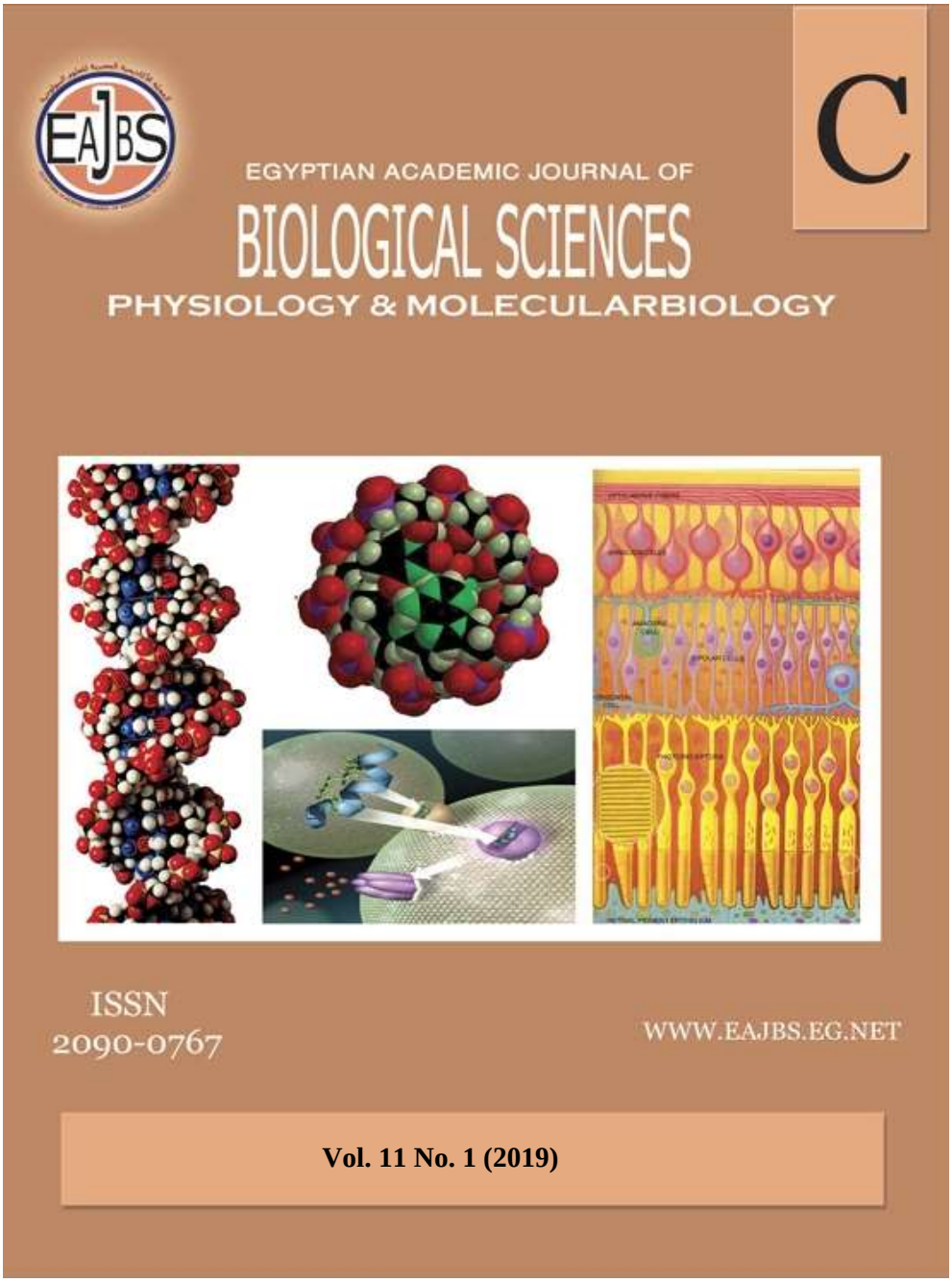

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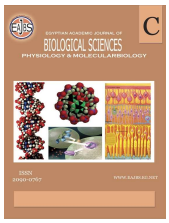

Role of DNA Polymerase κ in the Processing of DNA-protein Cross-link Damage Induced by 2′-deoxy-5-azacytidine and Formaldehyde

