DNA Interstrand Crosslinks Repair in Mammalian Cells

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We studied the formation of double strand breaks (DSBs) as intermediates in the repair of DNA interstrand crosslinks (ICLs) by homologous recombination (HR). The plasmid EGFP-N1 was crosslinked with trioxsalen to give one ICL per plasmid on average. HeLa cells were transfected with the crosslinked plasmids and the ICL repair was monitored by following the restoration of the GFP expression. It was accompanied by γ-H2AX foci formation suggesting that DSBs were formed during the process. However, the same amount of γ-H2AX foci was observed when cells were transfected with native plasmid, which indicated that γ-H2AX foci appearance could not be used to determine the amount of DSBs connected with the ICL repair in this system. For this reason we further monitored the DSB formation by determining the amount of linearized plasmids, since having one crosslink per plasmid on average, any ICL-driven DSB formation would lead to plasmid linearization. Native and crosslinked plasmids were incubated in repair-competent cell-free extracts from G1 and S phase HeLa cells. Although a considerable part of the ICLs was repaired, no linearization of the plasmids was observed in the extracts, which was interpreted that DSBs were not formed as intermediates in the process of ICL repair. In another set of experiments HR-proficient HeLa and HR-deficient irs3 cells were transfected with native and crosslinked plasmids, and 6 h and 12 h later the plasmid DNA was isolated and analyzed by electrophoresis. The same amount of linear plasmid molecules was observed in both cell lines, regardless of whether they were transfected with native or crosslinked pEGFP-N1, which further confirmed that DSB formation was not an obligatory step in the process of ICL repair by HR.

Key words: DNA Interstrand Crosslinks, Homologous Recombination, DNA Repair

Introduction

DNA interstrand crosslinks (ICLs) are extremely toxic DNA damages blocking essential cellular processes such as transcription and replication. ICLs are incurred during normal metabolism, by environmental agents and chemotherapy. Both prokaryotic and eukaryotic organisms have developed multiple ways to remove ICLs in which nucleotide excision repair (NER), mismatch repair, translesion DNA synthesis, nonhomologous end joining (NHEJ) and homologous recombination (HR) participate to different extent (Nojima et al., 2005; Zheng et al., 2006). However, the molecular mechanisms of these pathways are still poorly understood, especially in higher eukaryotic cells. Recent reports indicated that the choice of repair pathway could depend on the phase of the cell cycle, chromatin organization, chemical nature of the crosslinks, etc. Both in vitro and in vivo experiments have shown that the initial step of ICL repair in mammalian cells involves the ERCC1/XPF endonuclease, which nicks DNA on one or both sides of the ICL (Kuraoka et al., 2000; Mu et al., 2000; Niedernhofer et al., 2004). Then a HR step is involved in which an undamaged donor DNA sequence provides a homologous copy for the repair of the damaged DNA (De Silva et al., 2000). What lies between these two steps is not well established. It has been suggested that the initial nicks are transformed into double strand breaks (DSBs) presumably during DNA replica-
tion to facilitate the recombination (Niedernhofer et al., 2004; Rothfuss and Grompe, 2004). This model has been confirmed for yeasts, where the principle HR pathway, the gene conversion, indeed proceeds via DSB formation (Barber et al., 2005). However, the interdependence of ICL incision and DSB formation in mammalian cells is much less clear. There are several reports describing the generation of DSBs as intermediates in the repair of ICLs (De Silva et al., 2000). Most of the evidence for the formation of DSBs in the course of HR stems from the appearance of γ-H2AX foci in cell nuclei after treatment with cell-free extracts from G1 and S phase HeLa cells. Although ICL repair was taking place in the extracts, no specific linearization of the plasmids was observed, which was interpreted that DSBs were not formed as intermediates in the process. Native and crosslinked plasmids were incubated in repair-compe-
tent cell-free extracts from G1 and S phase HeLa cells. There are data, that ICLs are repaired by different pathways in mammalian cells. There are, that ICLs are repaired by the ATR-dependent pathway, while DSBs by the ATM-dependent pathway. Both ATM (ataxia telangiectasia-mutated) and ATR (ATM- and Rad3-related) proteins are phosphatidylinositol-3-OH-kinase-like kinases (PIKK), which however, are activated by different stimuli. The molecular structures that activate ATM proteins are blunt, or almost blunt ends of DSBs, while the molecular structures that activate ATR proteins are ssDNA stretches flanked by dsDNA.

