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Repair pathways independent of the Fanconi anemia nuclear core complex play a predominant role in mitigating formaldehyde-induced DNA damage

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ABSTRACT

The role of the Fanconi anemia (FA) repair pathway for DNA damage induced by formaldehyde was examined in the work described here. The following cell types were used: mouse embryonic fibroblast cell lines FANCA$^{-/-}$, FANCC$^{-/-}$, FANCA$^{-/-}$C$^{-/-}$, FANCD2$^{-/-}$ and their parental cells, the Chinese hamster cell lines FANCD1 mutant (mt), FANCGmt, their revertant cells, and the corresponding wild-type (wt) cells. Cell survival rates were determined with colony formation assays after formaldehyde treatment. DNA double strand breaks (DSBs) were detected with an immunocytochemical γH2AX-staining assay. Although the sensitivity of FANCA$^{-/-}$, FANCC$^{-/-}$ and FANCA$^{-/-}$C$^{-/-}$ cells to formaldehyde was comparable to that of proficient cells, FANCD1mt, FANCGmt and FANCD2$^{-/-}$ cells were more sensitive to formaldehyde than the corresponding proficient cells. It was found that homologous recombination (HR) repair was induced by formaldehyde. In addition, γH2AX foci in *FANCD1mt* cells persisted for longer times than in *FANCD1wt* cells. These findings suggest that formaldehyde-induced DSBs are repaired by HR through the FA repair pathway which is independent of the FA nuclear core complex.

1. Introduction

Formaldehyde is an aliphatic monoaldehyde, highly reactive, and a human carcinogen [1]. Formaldehyde is used as industrial applications, disinfectant, embalming agent, and miscellaneous applications. In view of its widespread use and toxicity, exposure to formaldehyde is a significant consideration for human health. Formaldehyde induces the formation of DNA–protein cross-links (DPCs) in target tissues. It has been reported that incomplete repair of formaldehyde-induced DPCs can lead to the formation of mutations, in particular chromosome mutations and micronuclei in proliferating cells [2]. It has been found that transcription-coupled repair (TCR)/nucleotide excision repair (NER) pathways are involved in repairing formaldehyde-induced DPCs [3]. Cellular pathways for DNA repair and/or tolerance of DPCs proceeds via the formation of NER-dependent single-strand break (SSB) intermediates [4]. In past studies, it has been also detected the formaldehyde-induced DSBs [5]. Homologous recombination (HR), but not NER, plays a pivotal role in the tolerance to DPCs in mammalian cells [6]. This implies that there may be two pathways for the repair of DPCs.

Fanconi anemia (FA) repair or DSB repair pathways are involved in the repair of DNA damage induced by alkylating agents [7–10]. FA is characterized by developmental abnormalities, susceptibility to certain cancers, and a sensitivity to DNA–DNA cross-linking agents [11]. Although the FA pathway was initially characterized with regard to the repair of DNA cross-linking agents [11], additional studies have found an increasingly detailed involvement in general recombination repair and in the resolution of replication

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arrest [12–14]. The regulation of the FA pathway is subject to an intricate system of control, and thirteen FA genes have now been identified [11], but the precise function of many of the FA proteins still remains to be elucidated. FANCD1 has been identified as the breast cancer susceptibility protein BRCA2 [15] which regulates RAD51 in HR repair [16]. The FA proteins (A, B, C, E, F, G, L and M), together with the novel FA elements FAAP24/100, are subunits of a nuclear core complex required for the monoubiquitylation of FANCD2 [17,18]. It has been confirmed that FANCD2 and other FA proteins, including FANCG, promote HR repair [15,19–21].

The present study was designed to examine which specific components of the FA repair pathway significantly contribute to formaldehyde sensitivity. The activity of the components of the FA repair pathway (FANCA, FANCC, FANCD1, FANCD2 and FANCG) leading to repair of DNA damage induced by formaldehyde was assessed using colony forming assays. The cells used in this study consisted of a panel of mouse embryonic fibroblasts (MEF) and Chinese hamster cell lines defective in specific components of the FA repair pathways.

