DNA Damage and Cellular Stress Responses

Constitutive Activation of Caspase-3 and Poly ADP Ribose Polymerase Cleavage in Fanconi Anemia Cells

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Abstract

Fanconi anemia (FA) is a rare syndrome characterized by developmental abnormalities, progressive bone marrow failure, and cancer predisposition. Cells from FA patients exhibit hypersensitivity to DNA cross-linking agents and oxidative stress that may trigger apoptosis. Damage-induced activation of caspases and poly ADP ribose polymerase (PARP) enzymes have been described for some of the FA complementation groups. Here, we show the constitutive activation of caspase-3 and PARP cleavage in the FA cells without exposure to exogenous DNA-damaging factors. These effects can be reversed in the presence of reactive oxygen species scavenger N-acetylcyestein. We also show the accumulation of oxidized proteins in FA cells, which is accompanied by the tumor necrosis factor (TNF)-α oversecretion and the upregulation of early stress response kinases pERK1/2 and p-P38. Suppression of TNF-α secretion by the extracellular signal-regulated kinase inhibitor PD98059 results in reduction of caspase-3 cleavage, suggesting a possible mechanism of caspases-3 activation in FA cells. Thus, the current study is the first evidence demonstrating the damage-independent activation of caspase-3 and PARP in FA cells, which seems to occur through mitogen-activated protein kinase activation and TNF-α oversecretion. Mol Cancer Res; 8(1); 46–56. ©2010 AACR.

Introduction

Fanconi anemia (FA) is a genetic disorder characterized by congenital abnormalities, bone marrow failure, and marked cancer susceptibility (1, 2). Currently, 13 FA complementation groups are known to exist, each of which represents a FA subtype abbreviated as A (FANCA), B (FANCB), C (FANCC), D1 (FANCD1/BRC2A2), D2 (FANCD2), E (FANCE), F (FANCF), G (FANCN), I (FANCI/KIAA11794), J (FANCJ/BRIP1), L (FANCL), M (FANCM), and N (FANCN; refs. 3, 4). With the exception of FANCD2 and FANCI, the encoded FA proteins have no similarities with each other but cooperate in a common FA/BRC pathway by the formation of subnuclear and nuclear complexes, where activation by monoubiquitylation of FANCD2 and FANCI seems to orchestrate a cascade of events in response to DNA damage (5).

Cells derived from FA patients are hypersensitive to cross-linking agents that are potent inducers of programmed cell death (PCD or apoptosis; ref. 6). Studies in cohorts of FA patients show that PCD is uncommon in the absence of damaging factors and the major cause of mortality is bone marrow failure (7-9). This can be mediated by several inducers, including dissolved oxygen, superoxide and hydroxyl radicals, and other reactive oxygen species (ROS), and results in cell growth arrest or cell death (10-12). FA cells are sensitive to ROS, thus acquiring abnormalities in several redox status end points (13-15). FANCA and FANCN are redox-sensitive proteins that are multimerized to form a nuclear complex in response to oxidative stress (16). Not surprisingly, the survival of human primary FA BM cells can be ameliorated by reducing oxidative stress (14).

Hypersensitivity to ROS often acts as a second messenger in cellular signaling and is mediated by specific cysteine proteases, known as caspases (17). Sequential activation of caspases, on induction of PCD, has been shown in FA hematopoietic progenitor cells in response to mutagenic stress (18). Caspases exist within the cell as zymogens (inactive proforms) that can be cleaved to form active enzymes following the induction of apoptosis (19). One of the first proteins identified as a substrate for caspases is poly ADP ribose (PAR) polymerase (PARP; also known as PARP1 and adenosine diphosphate ribosyltransferase; ref. 20). PARP is involved in DNA damage repair and catalyzes the synthesis and binding of PAR to DNA strand breaks and modifying nuclear proteins (21). The ability of PARP to repair damaged DNA is prevented following cleavage of PARP by caspase-3 (21).