In a previous paper we have shown that trioxsalen (4',5',8-trimethylpsoralen) crosslinks in plasmid DNA were removed by HR after transfection in mammalian cells (Atanassov et al., 2005). To establish the role of DSBs in this process, in the present paper we monitored the DSB formation by determining the amount of linearized plasmids, since having one crosslink per plasmid on the average, any ICL-driven DSB formation would lead to the plasmid linearization. Native and crosslinked plasmids were incubated in repair-competent cell-free extracts from G1 and S phase HeLa cells. Although ICLs repair was taking place in the extracts, no specific linearization of the plasmids was observed, which was interpreted that DSBs were not formed as intermediates in the process. In another set of experiments HR-proficient HeLa and HR-deficient irs3 cells were transfected with native and crosslinked plasmids, and 6 h and 12 h later the plasmid DNA was isolated and analyzed by electrophoresis. The same amounts of linear plasmid molecules were observed in both cell lines, regardless of whether the plasmid was native or crosslinked, which further confirmed that DSB formation was not an obligatory step in the process of ICL repair by HR in mammalian cells.

Materials and Methods

Cells and plasmids

Human HeLa-M cells and Rad 51C deficient hamster irs3 (Jones et al., 1987) cells were purchased from LGC Promochem and were cultured in D-MEM with 10% fetal calf serum, supplemented with antibiotics and incubated in 5% CO₂ atmosphere. To synchronize the HeLa cells in the G1 phase, they were grown to 60–70% confluency and treated with 0.5 mM mimosine (Sigma-Aldrich, Munich, Germany) for 12 h, transfected with pEGFP-N1 and kept in mimosine for another 12 h. To synchronize cells in the S phase, they were treated with mimosine as above for 12 h, washed twice and released in fresh medium 1 h before transfection. For FACS (fluorescence-activated cell sorting) analysis cells were pelleted, washed with phosphate buffered saline (PBS, 0.14 M NaCl, 0.01 M phosphate buffer, pH 7.0), treated with 20 μg/ml RNase for 30 min at 37 °C and stained with 20 μg/ml propidium iodide at room temperature for 30 min. 2·10⁴ cells/sample were analyzed with a FACScalibur cell sorter (BD Biosciences, Heidelberg, Germany), using WinMDI v2.9 software. Plasmid pEGFP-N1 containing the gene for the green fluorescent protein (GFP) was purchased from Clontech-Europe (Saint-Germain-en Laye, France) and was propagated in E. coli XL1-Blue (Inoue et al., 1990).

Trioxsalen treatment

10 μg of plasmid DNA were dissolved in TE buffer (50 μl 10 mM TRIS-HCl, 1 mM EDTA, pH 8). 10 μg of trioxsalen dissolved in dimethylsulfoxide were added. The mixtures were placed in 96-well plastic plates and irradiated in a Hanau UV illuminator box (W. C. Heraeus GmbH, Hanau, Germany) by two 15 W Sylvania black light tubes with an emission maximum at 364 nm from a distance of 15 cm for different times (Gunz et al., 1996). 1 μl NaCl was added to a final concentration of 0.2 M NaCl, and plasmid DNA was precipitated with 2 volumes of ethanol. The number of inter-
strand trioxsalen crosslinks was determined by denaturation and electrophoresis of the linearized plasmids as described by Thompson and Mosig (1990). Under these conditions plasmids without crosslinks moved as single strand DNA, while the plasmids containing ICLs migrated as double strand DNA.

