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Nucleotide excision repair : a multi-step mechanism required to maintain genome integrity

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ARSENIC INHIBITS THE SEALING OF UV-INDUCED CHROMOSOMAL DNA NICKS DURING NUCLEOTIDE EXCISION REPAIR BY INHIBITING DNA LIGASE IIIα

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Submitted

SUMMARY

Arsenic-induced carcinogenesis is a world-wide problem which is threatening to reach epidemic proportions. Epidemiological data and in vivo experiments suggest that arsenic exposure interacts with UV radiation exposure to increase the risk of skin cancer. Inhibition of DNA repair processes is one of the most prominent mechanisms in arsenic-induced carcinogenicity. Impaired gap filling and sealing of chromosomal DNA in nucleotide excision repair (NER) leads to genome instability. Here we show that low concentrations of arsenite (As(III)) disrupt the repair of both CPD and 6-4PP photolesions, and with equal efficiency. We found a slight reduction in accumulation of pre-incision factors accumulating at UV-damage sites in the presence of As(III), yet complex build-up was not significantly altered. Incisions were still made and accumulation of post-incision factors at UV-damage sites was undisturbed by the presence of As(III). The disassembly of pre-incision factors was however delayed in As(III) treated cells. Moreover, we provide data demonstrating that ligation and in particular, DNA ligase IIIα function is especially disrupted leading to impaired NER capacity in As(III) treated cells. Our data reveals new insights into the mechanisms involved in arsenic co-carcinogenesis and provides a possible explanation for the impact that As(III) exposure has on a variety of human cancers, including skin cancer.

INTRODUCTION

Naturally-occurring arsenic in drinking water poses a growing global health risk as millions of people are exposed to elevated levels of arsenic derivatives. According to recent reports chronic low-dose exposure of contaminated drinking water is thought to affect nearly 140 million people in more than 70 countries (Bagchi, 2008). In addition, arsenic contaminated water is widely used for irrigating crops during dry season rice production resulting in elevated arsenic uptake by crops (Meharg et al., 2003) and substantial human intake of arsenic due to consumption of rice (Williams et al., 2005). World Health Organisation guidelines set a safe limit of 10 µg/L of arsenic in water supplies, but millions of people drink water with higher arsenic content (WHO, 2001, ATSDR, 2007). High concentrations of arsenic in drinking water are predominantly found in certain regions of the United States as well as in India, Mexico, Taiwan, Chile and China (Hughes et al., 2007, Huang et al., 2004). However, at present Bangladesh is thought to be the worst-affected country. Epidemiology studies indicate that arsenic is a human carcinogen (IARC, 2004). Long term ingestion is associated with an increased risk of developing tumours of the skin, bladder, liver, kidney, prostate, while inhalation of arsenic compounds increases the risk of lung cancer (Tapio et al., 2006). Arsenic tends to accumulate in the skin, where characteristic non-malignant skin lesions such as hyperpigmentation and hyperkeratosis appear on the palms and soles before skin cancers such as Bowen's disease, squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) appear. (Centeno et al., 2002). Trivalent arsenites appear to be more toxic and are responsible for carcinogenicity; however, in many cases exposure to both trivalent and pentavalent inorganic arsenic occurs but the precise chemical speciation is usually not known (ATSDR, 2007). The underlying mechanisms of arsenic carcinogenicity in humans still remains elusive, mainly due to the fact that arsenic is not a potent mutagen by itself. Many different mechanisms of action have been proposed such as enhanced cell proliferation, altered DNA methylation patterns, induction of oxidative stress, co-carcinogenesis and tumour promotion. However, since arsenic can act as a co-mutagen by compromising the repair of DNA damage induced by a variety of genotoxic agents, several studies have indicated that impairment of DNA repair processes is one of the most prominent mechanisms in arsenic-induced carcinogenicity (Hartwig et al., 1997; Hartwig et al., 2002; Andrew et al., 2006). Low concentrations of arsenite, which are not mutagenic, are able to enhance the carcinogenicity of ultraviolet radiation (UVR) in mice (Rossman et al., 2001; Rossman et al., 2002). Studies suggest that the co-mutagenic and co-carcinogenic effect of arsenic is almost certainly due to an inhibitory effect on NER (Okui et al., 1986; Hartwig et al., 2002).

Nucleotide excision repair (NER) is the major pathway for the removal of UV-induced lesions such as pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) as well as bulky DNA damage induced by a variety of environmental mutagens. NER can operate via two sub-pathways: global genome repair (GGR) that acts on lesions throughout the genome, and transcription-coupled repair (TCR) which repairs lesions on the transcribed strand of active genes. The importance of NER for human health is illustrated when defects in genes encoding for GGR proteins give rise to the UV-sensitive and cancer-prone genetic

disorder xeroderma pigmentosum (XP) (Friedberg, 1996). In human cells, the NER reaction requires at least six core protein complexes for damage recognition and dual incision (XPC-hHR23B, TFIIH, XPA, RPA, XPG and XPF–ERCC1) (Volker et al., 2001; Gillet and Scharder, 2006; Mocquet et al., 2008). Other factors are required for repair DNA synthesis and ligation i.e. PCNA, RFC, DNA polymerase δ or ε and XRCC1-DNA ligase III or DNA ligase I (Kelman et al., 1995; Araujo et al., 2000; Moser et al., 2007; Overmeer et al., 2010). One potential group of molecular targets for arsenic are the zinc-finger containing NER proteins since As(III) has been shown to have a high affinity for vicinyl sulfhydryl groups (Hartwig, 2001). Schwerdtle and collegues (2003) demonstrated the ability of low micromolar concentrations of trivalent arsenic compounds to release zinc from a peptide consisting of 37 amino acids representing the zinc finger domain of the human XPA protein. Other important NER components with zinc finger domains include human replication protein A (RPA), DNA polymerases delta (pol δ) and epsilon (pol ε) and one of the most recently identified NER factors; DNA ligase IIIα (Lig3).