2. Materials and methods

2.1. Cell lines

MEF cell lines from FANC wild-type (*wt*) and deficient cells (FANCwt, FANCA$^{-/-}$, FANCC$^{-/-}$, FANCA$^{-/-}$C$^{-/-}$ and FANCD2$^{-/-}$) cells [22] were obtained from the Fanconi Anemia Cell Repository, Oregon Health and Science University (Portland, OR, USA). The Chinese hamster lung fibroblast cell lines used in this study were: V79 (FANCD1wt), V-C8 (FANCD1 mutant (mt)), V-C8 + FANCD1 (FANCD1 revertant (rev), V-C8 containing a BAC with the murine FANCD1 gene) [23,24]. The Chinese hamster ovary cell lines used in this study were: AA8 (FANCGwt), KO40 (FANCG null mt), 40BP6 (FANCGrev, complemented with genomic Chinese hamster FANCG) [25], and SPD8 [26]. All cells were cultured in DMEM-10 [Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 20 mmol/l 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, penicillin (50 units/ml), streptomycin (50 μg/ml), and kanamycin (50 μg/ml)]. The cells were cultured at 37 °C in a conventional humidified CO_2 incubator.

2.2. Drug treatments

Formaldehyde (Nacalai Tesque, Kyoto, Japan) was dissolved at a stock concentration of 10 mM in medium. Cells were exposed to medium containing formaldehyde at various concentrations for 1 h or 4 days and then rinsed twice with PBS.

2.3. Cell survival

Cell survival was measured using a standard clonogenic survival assay as previously described [27]. The sensitivity of each cell line was assessed from its D_{50} value, i.e. from the formaldehyde dose that reduced cell survival to 50%. In order to accurately compare formaldehyde sensitivities in the repair defective cell lines, the relative D_{50} values were normalized using the D_{50} value of the corresponding proficient cell lines.

2.4. Recombination assay

SPD8 cells were grown in the presence of 5 μg/ml 6-thioguanine in order to reduce the frequency of spontaneous reversions prior to treatments. The protocol for the reversion assay [26] with SPD8 cells involved the inoculation of flasks (75 cm^2) with 1.5×10^6 cells in DMEM 4 h prior to a 24 h treatment period in a humidified 5% CO_2 incubator. After a 50 μM formaldehyde treatment, the cells were rinsed three times with 10 ml of PBS, and 30 ml of DMEM was added to the cells, and they were to allow to recover from the treatment for 48 h. Selection of revertants was performed by plating three dishes/group (3×10^5 cells/dish) in the presence of hypoxanthine-L-azaserine-thymidine (HAsT; 50 mM hypoxanthine, 10 mM L-azaserine, 5 mM thymidine). Two dishes containing 500 cells each were plated for cloning. All plates were fixed. The cloning plates were harvested after 8 days of growth, and colonies were fixed with methanol and stained with a 2% Giemsa solution. The cells on the selection plates were grown for 10 days before fixation.

2.5. Histological study of histone H2AX phosphorylation

Mouse anti-phospho-H2AX (ser139) monoclonal antibodies (Upstate Biotechnology, Lake Placid, NY, USA) were used to detect γH2AX foci. The immunocytochemical methods have been described in detail previously [28,29]. γH2AX foci were observed with anti-γH2AX antibodies (green) and nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue).

2.6. Flow cytometry analysis of H2AX phosphorylation at ser139

To determine the extent of phosphorylation of H2AX at ser139, samples were analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, USA) as previously described [28,29]. Mean values of γH2AX expression (MV) were calculated according to the formula MV = Mx – Mc, where Mx is the mean γH2AX fluorescence at 30 min after treatment, and Mc is the mean γH2AX fluorescence in untreated controls.

2.7. Statistical analysis

Statistical analyses were performed using the Student's *t*-test.