**Protein extract**

Protein extracts were prepared as described by Wood et al. (1988) with modifications. Cells were rinsed twice in ice-cold PBS, resuspended in hypotonic buffer (10 mM TRIS-HCl, pH 8.0, 1 mM EDTA). A tablet of Complete™ protease inhibitor cocktail per 50 ml solution (Roche Diagnostics GmbH, Graz, Austria) was added, and the mixture was kept on ice for 20 min to swallow. Cells were then homogenized by 20–30 strokes in a Dounce homogenizer (Fisher Scientific). To each cell lysate 4 ml of an ice-cold solution containing 50 mM TRIS-HCl, pH 8.0, 10 mM MgCl₂, 2 mM dithiothreitol, 25% sucrose and 50% glycerol were added slowly under stirring. 1 ml of neutralized saturated ammonium sulfate was then added with gentle mixing. The mixture was kept on ice with occasional stirring for 30 min and then centrifuged at 107000 ¥ g for 3 h. The upper two thirds of the supernatant were withdrawn and the protein precipitated by addition of 0.33 g/ml neutralized ammonium sulfate. The precipitate was collected by centrifugation, resuspended in a minimal volume of 25 mM HEPES-KOH, pH 7.9, 0.1 M KCl, 12 mM MgCl₂, 1 mM EDTA, 2 mM dithiothreitol and 17% glycerol and desalted using a Sephadex G-25 coarse column. The protein concentration was determined by the Bradford reaction.

**γ-H2AX foci detection**

Cells were seeded onto coverslips, and 24 h later they were washed and fixed with 2% paraformaldehyde in PBS for 5 min in an ice bath, followed by permeabilization with methanol for 5 min at −20 °C. The unspecific binding was blocked using a 0.5% solution of gelatine from bovine skin (Sigma-Aldrich) in PBS for 30 min at room temperature, and the coverslips were incubated with anti γ-H2AX mouse monoclonal antibody at 1:200 dilution (Abcam plc, Cambridge, UK). The coverslips were washed with 0.1% gelatine, 1% goat serum and incubated with Texas red conjugated secondary antibody (Abcam plc). The slides were examined with a fluorescence microscope Axiovert 200M (Carl Zeiss MicroImaging GmbH, Hamburg, Germany).

**Host cell reactivation assay**

Control and trioxsalen-treated plasmids were introduced into cells with the liposome lipofectamine 2000 transfection kit (Invitrogen, Karlsruhe, Germany) as recommended by the manufacturer. Fluorescent cells were observed under a fluorescence microscope 12 h later. 600 cells were counted for each determination and the percentage of fluorescent cells was calculated (Li et al., 1999).

**Isolation and fractionation of DNA**

Low-molecular weight DNA was isolated from Hirt supernatants containing the low-molecular nuclear DNA as described by Choo et al. (1989). Briefly, cells were grown to 60–70% confluency and lysed in 0.6% sodium dodecyl sulfate. 5 mM NaCl was added to the lysate to a final concentration of 1 mM and the mixture was kept at 4 °C overnight. The supernatant was collected after centrifugation at 20000 ¥ g for 30 min, and after extraction with phenol/chloroform (1:1), pH 8, DNA was precipitated with 2 volumes of ethanol. DNA was fractionated in 1% agarose gels, stained with ethidium bromide and transferred onto Hybond N+ membranes by Southern blotting. The membranes were hybridized with a DIG-labeled DNA probe (495 bp fragment of pEGFP-N1). The hybridized membranes were visualized using DIG High Prime DNA labeling and detection starter kit II (Roche Diagnostics GmbH) as recommended by the manufacturer.

