Genotoxicity of alcohol is linked to DNA replication-associated damage and homologous recombination repair

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Introduction

Human exposure to acetaldehyde (AA) is widespread and occurs constantly through inhalation or gastrointestinal intake. Although, small amounts of AA are formed endogenous during catabolic processes, the main source of human exposure to AA is through the metabolism of ethanol after ingestion. Worldwide, 3.6% of all cancer deaths are attributable to alcohol intake (1). Alcoholics may have a blood concentration of AA as high as 2–20 mM in saliva (2) and it is estimated to increase up to 3.5 mM (4). In tobacco smoke, the AA concentration is up to 1400 µg per cigarette, which is at least 1000 times more than the concentrations of polycyclic aromatic hydrocarbons or tobacco-specific nitrosamines (5). Taken together, all gathered data, AA has recently been classified as a human carcinogen group 1 and also nominated as high priority agent to be evaluated by International Agency for Research on Cancer during 2010–14 (6).

Genotoxic effects of AA have been extensively studied during the last three decades and the evidences for AA as a mutagenic and carcinogenic are well documented (1). However, the mechanism underlying the genotoxic effect of AA is not fully understood (7). The possibility of AA to form more complex DNA lesions such as DNA–DNA cross links and DNA–protein adducts (8) represents an important aspect in genotoxicity. The present study was undertaken to study mechanisms of genotoxicity induced by AA. Such an issue is of great importance for evaluating the cancer risk of alcohol consumption as well as occupational exposure to AA. Studies were performed on cell cycle progression, cell survival, recombination and replication, induction of double-strand breaks (DSBs) in vitro as well as genomic instability in vivo in rats and in vitro.

Materials and methods

Materials

AA, hypoxanthine, 1-azaserine, thymidine, RNase and propidium iodide were purchased from Sigma–Aldrich (Stockholm, Sweden). Primary monoclonal mouse γH2AX antibody (clone JW301) was obtained from Millipore (Stockholm, Sweden), primary monoclonal rabbit RAD51 antibody (clone PC130) was obtained from Calbiochem, primary monoclonal rabbit 53BP1 antibody was obtained from Bethyl Lab (Montgomery, TX); Click-iT EdU Alexa Fluor® 488 Kit, secondary antibody Alexa Fluor® 555 donkey anti-mouse IgG (2 mg/ml) and Alexa Fluor® 488 anti-rabbit IgG (2 mg/ml), TOPRO-3 iodide and Prolong® Gold were purchased from Invitrogen (Stockholm, Sweden). Hoechst 33342 and Thiazole Orange were purchased from Molecular Probes (Pitcchford, OR). Ultima Gold XR scintillation cocktail was purchased from PerkinElmer (Waltham, MA).

Animals

Male Wistar rats (8 weeks old) obtained from the Nofer Institute Animal Husbandry were housed two animals per cage under standard laboratory conditions: 21 ± 2°C and 12:12 light–dark cycle. All animals had access toAIN93 rodent diet and water ad libitum. The study was approved by the Local Ethics Committee (resolution no. L/B/D223).

Cell lines

The Chinese hamster ovarian cell lines. AA8 and irs1SF cell lines, wild-type and deficient in XRCC3 protein involved in homologous recombination (HR) repair, respectively, were all obtained from L. Thompson, LNL Livermore, CA. The cell lines were cultured in medium (Dulbecco’s modified Eagle’s medium) with the addition of 9% fetal calf serum and penicillin-streptomycin (90U/ml) (DMEM), at 37°C and 5% CO2 in humidified air.

Chinese hamster lung fibroblasts. V79 cells were cultured at 37°C in DMEM. SPD8 cells were previously originally isolated from V79 cells. They carry a duplication of exon 7 of the HPRT gene, resulting in truncated HPRT protein and can be reverted by an exchange of Rad51-supported HR (9). SPD8 cells were thereafter cultured under the same conditions as the parental V79 cell line but supplemented with 6-thioguanine (5 µg/ml) in order to minimize the background frequency of spontaneous reversion prior to treatment.