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ABSTRACT

Genomic DNA that essential for cell survival is constantly undergoes various forms of DNA damages upon attacked by DNA-damaging agents from exogenous and endogenous sources. DNA-protein cross-links (DPCs) are super-bulky, steric hindrance and less characterized DNA damage among those so far identified. Currently known DPCs are classified into four main types depending on the way of attachment to DNA strands. Of these types, type 1 is the most ubiquities in which cross-linked proteins (CLPs) are covalently attached to an undistorted DNA strand. While several researchers worldwide start to be attention about DPC damage, the repair factors that are indispensable for the processing of type 1 DPC remain largely elusive. Therefore, in the present study, we analyzed the role of translesion synthesis (TLS) DNA polymerases κ and ι (polκ and ι) in the processing of type 1 DPC. Obviously, mouse cells deficient in polκ were highly sensitive to 2′-deoxy-5-azacytidine (azadC, a DNA methylating agent) and formaldehyde (FA, a simple aldehyde). Furthermore, the quantitative analysis of DPCs in *polκ* proficient and deficient cells using fluorescence labeling method which we have developed recently revealed that the amount of DPCs increased significantly in azadC and FA-treated cells compared to untreated control. In contrast, a DNA methylation inhibitor Zebularine (Zeb) does not enhance the sensitivity of *polκ* deficient cells compared to *polκ* proficient cells. Additionally, no DPC is formed upon treatment with Zeb in polκ cells. The most remarkable conclusion is that the sensitivity of *polκ* deficient cells to azadC is exclusively due to DPC and ruling out the involvement of polκ in DNA methylation. Based on the current findings, we suggested a possible repair model for type 1 DPC induced by azadC and FA. Wherein, small peptides result from breakage of large CLPs are bypassed by polκ and consequently the repair proceeds.

INTRODUCTION

DNA molecule contains vital genetic information for nearly all living organisms and required for cell survival and function. Thus, it is very essential to keep genetic information intact. However, DNA undergoes several forms of DNA

damages upon continuously exposed to physical or chemical agents. Some of these agents are endogenous byproducts of the cellular metabolism (e.g., reactive oxygen and nitrogen species), while others are exogenous such as ionizing radiation, ultraviolet (UV) light, chemotherapeutic agents and genotoxic chemicals (Friedberg et al., 2006). Various forms of DNA damages have been identified up to date including base damage, intra-strand cross-links, inter-strand cross-links (ICLs), DNA-protein cross-links (DPCs), single-strand breaks (SSBs), and double-strand breaks (DSBs). Accumulation of such DNA damages has been considered to contribute to some of the features of aging and cancer initiation and development (Loeb et al., 2001; Hoeijmakers et al., 2001 and Garinis et al., 2008). Consequently, cells have evolved DNA repair mechanisms in order to repair these damages and allow for normal DNA transactions such as replication and transcription. A variety of structural and regulatory proteins are associated with DNA in cells. However, these proteins are often covalently trapped in DNA to form DPCs when cells are exposed to DNA-damaging agents or the inhibitors of DNA-metabolizing enzymes used in chemotherapy (Barker et al., 2005 and Ide et al., 2011). DPCs are subdivided into four types depending on whether and how they are associated with flanking DNA nicks (Ide et al., 2011; 2015; 2018 and Nakano et al., 2017). Of these types, type 1 is the most ubiquitous form under physiological conditions in which proteins are covalently trapped to an undistorted DNA strand and can be produced by formaldehyde (FA) and 2'-deoxy-5-azacytidine (azadC). Formaldehyde induces DPC by reacts with the amino group of a DNA-binding protein and that of cytosine base in DNA, giving rise to a cross-link between DNA and protein. While, azadC is a potent DNA methylating agent, when cells uptake azadC, it metabolized and intercalated into DNA in place of cytosine, 5-azacytosine (the base moiety azadC) traps the reaction intermediate of DNA methyltransferase (DNMT) forming DPC (Ide et al., 2011). The cross-linked proteins (CLPs) to DNA that form DPCs are super-bulky therefore cause steric hindrance to proteins associated with DNA transactions and hamper their function. If steric hindrance of DNA associated proteins were eliminated, the DNA repair proteins were come freely to the DPC site and allowed for the repair process. Regarding the mechanisms involved in the active repair of DPCs, previously we reported that nucleotide excision repair (NER) does not play a role in the removal of genomic DPCs induced by FA and the unstable DPCs are removed by spontaneous hydrolysis but stable DPCs stay longer in the genome and affect DNA transactions (Shoulkamy et al., 2012). Another study from our laboratory reported that NER has a negligible role in the repair of DPCs in mammalian cells (Nakano et al., 2007 and 2009). However, homologous recombination (HR) deals with DPCs in mammalian cells (Nakano et al., 2009; Novakova et al., 2003 Reardon et al., 2006; and Ridpath et al., 2007). A recent study mentioned that proteasomes and proteases Wss1 (a yeast metalloprotease) and Sprtn (a metalloprotease in higher organisms) encounter type 1 DPCs and DNA-dependent protease is activated and turns large CLPs to smaller cross-linked peptides (Stingele et al., 2015; 2017 and Vaz et al., 2017). Small peptides that result from the proteolytic degradation of DPCs are

