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The FANCG Fanconi anemia protein interacts with CYP2E1: possible role in protection against oxidative DNA damage

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Fanconi anemia (FA) is a genetic disorder that leads to aplastic anemia and birth defects and predisposes to cancer. FA cells exhibit characteristic hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC), and FANCG is one of six known FA gene products. By immunocytochemical analysis of transfected cells, we discovered that although FANCG localized to both the nucleus and cytoplasm, there was an increase in cells with predominantly cytoplasmic staining after treatment with MMC. Concurrently, while searching by two-hybrid analysis for proteins that associate with FANCG, we identified a novel interaction between FANCG and cytochrome P450 2E1 (CYP2E1). A member of the P450 superfamily, CYP2E1 is associated with the production of reactive oxygen intermediates and the bioactivation of carcinogens. High constitutive levels of CYP2E1 were found in a FA-G lymphoblast cell line, whereas complementation of the FA-G line with wild-type *FANCG* was associated with decreased CYP2E1. These findings suggested that the interaction of FANCG with CYP2E1 might alter redox metabolism and increase DNA oxidation. Using a fluorescent assay, we found a dose-dependent increase in the oxidized DNA base, 8-oxoguanine (8-oxoG), after treatment of mutant FA-G cells with H_2O_2 or MMC. Conversely, significantly lower levels of 8-oxoG were detected in *FANCG*-complemented FA-G cells. We conclude that the unknown function of FANCG involves at least transient interaction with cytoplasmic components, possibly including CYP2E1, and propose a role for FANCG in protection against oxidative DNA damage.

Introduction

Fanconi anemia (FA) is a rare genetic disorder that leads to aplastic anemia and birth defects and predisposes to cancer. To date, at least six FA genes, *FANCA* (1,2), *FANCC* (3), *FANCD2* (4), *FANCE* (5), *FANCF* (6) and *FANCG* (7), have been identified, but the biochemical function of each encoded protein is still unclear. As FA cells are hypersensitive to agents such as mitomycin C (MMC), this function presumably involves recognition or repair of damaged DNA.

Five of the FA proteins, FANCA, FANCC, FANCE, FANCF and FANCG appear to interact in a common functional pathway and may form a molecular complex (8–13). Recently, *FANCD2* was cloned (4) and its gene product reported to undergo activation from a short (FANCD2-S) to a long (FANCD2-L) form after monoubiquitination in the presence of the intact FA protein complex (14). FANCD2-L was also found to co-localize with BRCA1 within nuclear foci or dots (14).

Several lines of investigation, however, have suggested additional extranuclear functions for at least some FA components (15,16). The first FA gene product to be identified, FANCC, was shown to bind to and to attenuate the catalytic activity of NADPH:cytochrome P450 reductase (RED; EC 1.6.2.4) (17). Cytochrome P450s (P450s) are a superfamily of hemoproteins that catalyze the oxidation of a wide variety of endogenous and xenobiotic chemicals, including therapeutic drugs and carcinogens (18,19). P450s have been found in all tissues, with the highest levels in the liver. P450-dependent metabolism requires two protein components, P450 and RED. Both enzymes are embedded in the membrane of the endoplasmic reticulum, and RED shuttles electrons from NADPH to P450. Interaction between FANCC and RED implicated abnormal microsomal detoxification in FA-C, suggesting that unopposed RED activity could lead to the generation of reactive oxygen intermediates (ROI) capable of damaging macromolecules such as DNA. Recently, FANCC has also been shown to prevent apoptosis in hematopoietic cells through redox regulation of glutathione *S*-transferase P1-1 (GSTP1), a phase II detoxification enzyme that catalyzes the conjugation of glutathione with various xenobiotics (15).

FANCG (7), identical to XRCC9 (20), is a 65 kDa protein that is present in both the nucleus and cytoplasm of cells. In order to gain insight into the function of FANCG, we performed a systematic determination of relative amounts of FANCG distributed to nuclear or extranuclear compartments. We discovered an increase in cells with predominantly cytoplasmic staining after treatment with MMC. In addition, FANCG was found to interact with cytochrome P450 2E1 (CYP2E1), suggesting involvement of FANCG in some aspect of redox metabolism.

Abbreviations: FA, Fanconi anemia; GST, glutathione *S*-transferase; MMC, mitomycin C; 8-oxoG, 8-oxoguanine; ROI, reactive oxygen intermediates.