Treatment conditions

In vivo. After 1 week of acclimatization period, the animals were separated into two groups (four animals per each group). Control group was given tap water, whereas the treatment group was given 10% ethanol for 4 weeks in drinking water ad libitum. During the experiments, body weight, water and fodder consumption were recorded twice a week during the experiment.

In vitro. AA was diluted in ice-cold Hank’s balanced salt solution with 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) (HBSS**) and immediately added to the cell cultures. AA treatments were conducted on ice with ice-cold tips followed by tightening the locks and incubation at 37°C. The treatment was stopped by rinsing the cultures twice with HBSS**.

Flow cytometric micronucleus assay

After anesthetization, about 100–200 µl of peripheral blood was drawn from orbital vein into heparinized tube using a Pasteur pipette. The procedure for sampling, fixation, staining and flow cytometric analysis of erythrocytes from the orbital vein was as follows. Peripheral blood was collected into a tube containing a heparin solution. After centrifugation, the supernatant was collected and centrifuged again. Finally, the supernatant was collected and resuspended in PBS (phosphate-buffered saline) containing 0.1% Triton-X 100 and 0.5% Bovine Serum Albumin. The suspension was filtered through a 150 µm nylon mesh to remove debris. The filtered cells were then used for flow cytometric micronucleus assay.
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Replication fork elongation assay

A (100 μM aphidicolin (Aph). The cells were pulse labeled with EdU either during the treatment or after. Samples were incubated with 488 in presence and absence of 10 mM ethynyl deoxyuridine (EdU) for 24 h. The medium was then removed and the cells were labeled with DMEM containing 0.1% 3 H-TdR (7.4 kBq/ml 3H-TdR) for 30 min for labeling of replication forks. Afterwards the labeling was terminated by incubating the medium to ICE-cold DMEM containing AA followed by incubation for 15 min at 37°C in sealed wells. After treatment, the cells were washed and incubated in DMEM. At each time point, samples were taken and washed with 500 μl ice-cold 0.15 M NaCl and put in a dark box on ice. Thereafter, 500 μl 0.03M NaOH was added and the cells were left to unwind for 30 min. Afterwards, the cells were treated as described previously (13).

Cytotoxicity assay

The day before treatment, 5 × 10^5 cells were seeded in 25 cm^2 flasks and cultivated in 8 ml DMEM over night. Thereafter, the cells were exposed to 1.2 mM AA for 24 h. After 24 h recovery, the cells were released by trypsinization and counted. The colony-forming ability was analyzed by plating duplicate dishes (200 cells per dish). Cells were then grown for 7 days before fixation, stained by methylene blue in methanol (4 g/l) and counted for colony formation.

Recombination assay

SPD8 cells have a non-functional HPRT gene, which renders them resistant to 6-thioguanine but sensitive to HAT (50 μM hypoxanthine, 10 μM l-azaserine, 5 μM thymidine) medium since l-azaserine blocks de novo purine biosynthesis. In order to survive in HAT medium, cells must be able to metabolize guanine into guanosine monophosphate under catalyzation of HPRT protein, i.e. HAT medium is selective for SPD8 cells that revert to a wild-type HPRT gene by deleting the duplicated part of the gene through a HR mechanism (14). The recombination assay involves inoculation of 1.5 × 10^6 SPD8 cells into flasks (75 cm^2) 24 h prior to treatment with 1.2 mM AA for 24 h. After 24 h recovery, HPRTr revertants were selected, fixed, stained and counted as described previously (14).