The zinc finger-containing nucleotide excision repair proteins clearly provide a potential target for toxic metal compounds. This mechanism is in agreement with arsenic acting as a co-carcinogen by compromising the repair of DNA damage induced by other DNA damaging agents. Herein, we provide direct evidence that the repair of UV-induced lesions is severely inhibited by the presence of As(III). More specifically, we find that As(III) inhibits repair patch ligation in NER, probably due to a direct inhibition of Lig 3 function.

RESULTS

Arsenite exposure impairs the repair of UV-induced lesions

We investigated the effects of arsenic on gene-specific repair of UV-induced photolesions under non-cytotoxic conditions. The induction and repair of UV-induced CPD (UV dose 10J/m^2) was measured (Moser et al., 2005) in confluent fibroblasts treated with 10μM As(III). A dose of 10μM As(III) is classed as non-cytotoxic in (clonal) cell survival assays carried out previously (Hartwig et al., 1997, Nollen et al., 2009). Repair was measured in a 3' located 18.5kb *Eco*RI fragment of the active ADA gene, in which both strands are transcribed. The intensity of the band representing the ADA gene immediately after irradiation (t=0hr) was diminished after treatment with T4 endonuclease V in both untreated and As(III) treated cells, indicating the presence of CPDs (Fig 1A-B). The intensity of the ADA band after treatment with T4 endonuclease V had completely returned by 24hr, indicating the near complete removal of CPDs from the ADA gene of untreated normal primary fibroblasts. In arsenic treated cells the intensity of the ADA band after treatment with T4 endonuclease V was significantly diminished compared to the intensity of the untreated band after 24hrs, indicating incomplete repair after 24hrs (Fig. 1A-B). Quantification of band intensities and application of the Poisson distribution revealed 90% and 50% repair of CPD after 24hrs in mock - and arsenic treated cells, respectively. These results indicate that there is a clear reduction in the ability of arsenic treated cells to repair CPDs in the active ADA gene compared to untreated normal primary fibroblasts.

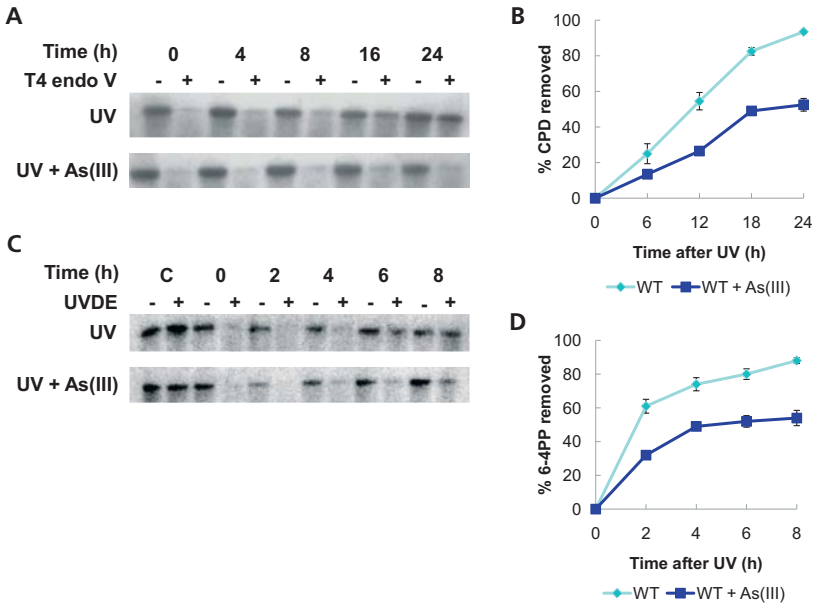

Fig 1. Normal human fibroblasts pre-incubated with As(III) show decreased repair of both CPD and 6-4PPs compared to untreated cells. Removal of CPD and 6-4PP at various times after global UV irradiation was measured in EcoRI restriction fragments of the active ADA gene using a probe recognizing both strands. A. Autoradiogram and B. histogram showing removal of CPD over a period of 24 h in primary normal human (VH25) fibroblasts treated with and without 10μM As(III). C. Autoradiogram and D. Histogram showing removal of 6-4PP over a period of 4 h in primary normal human fibroblasts (VH25) treated with and without 10μM As(III).

Gene specific removal of UV-induced 6-4PP was carried out as described previously (Moser et al., 2005). A high UV dose of 30J/m^2 had to be applied in order to induce sufficient levels of 6-4PP for analysis, i.e. 1-2 6-4PP per restriction fragment of ~20kb. Representative autoradiograms are shown in Figure 1C. The intensity of the ADA band after treatment with UVDE had significantly returned by 8hr and had a similar intensity to the untreated band indicating an almost complete repair (85%) of 6-4PP after 8hr in normal cells. In contrast, the intensity of the ADA band after UVDE treatment in As(III) treated cells was significantly reduced after 8hrs (Fig. 1C-D); the extent of repair was quantified as 50%. Together, these results indicate that there is a considerable reduction of the repair of CPDs and 6-4PPs in normal human cells treated with physiologically relevant concentrations of arsenic. Moreover, the level of repair inhibition of both photolesions was similar.