Materials and methods

Cell lines, transfection, anti-FANCG antibody, FANCG retroviral vector

HeLa and 293 cells (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) and 10% heat-inactivated fetal bovine serum (FBS). FA lymphoblastoid cell lines (kindly provided by Dr H.Joenje, Free University of Amsterdam) were grown in RPMI 1640 and 15% FBS: EUFA143 (FA-G), HSC72 (FA-A) and HSC536 (FA-C). All FA cell lines were sensitive to MMC (Calbiochem, La Jolla, CA). The h2E1/OR cell line constitutively expresses human CYP2E1 (Gentest, Woburn, MA) and was maintained in RPMI 1640 with L-histidinol and 2 mM L-glutamine.

For transient transfection, HeLa or 293 cells were transfected with 5 μg of plasmid DNA using SuperFect (Qiagen, Valencia, CA), followed by immunoprecipitation and immunoblotting analysis or immunocytochemical staining. The pcDNA3-Flag-FANCG plasmid (21) was generated by insertion of the full-length FANCG cDNA (kindly provided by Dr H.Joenje) into the expression vector pcDNA3-Flag. Rabbit polyclonal antiserum against FANCG was generated as described previously (21).

To obtain a FANCG-expressing retroviral vector, the full-length FANCG cDNA was subcloned into the retroviral vector pLNCX2 (Clontech, Emeryville, CA). FANCG/pLNCX2 was then transfected into the PT67 packaging cell line (Clontech), and a stable virus-producing cell line was established. The FA-G cell line, EUFA143, lacks detectable FANCG, and was transduced with supernatant from FANCG/pLNCX2. Similarly, we established a stable clone of h2E1/OR cells overexpressing FANCG.

Immunocytochemical staining and confocal microscopy

Immunocytochemical staining of transfected HeLa or 293 cells and confocal fluorescent microscopy were performed as described previously (21). After 6 h, the cells were incubated with MMC (100 nM) to evaluate the effect of MMC on the subcellular localization of FANCG. Fixed cells were stained with anti-FLAG antibody or anti-FANCG antibody, followed by rhodamine-conjugated anti-mouse IgG or fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (both from Chemicon International, Temecula, CA), respectively. Control slides were stained with secondary antibody alone.

Two-hybrid methodology

Yeast two-hybrid screening was performed using the MATCHMAKER two-hybrid system (Clontech) (22). To construct bait plasmids, overlapping fragments encoding amino acids 1–275, 225–511 and 401–622 of FANCG were created by polymerase chain reaction (PCR) with FANCG cDNA as a template and appropriate primers. Individual PCR fragments were cloned in-frame into the pAS2-1 plasmid vector (Clontech). The yeast strain (PJ-69-2A) was first transformed with these bait plasmids and then mated with the pre-transformed strain (Y187) containing pools of fetal liver cDNA cloned in pACT-2 plasmid (Clontech). Colonies capable of growth on the minimal synthetic dropout plates lacking histidine, tryptophan and leucine were selected for further analysis for β-galactosidase activities.

Immunoprecipitation and immunoblotting

Whole-cell extract preparation and immunoblotting were performed as described previously (21). For immunoprecipitations, cell lysates containing equal amounts of protein were incubated with anti-FLAG antibody (Sigma Chemical, St Louis, MO), anti-FANCG antibody or normal rabbit Ig (Santa Cruz Biotechnology) at 4°C for 2 h. Immune complexes were collected by incubating with protein A/G PLUS-agarose (Santa Cruz Biotechnology) for an additional hour, followed by SDS–PAGE and western blotting.

Protein blots were first probed with anti-FANCG antibody, anti-CYP2E1 antibody (Panvera, Madison, WI), and anti-β-tubulin antibody (Santa Cruz Biotechnology). They were then probed with appropriate second antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Specific signals on the blot were detected with an enhanced chemiluminescent method (Amersham Pharmacia Biotech).

Glutathione S-transferase (GST) pull-down assay

G3 (C-terminal region of FANCG, amino acids 401–622) and full-length FANCG were cloned into pGEX-5X-1 (Amersham Pharmacia Biotech), creating an in-frame fusion to GST. GST, GST–G3 and GST–full-length FANCG fusion proteins were induced by isopropyl-β-D-thiogalactose in DH5α *Escherichia coli* cells, and purified with glutathione–Sepharose beads. The amounts of the purified protein were checked using GelCode Blue Stain Reagent (Pierce Chemical). The beads were incubated with recombinant CYP2E1 (Gentest) for 2 h. The beads were washed five times (1% Triton X-100, 10 mM Tris, 150 mM NaCl, 1 mM EDTA and protease inhibitors) and then subjected to SDS–PAGE and western blotting with anti-CYP2E1 antibody or anti-FANCG antibody.

