Poly (ADP-Ribose) Polymerase is Involved in the Repair of DNA Damage Due to Sulfur Mustard by a Mechanism Other Than DNA Ligase I Activation

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ABSTRACT

Poly (ADP-ribose) polymerase (PARP) modulates several cellular functional proteins by a mechanism in which the proteins are poly-ADP-ribosylated by transferring the ADP-ribose moieties from the enzyme substrate NAD^+ to the proteins. PARP is activated following damage to cellular DNA by alkylating agents including sulfur mustard (SM). We observed concurrent activation of PARP and DNA ligase in SM-exposed human epidermal keratinocytes (HEK). Previous reports from other laboratories suggested that DNA ligase activation could be due to its modification by PARP. In humans, there are three distinct DNA ligases, I, II and IV of which DNA ligase I participates in DNA replication and repair. By metabolically labeling HEK using ^{3}H-adenosine (adenosine 2,8-^{3}H) to generate intracellularly radiolabeled NAD^+ (^{3}H-adenine) and then exposing the cells containing ^{3}H-NAD^+ to 1 mM SM, we found that isolated DNA ligase I was not ^{3}H-labeled. This result indicates that DNA ligase I is not a substrate for ADP-ribosylation by PARP. Interestingly, our results show an effect of PARP inhibition on the decay of activated DNA ligase following exposure of HEK to SM. In the presence of 2 mM 3-amino benzamide (3-AB), a PARP inhibitor, the activated DNA ligase has a half-life that is four-fold higher than that observed in the absence of 3-AB. SM-induced DNA damage repair is dependent on DNA ligase I activation, which we have found to occur via phosphorylation catalyzed by DNA-dependent protein kinase (DNA-PK). The longer half-life of DNA ligase I observed when PARP is inhibited suggests that DNA repair requires PARP, and that DNA ligase I remains activated until DNA damage repair is complete. Therefore, PARP is involved in DNA repair mechanisms other than that of DNA ligase I activation. PARP inhibitors have been suggested as prospective SM antidotes. We are interested in developing mechanism-based intervention of SM injury and, therefore, the knowledge about how PARP participates in DNA repair should be useful for this objective. By using the DNA ligase I phosphorylation assay and inhibiting PARP chemically and also in a separate system through induction of PARP antisense mRNA in the cells, we have confirmed that DNA ligase I is not the target of PARP action. Based on our results and the information available in the literature, we propose that in DNA repair, PARP participates at the stage of altering the chromosomal structure to make the damage accessible to the repair enzymes. Overall, the results of our studies suggest the interplay of PARP and DNA ligase I in the repair of chromosomal DNA damaged by SM.

INTRODUCTION

Sulfur mustard (SM, bis-(2-chloroethyl) sulfide) inflicts debilitating injury to skin (vesication) and to other organs exposed to it. At the molecular level, SM targets chromosomal DNA, RNA and proteins. Following damage by alkylating agents such as dimethyl sulfate (1) or sulfur mustard, stimulation of poly (ADP- ribose) polymerase (PARP) (2) and DNA ligase activity have been observed (3). It has been suggested that in response to DNA damage, DNA ligase is stimulated by ADP-ribosylation (4,5). SM injury initiates two processes, namely, DNA repair and apoptosis. SM produces alkyl adducts, cross-links, and double strand breaks in the chromosomal DNA (6). Repair of these damages can restore DNA integrity and cell viability. On the other hand, apoptosis leads to caspase-3-mediated PARP degradation, DNA fragmentation, and cell death.