Detection of RAD51 foci, γ-H2AX foci, 53BP1 foci, micronuclei and catastrophic mitosis by confocal microscopy

V79 cells were seeded with a density of 40 000 cells per cover slip and transferred to 75 cm^2 cell flask. The next day, cells were treated with AA in 15 ml ice-cold DMEM. The flashes were kept on ice and the lights were tightly closed immediately after treatment. The cells were incubated for 30 min or 24 h at 37°C in 5% CO2 in humidified air along with the untreated samples. The media was removed and the cover slips were transferred to a 12-well plate. One milliliter media was added with or without 10 mM ethynyl deoxyuridine (EdU) 488 in presence and absence of 10 μM aphidicolin (Aph). The cells were pulse chased with EdU either during the treatment or after. Samples were incubated at 37°C, 5% CO2 and the treatment was terminated by washing in PBS and fixation in 4% paraformaldehyde. The immunocytoology and image acquisition are described in detail in (15). In short, primary antibodies used were rabbit polyclonal RAD51 (H-92, Santa Cruz 1:10000), rabbit polyclonal 53BP1 (ab21083, Abcam 1:1000) and mouse monoclonal anti-γ-H2AX (clone JBW301, Millipore 1:1000). The secondary antibodies used were AlexaFluor 555 donkey antirabbit IgG and AlexaFluor 488 donkey antimouse IgG (Molecular Probes). Antibodies were diluted 1:500 in PBS containing 3% bovine serum albumin and 0.05% Tween20. The DNA was counterstained with TOPRO-3 iodide (diluted 1:100). Images were obtained with a Zeiss LSM 510 confocal microscope.

Results

Rats exposed to alcohol in their drinking water have increased level of genomic instability

AA, a main metabolite of alcohol, has previously been shown to be genotoxic (1). The understanding of the genotoxic impact of alcohol intake brings AA into focus due to the fact that so many individuals are exposed. Therefore, it is of great public interest to investigate the effect of alcohol consumption at doses relevant to human exposure. Here, we investigated the induction of MPCEs in peripheral blood in rats given 10% ethanol in their drinking water. A typical dot plot of reticulocytes representing RNA content (FL1 axis) versus DNA content (FL4 axis) is presented in Figure 1A. We observed that animals exposed to ethanol for 4 weeks showed on average 3.5 times significantly increased levels of MPCEs compared with untreated animals (Figure 1B). Monitored body weight, water and fodder consumption were stable during the experiment.

AA treatment inhibits replication fork progression and is cytotoxic in a time-dependent manner

The nature of a possible cancer initiating lesion induced by AA, and the mechanisms of triggered repair have not yet been characterized. We investigated a possible effect of AA on cell cycle progression.

![Fig. 1](image1.png)

**Fig. 1.** (A) Dot plot of reticulocytes representing DNA content (FL4) versus RNA content (FL1) in enriched reticulocytes of rats. The reticulocytes lying above the main population of PCEs represent a micronuclei-containing cells (MPCEs) as was verified by sorting and microscopy. (B) The rats given 10% ethanol in drinking water for 4 weeks showed significantly increased levels of frequency of MPCE (MPCE) (Student’s t-test, two-tailed, **P < 0.05**). The standard deviation of three independent experiments is depicted.

![Fig. 2](image2.png)

**Fig. 2.** Induction of cell cycle arrest in G2 phase in V79 cells treated by 1.2 mM AA for 24 h. The white columns represent control cells and the gray columns represent AA-treated cells. The standard deviation of three independent experiments is depicted.
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Indeed, we found that AA-treated cells accumulated in S or G2/M phase as a result of 1.2 mM AA treatment for 24 h at 37°C (Figure 2). This might indicate a repair mechanism involved in termination of the S phase and/or progress into mitosis. The data on cell cycle progression mentioned above suggest that AA-treated cells face lesions that interact with the replication process. It is known that AA gives rise to DNA interstrand cross links [ICLs (16)], which have a potential to disrupt replication progression. Therefore, we investigated the effect of AA on replication fork progression. For analysis of replication fork progression, a method based on the alkaline DNA unwinding technique was applied (13). As shown in Figure 3A, AA treatment delayed replication fork progression in a dose-dependent manner. In agreement with this observation, we found that prolonged treatment with AA led to increased cytotoxicity (Figure 3B and 3C). Treatments performed for 1, 4 and 24 h at AA concentrations up to 60 mM resulted in dose-dependent cell survival (Figure 3B). The cytotoxicity induced by AA was also time dependent, which is illustrated in Figure 3C representing the cytotoxicity at different dose rates, e.g. the initial concentration given over the time of treatment expressed in mM × h.