3. Results

3.1. Repair genes which respond to formaldehyde-induced DNA damage

Cellular responses to formaldehyde were examined using colony formation assays. The formaldehyde sensitivity of FANCA$^{-/-}$, FANCC$^{-/-}$ and FANCA$^{-/-}$C$^{-/-}$ cells was comparable to that seen in proficient cells (Fig. 1A). On the other hand, FANCD1mt, FANCGmt and FANCD2$^{-/-}$ cells were more sensitive to formaldehyde than the corresponding proficient cells (Fig. 1A). The relative D_{50} values after exposure to formaldehyde are: FANCD1mt cells (0.45) ≅ FANCGmt cells (0.50) ≅ FANCD2$^{-/-}$ cells (0.52)<<FANCA$^{-/-}$C$^{-/-}$ cells (0.78) ≅ FANCA$^{-/-}$ cells (0.80) ≅ FANCC$^{-/-}$ cells (0.82) (Fig. 1B).

3.2. Frequency of HR induced by formaldehyde

Since HR repair resembles the FA repair pathway through a step involving FANCD1, it is of interest to know whether HR in SPD8 cells is specifically induced by formaldehyde. After exposure to 50 μM formaldehyde, HR frequencies increased by a factor of 2.4 when compared to control cells (Fig. 2).

3.3. Immunocytochemical staining of γH2AX foci

γH2AX immunocytochemical staining, a very sensitive method of detecting DSBs, was used to examine the presence of γH2AX foci. Fig. 3 shows a typical photograph of γH2AX foci in FANCD1wt

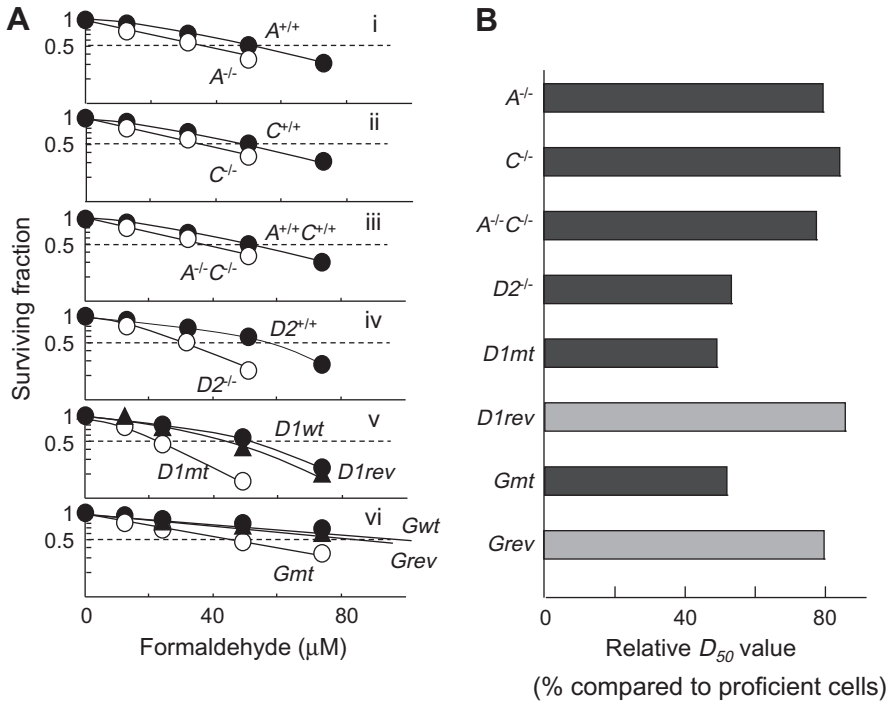

Fig. 1. Cellular sensitivity to formaldehyde. Panel A: (i) closed circles, FANCA$^{+/+}$ cells; open circles, FANCA$^{-/-}$ cells. (ii) Closed circles, FANCC$^{+/+}$ cells; open circles, FANCC$^{-/-}$ cells. (iii) Closed circles, FANCA$^{+/+}$C$^{+/+}$ cells; open circles, FANCA$^{-/-}$C$^{-/-}$ cells. (iv) Closed circles, FANCD2$^{+/+}$ cells; open circles, FANCD2$^{-/-}$ cells. (v) Closed circles, FANCDwt cells; closed triangles, FANCDrev; open circles, FANCDmt cells. (vi) Closed circles, FANCGwt cells; closed triangles, FANCGrev; open circles, FANCGmt cells. Each point represents the mean of three independent experiments. Panel B: relative D_{50} values (% compared to proficient cells) for formaldehyde sensitivity.