Present research is aimed at studying caspase-3 activity as well as PARP status in FA cells of several complementation

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groups in the absence of exogenous DNA damage. We have shown higher caspase-3 activity and PARP cleavage in FA-D2 and other FA complementation groups compared with their corrected counterparts or in the small interfering RNA (siRNA) FANCD2–depleted normal cells versus scRNA-transfected cells. Interestingly, the level of PARP cleavage and caspase-3 upregulation could be reduced on treatment of cells with ROS inhibitor, suggesting that oxidative stress is one of the mediators in these events. We also showed higher level of apoptosis in FA cells with corresponding accumulation of oxidized proteins. This was accompanied by tumor necrosis factor (TNF)-α over-secretion and upregulation of early damage-response kinases p-ERK1/2 and p-P38. Moreover, suppression of TNF-α secretion by extracellular signal-regulated kinase (ERK) inhibitor PD98059 results in the reduction of caspase-3 cleavage, suggesting a possible mechanism of caspases-3 activation in FA cells. The blocking of TNF receptor decreased caspase-3 activity in D2/−/−, but not in D2/+/−–corrected cells, suggesting that caspase-3 response is TNFα/TNFα1 mediated. The intracellular ROS seemed to be TNFα/TNFα1-dependent for D2/−/− but not for D2/+/−–corrected cells, suggesting that overproduction of TNFα, rather than ROS accumulation, causes caspase-3/PARP response. Thus, the current study is the first evidence demonstrating the damage-independent activation of caspase-3 and PARP in FA cells, and that this activation probably occurs through mitogen-activated protein kinases (MAPK) activation and TNF-α oversecretion.

Materials and Methods

Cell Lines and Culturing

The following human transformed fibroblast cell lines were used in this study: PD20 (FANCD2−/−), PD20 retrovirally corrected with pMMp-FANCD2 cDNA, FANCA−/− (PD220)–transformed fibroblasts and corrected counterparts (FA consortium),4 FANC-G− and FANC-G–corrected primary fibroblasts (gift of Dr. D. Schindler, Würzburg University, Würzburg, Germany). In addition, we used primary human fibroblasts derived from a healthy individual and lymphoblastoid cells from a non-FA patient [FANCL (EUFA 868), FANC] (LFA 177), and FANCA (LFA 82)–transformed lymphoblastoid cells]. FANCC (PD331.1)–transformed fibroblasts and corrected counterparts were cultured at 70% confluence as described previously (22). FANCI and FANCNM human lymphoblasts were kind gifts from Juan Bueren’s laboratory (Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, Madrid, Spain). The PARP inhibitor 3-aminobenzoamide (3AB; Sigma) was dissolved in DMSO and was added to cell medium at a final concentration of 10 mmol/L when required. ERK inhibitor PD98059 (Calbiochem), dissolved in DMSO, was used at 50 μmol/L final concentration. ROS inhibitor NAC, from Sigma, was used at 2 mmol/L final concentration. WPDQY (Abgent, Inc.), an inhibitor of TNF receptors, was used at 1 μmol/L concentration.

Cells Treatment and Preparation of Cell Lysates

For siRNA experiments, we followed previously described procedures with the same FANCD2 siRNA sequences (Invitrogen; ref. 22). Whenever necessary, the PKC inhibitor bisindolylmaleimide I (GF109203X from Calbiochem) was added directly to the cell medium as DMSO-dissolved stock solution. Cells were washed with ice-cold PBS and resuspended in buffer C consisting of 20 mmol/L HEPES (pH 7.9), 420 mmol/L KCl, 25% glycerol, 0.1 mmol/L EDTA, 5 mmol/L MgCl2, 0.2% Nonidet P-40, 1 mmol/L DTT, and a 1:40 volume of protease inhibitor mixture (Sigma) on ice for 30 min. After centrifugation at 12,000 × g for 10 min at 4°C, the supernatant was collected as the total cell lysate. The pellet was resuspended in buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 2% glycerol, 1 mmol/L DTT, and a 1:40 volume of protease inhibitor mixture containing 4-(2-aminoethyl)benzenesulfonfyl fluoride, pepstatin A, E-64, bestatin, leupeptin, aprotinin, and sodium EDTA] and homogenized on ice by sonication. After centrifugation at 1,000 × g for 10 min at 4°C, the supernatant was collected as the cytoplasmic fraction. The resulting nuclear pellet was then washed with buffer A twice and resuspended in TNE buffer [10 mmol/L Tris-HCl (pH 7.5), 0.1 mol/L NaCl, 1 mmol/L EDTA]. For dephosphorylation experiments, 100 units of λ-phosphatase (Calbiochem) were added to the appropriate cell lysates following incubation for 30 min at 37°C. For the degradation assay (Supplementary Fig. S1), siRNA FANCD2–depleted samples were incubated in parallel with the mixture of sodium vanadate and sodium pyrophosphate (both from Sigma). To test for IFN response, we analyzed siRNA-transfected primary cells or cells stimulated with 2 μg Poly-I-poly-C were followed by Western blot analyses of eIF2λ/eIF2α antibodies or real-time PCR experiments (22) to test the expression of the IFN pathway–related gene STAT1.