bypassed by Rev3 (a catalytic subunit of DNA polymerase ζ) as proposed by a genetic study in yeast (Stingele et al., 2014). Moreover, DNA polymerase ζ catalyzes TLS through a defined DPC in *Xenopus* egg extracts (Duxin et al., 2014). In addition, TLS DNA polymerases η, κ, ν, and ι are completely blocked in DPC containing 23-mer peptides *in vitro*, however, a 10-mer peptide is bypassed (Yeo et al., 2014). However, it has not been fully clarified how mammalian cells deal with type 1 DPCs and whether TLS DNA polymerases are required for the processing of DPCs. To address this issue, we analyzed the sensitivity of a panel of mammalian cells deficient in TLS DNA polymerases, κ, η and ι to a type 1 DPC inducing agents (azadC and FA). The results indicate that polκ has a substantial role in palliating the cytotoxic effect of azadC and FA. A quantitative analysis of DPCs indicates that the capacity of DPC induction is significantly higher in polκ treated cells compared to untreated control. The data obtained here suggested a substantial role of DNA polκ in the processing of DPCs in mammalian cells.

MATERIALS AND METHODS

Chemicals and Cell Culture:

Mouse embryonic fibroblast (MEF) cells proficient and deficient in DNA polymerases were used for the experiment. Cells were available at our laboratory (Hiroshima University, Japan) (Table 1) and were established as explained previously (Ohkumo et al., 2006 and Shimizu et al., 2003 and 2005). Cells were cultivated in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% inactivated fetal bovine serum (Corning) and L-glutamine. Cells were maintained in a humidified incubator at 37°C with 5% CO_2 atmosphere, and harvested with 0.05% trypsin-EDTA. AzadC, FA and Zebularine (Zeb) were purchased from Wako Pure Chemical Industry.

Cell Survival Assay:

Cell survival was measured by clonogenic assay. Cells were plated into 100-mm culture dishes (Corning) and incubated for 12 hr. AzadC and FA were added to cells and incubated for 24 and 2 hr, respectively. AzadC requires DNA synthesis to extend its cytotoxic effects, so 24 hr treatment time is strictly needed. After the time of treatment is passed, cells were washed with fresh medium and incubated for 6 days until the colonies were formed. Colonies were fixed in 10% formalin solution and visualized by staining with 0.1% Crystal violet. Colonies with more than 50 cells were scored and the survival curves were plotted. The physiologically relevant doses that gave a 10% survival (LD_{10}) were determined from the survival curves.

Detection of DPC Damage

Purification of DNA Contains CLPs from the Treated Cells:

Cells ($Polκ^{+/+}$ and $Polκ^{-/-}$) in the mid-logarithmic phase (three 150-mm dishes) were treated for 24 hr with azadC and Zeb (inhibitors of DNA methylation) or 2 hr with FA at LD_{10} concentrations (Table 2). Following treatment periods, the culture medium was removed and cells were collected, washed with cold phosphate buffer saline and stored at -80°C until use. DNA containing CLPs was isolated by CsCl-density gradient centrifugation as described previously (Shoulkamy et al., 2012) with some modifications. Cells were suspended in 900 µl of a buffer containing 10 mM phosphate buffer (PB, pH 7.5), 1 mM EDTA, 20 µg RNase A (Sigma),

and protease inhibitor cocktail (Roche), and kept on ice for 10 min. The cell suspension was mixed with sarkosyl (final concentration 1%) and kept on ice for 30 min. The samples were subjected to CsCl density gradient ultracentrifugation at 20°C for 4 hr. DNA was purified by two rounds of ultracentrifugation to get pure DNA with cross-linked proteins and ensure complete removal of free proteins. After centrifugation, fractions containing DNA as determined by agarose gel electrophoresis were combined and dialyzed against PB + 1 mM EDTA (3 hr × 2), PB + 2 M NaCl (3 hr × 2), and finally MilliQ water (3 hr × 2) at 4°C. The DNA samples were concentrated by using a centrifugation evaporator without applying heat. The DNA concentration was measured on a Thermo Scientific Nanodrop 2000c, and the DNA was stored at -20°C until labeling procedures.