Detection of oxidative damage to DNA

For the detection of oxidative damage to DNA *in vitro*, we used the Oxy DNA assay kit (Calbiochem), which is based on the direct binding of a fluorescent probe to 8-oxoguanine moieties in the DNA of fixed cells. Lymphoblastoid cells were washed in phosphate-buffered saline (PBS) and resuspended in 1% FBS-containing medium for 1 h before addition of test agents. Hydrogen peroxide (H_2O_2) (Sigma) was added at concentrations ranging from 300 μM to 1 mM for 2 h. MMC was added at concentrations ranging from 10 to 100 nM for 2 h. After exposure to test agents, cells were washed with ice-cold PBS and then fixed with 2% paraformaldehyde and 70% ethanol. Cells were blocked and stained with FITC conjugate. Fluorescence was analyzed using the FACS cytofluorometer and CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Results

In order to investigate the subcellular localization of FANCG, we transfected 293 cells with pcDNA3-Flag-FANCG and performed indirect immunofluorescence staining and confocal microscopy. In these experiments, we noticed much greater cytoplasmic staining after cells had been treated with MMC. In order to quantitate this tendency, we analyzed (by visual inspection) the pattern of FANCG immunostaining in the nucleus and cytoplasm after transient transfection. The subcellular localization of FANCG was variable, ranging from predominantly nuclear staining to predominantly cytoplasmic staining. However, we found greater cytoplasmic staining after incubation with MMC (100 nM) for 12 h (Figure 1). This dose of MMC was chosen because it is highly cytotoxic to mutant FA-G cells but not to 293 or HeLa cells (>90% viability).

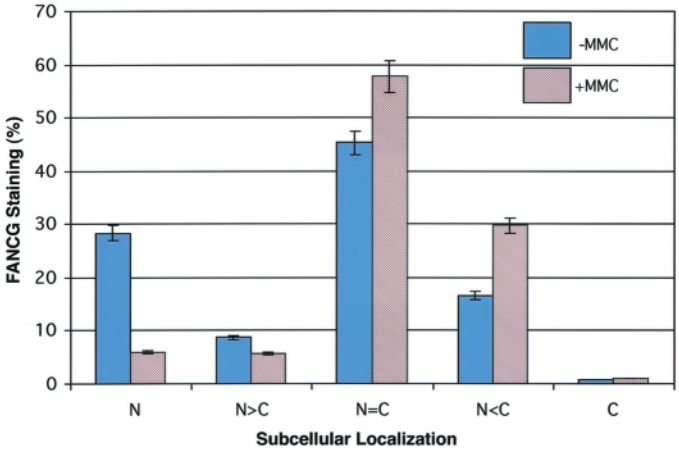

Fig. 1. The percentage of FANCG immunostained cells distributed in subcellular compartments. We analyzed (by visual inspection) the distribution of FANCG immunostaining in the nucleus and in the cytoplasm and evaluated the effect of MMC treatment on the localization pattern. Approximately 1000 cells were counted. The stained cells were divided into five groups as follows: N, nuclear staining with absent staining in the cytoplasm; N > C, much greater nuclear expression than cytoplasmic expression; N = C, equal amounts in the cytoplasm and the nuclei; N < C, much greater cytoplasmic expression than nuclear expression; C, cytoplasmic staining with absent staining in the nucleus.

Among the possible explanations that might account for the observed variability in FANCG distribution, we first wanted to assess whether the staining pattern correlated with transit through the various cell-cycle phases. For these experiments, we created a polyclonal 293-cell line that stably expressed FANCG. We had earlier determined that these cells also showed greater cytoplasmic FANCG staining after incubation with MMC (data not shown). These cells were then synchronized using a hydroxyurea block. At 0 h after release from the block (with 98% of cells in S phase), most of the cells appeared to display N > C or N = C patterns of staining (data not shown). Although this pattern was slightly different from that of asynchronous cells (which displayed mostly N or N = C), we concluded that cell cycle-specific changes did not necessarily account for the cytoplasmic staining pattern induced by MMC.