We have been studying the repair of SM-inflicted DNA damage in a model system, cultured human epidermal keratinocytes (HEK). DNA ligase is an essential enzyme for DNA replication and repair. In mammals, three distinct DNA ligases have been identified and characterized. Among these, the ~120 KDa DNA ligase I has been studied the most. This enzyme is DNA substrate specific and is involved in DNA repair and replication. DNA ligases III and IV have similar substrate specificity in that they can ligate both DNA-DNA and RNA-DNA substrates (7). DNA ligase IV is a ~ 100 kD enzyme (8) and is considered to be specific for V(D)J recombination (9), a process through which immunoglobin and T cell receptor genes are assembled. A possible mechanism of DNA ligase stimulation may be through (ADP-ribosyl)ation, as suggested previously (4,5), or through phosphorylation by a kinase activity. We have reported that the stimulation of DNA ligase I activity in HEK exposed to SM is specific to double strand breaks in DNA and is mediated by DNA-dependent protein kinase (DNA-PK) (10). We also observed that the half-life of activated DNA ligase is four-fold greater in the presence of 3-amino benzamide (3-AB), a PARP inhibitor, suggesting that PARP is needed for DNA repair (3,12). If DNA ligase is not activated by ADP-ribosylation, it is puzzling that PARP inhibition influences DNA ligase activity. A similar observation was also made in the presence of an intracellular Ca^{2+} chelator, BAPTA AM (1,2 Bis (2 amino phenoxy) methane N, N, N', N'- tetraacetic acid methyl ester) (3). These observations indicate a need for a better understanding of the role of PARP in the repair of DNA damage.

DNA ligase I activation by phosphorylation appears to be a usable biomarker of SM-induced chromosomal DNA damage and the initiation of DNA repair. This biomarker may be useful in monitoring DNA damage and repair associated with effects such as involvement of PARP in vesicant damage intervention. In this report, we present evidence that DNA ligase I is not the target of PARP action. We suggest that PARP is essential for efficient DNA repair, and that the purpose of PARP action appears to be to unfold the chromatin through poly(ADP-ribosyl)ation and make damaged chromosomal DNA accessible to DNA repair enzymes.

MATERIALS AND METHODS

Materials

SM (> 98% pure) was obtained from the US Army Research, Development and Engineering Command (USARDEC), Edgewood, Aberdeen Proving Ground, MD. HEK and keratinocyte growth medium (KGM) were purchased from CAMBREX, Walkersville, MD, USA. OligodT cellulose and poly dA were purchased from Pharmacia LKB, Piscataway, NJ, USA. Protein assay reagent was the product of Biorad, Richmond, CA, USA. Protease inhibitors and 3-amino benzamide (3-AB) were purchased from Sigma-Aldrich, St. Louis, MO. All other chemicals were of analytical grade. Monoclonal antibodies to bovine DNA ligase I was a kind gift from Dr. Tomas Lindahl, Imperial Cancer Research Fund, UK. Radiolabeled (^{33}P) orthophosphoric acid was obtained from PerkinElmer-NEN, Boston, MA. The apoptosis inhibitor Z-VAD-FMK (benzyl oxycarbonyl-Val-Ala-Asp-fluoromethylketone) and AC-DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp-CHO (aldehyde)) were purchased from BD Biosciences, San Diego, CA. The CD95 antibody secreting hybridoma (HB-11726) was obtained from American Type Culture Collection (ATCC), Gaithersburg, MD. The dexamethasone inducible PARP (-) human keratinocyte cell line was a kind gift from Dr. Dean Rosenthal and Dr. Mark Smulson, Georgetown University School of Medicine, Washington, D.C.

Methods

Cells: Stock HEK from adult skin from a single donor at passage 2 were cultured in keratinocyte growth medium (KGM) in 5% CO_2 at 37°C in a humid atmosphere to almost 100% confluence in 150 cm^2 flasks before exposure to SM and labeling with $^{33}PO_4^{-3}$.

Exposure of HEK to SM in the Presence of $^{33}PO_4^{-3}$ and Inhibitors of PARP and Apoptosis: HEK in monolayers were grown to confluence, the medium was removed by aspiration and the cells were then washed with 37°C saline (3 x 25 mL). Frozen stock of SM in saline was thawed and vortexed to obtain a 4 mM solution. This stock solution was diluted in the flasks containing the cells using phosphate-free medium A (148 mM NaCl, 5.4 mM KCl, 10 mM $NaHCO_3$, 10 mM D-glucose and 25 mM Hepes, pH 7.4; 339 mOsM) containing 0.75 mCi of $^{33}PO_4^{-3}$ (New England Nuclear, specific activity, 5280 Ci/mMol) to 1 mM. The flasks were incubated for 30 min at room temperature and then transferred to a 37°C CO_2 incubator for 1.5 hr. Following this incubation, cells were harvested. To study the effects of PARP and apoptosis inhibitors, the cells were incubated with either 3-AB (PARP inhibitor) or AC-DEVD-CHO (caspase-3 inhibitor) or Z-VAD-FMK (general caspase inhibitor) or CD95 antibody (inhibitor of apoptosis via the CD95 (Fas) receptor pathway) for 30-60 min in the phosphate-free medium before SM exposure.