FITC-labeling for CLPs and Fluorescence Measurement:

Fluorescein isothiocyanate (FITC, Dojindo) was dissolved in dimethylformamide to a final concentration of 10 mM. Thirty µg of DNA in 20 mM borate buffer (pH 8.0) was mixed with 0.1 mM FITC solution and incubated for 1 hr at room temperature in dark. DNA was precipitated by ethanol, and the resulting DNA pellet was washed twice with 70% ethanol, air dried and dissolved in MilliQ water. The concentration of DNA was measured on a Thermo Scientific Nanodrop 2000c. The fluorescence intensity of thirty µg of FITC-labeled DNA was measured on a Hitachi F-2500 fluorescence spectrophotometer.

RESULTS

The Sensitivity of Cells Deficient in *polκ* and *polι* to azadC and FA:

To analyze the cytotoxic effects of azadC and FA on cells deficient in DNA polymerases (Polκ and ι), the sensitivity of a series of MEF cells deficient in DNA polymerases (Table 1) were tested. Cells were exposed to different doses of azadC and FA, and cell survival was tested using clonogenic assay. The sensitivity results demonstrate that cells deficient in *Polκ* (Fig. 1 A) but not *Polι* or h (η) (Fig. 1B) are exceedingly sensitive to azadC compared to wild type cells. Similarly, cells deficient in *Polκ* (Fig. 2 A) but not *Polι* or h (η) (Fig. 2B) show a high sensitivity upon treatment with various FA doses. The high sensitivities of *Polκ* deficient cells observed with both azadC and FA and the shared sensitivity of Polι mutants with wild type cells indicate that Polκ but not ι has a substantial role in palliating the cytotoxic effect of a DNMT inhibitor (azadC) and FA strongly suggesting a role for Polκ in the repair of DNA damage induced by both azadC and FA. Conversely, treatment of polκ$^{-/-}$ (mutant) and polκ$^{+/+}$ (wild type) cells with different doses of Zeb (a cytidine analog that inhibits DNA methylation) cause no increase in the cell sensitivity, excluding the role of polκ in DNA methylation (supplementary figure 1 A). The lethal dose that gave 10% survival (LD_{10}) was determined in polκ-/- cells for azadC, FA and Zeb and used for the subsequent experiment (Table 2).

Table 1: Cells proficient and deficient in DNA polymerases

Cell name	Mutation
B3	**wild type**
B6	*Poli*$^{-/-}$
2-1	*Polη* $^{-/-}$
B8#	*Po η* $^{-/-}$ *Poli*$^{-/-}$
Polκ$^{+/+}$	**wild type**
Polκ$^{-/-}$	*Polκ*$^{-/-}$ *Polκ*$^{-/-}$
2092	*Poli*$^{-/-}$ $^{-/-}$ *Polκ*$^{-/-}$
2095	*Polη* $^{-/-}$ *Poli*$^{-/-}$ *Polκ*$^{-/-}$

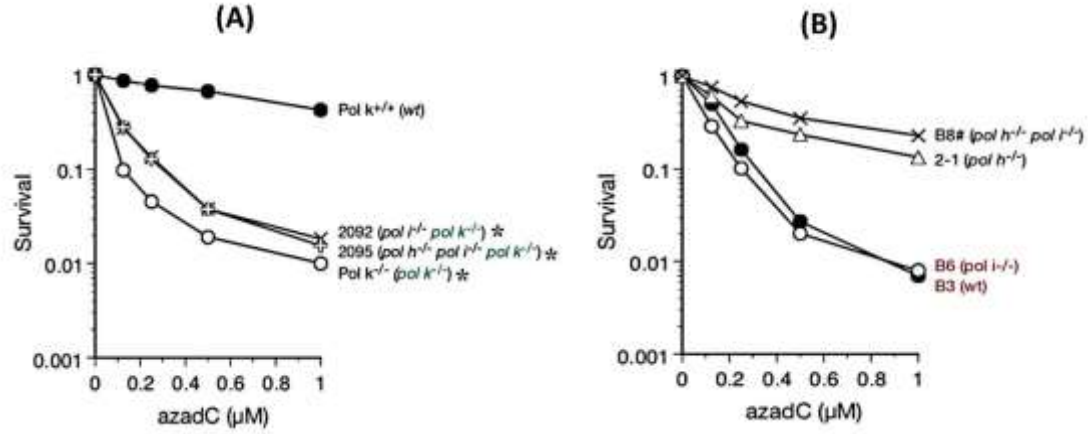

Fig. (1): Survival of azadC-treated MEF cells. Wild-type and repair-deficient MEF cells were treated with the indicated azadC doses for 24 hr and their survival was measured using a clonogenic assay. Data points are the means of three independent experiments, and standard deviations are not shown for the clarity of plots. Statistically significant differences in sensitivity (t-test, $p < 0.05$) for Polκ$^{+/+}$ and Polκ$^{-/-}$ are indicated by an asterisk.