Core NER components are able to accumulate but persist at UV-lesions in the presence of arsenite

We examined the effect of As(III) on NER complex formation to elucidate which steps are target to inhibition. We applied local UV irradiation and immunofluorescence microscopy

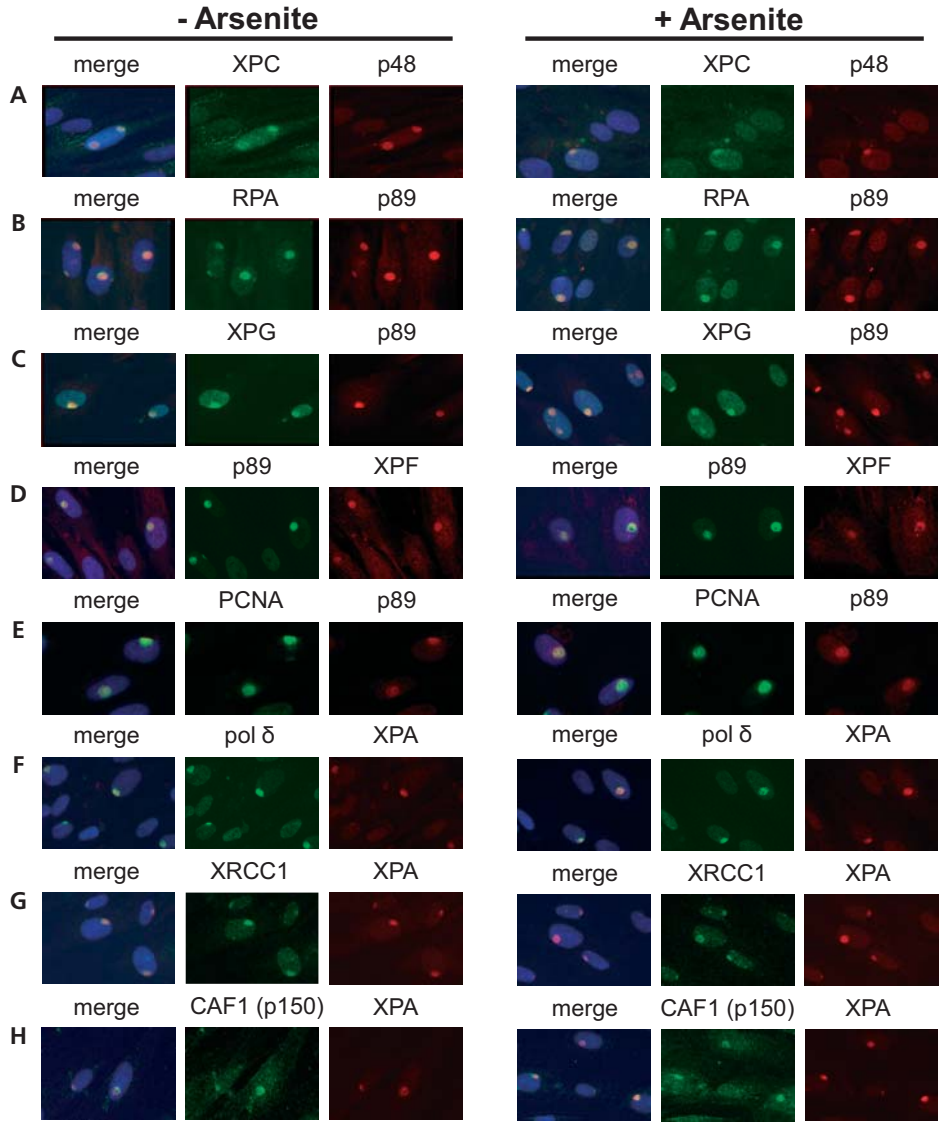

Figure 2. Core NER factors are able to bind to the NER complex despite 24hr treatment with 10μM As(III). Images A-G, were obtained with locally UV-irradiated (30 J/m^2) confluent primary normal fibroblasts. "Merge" refers to the combined image of DAPI and the pair of antibodies used for each panel. Immunolocalization of A, XPC and DDB2(p48) B, RPA(p70) and TFIIH(p89) C, XPG and p89 D, p89 and XPF E, PCNA and p89 F, DNA pol δ and XPA G, XRCC1 and XPA H, CAF1(p150) and XPA.

to investigate the accumulation of core NER components at damage sites in As(III) treated, as well as mock-treated normal human fibroblasts. To assess the impact of arsenic on the pre-incision step we examined the damage recognition factors, UV-DDB and XPC at local UV sites in the presence of As(III) (Fig. 2). As shown in Fig.2, UV-DDB and XPC accumulate at UV spots. In fact, other pre-incision factors such as p89 (TFIIH), XPA, and endonucleases XPG and ERCC1-XPF were also able to accumulate in the presence of As(III) (Fig. 2). Likewise, the core post-incision factors, RFC, PCNA, p21, DNA polymerase δ, the XRCC1-DNA ligase III complex and CAF-1 were also able to accumulate at local UV-induced sites in the presence of As(III) (Fig. 2 and data not shown).

Pre-incision NER components assemble with a slightly reduced efficiency into the NER complex in the presence of As(III). A 24hr pre-incubation with 10μM As(III) led to a slight decrease (approx. 14%) of XPA and XPB/p89 accumulation at local UV damage sites (Fig. 3A). We also checked the disassembly of the NER pre-incision complex in the absence