SDS-PAGE and Western Blot Analysis

Cell lysates (25 μg/lane) or purified proteins were resolved on 4% to 8% or 4% to 13% SDS-polyacrylamide gels under reducing conditions (10 mmol/L DTT or 1% β-mercaptoethanol). Proteins were then transferred to a polyvinylidene difluoride membrane and were probed with an anti-PARP, cleaved anti–caspase-3, cleaved anti–caspase-8, anti–glyceraldehyde-3-phosphate dehydrogenase, anti-vinculin (all from AbCam), anti-PAR antibody (a rabbit polyclonal IgG from AbNOVA or SantaCruz), or with an anti-FANCD2 antibody (monoclonal mouse IgG, Novus Biologicals) followed by a horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences). Proteins were visualized by using the enhanced chemiluminescence system (Amersham Biosciences).

4 http://www.fanconi.org
Immunoprecipitation

Cells were washed with PBS and resuspended in 1 mL of lysis buffer [50 mmol/L HEPES (pH 7.0), 100 mmol/L NaCl, 1 mmol/L EDTA, and 0.1% NP-40] followed by shearing with a 23.5-gauge needle. Clarified supernatants (2 mg) were mixed with 1 μg of affinity-purified antisera against PARP overnight at 4°C. Immunocomplexes were precipitated with protein-A-Sepharose beads (Pierce), washed thrice with lysis buffer, and boiled in 1× Laemmli buffer. All precipitated proteins were resolved by 4% to 12% SDS-PAGE and transferred to Immobilon-P membranes (Millipore), followed by Western blot analysis with phospho-PARP antibodies (MBL Laboratories).

Assay of Caspase-3 Activity

After the incubation with or without caspase-3 inhibitor, cells were washed thrice in PBS and cell extracts were prepared as above. Equal-volume STKM buffer [0.25 mol/L sucrose, 50 mmol/L Tris-HCl (pH 7.5), 25 mmol/L KCl, 5 mmol/L MgCl2, and 0.25% Triton X-100] was added and the lysates remained on ice for 15 min. Cell lysates were stored at -80°C until assay. Caspase-3 activity was colorimetrically measured using Ac-DEVD-AFC fluorometer (Bio-Rad), according to the manufacturer’s instructions. Corresponding levels of caspase-3 activity were determined by comparing the levels of control or normal cells with deficient or depleted counterparts, with the number of relative fluorescence units detected in controls set at 100%. Corresponding levels of caspase-3 activity in the presence of caspase-3 inhibitors were subtracted with the number of relative fluorescence units detected in controls.

Analysis of Apoptosis in FA Cells

Apoptosis was assessed using slightly modified protocol described in the Apoptosis Detection Manuel (Promega), which uses the principle of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling to detect fragmented DNA in apoptotic cells. Briefly, cells were grown on slides or prepared as cytospins and fixed in 3% paraformaldehyde, were permeabilized, were washed, and were treated with the TdT incubation buffer at 37°C for 60 min. After thorough washing in PBS, the slides were counterstained with 1 μg/mL propidium iodide (Sigma) in PBS (15 min) and were washed in distilled water before mounting in fluorescence-free mountant (DAKO). Slides were then scanned with a Typhoon Laser Scanner at 520 nm to identify signals of incorporated fluorescein-12 dUTP (apoptotic cells) or with 620 nm for identification of total cell numbers from propidium iodide staining. The apoptotic index was counted by 2D-Bio-Rad software as a result of three independent measurements. To confirm the above data, several cell samples were assessed for apoptosis using an Annexin V-fluorescence-activated cell sorting protocol, and for staining using an Annexin V and 7-aminomycin D with a BD ApoAlert Annexin V kit (BD Pharmingen) in accordance with the manufacturer’s instructions. Apoptosis was analyzed by quantification of Annexin V-positive cell population by flow cytometry.