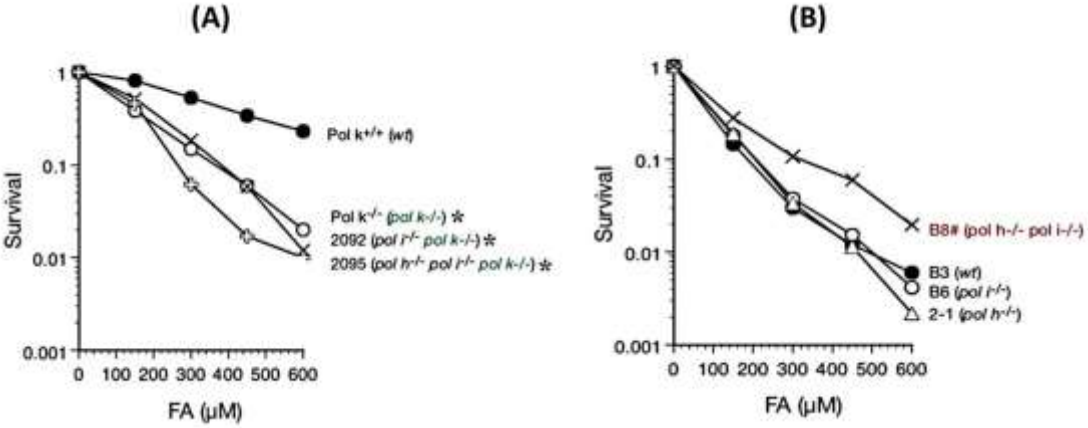

Fig. (2): Survival of FA-treated MEF cells. Wild-type and repair-deficient MEF cells were treated with the indicated FA concentrations for 2 hr, and their survival was measured using a clonogenic assay. Data points are the means of three independent experiments, and standard deviations are not shown for the clarity of plots. Statistically significant differences in sensitivity (t-test, $p < 0.05$) for Polκ$^{+/+}$ and Polκ$^{-/-}$ are indicated by an asterisk.

Table 2: The physiologically relevant doses that gave a 10% survival (LD10) for Polκ-/- mutant

Drug or chemical (abbreviation)	LD_{10} (μM)
2'-deoxy-5-azacytidine (azadC)	0.125
Formaldehyde (FA)	200
Zebularine (Zeb)	72

AzadC and FA Both Induce DPCs in Polκ Cells:

To explore whether azadC and FA induce DPCs in polκ cells, both polκ$^{-/-}$ (mutant) and polκ$^{+/+}$ (wild type) cells were combined immediately after treatment with azadC and FA at LD_{10} concentrations and the initial amount of genomic DPCs were quantified by the FTIC-labeling method as described above. The results revealed that both azadC and FA are significantly inducing DPCs in both cell types as quantified by fluorescence intensity estimation for CLPs (Fig. 3). It is well known that azadC is a DNA methyltransferase (DNMT) inhibitor and cause its effect through incorporation into DNA nucleotides, substitute cytosine, and subsequently methylate DNA (Ide et al., 2011). Keeping this in mind, we investigated whether the sensitivity observed with polκ$^{-/-}$ cells toward azadC accounts for DPC formation or DNA methylation. Therefore, polκ$^{+/+}$ (wild type) and polκ$^{-/-}$ (mutant) cells were treated with a well-known DNA methylation inhibitor (Zeb) at LD_{10} (Table 2) for 24 hr and the amount of DPCs was quantified. The fluorescence intensity of the CLPs displayed that the amount of DPCs is not increased compared to untreated control upon treatment with Zeb (Fig. 3), strongly suggesting that DPC but not inhibition of DNA methylation is the master damage associated with the cytotoxicity of azadC. From the above-mentioned results, a possible role for polκ in the processing of DPCs induced by azadC and FA was illustrated in Fig. 4. In which, the stalled replication fork by DPCs can be proceeds when polκ bypass the small cross-linked peptides resulting from proteolysis of large CLPs.