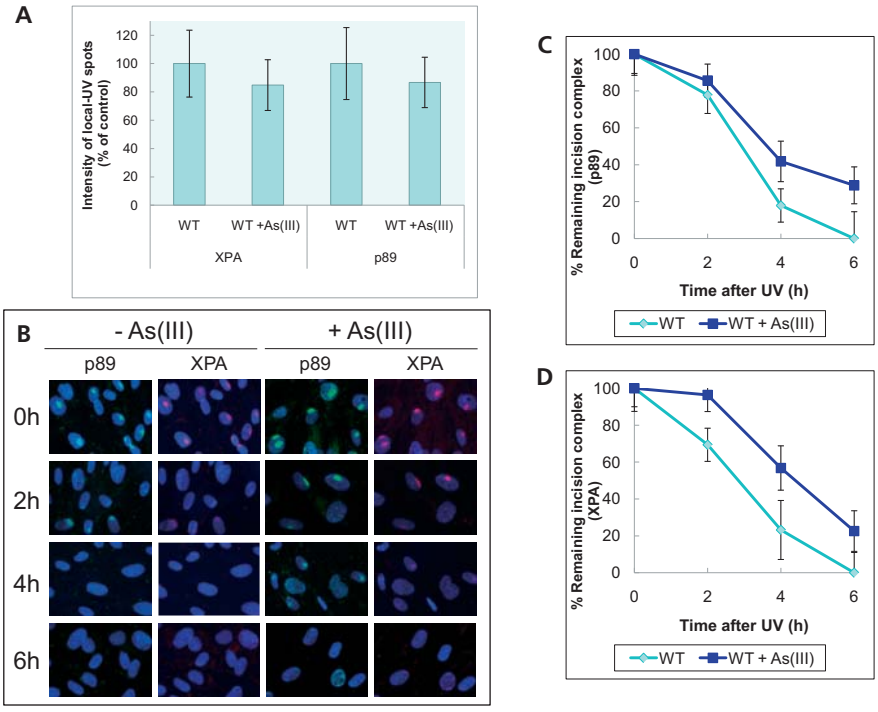

Fig 3. The disassembly kinetics of the NER complex is disrupted in As(III) treated fibroblasts compared to untreated fibroblasts. A, Fluorescent immunostaining of XPA and p89 in the presence or absence of 10μM As(III) 20min after local UV-irradiation. The average intensities of XPA and p89 spots after preincubation with As(III) are normalised to control cells without As(III) incubation (100%). B, Disassembly of the NER complex was monitored by fluorescent immunostaining of both p89 and XPA spots at various time points after local UV irradiation in both untreated primary confluent fibroblasts cells treated for 24hrs with 10μM As(III). C, The removal of p89 from the incision complex over a period of 6h, D, The removal of XPA from the incision complex over a period of 6h. Error bars represent the SEM values of 40 nuclei.

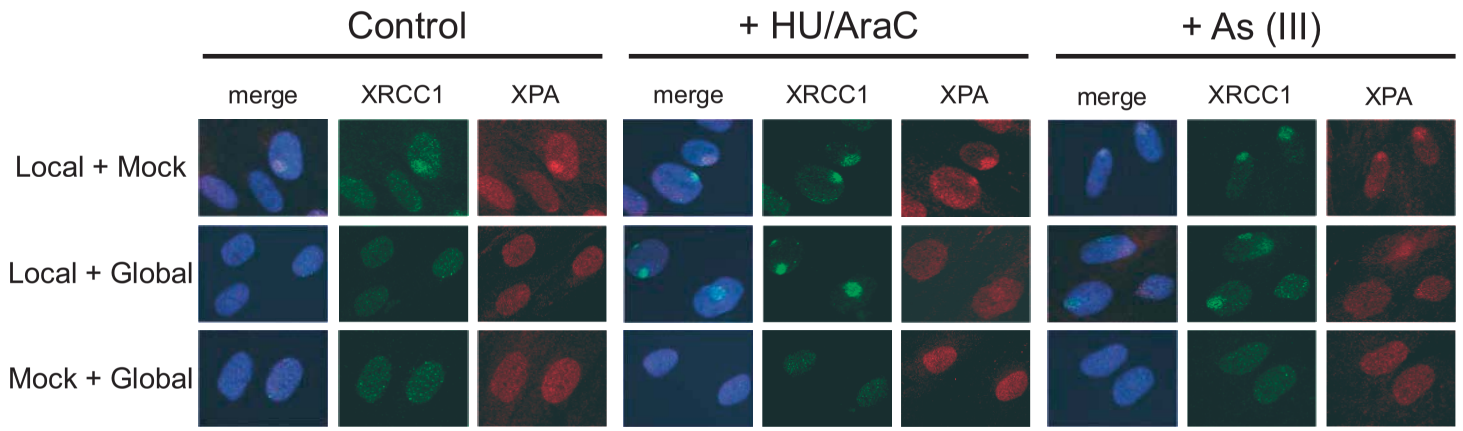

Fig 4. The DNA resynthesis/ligation step is disrupted by As(III) in living cells. A, Normal human fibroblasts, B, Normal human fibroblasts treated with HU and AraC and C, normal human fibroblasts treated for 24hr with10μM As(III) were locally UV-irradiated with 30 J/m^2, incubated for 1 hr, and subsequently globally irradiated with 30 J/m^2. Proteins were immunofluorescently labeled 1hr after the second irradiation showing immunolocalization of XRCC1 and XPA. Controls included cells which had been given only the first local UV-irradiation (25 J/m^2), followed by a mock UV-irradiation, and cells which had been given a mock irradiation followed by global UV-irradiation (25 J/m^2).

and presence of As(III) using local UV-irradiation and immunofluorescence. Previous work has shown that normal cells treated with DNA synthesis inhibitors (HU and AraC) are unable to proceed with the last stages of NER and therefore display retarded disassembly of the NER pre-incision complex and impaired removal of photolesions (Moser et al., 2007). Similarly, the disassembly kinetics of the NER pre-incision complex in As(III) treated cells (monitored by XPA or XPB/p89 TFIIH at local UV spots) was significantly retarded in comparison to that in mock-treated cells (Fig. 3B-3D).

Arsenic inhibits the DNA resynthesis/ligation step of NER

When cells were treated with DNA synthesis inhibitors HU and AraC, the post-incision factor XRCC1 accumulated at sites of local UV-damage (Fig 4B). To establish whether the last steps of NER were disrupted by As(III), we carried out in vivo competition experiments as described in detail elsewhere (Overmeer et al., 2010). Local UV-irradiation to cells in the presence of HU and AraC followed by global UV-irradiation revealed that XRCC1 remained confined to local UV-induced damage spots even after the application of a second competing global UV-irradiation (Fig. 4B). Pre-incision proteins such as XPA are able to leave the NER complex under these conditions (Fig. 4B) (Overmeer et al., 2010). It is therefore likely that the entire DNA re-synthesis/ligation complex will remain assembled at the site of repair and only dissociate after the completion of repair synthesis and ligation. We carried out similar competition experiments in cells which had been exposed to As(III) and found that the post-incision protein XRCC1 was confined to local UV-damage spots despite a second global UV-irradiation (Fig. 4C).