Cell Harvesting and Preparation of Cell-free Extracts: The flasks were removed from the incubator and the radioisotope containing medium was removed and the flasks were washed with ice cold saline (3x25 mL). The cells were scraped into ice-cold saline and pooled cell suspension was centrifuged to pellet the cells. The pellet was suspended in a

small volume (1-1.5 mL) of an extraction buffer containing the following: 300 mM NaCl, 50 mM tris.HCl, pH 7.5, 1 mM EDTA, 0.1% Triton x-100 and 10% glycerol. In addition, the following protease inhibitors were also present: Phenyl methyl sulfonyl fluoride, 1 mM; pepstatin, 5 µg/mL; aprotinin, 2 µg/mL; leupeptin, 1.5 µg/mL and N-alpha-P-tosyl-L-chloromethyl ketone, 0.5 µg/mL (11). The suspended cells were disrupted by four freeze-thaw cycles. A clear cell-free extract was obtained following centrifugation of the suspension at $4^{o}C$ using a Sorval centrifuge (SS-34 rotor) at 5000 rpm for 20 min. The pellet obtained was re-extracted with another 1 mL of extraction buffer, and the clear extract obtained after centrifugation as before was combined with the first and stored at -$20^{o}C$ before further processing.

Analysis and Quantitation of DNA ligase I phosphorylation: Previously, we published the experimental procedures and results to show that DNA ligase I is phosphorylated after exposure to SM (10). The phosphorylated DNA ligase I was isolated by affinity chromatography using a bovine DNA ligase I monoclonal antibody affinity column. Under identical conditions the extent of phosphorylation can be directly correlated to the chromatographic peak area. These criteria were used to evaluate the effects of the apoptosis inhibitors on DNA ligase I phosphorylation due to SM.

DNA ligase and Protein Assays: DNA ligase was assayed using poly dA- ^{3}H oligo dT substrate as previously described (3). Protein was assayed using the Biorad protein assay reagent and γ-globulin as the standard.

RESULTS AND DISCUSSION

Based on the results of our previous studies, we suggested that PARP might have a role in DNA ligase activation (12). In later studies, we demonstrated that DNA ligase I activation is by phosphorylation mediated by DNA-PK in response to SM-induced DNA double strand breaks (10). However, in the presence of 3-AB, the rate of repair is slower, and the rate constant for the repair of SM-induced DNA damage in HEK is about one third of that measured in the absence of 3-AB (12). A parallel observation has also been made about the decay rate of activated DNA ligase in SM-exposed HEK (3). These observations suggest that PARP is required for rapid repair of SM-induced chromosomal DNA damage. To clarify the role of PARP and the interaction between PARP and DNA ligase activities during SM-induced DNA damage repair, DNA ligase I phosphorylation was studied in cells in which PARP was specifically eliminated by dexamethasone-induced PARP anti-sense expression. In these cells, over 90% of PARP was inhibited (13) offering a better specific inhibition compared with 3-AB where non-specific effects could be due to its metabolites.

In *Figure 1* is shown a typical elution profile of phosphorylated DNA ligase I from a bovine DNA ligase I monoclonal antibody column. The data were generated by comparing phosphorylation in the presence of $^{33}PO_4^{-3}$ in SM-exposed and unexposed HEK as described in the Methods section. The areas under the curves obtained by Area integration using “PRIZM” software are directly proportional to the extent of

phosphorylation. Using this method, the ratio obtained for SM-exposed HEK compared with unexposed control was 2. This result indicated a high level of DNA ligase I phosphorylation due to SM exposure in HEK. Using a similar approach, we examined ADP-ribosylation to determine whether concurrent phosphorylation and poly(ADP-ribosyl)ation is required for DNA ligase I activation.

We used HEK in which NAD^+ was metabolically ^{3}H-labeled at adenine and then exposed the cells to SM. DNA ligase I eluted from the antibody column did not have excess ^{3}H label when compared with the SM-unexposed control cells (*Table I*). These results indicate that DNA ligase I is not ADP-ribosylated following SM exposure of HEK. Therefore, phosphorylation is the only modification undergone by DNA ligase I after SM exposure.