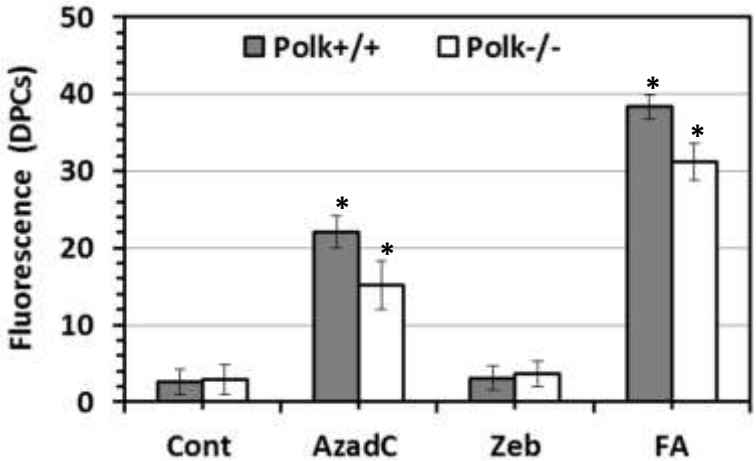

Fig. (3): Analysis of DPC induction in azadC, Zeb and FA-treated cells. MEF (Polκ$^{+/+}$ and Polκ$^{-/-}$) cells were treated without (Cont) or with azadC and Zeb for 24 hr or FA for 2 hr at LD_{10}, and the resulting DPCs were quantified by the FITC-labeling method. Data points are the means of three independent experiments with standard deviations. Statistically significant differences in the amounts of DPCs (t-test, $p < 0.05$) for untreated (Cont) and treated cells are indicated by an asterisk

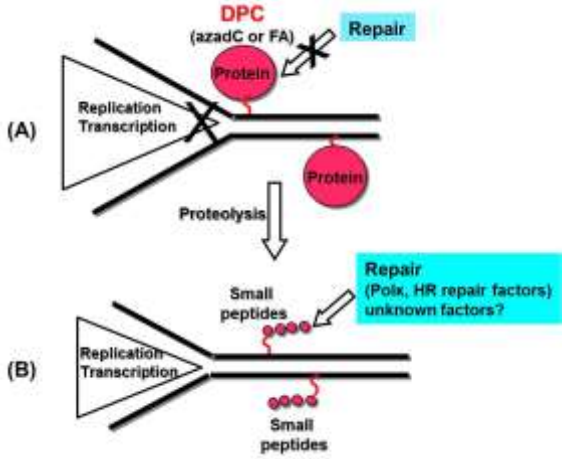

Fig. (4): Prospective repair model of type 1 DPC induced by azadC and FA. (A) Proteins around DNA are often covalently trapped on undistorted DNA strand resulting in type 1 DPC formation when cells are exposed to azadC and FA. CLPs that form DPC are super-bulky and likely obstruct DNA transactions such as replication, transcription and repair. (B) CLPs are subjected to proteolysis that turns large CLPs to small peptides. The small peptides are passed by TLS DNA polκ and consequently replication fork reactivated and replication, transcription and repair proceed.

DISCUSSION

DNA contains the genetic information an organism needs to develop, live and reproduce. DNA is continuously undergoing spontaneous modifications and various types of DNA damage when cells are subjected to DNA damaging agents from exogenous or endogenous sources (Friedberg et al., 2006). Of these damages, DPCs are ubiquitous but less characterized damage among those so far identified. They are super-bulky formed by a variety of genotoxic agents and are proposed to have therapeutic significance (Ide et al., 2011). Previously, we developed a quantitative and highly sensitive detection method of DPCs based on fluorescence labeling of CLPs that bind to DNA to follow the induction and repair of DPCs in mammalian cells (Shoulkamy et al., 2012). Up to date, the active repair mechanism for DPCs has not been fully determined. Accordingly, it is important to give insights about the possible factors involved in the repair of DPCs. Therefore, in the present study, we checked the role of mammalian TLS DNA polymerases in the repair of DPCs. To clarify whether these TLS DNA polymerases (κ, ι and η) are involved in the processing of DPCs, MEF cells deficient and proficient in TLS DNA polymerases (κ, η and ι) were treated with various doses of azadC and FA for 24 and 2 hr respectively, and the sensitivity was analyzed using clonogenic assay. The genetic analysis demonstrated that TLS DNA polymerases factor (polκ) but not (ι or η) is pivotal in palliating the cytotoxicity of azadC and FA and critical for cell survival. This result is in consistency with that data with DT40 cells deficient in TLS polymerases (Rev1 and 3) that reported sensitivity to FA and suggested a role of Rev1 and 3 in DPC tolerance (Ridpath et al., 2007). Contrariwise, another study on DT40 cells revealed that TLS polymerases