DNA ligase I cells are sensitive to both UV-irradiation and As(III)

To examine which post-incision protein is inhibited by arsenic, we choose a genetic approach. We evaluated the effect of a DNA ligase I deficiency on the UV sensitivity of human fibroblasts pre-treated with As(III). Wild-type and Lig1 (46BR) cells were either mock or pre-treated with low doses (10μM for 24hrs or 40μM for 4hrs) of As(III) prior to UV-irradiation. We find a slight sensitivity of Lig1 cells to UV-irradiation when compared to normal human fibroblasts (Fig. 5A) as reported previously (Teo et al., 1983, Bentley et al., 2002). Treatment of human cells with As(III) in the absence of Lig1, leads to clearly enhanced sensitivity to UV-irradiation (Fig. 5A). We examined the possibility that As(III) may target Lig3 thereby inhibiting the post-incision step of NER. We carried out similar survival assays in HeLa cells and HeLa cells which have a stable Lig3 knock-down (HeLa-L3 KD) but have normal levels of Lig1. We find that the absence of Lig3 did not lead to an increased sensitivity to As(III) pre-incubation prior to UV-irradiation (Fig. 5B). Since we observe residual repair and intermediate UV sensitivity of normal cells treated with As(III) we wondered whether a certain population of cells may be proliferating due to As(III) treatment. Lig1 is highly expressed in proliferating cells and our previously published work indicated that Lig1 is able to take over the role of Lig3 allowing NER-mediated ligation (Moser et al., 2007).

Arsenite impairs ligation of NER-mediated DNA breaks and inhibits DNA ligase IIIα

We assessed the effect of As(III) on repair patch ligation by using the alkaline comet assay. In the absence of UV irradiation, the comet tail size of normal or Lig1-deficient cells did not differ significantly from the tail of As(III) treated cells; in all cells, UV irradiation induced an increase in tail size 30 min after UV irradiation. Normal and Lig1-deficient cells showed efficient sealing of breaks 4 hr after UV irradiation, to a level similar to non-irradiated controls (Fig. 5D). However, normal human cells treated with As(III) showed impaired sealing of UV-induced breaks, coinciding with the observed (partial) inhibitory effect on the repair of UV-induced photolesions. Moreover Lig1 deficient cells displayed severely abolished ligation when treated with As(III) and UV (Fig. 5D). To test for a specific ligase deficiency resulting from As(III) exposure, we specifically analyzed the effect of As(III) on the activities of various purified DNA ligases and DNA ligation activity. For the doses used *in vivo* (around 40 μM), the ligation by DNA Ligase I and III is barely reduced; in addition, no effect on T4 DNA ligase was observed (data not shown).

As shown in figure 6, for higher doses (1-9 mM), we observed a specific effect on DNA ligase III whereas DNA ligase I and T4 DNA ligase activities were unaffected by As(III) pre-incubation. The results of the joining assay were not affected by either pre-incubation of the ligases with arsenite or by omitting DTT from the ligation buffer.

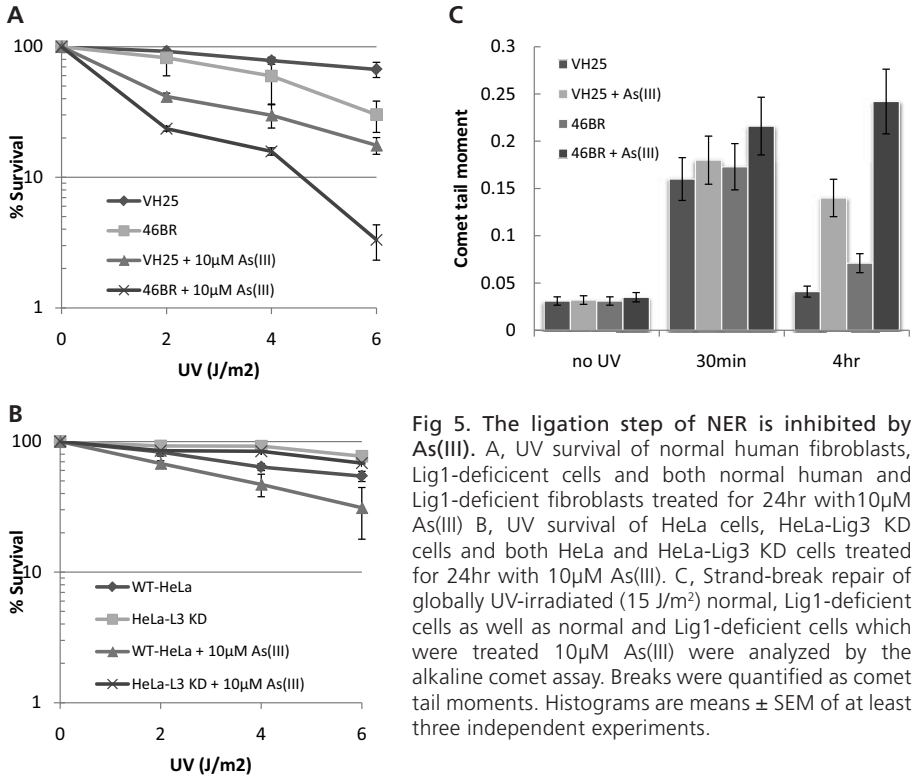

Fig 5. The ligation step of NER is inhibited by As(III). A, UV survival of normal human fibroblasts, Lig1-deficicent cells and both normal human and Lig1-deficient fibroblasts treated for 24hr with10μM As(III) B, UV survival of HeLa cells, HeLa-Lig3 KD cells and both HeLa and HeLa-Lig3 KD cells treated for 24hr with 10μM As(III). C, Strand-break repair of globally UV-irradiated (15 J/m^2) normal, Lig1-deficient cells as well as normal and Lig1-deficient cells which were treated 10μM As(III) were analyzed by the alkaline comet assay. Breaks were quantified as comet tail moments. Histograms are means ± SEM of at least three independent experiments.