In *Table I*, also is shown the effect of decreasing PARP activity in HEK on the extent of DNA ligase I phosphorylation due to SM. In these experiments, PARP was decreased either by using a chemical inhibitor, 3-AB (2 mM), or by expression of dexamethasone inducible PARP antisense mRNA in an immortalized HEK cell line (13). The ratios of the areas under the elution curves of SM-exposed to unexposed are given. An enhanced DNA ligase I phosphorylation was observed in SM-exposed cells compared with unexposed controls in both 3-AB treated cells as well as in PARP (-) cells. A relatively lower level of phosphorylation observed in PARP (-) cells may be attributed to these cells not being primary human epidermal keratinocytes. However, the results suggest that PARP activity is not required for DNA ligase I activation via phosphorylation, and that DNA ligase I is not a target of PARP action during SM-induced chromosomal DNA damage repair.

Caspase-3-catalyzed PARP degradation is one of the major events during SM-induced apoptosis. In SM-exposed cells, DNA damage/repair and apoptosis are dependent on SM concentration and time after SM exposure. At a high SM concentrations (100 μM or higher), DNA repair and apoptosis are interdependent, i.e., one influences the other. If PARP is essential for DNA repair, apoptotic PARP degradation is expected to retard DNA repair. Therefore, inhibition of apoptosis should enhance DNA repair and cell viability. We tested the effects of a specific caspase-3 peptide inhibitor, AC-DEVD-CHO, and a general caspase inhibitor, Z-VAD-FMK on the extent of SM-induced DNA ligase I phosphorylation compared with unexposed controls. The extent of phosphorylation was reduced about 17% by the specific caspase-3 inhibitor and about 45% by the general caspase inhibitor (*Table II*). In SM-exposed HEK, the apoptotic signal is also transmitted through a cell surface death receptor CD95 (Fas) (13). Therefore, we studied the effect of CD95 antibodies on SM-induced DNA damage and, therefore, DNA ligase I activation. Interestingly, the CD95 antibodies reduced DNA ligase I phosphorylation (about 60%) that requires DNA double strand breaks (*Table II*)**.** Since DNA ligase I phosphorylation is an indicator of DNA damage and possibly DNA repair, these results show inhibition of apoptosis influences DNA damage and repair.

In this report, we have presented data indicating that in SM-exposed HEK, PARP is not directly involved in DNA ligase I phosphorylation, i.e., its activation (*Table I*). However, PARP is necessary for efficient repair of DNA damage due to SM (12). The following is a discussion of how PARP may be involved in DNA repair.

In apoptosis, activated caspase-3 degrades PARP. Therefore, inhibiting apoptosis in SM-exposed HEK, e.g., by using the general caspase inhibitor Z-VAD-FMK should prevent PARP degradation. Data presented in *Table II* indicate that in SM-exposed HEK, when apoptosis is inhibited, i.e., PARP is maintained at a higher level, DNA ligase I phosphorylation-cum-activation is reduced. Previously published results from our laboratory showed that DNA ligase I activation via phosphorylation occurs in response to DNA double strand breaks, and that the level of DNA ligase activity reflects the status of DNA damage and its repair (3, 10).

An active role of PARP in DNA repair was recently postulated by Sanderson and Lindahl (14). According to this report, although several enzymes with poly(ADP-ribosyl)ation have been identified, PARP-1 accounts for over 90% of the total activity detected in mammalian cells following DNA damage. This report also suggested that PARP-1 has the unusual property to act as a "nick sensor" and binds tightly to DNA single- and double-strand breaks. Upon DNA binding, PARP-1 is activated to ADP-ribosylate itself, as well as to a lesser degree, a limited number of other proteins involved in chromatin organization, DNA repair, and DNA metabolism. These authors discussed evidence to support the notion that PARP-1 or PARP-1 dependent (ADP-ribosyl)ation might contribute directly or indirectly to efficient DNA repair and the maintenance of genomic stability. In another recent report, d'Erme et al. discussed the role of poly(ADP-ribosyl)ation in modulation of chromatin structure (15). These authors discussed evidence to suggest that the process of poly(ADP-ribosyl)ation is involved in the dynamic transition of chromatin fibers between a condensed and a decondensed state through the addition of a negative charge to the nuclear proteins. Their results obtained by using scanning force microscopy revealed that poly(ADP-ribosyl)ation of chromatin fiber induces fiber relaxation and decondensation. Based on these concepts, we propose that in SM-exposed HEK, when apoptosis is inhibited, the higher level of PARP facilitates DNA repair possibly via the mechanisms discussed above and, therefore, the reduced level of DNA damage is indicated by the decrease in DNA ligase I phosphorylation (*Table II*).