**Results**

**γ-H2AX foci formation**

In a previous paper we have shown that S phase HeLa cells repair trioxsalen ICLs more efficiently than G1 phase cells (Mladenova and Russev, 2006). The current models for ICL repair include initial formation of nicks in the vicinity of ICLs, which are later convert into DSBs. Since there is evidence that ICLs are recognized and initial incisions are made in all phases of the cell cycle (McHugh and Sarkar, 2006; Niedernhofer et al., 2004), we suggested that the observed cell cycle
phase-dependent difference in the repair efficiency could reflect the higher efficiency with which the nicks were converted into DSBs in the S phase. To clarify this point we transfected HeLa cells with pEGFP-N1, randomly crosslinked with trioxsalen to give one ICL/plasmid on average. Trioxsalen is a chemical agent commonly used to crosslink DNA. It represents a bifunctional furcoumarin, which intercalates between the bases and, upon irradiation with 364 nm UV light, forms chemical bonds with pyrimidine bases in a two-step reaction. In the first step monoadducts are formed, which in the second step are transformed into crosslinks provided a pyrimidine base is available at the opposite DNA strand. Since the crosslinked plasmids are repaired by HR in HeLa cells quite efficiently (Atanassov et al., 2005; Mladenova and Russev, 2006), we supposed that this will be a good system to study the formation of DSBs in the plasmid DNA in the course of its repair. As an indication of the DSBs formation we used the appearance of γ-H2AX foci since DSBs summon immediate and massive phosphorylation of the histone H2AX variant (Rogakou et al., 1999). Thus, at different times after transfection the number of γ-H2AX foci was determined. Surprisingly, both in the transfected with crosslinked plasmid and in the transfected with control native plasmid HeLa cells soon after transfection γ-H2AX foci began to appear. Moreover, the kinetics of γ-H2AX foci appearance and disappearance as well as their number and sizes did not particularly differ in transfected with native plasmid and transfected with crosslinked plasmid cells (not shown), which suggested that in this case γ-H2AX foci were not formed on DSBs generated in the process of ICL repair. The reason for the appearance of γ-H2AX foci in the transfected with native pEGFP-N1 cells was not clear, but we later found that part of the plasmid molecules was linearized upon entering the cells regardless of whether they were crosslinked or not, which could explain this result.

**Incubation of plasmids in protein extracts**

After we have found that the γ-H2AX foci could not be used to monitor the ICL-driven DSB appearance in our system, we tried to determine the formation of DSBs as intermediates in the ICL repair, by more direct means. It has been shown that protein extracts were able to carry out the ICL repair reaction with a reasonable efficiency (Kucherlapati et al., 1985; Li et al., 1999). For this reason we tried to monitor the formation of DSBs during the ICL repair in vitro in G1 and S phase HeLa cell extracts. Mimosine was chosen as a synchronizing agent, since it does not affect the DNA synthesis and its action is fully reversible immediately after withdrawal (Lalande, 1990). The synchronization was monitored by FACS analysis (Fig. 1). The analysis of exponentially growing HeLa cells shows that the normal distribution of cells in the cell cycle was: 56% in the G1, 20% in the S, and 24% in the G2/M phase. G1 phase extracts were prepared from cells blocked with mimosine for 24 h. At this point over 90% of the cells were in the G1 phase. Alternatively, S phase extracts were prepared from cells 6 h after release from the mimosine block. At this point over 80% of the cells were in the S phase. Native and crosslinked plasmids were incubated in the extracts for 1 h, and then the plasmid DNA was recovered. It was used to transfect *E. coli* to determine the repair, since only plasmids without crosslinks would ensure the growth of *E. coli* colonies on selective (50 μg/ml kanamycin) medium (Atanassov et al., 2003). As negative control, *E. coli* were transfected with crosslinked plasmid incubated in buffer without protein extract for 1 h. Practically no colonies were detected in this case, while the crosslinked plasmid incubated in the extract of S phase cells gave 24% colonies compared with the native plasmid (100%), which showed that effective repair of the crosslinked plasmids has taken place in the extract. Plasmid DNA was also subjected to agarose gel electrophoresis, to monitor the DSB appearance with the presumption that in most of the plasmids carrying a single ICL, any ICL-related DSB will be linearized. However, when plasmid DNAs isolated from the extracts