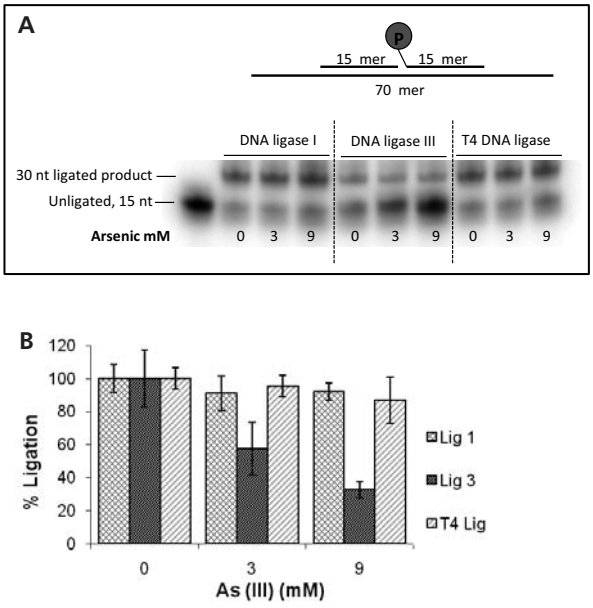

Fig 6. As(III) specifically inhibits Lig3 in vitro. A, The effect of As(III) on DNA ligases (Lig1, Lig3 and T4 DNA Lig) and DNA ligation activity was analyzed as described in the materials and methods. Samples were electrophoresed through a 10% denaturing polyacrylamide gel and autoradiography results quantified. B, quantification of ligation efficiency.

DISCUSSION

Environmental agents may compromise the DNA repair capacity of cells thereby increasing the risk of mutations and cancer from simultaneous exposure to genotoxic agents. Arsenic has been shown to act synergistically with other environmental contaminants, such as tobacco smoke, ionizing radiation and sunlight in causing increased incidence of internal cancers (Rahman et al., 2009, Ferreccio et al., 2000) and skin cancers (Centeno et al., 2002) respectively observed in arsenic-exposed populations. Experimental studies have also shown that low concentrations of arsenite enhanced the tumorigenicity of solar UV irradiation in hairless mice (Rossman et al., 2001, Burns et al., 2004). It is likely that arsenic may act as a human carcinogen, at least in part, through inhibition of DNA repair mechanisms. Previous work using UV-irradiated human repair-proficient and repair-deficient XPC fibroblasts showed that the incision as well as the ligation step of NER were inhibited by As(III) (Hartwig et al., 1997). Herein, we show a clear and similar reduction (around 50%) of the repair of UV-induced CPD and 6-4PP in As(III) treated cells as compared to mock-treated fibroblasts consistent with a 30-40% reduction of UV-induced repair replication in As(III) treated human fibroblasts (Hartwig et al., 1997). In accordance with these observations we find that As(III) treated cells display retarded disassembly of the NER pre-incision complex.

Arsenic may directly impair DNA repair processes by affecting binding to DNA damage and altering the function of proteins within the DNA repair complex. However, in vitro studies have shown that high concentrations of arsenic are needed to disturb purified DNA repair enzymes (Li and Rossman, 1998, Hu et al., 1998). One potential group of molecular targets are the zinc finger proteins. In this family of proteins, zinc is complexed through

four invariant cysteine and/or histidine residues forming a zinc finger domain involved in DNA binding and protein-protein interactions. NER core-factors with zinc finger domains include the pre-incision factor XPA and a recently identified post-incision factor, DNA ligase IIIα (Lig3). In the XPA protein, the zinc finger motif is part of the minimal DNA binding domain and consists of zinc complexed to four cysteine residues; substitution of any of the four cysteines leads to severe reduction in NER activity (Miyamoto et al., 1992). Previous studies have investigated the impact of As(III) on XPA repair protein and its binding to damaged DNA. Trivalent arsenicals have been shown to release zinc from a 37 amino acid-containing XPAzf peptide, representing the zinc finger domain of the human XPA protein (Schwerdtle et al., 2003). More detailed analysis revealed that a ten-fold excess of arsenite partially oxidized the zinc finger structure of XPAzf (Piatek et al., 2008). Thus, zinc binding structures may be sensitive targets for arsenicals. With respect to XPA function, subcellular studies observed no or only a slight decrease on XPA binding to UVC- (Asmuss et al., 2000;) or MMC-damaged (Mustra et al., 2007) oligonucleotides by arsenite.

The current study demonstrates that XPA function is not significantly disrupted by the presence of physiologically relevant concentrations of As(III). XPA is still able to bind to the NER complex in As(III) exposed cells, albeit, with a slightly reduced efficiency. Interestingly, p89 which localises to the NER complex prior to XPA, also binds to the NER complex with slightly reduced efficiency in As(III) treated cells. Similarly, reduced association and dissociation of XPC at sites of local UV-induced DNA damage has recently been reported (Nollen et al., 2009). The latter effect was accompanied by reduced XPC gene expression in the presence of arsenite. Since XPC binds prior to XPA and p89, reduced levels of XPC may account for slightly reduced level of XPA (and p89) at local-UV damage and may also explain reduced incision frequencies after UVC irradiation observed previously (Hartwig et al., 1997). However, although recruitment of XPA to the NER pre-incision complex is only slightly disturbed in the presence of As(III), its function may be impaired. In the absence of XPA function, NER specific incisions would not be introduced and post-incision proteins such as pol δ and XRCC1-Lig3 would be unable to localize at local UV damage sites (Moser et al., 2007). The efficient assembly of post-incision proteins into the NER complex shown in this study and the induction of UV specific breaks in arsenic treated cells also described previously (Hartwig et al., 1997) implies efficient dual incision, even though although reduced as compared to control cells. The observations that all core NER factors are recruited to UV damage and the persistent accumulation of pre-incision proteins in As(III) exposed cells demonstrate that the disassembly process is disrupted. This coincides with the observed NER defect and suggests that a late, post-incision event is likely to be affected by As(III) exposure. These observations parallel previous findings, UV–irradiated human fibroblasts exposed to the DNA polymerase inhibitors HU and AraC, were shown to display persistent accumulation of pre-incision NER proteins at UV damage (Overmeer et al., 2010).