Concurrently with these localization experiments, we searched for proteins that might interact with FANCG, in an effort to gain further insight into its cytoplasmic or nuclear function. By yeast two-hybrid screening using a human fetal liver cDNA library, we isolated a clone that strongly interacted with the C-terminal 222 amino acids of FANCG (amino acids 401–622, which we termed G3). The sequence of this clone was identical to that of the C-terminal region of cytochrome P450 2E1 (CYP2E1) (23). We decided to focus on CYP2E1

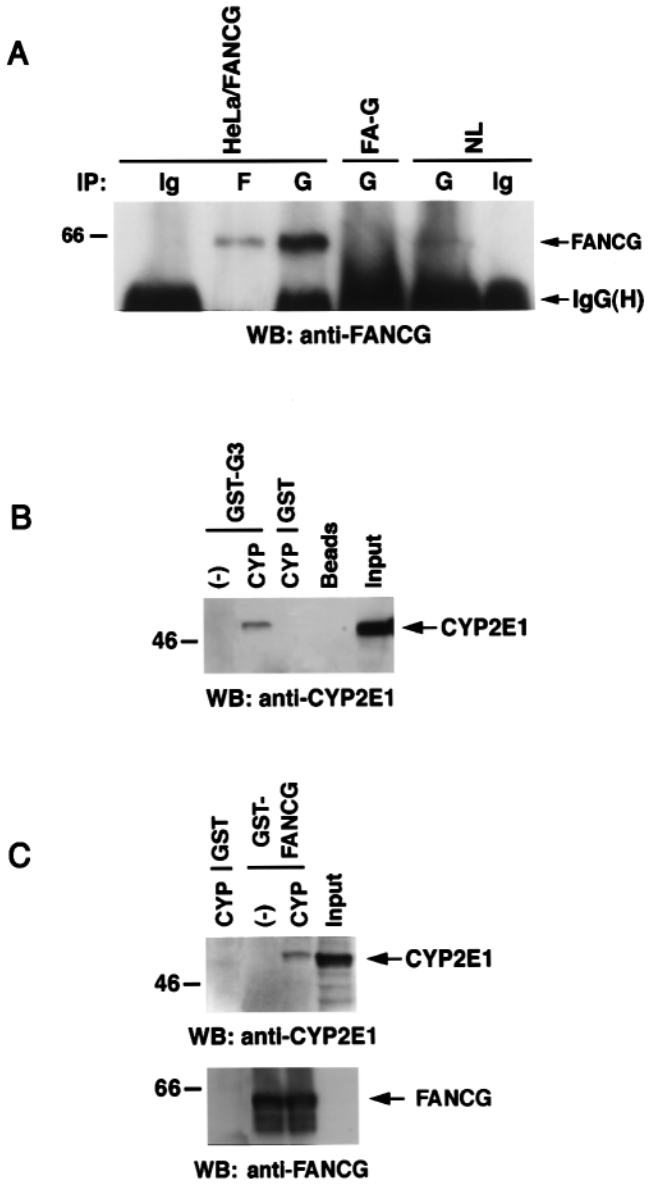

Fig. 2. (**A**) HeLa cells transiently transfected with a construct encoding for Flag-tagged FANCG, normal control lymphoblasts (NL), and FA-G cells (EUFA 143) were analyzed for expression of FANCG. Proteins from the indicated cells were immunoprecipitated (IP) with anti-Flag (F), anti-FANCG (G) or rabbit IgG (Ig) antibodies. The respective immune complexes were washed extensively and subjected to western blot analysis with the anti-FANCG antibody. The arrowheads indicate the position of FANCG and Ig heavy chains [IgG(H)]. (**B**) Binding of CYP2E1 to FANCG. Purified GST–G3 and GST alone were immobilized onto glutathione–Sepharose beads and were incubated with or without recombinant CYP2E1 (lanes 1–3). After washing, the proteins associated with GST–G3 and GST beads were analyzed by SDS–PAGE and western blotting using anti-CYP2E1 antibody. Control beads were also incubated with recombinant CYP2E1 (lane 4). (**C**) The upper panel shows the binding of FANCG to CYP2E1. GST–FANCG and GST beads (lanes 1–3) were analyzed by SDS–PAGE and western blotting using anti-CYP2E1 antibody. The lower panel shows the same blot re-probed with anti-FANCG antibody.