Involvement of HR in repair of AA-induced DSBs

By using confocal microscopy, we observed an approximately 4-fold increase of γH2AX foci induced by 1.2 mM AA treatment during 3 h at 37°C (Figure 4A). In agreement with findings by others (17), these results imply that DSBs are formed although the mechanism remains unclear. It has previously been shown that RAD51 is involved in repair of DNA damage associated with replication in mammalian cells (18). The responses to AA in the HR assay together with the observed induction of γH2AX foci prompted us to further analyze possible induction of RAD51. As expected, the AA treatment enhanced the levels of RAD51 foci formation approximately 6-fold compared with untreated cells as investigated by confocal microscopy (Figure 4A). The induction of RAD51 and γH2AX foci observed here clearly

Fig. 3. (A) Delay of replication fork progression in AA8 (parental CHO cell line) treated with 0 mMh (open diamond), 0.16 mMh (open square), 0.32 mMh (filled triangle) and 1 mMh (filled square) AA was dose-dependent. (B) Clonogenic survival as a colony outgrowth following exposure to AA for 1 h (filled diamond), 4 h (filled square) and 24 h (filled triangle) was studied in V79 cells. (C) Clonogenic survival as a colony outgrowth following exposure to AA for 1 h (filled diamond), 4 h (filled square) and 24 h (filled triangle) was studied in V79 cells (dose was calculated as the initial concentration given over time, e.g. mM × h). Survival is plotted as the log of the percentage of living cells. The standard deviation of at least three independent experiments is depicted.

Fig. 4. (A) AA treatment (1.2 mM for 24 h) (gray bars) enhanced the levels of RAD51 and γH2AX foci compared with untreated (white bars) V79 cells. The standard error of three independent experiments with 300 cells counted for each experiment is depicted. The statistical significance compared with untreated cells was determined by Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001). (B) Clonogenic survival as a colony outgrowth following exposure to AA for 24 h was studied in parental cell line (AA8, filled diamond) and DNA repair deficient derivative irs1SF (XRCC3 mutated, filled square). Dose was calculated as the initial concentration given over time, e.g. mM × h. Survival is plotted as the log of the percentage of living cells. The means and standard errors (bars) of least three independent experiments are depicted. (C) Recombination frequency in SPD8 cells treated with AA for 24 h (R² = 0.8). Dose was calculated as the initial concentration given over time, e.g. mM × h. The statistical significance was calculated using linear regression analysis (P < 0.001). The standard error of at least three independent experiments is depicted.
supports the hypothesis that repair of AA-induced DSBs is processed by a recombination mechanism. Chinese hamster ovarian (CHO) cell lines AA8 (wild-type) and deficient in XRCC3, the RAD51 paralog (irs1SF), were employed to get further support for this suggestion. The cytotoxic effect of AA in CHO cells is shown in Figure 4B. It was found that irs1SF cells were 50 times more sensitive than wild-type cells. In order to further support involvement of HR, we studied the effect of AA by utilizing the SPD8 assay to detect functional HR by an exchange mechanism (19). A concentration-dependent and statistically significant increase in the level of recombination by an exchange mechanism was observed at doses applied (Figure 4C) with the corresponding clonogenic survival 70.2 ± 12.2%, 69.9 ± 6.1% and 24.9 ± 9.4%, respectively.