Similarly, inhibition of polδ/pol in confluent normal human cells by HU/AraC leads to accumulation of DNA strand breaks and abolishment of 6-4PP repair (43, 24). Moreover,

components involved in the DNA re-synthesis/ligation step of NER remain assembled at the site of repair and only dissociate after the completion of repair synthesis and ligation (Overmeer et al., 2010). Our in vivo competition experiments as well as reduced repair synthesis (Hartwig et al., 1997) show that As(III) functions with striking similarity to DNA synthesis inhibitors HU and AraC, disrupting the resynthesis/ligation step of NER. Lig3 together with XRCC1 are indispensable for the repair of UV-lesions in quiescent cells, whereas both XRCC1-Lig3 and Lig1 are engaged in the sealing step of NER in dividing cells. The severely impaired repair of UV damage in arsenic treated human cells lacking Lig1 (46BR cells) points to arsenic mediated inhibition of Lig3. In fact, cells lacking functional Lig1 and Lig3 display synergistic impairment of NER-mediated repair. This explains the enhanced sensitivity of UV-irradiated Lig 1 defective cells to As(III) as these cells are solely dependent on Lig3. Proliferating cells involve ligase1 in the post-incision step of NER and hence might be less sensitive to the effects of As(III). The residual repair manifest from the gene-specific repair assay and comet assay experiments as well as the intermediate UV sensitivity of cells lacking Lig3 suggests that there is a percentage of cells which are able to complete NER or that NER is delayed. We observed a higher percentage (50%) of cells expressing high levels of PCNA, a marker for proliferation, in cells which were exposed to As(III) compared to control cells (data not shown). In line with these observations, it is well known that As(III) induces cell proliferation in cultured cells as well as in vivo (Germolec et al., 1996; Hamadeh et al., 2002; Rossman et al., 2004). In these cells, Lig1 is strongly expressed and may partly compensate for the Lig3 deficiency which is induced through As(III) exposure. Taken together, the data suggests that Lig3 function is impaired by As(III) which ultimately results in impaired NER capacity. The zinc finger motif of Lig3, located at the N-termini, has extensive sequence similarity to zinc fingers present in the base excision repair factor, poly(ADP-ribose) polymerase (PARP). Interestingly, PARP activity has been shown to be inhibited by very low, non-cytotoxic concentrations of arsenite (Yager and Wiencke, 1997; Hartwig et al., 2003; Qin et al., 2008). Our in vivo findings are in agreement with previous in vitro results showing that As(III) inhibits the ligation step of base excision repair (Li and Rossman, 1998; Hu et al., 1998) and DNA strand break rejoining in MMS-treated cells (Lynn et al., 1997) attributed primarily to inhibition of Lig3. The specific inhibitory effect of As(III) on Lig3 might support this conclusion although this inhibition is only achieved at millimolar doses of As(III). Previous in vitro studies on DNA repair enzymes showed that Lig1 and Lig3 were both inhibited by 10mM arsenite (37). However, we observe a specific effect on Lig3 only using similar millimolar doses of As(III), Lig 1 was unaffected. For As(III) doses such as those used *in vivo* (10 - 40 μM), the ligation by DNA Ligase I and III is barely reduced and there is no effect on T4 DNA ligase. These data tend to suggest that in vitro experiments only partly mimic the cellular conditions; differences may be due to the formation of more inhibitory arsenic species within cells, by reduced gene expression of lig3 as opposed to direct interactions, by inhibition of poly(ADP-ribosyl)ation as reported previously (Hartwig et al., 2003) or simply by artifical buffer conditions present in the in vitro system, such as the presence of 1 mM DTT which may bind arsenite. Arsenic has been shown to down-regulate the expression of several

DNA repair genes in a time- and dose-dependent manner (Hamadeh et al., 2002; Andrew et al., 2006; Nollen et al., 2009). However, whether XRCC1 or Lig3 are differentially down-regulated as a result of arsenic exposure is currently unknown. Further studies are therefore required to fully understand how As(III) inhibits the ligation step of NER.

In conclusion, we find that As(III) induces its co-carcinogenic effects at least in part by inhibiting the sealing of chromosomal DNA nicks that arise during NER. Our data suggests that the ligation step of NER is significantly inhibited by As(III) and that Lig3 function is particularly impaired. Taken together, our data reveal new insights into the mechanisms involved in arsenic carcinogenesis and provides a possible explanation for the impact that As(III) exposure has on a variety of human cancers, including skin cancer.

MATERIAL AND METHODS

Cell Culture. Cells used in this study were cultured in DMEM (without hypoxanthine and thymidine) supplemented with 10% fetal calf serum and antibiotics at 37°C in a 5% CO_2 atmosphere and include primary normal (VH25) as well as Lig1 (46BR) deficient human fibroblasts, as well as parental HeLa cells and HeLa cells with stable Lig3 knockdown (HeLa-Lig3-KD) (Moser et al., 2007).

Global and Local UV-irradiation. Cells were treated with either medium containing 10μM arsenite ($NaAsO_3$) or medium containing the equivalent amount of water for 24hr or 40μM arsenite for 4hr. Prior to irradiation, the medium was removed and kept at 37°C. The cells were subsequently rinsed briefly with PBS and exposed to global UV irradiation at a dose rate of 0.2 J/m²/s with a Philips TUV lamp (predominantly 254nm). Cells were locally UV-irradiated through a filter with pore size 8μm as described previously (Volker et al., 2001). After irradiation, the cells were returned to culture conditions for the time periods as indicated. When required, cytosine-β-arabinofuranoside (AraC, Fluka) and hydroxyurea (HU, Fluka) were added to the medium 30 minutes (min) prior to irradiation and remained present throughout the time course of the experiment. Final concentrations were 10 μM for AraC and 100 mM for HU.