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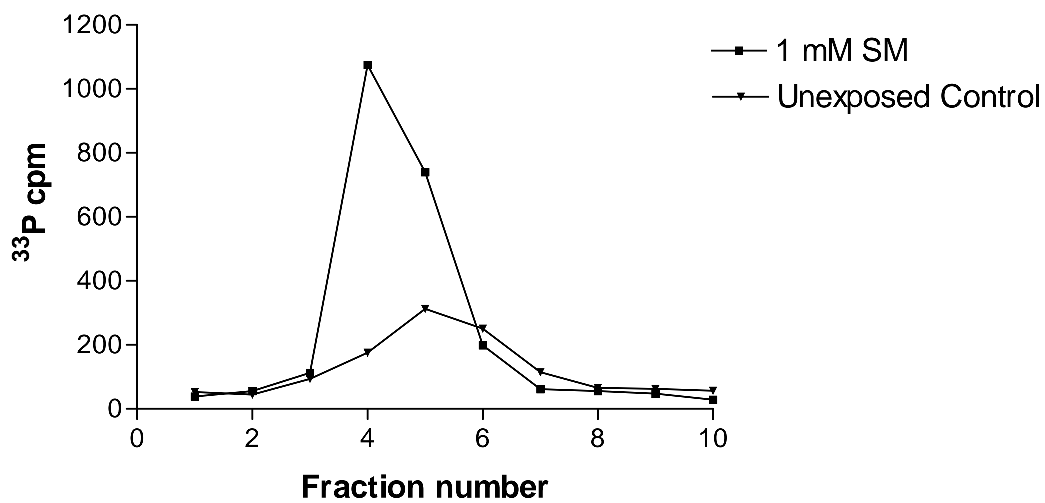

Figure 1. Elution profile of (^{33}P) phosphorylated DNA ligase I in HEK from a monoclonal DNA ligase I antibody affinity column. The extent of phosphorylation is indicated by the area under the curve. The ratio of the areas corresponding to SM-exposed HEK (2374) vs. SM-unexposed control (1169) equals 2.03.

Table I

Poly(ADP-ribosyl)ation or Phosphorylation of DNA Ligase I in SM-Exposed HEK: Effect of Decreased PARP Activity

Cell Type	DNA Ligase I Modification	Ratio of Areas Under the Curve
HEK* with ^{3}H label in NAD^{+}	Poly(ADP-ribosyl)ation	1.0
HEK (no treatment)	Phosphorylation	2.0
HEK* treated with 2 mM 3-AB	Phosphorylation	1.8
PARP (-) HEK** cell line	Phosphorylation	1.3

*Primary HEK; ** Dexamethasone inducible PARP (-) cell line

Extent of ^{3}H (line 1) or ^{33}P (lines 2 - 4) incorporation observed under different conditions specified. The data show the ratio (SM-exposed to unexposed controls) of the areas under the curves representing DNA ligase I elution from a bovine DNA ligase I monoclonal antibody affinity column (see *Fig. I*). For ^{3}H-NAD^{+} experiments, the eluted samples corresponding to both SM-exposed and unexposed control showed only background ^{3}H counts without any stimulation due to SM.

Table II

Effect of Apoptosis Inhibitors on DNA Ligase I Phosphorylation in SM-Exposed HEK

Apopotosis inhibitor added*	Ratio of Areas Under the Curve
None	1.0
Z-VAD-FMK (4 µM)	0.55
AC-DEVD-CHO (100 µM)	0.83
CD95 antibody (2 µg/mL)	0.40

*Primary HEK

The extent of ^{33}P incorporation into DNA ligase I (phosphorylation) due to SM in the absence or presence of apoptosis inhibitors was compared. The data show the ratio (apoptosis inhibitor treated vs. untreated) of the areas under the curves representing DNA ligase I elution from a bovine DNA ligase I monoclonal antibody affinity column.