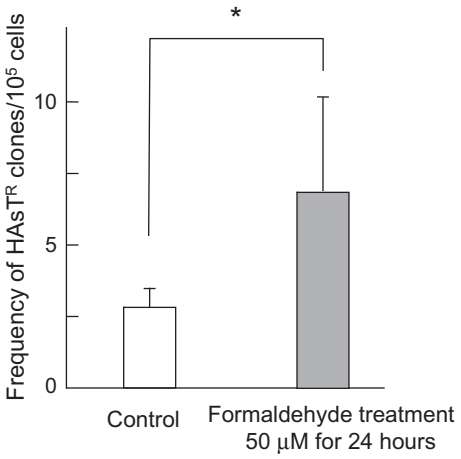

Fig. 2. Formaldehyde-induced HR repair in SPD8 cells. The reversion frequency from a non-functional to a functional hprt gene, giving resistance to HaST following a 24 h treatment with 50 μM formaldehyde is shown. Columns show the mean of two independent experiments; bars indicate the SD. An asterisk (*) indicates the differences are statistically significant ($P < 0.05$).

and FANCD1mt cells after a 1 h treatment with 500 μM formaldehyde.

3.4. Phosphorylation of histone H2AX

Since it is difficult to quantify γH2AX-positive foci, the intensity of the γH2AX signals was assayed with flow cytometry. When cells were fixed immediately after a 1 h treatment with formaldehyde, the dose–response for γH2AX was similar in FANCD1wt cells and FANCD1mt cells (Fig. 4A). There was no significant difference in the number of γH2AX foci induced by formaldehyde in FANCD1wt cells and FANCD1mt cells (Fig. 4A). There were also no large changes at 24 h after formaldehyde exposure in FANCD1mt cells. On the other hand, in FANCD1wt cells, the number of γH2AX foci decreased by approximately 45% shortly after processing (Fig. 4B).

4. Discussion

The aim of this study was to determine which FA proteins play important roles in the repair of formaldehyde-induced DNA damage. FANCA and FANCC appear to play only minor roles in the repair pathway for formaldehyde-induced DNA damage (Fig. 1) while FANCD1, FANCG, and FANCD2 appear to be more important (Fig. 1). The data obtained here agree with recent reports showing that FANCD1 or FANCD2 deficient DT-40 (chicken) cells and FANCG deficient human cancer cells are hypersensitive to formaldehyde [30]. In addition, these results are consistent with previous studies which examined the sensitivity of these cells to another cross-linking agent (mitomycin C) or methylating agent (methylmethanesulfonate) [25,27]. Formaldehyde-induced DNA damage may be repaired through the FA repair pathway which is independent of the FA nuclear core complex. Recently, it has been found that FANCD2 interacts with FANCD1 independently of the FA nuclear core complex [31]. In addition, FANCG has a role which is independent of the FA nuclear core complex. Once FANCG is phosphorylated at serine 7, it forms a complex comprising FANCD1, FANCD2, FANCG, and XRCC3, in this complex promotes HR repair [31]. In fact, it was found that HR repair was induced by formaldehyde (Fig. 2). The data obtained here are in agreement with recent reports that HR repair plays a pivotal role in the repair of formaldehyde-induced DPCs in mammalian cells [6]. Although it was not detected the accumulation of formaldehyde-induced DSBs using pulsed-field gel electrophoresis [4], it is currently believed that the detection method used in these studies is not sufficiently sensitive. Visualization of