Cells treated with AA are characterized by induction of γH2AX foci and genomic instability

In order to investigate whether the AA-induced DSBs were related to the replication process, cells were analyzed for γH2AX foci formation after inhibition of replication by Aph (20). EdU was administered 10 min before treatment by Aph in order to identify S-phase cells, since EdU is incorporated during DNA replication and repair (21). As a result of AA treatment for 30 min, a significant increase in γH2AX foci positive cells compared with untreated cells was observed 210 min after initiation (Figure 5A). AA-treated cells showed a four times increase in γH2AX foci formation compared with the control, whereas treatment with Aph constrained the foci formation to background level (Figure 5B). This clearly indicates that AA-induced γH2AX foci formation is related to the replication process. This finding could also be confirmed in terms of EdU incorporation suggesting that the major part of the AA-induced damage occurs in replicating cells (Figure 5C). Radiation-induced DSBs are reported to be processed both by non-homologous end-joining and HR (22). Failure to perform efficient repair of DSBs remaining during S phase might result in the loss of a chromosome arm, which can be monitored as micronuclei (MN) formation, chromosome rearrangements, formation of dicentric chromosomes and mitotic catastrophe. Thus, as expected, we observed an induction of both MN and catastrophic mitosis in cells treated with 1.2 mM AA for 24 h (Figure 5D). The induction of MN, γH2AX and RAD51, as a marker for DSBs repair, was found to be dose dependent (Figure 5E–G).

AA-induced DSBs require HR for repair during replication

From the results mentioned above, we concluded that AA gives rise to replication-associated DSBs. By using confocal microscopy, we studied the induction of p53 binding protein (53BP1) and γH2AX foci as
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AA gives rise to genomic instability (Figure 5D) in agreement with what has been reported by others (23,24). Of mechanistic interest and concern is the observation that the cytotoxic effect induced by AA was achieved in response to long-term exposure at low doses, rather than at high doses for short exposure times (Figure 3B). At the moment, the molecular event(s) responsible for DNA damage response induced by AA is not well known. By this reason, the repair of the damage resulting in genetic instability can only be a matter of speculation. It could be expected, though, that the repair occurs by a variety of repair pathways due to the fact that many different AA-induced DNA lesions including ICLs have been identified (16,23–25).

The importance of Fanconi anaemia DNA repair pathway, which is thought to coordinate HR repair, in counteracting AA-induced genotoxicity has been shown previously in vivo and in vitro (17,26,27). Our observations imply that AA induces lesions that interfere with DNA replication where HR is the most important pathway in the repair of replication-associated DNA lesions (9,28). Here, we indeed show a crucial role for HR in the repair of AA-induced lesions. The RAD51 paralog XRCC3 not only facilitates loading of RAD51 onto DNA but also participates in the late stages of resolution of Holliday junctions (29). Here, we found that the cell line deficient in XRCC3 was 50-fold more sensitive to AA treatment compared with wild-type cells (Figure 4B) in agreement with findings by others (23). As expected, AA also showed recombinogenic activity (Figure 4C) and induced RAD51 foci formation, which is believed to be a signum for DSB repair by HR (30). Results suggest unrepaird ICL as a likely candidate responsible for observed DNA lesions. Further support for this is the evidence that DNA replication arrested by ICLs creates one-ended DSBs (31), in agreement with our results on γH2AX and 53BP1 foci formation (Figures 4A, 5A–C and 6A and B). In addition, we were able to demonstrate that these ICLs were formed in relation to replication (Figure 5B and 5C). They are expected to be repaired by template switching through HR (22) visualized in the present study by colocalization of γH2AX and RAD51 foci (Figure 6B). Interestingly, when investigating γH2AX foci formation induced by short-term AA treatment after different recovery times, it is evident that the damage and subsequent phosphorylation were not observed during treatment but rather afterwards (Figure 5A). Moreover, after treatment with DNA polymerase inhibitor Aph (20), the γ-H2AX foci formation was eliminated (Figure 5B). The replication dependence was further supported by the fact that the major part of the AA-induced damage occurs in replicating cells (Figure 5C). Interestingly, when investigating the RAD51 foci formation as a result of short-term treatment with AA, a significant induction was only seen 8h after treatment and was associated with γH2AX foci (Figure 6). This observation might be explained by a previous suggestion that a stalled replication fork converging with another fork generates a two-ended DSB (22), which could explain the late generation of γH2AX and 53BP1 foci. The main function of RAD51 protein is to promote strand invasion during HR (32). It has previously been suggested that DDS gives rise to instant phosphorylation of histone H2AX by Ataxia telangiectasia mutated (ATM) (33). This triggers repair mechanisms such as activation of S-phase cell cycle checkpoint (34) and the induction of other signaling factors involved in DNA repair (35). Several other proteins are known to colocalize with γH2AX at a latter point, such as a p53 interacting protein 53BP1 (36), also used as more specific marker for DSBs. Indeed, we observed a colocalization of γ-H2AX and 53BP1 in AA-treated cells (Figure 6B). The final damage outcome in terms of genomic instability induced by AA in vitro was monitored as MN and catastrophic mitosis induction by approximately 5-fold compared with untreated cells (Figure 5D). These results are in agreement with the findings of Kayani et al. (37) who observed similar levels of MN induction. Our data imply that AA-induced DNA damage response occurs mostly in replicating cells and results in MN formation and catastrophic mitosis. The results on structural chromosomal changes induced by AA in vitro were challenged in vivo by giving rats 10% alcohol in the drinking water for 4 weeks. The method for scoring MN in young PCEs reflects chromosomal damage during maturation step.