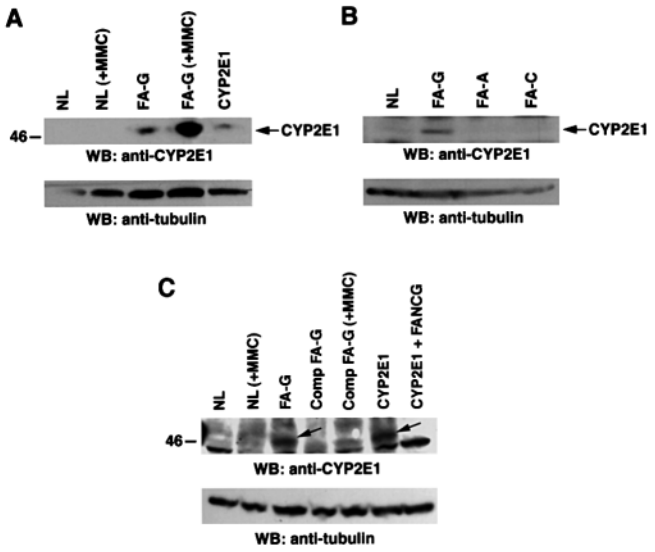

Fig. 3. Analysis of CYP2E1 expression in cell lines. Equal amounts of cell lysates were analyzed for CYP2E1 expression using an anti-CYP2E1 antibody (each upper panel). Each lower panel shows the same blot reprobed with anti-β-tubulin, indicating equivalent loading. Cell lines analyzed included: normal control (NL); FA-G, FA-A, FA-C lymphoblast cell lines; the h2E1/OR cell line that overexpresses CYP2E1 (CYP2E1), and the complemented FA-G cell line (Comp FA-G). Concurrent overexpression of FANCG and CYP2E1 was also analyzed (CYP2E1+FANCG). In addition, we tested the effect of adding MMC (100 nM for 12 h) on CYP2E1 expression. (**A**) Higher constitutive expression of CYP2E1 in the FA-G cell line. (**B**) Expression of CYP2E1 in FA cell lines. (**C**) CYP2E1 expression was down regulated in cell lines concurrently expressing FANCG.

because of its relationship to NADPH:cytochrome P450 reductase, which had been found previously to interact with FANCC (17).

In order to validate the usefulness of our anti-FANCG antibody in immunobinding assays, we tested its ability to specifically immunoprecipitate FANCG in HeLa cells transiently transfected with pcDNA3-Flag-FANCG. The 65 kDa FANCG protein was detected in both FANCG-transfected HeLa cells and (at very low levels) in a normal lymphoblast cell line (Figure 2A). To confirm that FANCG and CYP2E1 interact *in vitro*, we performed GST pull-down assays (Figure 2B). GST, GST–G3 (FANCG, amino acids 401–622) and GST–full-length FANCG fusion proteins were expressed and purified on glutathione–Sepharose beads. The beads were incubated with recombinant CYP2E1. After extensive washing, the beads were subjected to SDS–PAGE and western blot with an anti-CYP2E1 antibody. CYP2E1 was specifically co-precipitated by GST–G3 but not by GST alone (Figure 2B). CYP2E1 also did not bind to naked control beads. Interaction between GST–full-length FANCG and CYP2E1 was also detected (Figure 2C). In order to assess the potential for non-specific binding, we also performed pull-down assays between GST–FANCG and recombinant ferritin light chain (~30 versus 50 kDa for CYP2E1). No such interaction could be detected using the same conditions as described for FANCG and CYP2E1 (data not shown).

CYP2E1 is constitutively expressed in human liver and can be induced in a number of extra-hepatic tissues following various stimuli. We examined the expression of CYP2E1 in the FA-G lymphoblast cell line, EUFA143, by western blot (Figure 3A). Higher constitutive levels of CYP2E1 were found in the FA-G cell line than in a normal lymphoblast control line. After incubation with MMC (100 nM) for 12 h, expression of CYP2E1 in the FA-G cell line was further induced. Cell lysates of FA-A and FA-C cell lines yielded lower amounts of CYP2E1 when compared with the FA-G cell line (Figure 3B). We also analyzed CYP2E1 expression in our retrovirus-