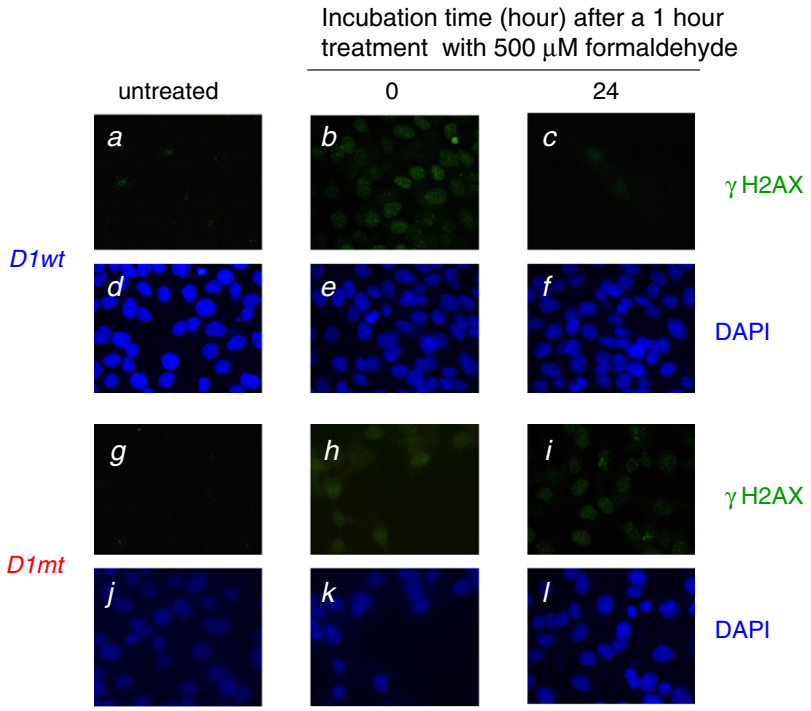

Fig. 3. Typical photographs of γH2AX in FANCD1wt and FANCD1mt cells after a 1 h treatment with 500 μM formaldehyde. (a–f) FANCD1wt cells; (g-l), FANCD1mt cells; (a–c) and (g–l) show γH2AX; (d–f) and (j–l) are stained with DAPI.

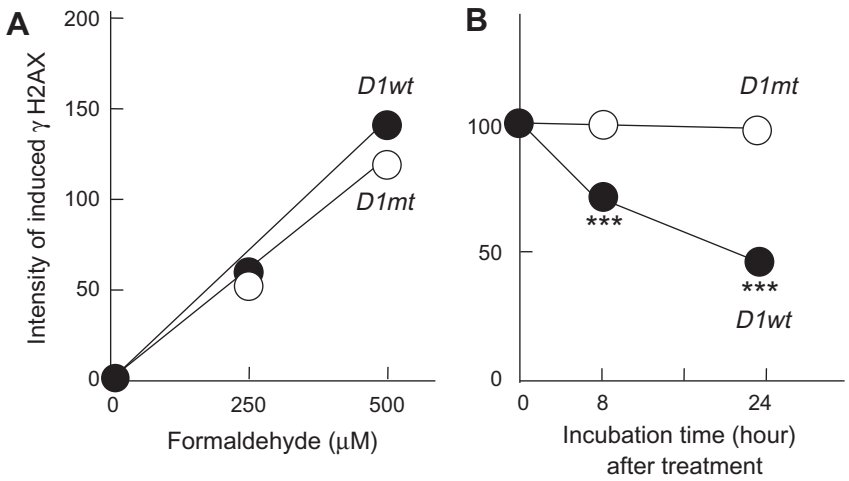

Fig. 4. Flow cytometry analysis of H2AX phosphorylation induced by formaldehyde. (A) mean values of γH2AX intensity vs. dose immediately after a 1 h treatment with formaldehyde. (B) mean values of γH2AX intensity at times after a 1 h treatment with 500 μM formaldehyde. The relative inducible γH2AX levels at different time points were normalized against the γH2AX levels measured immediately after treatment. Closed circles, FANCD1wt cells; open circles, FANCD1mt cells. Three asterisks (***) indicate the difference is statistically significant at $P < 0.001$.

formaldehyde-induced DSBs in the nuclei of cells was achieved by utilizing immunocytochemical methods with antibodies recognizing γH2AX (Fig. 3). This assay is extremely sensitive and is a specific indicator for the existence of a DSB [32,33]. Analysis of the post-treatment kinetics of γH2AX fluorescence with flow cytometry revealed a pattern suggesting that damage is processed more slowly in FANCD1mt cells than in FANCD1wt cells (Fig. 4B).

In conclusion, these findings support the idea that formaldehyde-induced DSBs may be repaired by HR through the FA repair pathway which functions independently of the FA nuclear core complex.

Conflict of interest statement

The authors declare that they have no conflicts of interest associated with this work.

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