by fluorescence microscopy. (f) Analysis of NHEJ repair at 10, 14 and 18 h, respectively in BCBL-1 cells treated as described in (e). y-axis represents the cell number that GFP is expressed due to the repair of the NHEJ reporter plasmid. Statistical significance is indicated.](http://vir.sgmjournals.org)
We speculated that KSHV PF-8 may have functions in addition to playing a role in viral DNA replication. To identify cellular proteins that interact with PF-8, a glutathione S-transferase (GST) pull-down assay and mass spectrometric analysis were performed, and the multifunctional DNA repair proteins Ku70 and Ku86 were identified as interacting with PF-8 (data not shown). To confirm the interaction between PF-8 and the Ku complex, Western blot analysis was performed with antibodies specific to Ku70 and Ku86 following the GST pull-down assay using cell extracts from 293T cells treated with BLM (200 μg ml\(^{-1}\)) for 24 h or with extracts of untreated cells. A GST pull-down assay was performed using glutathione–Sepharose beads followed by immunoblotting with anti-Ku70, anti-Ku86 and anti-GST antibodies. 

To differentiate DNA-dependent protein–protein interactions from DNA-independent interactions, ethidium bromide (EB) was added to one set of aliquots of the cell lysates and maintained at a concentration of 100 μg ml\(^{-1}\) throughout the co-IP process. The other set of aliquots was used in the co-IP assay without EB. Our results showed that PF-8 could be co-immunoprecipitated by a Ku70 antibody in the absence of EB, and it could not be co-immunoprecipitated in the presence of EB, indicating that PF-8 interacts with the Ku complex and that the interaction is DNA dependent (Fig. 2b). Thus, we confirmed that PF-8 interacts with the Ku complex, and that the interaction is dependent on both DSBs and DNA.
In the NHEJ pathway, DNA-PKcs is recruited to Ku-bound DNA ends in the DSB sites (Deriano et al., 2005). Through the interaction with the Ku–DNA complex, DNA-PKcs is activated and phosphorylates six of the seven known NHEJ proteins: Ku70, Ku86, XRCC4, Artemis, XLF and DNA-PKcs itself. Finally, DSB ends can be ligated by a complex of XLF, XRCC4 and DNA ligase IV (Pastwa & Błasiak, 2003). To determine whether the interaction between KSHV PF-8 and the Ku complex interferes with the recruitment of DNA-PKcs to the DSB sites, a co-IP assay was performed. BJAB cells, and uninduced and TPA-induced BCBL-1 cells were treated with BLM (50 μg ml⁻¹), followed by cell lysis in co-IP buffer, and were subsequently bound to Ku70 antibody pre-coupled to Sepharose beads at 4 °C for 4 h. A mouse-derived Flag antibody was used as a control. The results showed that DNA-PKcs is associated with Ku70 in BJAB and BCBL-1 cells but not in TPA-induced BCBL-1 cells (Fig. 2c). We reasoned that the interaction between the Ku complex and DNA-PKcs might be impaired by KSHV PF-8, which interacts with the Ku complex. Because PF-8 is a DNA-binding protein, when KSHV enters the lytic stage, multiple virus-encoded replication proteins, including PF-8, form a replication complex and support viral DNA synthesis. We showed above that the interaction between PF-8 and the Ku complex is DNA dependent and can be disrupted by EB. To verify whether blocking of the recruitment of DNA-PKcs by PF-8 was also DNA dependent, the co-IP assay was repeated in the presence and absence of EB using the Ku70 antibody to immunoprecipitate DNA-PKcs from the lysates of TPA-treated BCBL-1 cells. As shown in Fig. 2(d), the interaction between DNA-PKcs and Ku70 was impaired in the absence of EB, whilst the interaction was restored in the presence of EB. We concluded that KSHV
PF-8 prevents DNA-PKcs from interacting with the Ku complex at the DSB sites on the DNA.