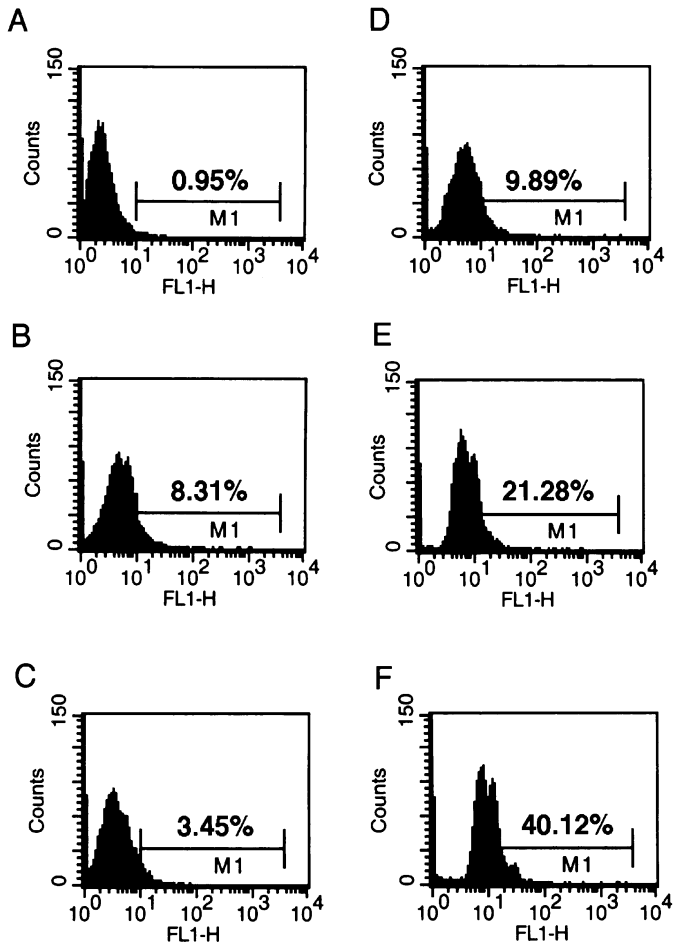

Fig. 4. Flow cytometric analysis of oxidative damage of DNA in lymphoblastoid cell lines using a fluorescent probe to 8-oxoG. The fixed cells were incubated with the FITC-conjugate probe and the fluorescence intensity was measured using flow cytometry. Shown are six univariate histograms: cell number (*y* axis) versus fluorescence intensity of FITC-filter channel (*x* axis) from a representative experiment. Complemented EUFA143 cells (**A**–**C**) and mutant EUFA143 cells (**D**–**F**) were examined for oxidative DNA damage following either H_2O_2 or MMC. Untreated cells are shown in (A) and (D). (B and E) Data from cells after exposure to 300 mM H_2O_2 for 1 h. (C and F) Data from cells after exposure to 100 nM MMC for 1 h. Numbers indicate the percentage of gated cells displaying ROI damage.

complemented FA-G cell line (Figure 3C). Transduction of EUFA143 with FANCG/pLNCX2 corrected the mutant line's MMC sensitivity (data not shown). Complementation of the FA-G line was associated with down-regulation of CYP2E1 expression (compare lane 4 with lane 3). After incubation with MMC, the complemented FA-G line also expressed relatively less CYP2E1, although not down to the basal level of normal cells (compare lane 5 with lanes 3 and 2). Furthermore, we examined the effects of concurrent (enforced) expression of FANCG in the h2E1/OR cell line, which was engineered to overexpress CYP2E1. After transduction with FANCG/pLNCX2, the level of detectable CYP2E1 was also reduced (Figure 3C). Taken together, our data suggest that wild-type FANCG regulates protein expression of CYP2E1 in at least certain cell types.

Our findings thus far led us to propose that the interaction of FANCG with CYP2E1 might alter redox metabolism and increase DNA oxidation. 8-Oxoguanine (8-oxoG or 7,8-dihydro-8-oxoguanine) is one of the most damaging lesions among the oxidized bases because of the formation of a stable base pair with adenine. It is possible to assess 8-oxoG reactivity by cellular fluorescence assays. Figure 4 shows representative data of 8-oxoG damage in mutant and in complemented EUFA143 cells as determined by flow cytometry. We found dose-dependent increases in fluorescence after treatment with H_2O_2 or MMC. (With these doses of H_2O_2 or MMC, >80% of mutant cells die within 3 days, whereas >50% of complemented cells are viable.) However, for all conditions tested, the levels of 8-oxoG reactivity present in complemented cells were significantly lower than were found in mutant EUFA143 cells.