![Fig. 1. FACS analysis of HeLa-M cells. (A) Exponential culture; (B) cells synchronized at the G1/S boundary by treatment with 0.5 mM mimosine for 12 h; (C) cells synchronized in the S phase by treatment with 0.5 mM mimosine for 12 h and then cultured in fresh medium for 6 h.](image-url)
were subjected to agarose gel electrophoresis it became evident that both native and crosslinked plasmids have been nicked to some extent shortly after introduction into the extracts, but no specific linearization was detected in the crosslinked plasmids in comparison with the native ones (Fig. 2A). When the plasmids isolated after incubation in the HeLa S phase extract were treated with the single strand specific S1 nuclease, which could convert SSBs (single strand breaks) into DSBs, most of the relaxed forms were linearized (Fig. 2B). This showed that, if the nicks have been converted into DSBs at any time in the course of the incubation, the obtained linear forms would have been clearly visible on the gels.

**HCR assay**

To check whether DSBs are legitimate intermediates in ICL repair *in vivo* we applied the host cell reactivation (HRC) assay in which damaged plasmid molecules are introduced into host cells where their repair takes place. A similar assay has been applied to show that HR-independent mechanisms for the removal of ICLs exist (Wang *et al.*, 2001; Zheng *et al.*, 2003). However, these experiments were poorly suited to study the HR pathway since in this case all plasmids carried the same ICL at the same place and undamaged homologous DNA was not available to serve as template. Here we used randomly crosslinked plasmid molecules, carrying one ICL per plasmid on average. In this case every lesion site on any of the plasmids would have multiple native sequences on the other plasmids to recombine (Atanassov *et al.*, 2003). Repair-proficient HeLa cells and repair-deficient irs3 cells were transfected with native and crosslinked pEGFP-N1 and 12 h after transfection the numbers of fluorescent cells were counted. In agreement with our previous results we found that HeLa cells were able to repair trioxsalen crosslinks quite efficiently, while the irs3 cells were not (Fig. 3). 6 and 12 h after transfection, Hirt supernatants were prepared and the low-molecular DNA was isolated and analyzed by electrophoresis. Blots were prepared from the gels and they were probed with a labeled 495 bp probe from the plasmid molecule to avoid any interference with the host chromosomal DNA. In agreement with the published results (Kucherlapati *et al.*, 1985), four bands appeared on the blots prepared from HeLa cells, corresponding to the supercoiled, linear and relaxed forms, respectively.
We did not detect any difference in the amounts of linear forms of the control and crosslinked plasmids, which was an indication that the DSBs were not formed in connection with the crosslinks repair. There are data that upon transfection the bigger part of the plasmid molecules entering the eukaryotic cells are cut and degraded (Lechardeur and Lukacs, 2006), which could explain the presence of linear plasmid molecules on the blots. On the other hand the presence of dimeric forms, which represent recombination intermediates, shows that HR has taken place both among the native and crosslinked plasmids. However, with these experiments we could not completely exclude the possibility that very short-lived DSBs are formed as specific intermediates of the ICL repair but we were not able to identify them because their concentration was very low. To rule out this possibility we repeated the HCR assay using recombination-deficient hamster irs3 cells. These cells have the mutated Rad 51C protein and are not able to carry out HR, but all other enzyme activities are intact (Jones et al., 1987). We reasoned that, if DSBs are normal ICL repair intermediates, DSBs would accumulate in these cells, since they could not be further processed. As expected the plasmid DNA isolated from the Hirt supernatants of the irs3 cells gave only 3 bands corresponding to the supercoiled, linear and relaxed forms. Dimeric forms in this case were absent which is quite natural having in mind that these cells were HR-deficient. There were no difference in the amount of linear plasmid forms present in the native and crosslinked plasmids, and they were very similar to those isolated from the HeLa extracts (Fig 4). This shows that the observed linearization of a part of the plasmid molecules after introduction in eukaryotic cells is rather irrelevant to the process of ICL repair by homologous recombination.