(Rev1 or 3) are crucial in the repair of ICLs induced by cisplatin (Niedzwiedz et al., 2004). Consequently, there are conflicting data on the involvement of these factors in the repair of DNA damage. A recent study on yeast has reported that type 1 DPCs that results from proteolytic degradation of DPCs are bypassed by Rev3 (Stingele et al., 2014) and DNA polymerase ζ catalyzes TLS through a defined DPC in *Xenopus* egg extracts (Duxin et al., 2014). In addition, TLS DNA polymerases η, κ, ν, and ι are completely blocked in DPC containing 23-mer peptides *in vitro*, however, a 10-mer peptide is bypassed (Yeo et al., 2014). The quantitative analysis of DPC formation in azadC and FA treated cells at LD_{10} strongly confirmed DPC formation, and these results are consistent with our previous data that demonstrated that FA is a potent DPC inducer (Shoulkamy et al., 2012 and Xie et al., 2016). Moreover, FA induces ICLs as determined from the sensitivity of cells deficient in Fanconi anemia pathways and NER repair factor (*XPF*) (Xie et al., 2016) as well as FA induces a weak DSB fragment in polκ cells as measured by static field gel electrophoresis (Supplementary figure 2). AzadC induces DPCs exclusively in polκ cells as measured by FITC labeling and doesn't induce DSBs (Supplementary figure 2). The sensitivity of HR repair factors (*Rad51* and *XRCC3*) but not NER repair factor (*XPF*) (Supplementary figure1 B) strongly excludes the ICL formation by azadC. In addition, the shared sensitivity of polκ$^{-/-}$ (mutant) and polκ$^{+/+}$ (wild type) cells to a DNA methylation inhibitor (Zeb) (Supplementary figure 1 A) ruling out the involvement of TLS polκ in DNA methylation and strongly indicating that the high sensitivity observed with azadC is exclusively due to DPCs. Based on the current findings in this study and the available data from other studies we have suggested a possible model for the processing of DPC (Fig 4). In this model, the replication fork stalled when encountered by CLPs that form DPC thereby impairing replication, transcription and repair machinery. CLPs are likely subjected to proteolysis that turns large CLPs to small peptides which are passed by polκ and consequently the stalled replication fork reactivated and the repair proceeds. Further studies are needful to examine other factors involved in the active repair of DPCs.

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SUPPLEMENTARY DATA

Supplementary data information associated with this article can be found, in the online version.

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ARABIC SUMMERY

دور إنزيم البلمرة κ في معالجة التلف الناتج عن إرتباط الدنا مع البروتين والمستحث بواسطة دواء
٢′- deoxy-5-azacytidine والفورمالدهيد

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الحمض النووي الدنا ضروريا لبقاء حياة الخلية ولكنه يتعرض بإستمرار لأشكال مختلفة من التلف لدى مهاجمته من قبل العوامل المسببة للتلف والتى تكون من مصادر خارجية أو داخلية. إن إرتباطات البروتين بالدنا (DPCs) هي عوائق كبيرة وتعتبر أقل أنواع التلف تميزًا من بين تلك التي تم تحديدها حتى الآن. تصنف DPCs المعروفة حاليا إلى أربعة أنواع رئيسية إعتمادا على طريقة الإرتباط بشريط الحمض النووي. من بين هذه الأنواع ، يعد النوع ١ للDPC هو الأكثر شيوعًا والذي ترتبط فيه البروتينات (CLPs) تساهميًا بشريط الDNA الغير مشوهة. في الوقت الذي بدأ فيه العديد من الباحثين في جميع أنحاء العالم بالاهتمام ب DPC ، فإن عوامل الإصلاح التي لا غنى عنها لمعالجة النوع ١ من الDPC تبقى بعيدة المنال إلى حد كبير. لذلك ، فإنه في هذه الدراسة قمنا بتحليل دور إنزيمات البلمرة κ و ι (DNA polymerases κ and ι) في معالجة النوع ١ للDPC. وقد أوضحت نتائج الدراسة الحالية أن خلايا الفئران الناقصة في جين إنزيم البلمرة κ (polκ) كانت حساسة للغاية لدواء ٢′-deoxy-5-azacytidine (azadC) والفورمالدهيد (FA). وعلاوة على ذلك، فقد أظهر التحليل الكمي للDPCs في الخلايا بإستخدام طريقة Fluorescence labeling التي قمنا بتطويرها مؤخرًا أن كمية DPCs زادت بشكل كبير في الخلايا التي تمت معالجتها بـ azadC وFA مقارنةً بالخلاياغير المعالجة. وعلى النقيض من ذلك، لا يسبب دواء Zebularine (Zeb) والذى يعتبر مثبط لل DNA methylation زيادة في حساسية الخلايا الناقصة في جين إنزيم البلمرة κ (polκ) مقارنة بالخلايا التى تحتوى على جين إنزيم البلمرة κ. بالإضافة إلى ذلك ، لم تزداد كمية DPC فى الخلايا المعالجة ب Zeb. إن الإستنتاج الأكثر إثارة للإهتمام هو أن حساسية الخلايا الناقصة في جين إنزيم البلمرة κ (polκ) إلى azadC تعود حصريا إلى تكوين الDPC وتستبعد دور إنزيم البلمرة κ في DNA methylation .إستناداً إلى النتائج الحالية، إقترحنا نموذج محتمل لإصلاح النوع ١ للDPC الناتج من معالجة الخلايا ب azadC و FA. حيث يتم تجاوز البتيدات الصغيرة الناتجة عن تكسير البروتينات الكبيرة المرتبطة بالدنا (CLPs) بواسطة إنزيم البلمرة κ (polκ) وبالتالي يسهل عملية الإصلاح.