To evaluate the impact of PF-8 on NHEJ, HeLa cells were transfected with eGFP–PF-8 or eGFP expression plasmids. At 24 h post-transfection, the cells were treated with BLM for 1 h at 4 °C to induce DSBs whilst suppressing NHEJ activity (Mladenov et al., 2009; Pastwa et al., 2003). The cells were incubated in a CO₂ (5 %) incubator at 37 °C to allow for repair. At each repair time point, the cells were washed and fixed for an immunofluorescence assay (IFA) to stain the γH2AX foci. We found that, in the eGFP-expressing cells, the DSBs were mostly rejoined by 10 min and almost completely repaired at 60 min, whereas a significantly higher fraction of DSBs remained unrepaired in the eGFP–PF-8-expressing cells at 10 and 60 min of repair (Fig. 3a). The representative cells at different repair time points were chosen to show the γH2AX foci in cells, and a delayed NHEJ was observed in eGFP–PF-8-expressing cells compared with eGFP-expressing cells (Fig. 3b, c).

To determine whether there was a dose response in the inhibition of NHEJ versus the expression level of PF-8, the GFP–PF-8 and GFP-expressing cells were divided into three groups: high, medium and low (Fig. 3d, open bars), within cells expressing GFP–PF-8 (or GFP) and with DSB foci. The intensities of the γH2AX foci based on relative fluorescence units (Fig. 3d, shaded bars) were compared. As shown in Fig. 3(d), at repair time 0 min, the intensities of the γH2AX foci in cells with different expression levels of eGFP–PF-8 or eGFP were comparable and at a high level. At repair times of 5, 10 and 60 min, the intensities of the γH2AX foci decreased rapidly over time to a basal level in cells expressing different amounts of eGFP, whilst the intensities of γ-H2AX foci in GFP–PF-8-expressing cells decreased much more slowly over time compared with GFP-expressing cells. Furthermore, the higher the expression level of GFP–PF-8, the slower the repair rate of NHEJ, indicating that expression of PF-8 impairs NHEJ in a dose-dependent manner in HeLa cells (Fig. 3d).

Many studies have revealed that the pathogenesis of KSHV is related to the lytic replication of the virus (Fowler & Meyskens, 1978; Green & Wold, 1976; Hayward, 2003; Valladares, 1967), but the mechanism remains unclear. Our results indicate that lytic infection by KSHV induces DSBs and impairs NHEJ in host cells. Recently, Jha et al. (2013) found that de novo infection of KSHV in human PBMCs induces the phosphorylation of H2AX and that γH2AX is required for the establishment of latent infection of KSHV. We expect that a lytic infection by KSHV induces DSBs and impairs NHEJ in HeLa cells expressing different amounts of eGFP, whilst the intensities of γ-H2AX foci in GFP–PF-8-expressing cells decreased much more slowly over time compared with GFP-expressing cells. Furthermore, the higher the expression level of GFP–PF-8, the slower the repair rate of NHEJ, indicating that expression of PF-8 impairs NHEJ in a dose-dependent manner in HeLa cells (Fig. 3d).

Our results indicate a link between lytic infection by KSHV and the impaired NHEJ repair of DSBs and provide a basis for understanding the mechanism of tumorigenesis associated with KSHV infection. Further work is needed to determine how virally encoded proteins contribute to the induction of DNA DSBs, which proteins are involved and, more importantly, what the correlation between impaired NHEJ and chromosomal instability is. Finally, because PF-8 plays a pivotal role in the lytic replication of KSHV, and the pathogenesis of KSHV is related to the lytic replication of the virus, KSHV PF-8 may serve as a potential target for both antiviral and antitumour therapy.

ACKNOWLEDGEMENTS

We thank Dr Vera Gorbunova of Department of Biology, University of Rochester, Rochester, NY, USA, for providing the NHEJ reporter plasmid pEGFP-Pem1-Ad2. This work was funded by the National Natural Science Foundation of China (grant 31070151).

REFERENCES