Clonogenic survival assays. Cells were trypsinized and 500 fibroblasts were plated in P_{90} dishes in duplicate (controls in triplicate), allowed to attach for 4 h, and subsequently treated with 10μM As(III) for 24 h or 40 μM for 4 h. Cells were irradiated with different doses of UV as described above. After 14 days the dishes were rinsed with 0.9% NaCl, dried, stained with methylene blue (0.25%) and colonies were counted under a light microscope (LW Scientific).

Gene specific determination of CPD. Cells grown to confluency were treated with either medium containing 10mM arsenite ($NaAsO_3$) or medium containing the equivalent amount of water for 24hr. The cells were subsequently irradiated with UV (254nm) at 10J/m² incubated in culture medium and lysed. Gene specific repair measurements were performed as described (Venema et al., 1991). Briefly, high-molecular-weight DNA was purified by phenol and chloroform extractions followed by ethanol precipitation. The DNA was restricted with *Eco*RI, further purified and ethanol precipitated before dissolving in TE. Equal amounts of DNA (5mg) were either treated or mock treated with the CPD specific enzyme T4 endonuclease V and electrophoresed in 0.6% alkaline agarose gels. The DNA was transferred to Hybond N+ membranes by vacuum blotting and hybridised with ^{32}P-labeled gene-specific probes. Radioactivity in full size fragments was quantified using a Cyclone Storage Phosphor Scanner (PerkinElmer). The number of CPD was calculated from the relative band densities in the lanes containing DNA either treated or not treated with T4 endonuclease V, using the Poisson expression.

Gene specific determination of 6-4PP. Confluent cells were treated with arsenite as mentioned above and subsequently irradiated with UV (254nm) at 30 J/m². CPD photoproducts were removed from the *Eco*RI digested DNA by in vitro photoreactivation employing the photolyase derived from *Anacystis nidulans*, (kindly provided Dr. A. Eker, Erasmus University, Rotterdam, The Netherlands). Photoreactivation was checked for completeness by treatment of DNA samples with T4 endonuclease V and Southern blotting analysis. Equal amounts of DNA (5mg) were either treated or mock treated with Δ228-UVDE endonuclease (5pmol/μg DNA),(kindly provided by M. de Ruiter, Leiden University,

Leiden, The Netherlands). After incubation, 10mM EDTA and 0.1% SDS were added and the DNA purified and dissolved in TE as described above. The samples were electrophoresed in 0.6% alkaline agarose gels and the DNA transferred to Hybond N+ membranes by vacuum southern blotting. The filters were hybridised with ^{32}P-labeled gene-specific probes. Radioactivity in full size fragments was quantified and the number of 6-4PP was calculated as described above.

Antibodies. The following primary antibodies were used in this study: rabbit polyclonal α-XPA, α-p89, α-p21, α-ERCC1, and mouse monoclonal α-DNA pol δ, α-p21 (Santa-Cruz), mouse monoclonal α-PCNA (PC10), α-XRCC1, α-XPA (Abcam), mouse monoclonal α-DNA lig3α (Genetex), mouse monoclonal α-DNA Lig1 (MBL), mouse monoclonal α-XPG (8H7, Molecular Probes), mouse monoclonal α-RPA (Ab-1, Oncogene) and rabbit polyclonal DDB2 (Acris antibodies). Secondary antibodies used were Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated donkey α-mouse (Jackson Laboratories) and Alexa Fluor 488 goat anti-mouse IgG and AlexaFluor 555 goat α –rabbit IgG (Molecular Probes). All secondary antibodies were used according to the manufacturer's instructions.

Immunofluorescence. The fluorescent labelling was performed essentially as described previously (Volker et al., 2001). Briefly, cells were washed with cold PBS, fixed and subsequently lysed on ice by either addition of 100% methanol (10 min), or by 2% formaldehyde and 0.2% Triton X-100 (15 min), or alternatively by addition of 2% paraformaldehyde (20 min at room temperature (RT) followed by 0.2% Triton X-100 and incubation for 5 min at RT. Cells were washed twice with PBS and incubated with 5% bovine serum albumin (BSA) in PBS for 30 min at RT. Subsequently, the cells were incubated with the primary antibodies diluted in wash buffer (WB) (PBS containing 0.5% BSA and 0.05% Tween-20) for 2 h at RT. The cells were washed 3 x with WB and incubated with the secondary antibody (diluted in WB) for 1 h at RT. In experiments requiring double labelling, antibodies were mixed in WB and incubated simultaneously. To visualise chromatin-bound PCNA, soluble PCNA was extracted by Triton X-100 treatment prior to methanol fixation. Cells were mounted in Aqua/polymount mounting medium containing DAPI (1.5 µg/ml) (Polysciences Inc., Warrington, PA). Microscopy and quantification of fluorescent signal has been described elsewhere (Moser et al., 2005).

Comet Assay. Alkaline comet assay was performed as described (Moser et al., 2007) with minor modifications. Cells grown to confluency were treated with either medium containing 10mM arsenite ($NaAsO_3$) or medium containing the equivalent amount of water for 24hr, globally UV-irradiated (15 J/m^2), and then incubated in fresh medium for the appropriate repair time. Prior to scoring, DNA was stained with 10 ug/ml ethidium bromide. Mean tail moments were quantified for 130 cells per sample in each experiment, with ColourProc software program as described previously (Moser et al., 2007). Histograms are the average mean tail moment per sample of at least 3 experiments (reported as means ± s.e.m).

DNA Nick Ligation Assay. The DNA ligation assay was performed essentially as described (Mackey et al., 1999). In brief, purified ligases I, III and T4 (3pmol of each) were incubated at 37^{0}C in ligation buffer containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.25 mg/ml BSA, 100 µM ATP and 100 mM NaCl without or with arsenite (0-9mM) for 10min. Ligation substrate and product were separated by electrophoresis through a 10% polyacrylamide denaturing gel.

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9