**Discussion**

The current models suggest that in the process of ICL repair, nicks are first made in their vicinity, which are later converted to DSBs to serve as substrate in HR. The formation of nicks or incisions is established beyond any doubt both in vitro and in vivo. In contrast with the great majority of nicks and small gaps formed and sealed at any moment by the normal metabolism of DNA, these nicks are probably persisting because of their location close to ICLs, which would prevent their efficient repair. The conversion of these nicks into DSBs and the relevance of such a conversion to the process of HR are less firmly established. Two are the arguments in support of the participation of DSBs as intermediates in ICL repair. The first one is the inevitable appearance of γ-H2AX foci in cell nuclei after the treatment with crosslinking agents (Mladenov et al., 2007). Since it is well established that γ-H2AX foci are formed at DSBs (Kruhlak et al., 2006), their appearance was taken to prove that DSBs have been formed. This conclusion, however, is not very sound since it has been recently reported that γ-H2AX foci could arise after treatment with MMS which gives monoadducts (Stojic et al., 2004), or even without any treatment (McManus and Hendzel, 2006). The results reported here also support the idea that γ-H2AX foci could appear concomitantly with, but not necessarily connected to the process of ICL repair. The second argument stems from the understanding that DSBs are recombinogenic lesions, while SSBs are not. Actually, back in the 1970s and even 1980s, SSBs were considered as initiators of HR since upon unwinding single stranded ends could invade homologous dsDNA sequences and lead to recombination (Holliday, 1964). The shift in the opinion from SSBs to DSBs being initiators of HR came after the finding that in S. cerevisiae the mating type switching which represents an act of non-
reciprocal recombination was initiated by a DSB (Szostak et al., 1983). There were many experimental results showing that nicks and single stranded gaps can also initiate HR (Rauth et al., 1986), but they were generally discarded because it was very difficult to prove that these nicks were not ultimately converted into DSBs immediately before the recombination event. However, recently increasing experimental evidence indicated that SSBs are indeed the major HR initiator in mammalian cells (Lee et al., 2004; Pascucci et al., 2005; Zheng et al., 2006), while DSBs are mended mostly by NHEJ (Brugmans et al., 2006; Lundin et al., 2002). This role of the SSBs is consistent with current recombination models and with the biochemical properties of the recombination proteins. The premature aging and cancer-prone Werner syndrome (WRN) is caused by defects in the RecQ helicase enzyme WRN, and WRN cells are highly sensitive to DNA crosslinking agents. Recently several papers describing the properties and the role of the WRN protein have shown that it has exonuclease, unwinding and annealing activities and participates along with BRCA1 in the repair of ICLs. Single strand DNAs such as in 3′-tailed, forked and D-loop regions are preferable substrates for the WRN. It could easily use nicked DNA to proceed with HR and subsequent gap filling, without first converting the nicks into DSBs (Cheng et al., 2006; Harrigan et al., 2006; Machwe et al., 2006). Thus a pathway to repair ICLs by HR in mammalian cells emerges in which DSBs are not intermediates.

The results described here are consistent with a model for ICL repair in which (a) incisions are made on both sides of the ICL thus transforming it into a gap in one strand and an adduct on the other strand opposite to the gap; (b) the nicked DNA is engaged in HR with native homologous sequence, forming intermediate dimeric structures containing two Holliday junctions, in which both damaged strands are paired with native strands; (c) the adduct and the gap are repaired by NER and gap filling DNA synthesis, respectively; and (d) the dimeric structures are resolved to restore the repaired DNA molecules. This ICL repair pathway has the advantage over the alternative pathway in which DSBs are formed since it is error-safe and not mechanistically connected with the progression of the replication forks, although it could be S phase-specific.

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