SUPPLEMENTARY DATA INFORMATION

Role of DNA polymerase κ in the processing of DNA-protein crosslink damage induced by 2′-deoxy-5-azacytidine and formaldehyde

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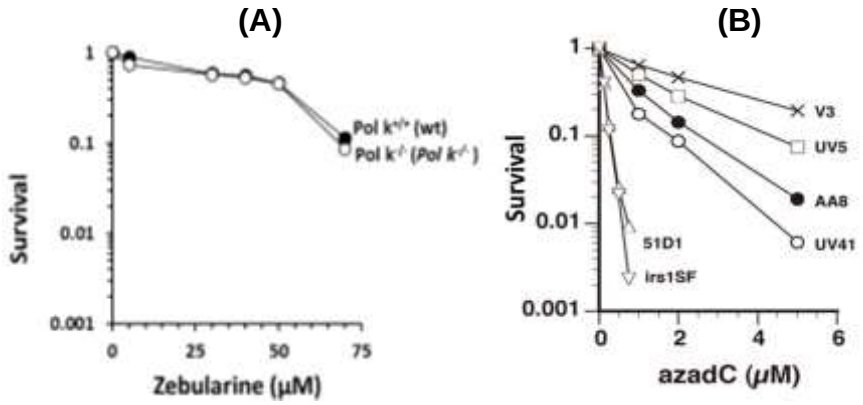
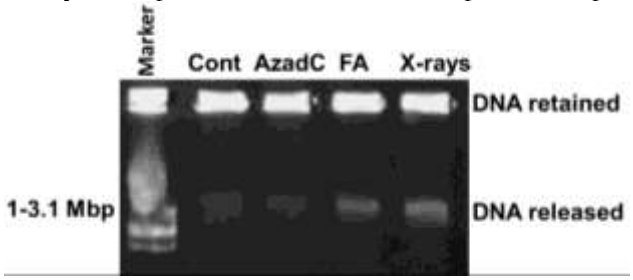


Supplementary figure 1: Sensitivity of azadC and Zebularine (Zeb) -treated cells. (A) Survival curves of repair-deficient Chinese hamster ovary (CHO) cells treated with Zeb: Repair-proficient AA8 cells and those deficient in NER (UV5 (XPD) and UV41 (XPF)), HR (51D1 (RAD51D) and irs1SF (XRCC3), and NHEJ (V3 (DNA-PKcs) were treated with the indicated concentrations of azadC for 24 hr. The colonies were scored for survival fractions. Data points are means of 2 independent experiments. **(B) Survival curves of MEF ((Polκ$^{+/+}$ and Polκ$^{-/-}$) cells treated with azadC:** MEF cells were treated with the indicated doses of Zeb for 24 hr and their survival was measured using a clonogenic assay. Data points are means of 2 independent experiments.



Supplementary figure 2: Detection of DNA-double strand breaks (DSBs) in MEF cells upon treatment with 2'-deoxy-5-azacytidine (azadC) and formaldehyde (FA). Polκ $^{+/+}$ cells were treated with azadC or FA (LD_{10}) for 24 and 2 hr, respectively. Cells were collected immediately after treatment and DSBs were analyzed by static field gel electrophoresis. The quantity of DNA released from the plug relative to total DNA (*i.e.*, released and retained DNA) was calculated. X-rays irradiated cells (5 Gy) were used as a positive control and analyzed in parallel with azadC and FA treated cells.