Discussion

FANCC was reported previously to interact with NADPH: cytochrome P450 reductase, a microsomal membrane protein involved in electron transfer (17). FANCC was found to suppress the ability of RED to reduce cytochrome *c* in the presence of NADPH, suggesting that mutations in FANCC might lead to the constitutive activation of RED. Our work here suggests that FANCG also appears to interact with a member of the cytochrome P450 pathway, CYP2E1. Our findings are in general accord with those of Ruppitsch *et al.*, who found that P450 enzymes (particularly CYP1A2) played a role in the altered metabolism of ROI in FA cells (16).

FANCA and FANCG have been reported to form protein complexes in both the cytoplasm and nucleus (10,11). However, nuclear localization of the FANCA–FANCG complexes was found to be critical for cellular resistance to MMC (24). Whereas these experiments argue for a specific nuclear function for FANCG, our data indicate greater extranuclear distribution of FANCG in cells following exposure to MMC. Therefore, we propose that FANCG may have both nuclear and cytoplasmic roles that are not mutually exclusive. This model would be highly analogous to the ataxia telangiectasia paradigm (reviewed in ref. 25), in which the ataxia telangiectasia mutated (ATM) gene product has a pleiotropic role in both DNA repair processing and in detoxification of ROI.

A number of caveats to our data should be considered. First, the addition of the FLAG tag could theoretically alter the localization patterns of FANCG. Secondly, endogenous FANCG may behave differently from transfected FANCG. In our report, we have also not distinguished the exact mechanism by which FANCG alters CYP2E1 levels. Finally, we have not measured levels of NADPH:cytochrome P450 reductase, which could modulate the level of P450 holoproteins without affecting immunodetectable levels.

In addition to its well-documented role in redox metabolism, CYP2E1 has clearly been shown to activate procarcinogens. Two, in particular, have relevance to the FA phenotype. 1,3-Butadiene is a volatile organic compound widely used in the production of resins and plastics (26). Butadiene is oxidized by cytochrome P450 enzymes (including CYP2E1) to the monoepoxide 1,2-epoxy-3-butene (EB) and its diepoxide 1,2:3,4-diepoxybutane (DEB) (27). DEB is a well-known clastogen that is commonly used *in vitro* to diagnose FA and would be expected to be harmful to FA patients if disseminated in the environment. Benzene, another ubiquitous pollutant, is known to cause leukemia and aplastic anemia in humans as well as hematotoxicity in rodents (28). Toxicity is thought to occur as result of oxidative metabolism in the liver and lung, again primarily via pathways mediated by CYP2E1 (29). We

hypothesize that abnormal metabolism of carcinogens such as butadiene and benzene may play a role in cancer development in FA patients.

Both butadiene and benzene cause oxidative DNA damage. Characteristic lesions, such as 8-oxoG, occur in DNA after both exogenous and endogenous oxidative stress. A relationship between FA and oxidative stress has long been recognized, and a defect in repair of oxidative DNA damage has been suggested. In this regard, there have been a number of reports measuring 8-hydroxy-2′-deoxyguanosine (8-OHdG). In one study, FA-A cells formed two to three times more 8-OHdG than control cells after incubation with H_2O_2 (30). In another study, 8-OHdG levels in FA homozygotes (and to a lesser extent, heterozygotes) were found to be significantly higher than those of age-matched controls (31). A third report showed an increased basal level of 8-OHdG in cells from complementation groups FA-C and FA-E (32). Removal of 8-OHdG after H_2O_2 treatment was significantly reduced in FA-E cells. With one exception (33), these reports have generally led to the suggestion that FA proteins may participate in the repair of oxidative DNA damage (34,35).

A monoubiquitinated form of FANCD2 was recently described to co-localize with the breast cancer susceptibility gene product, BRCA1, within nuclear foci (14). BRCA1 may be required for transcription-coupled repair of oxidative DNA damage (36). Deficiency of either BRCA1 or BRCA2 in human cancer cells leads to a block of the RNA polymerase II transcription machinery at the 8-oxoG site and impairs the transcription-coupled repair of the lesion (37). Expression of wild-type BRCA1 was able to fully complement the repair defect in BRCA1-deficient cells. These results suggest the possibility that one important function of FANCD2 and of FANCG is in either repair of or protection against oxidative DNA damage.

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