Discussion

Although alcohol is classified as a human carcinogen, the mechanisms behind initiation of alcohol-induced cancers are not very well understood. Here, we report that alcohol consumption in rats is directly genotoxic (Figure 1). As AA is the main metabolite produced during ethanol metabolism, we have focused to find an explanation to the genotoxic outcome induced by AA exposure. We have observed that markers of DSB formation as well as RAD51 foci induction as a marker of HR at different time points after AA treatment. As a result of 60 mM AA treatment for 30min, we observed a 6-fold increase in the formation of 53BP1 and a 3-fold increase in γH2AX at 4 and 8h as well as colocalization of these proteins (Figure 6A and 6B). The levels of RAD51 foci were also enhanced by AA treatment, starting 4h after treatment and reaching a 5-fold increase after 8h (Figure 6A). RAD51 and γH2AX foci colocalization was also observed (Figure 6B), suggesting that DSB formation during replication recruits HR for the repair process.

Fig. 6. (A) Percentage of γ-H2AX (black columns), RAD51 (white columns) and 53BP1 (gray columns) positive V79 cells fixed at 0, 4 and 8h after 30 min of treatment with 60 mM AA. Bars represent standard error for three independent experiments. (B) Colocalization of RAD51/53BP1 and γ-H2AX foci in V79 cells fixed at 8h after 30 min of treatment with 60 mM AA visualized by confocal microscopy. The statistical significance comparing with untreated cells was determined by Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001). The standard error of three independent experiments with 200 cells counted for each experiment is depicted.
of erythroblasts in the bone marrow (38). The genomic instability observed in rats drinking 10% alcohol was increased 3-fold in terms of the level of MPECs compared with control animals (Figure 1B). This result confirms the present findings in vitro and suggests that alcohol concentrations as low as those found in wine are sufficient to cause genomic instability and thus may lead to an increased cancer risk in humans. The observed induction of MN by AA could be the result of either of two mechanisms: the formation of chromosomal aberration due to strand breaks or lagging of chromosomes due to spindle dysfunction. Since DSBs were found to be associated with the replication process, we suggest the first mechanism. Further support for this assumption is that AA has been reported to produce its genotoxic effects mainly through a clastogenic rather than aneugenic mechanism (37), and cell lines deficient in HR exhibit an increase in chromosomal aberrations as a result of AA treatment (23,24).

Altogether, our data indicate that AA-induced DNA damage occurs mostly in replicating cells and the triggered DNA damage response is not immediate since we could observe late RAD51 and 53BP1 foci formation. The proposed mechanism of AA-induced genotoxicity is the induction of replication-associated DSBs induced by AA exposure which, as a late response, lead to genomic instability in terms of catastrophic mitosis and MN formation.

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References

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