OxyBlot Analyses

To assess the formation of protein carbonyl groups, the OxyBlot protein oxidation detection kit (Chemicon Int., Integen) was used according to the manufacturer’s detailed protocol. Briefly, total protein extracts were denatured by mixing with equal volumes of 12% SDS and then with the derivatized by adding 2,4-dinitrophenylhydrazine solution or control for 15 min at room temperature. After neutralization, samples were resolved on 4% to 12% Tris-glycine gels followed by Western blotting. Blots were probed with primary antibody recognizing specific derivatized dinitrophenyl moieties of the proteins. Proteins that underwent oxidative modification (i.e., carbonyl group formation) were identified as a band in the derivatized sample, but not in the control. Levels of oxidatively modified proteins were quantified and expressed as fold increase versus normal controls through measurement by scanning densitometry with the TotalLab 2.0 image program.

Assay for Glutathione Peroxidase, Catalase, and Intracellular ROS

Glutathione peroxidase activity was determined spectrophotometrically by monitoring the oxidation of NADPH at 340 nm (23). The Cellular Glutathione Peroxidase Assay kit (Calbiochem) was used to achieve this goal. Briefly, samples containing 0.2 μg of total proteins were dissolved in 1:10 (v:v) assay buffer [1 mmol/L reduced glutathione, 0.22 mmol/L tert-butyl hydroperoxide, and 0.4 UY/mL glutathione reductase (pH 7.6)]. Immediately before assay, samples were mixed with NADPH reagent in a 96-well microtiter plate and the absorbances at 340 nm were measured over 3-min intervals using a recording spectrophotometer. An extinction coefficient of 6.1 mmol/L was used for the calculation. One unit is the amount needed to oxidize 1 mmol of NADPH per minute. Catalase activity was determined spectrophotometrically at 240 nm by the reduction of H2O2 as described previously (24). GPX activity was measured by monitoring NADPH oxidation. A standard reaction mixture (0.2 mL) containing 100 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, 0.2 mmol/L β-NADPH, 1 mmol/L NaN3, 3 mmol/L glutathione, 0.1% (v/v) Triton X-100, 1.4 units of glutathione reductase, and 2.5 μmol/L TeGPXII was incubated at 30°C for 5 min. The background rate of NADPH oxidation was determined and...
the reaction was initiated by the addition of the hydroperoxide substrate. The nonenzymic activity due to the autoxidation of glutathione was also examined. The enzyme activity was calculated using $e = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Evaluation of intracellular ROS levels was estimated using the oxidant-sensitive H2-DCFDA probe as described by Royall and Ischiropoulos [Royall, J.A. and Ischiropoulos, H. (1993). Evaluation of 2′,7′-dichlorofluorescein and dihydrodorhamine 123 as fluorescent probes for intracellular $H_2O_2$ in cultured endothelial cells. Arch. Biochem. Biophys. 302, 348-355]. Cells were grown in 6-multiwell plates, then incubated in serum-free (phenol red–free RPMI 1640) medium with H2-DCFDA (5 $\mu$mol/L) for 30 min as described above. Cells were then carefully washed in PBS, scraped off into 1 mL of water, and the fluorescence was measured (excitation at 495 nm and emission at 520 nm).

**TNF-α Titration**

Overproduction of TNF-α in the supernatant of exponentially growing FA cells with or without NAC inhibitor was done according to Quantikine ELISA as described by the supplier (R&D Systems). Briefly, cells (either scrapped by rubber policemen or in suspension) were collected by centrifugation and supernatants were filtered through a 0.45-μm Millipore Filter (Millipore) and were immediately used for assay or stored at −80°C. Samples were diluted in appropriate buffer and were added to the anti–TNF-α-antibody precoated microwell strips. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α was added to the wells. Following incubation and washing steps, a substrate solution was added to the wells and color developed in proportion to the amount of TNF-α bound in the initial step. After stopping the reaction, the intensity of the color was measured by microplate reader at 450 nm with the correction wavelength set at 540 nm.

**Immunofluorescence**

Cells grown on coverslips were washed with PBS and subsequently fixed by adding PBS containing 4% formaldehyde (Sigma-Aldrich) for 10 min at room temperature. Cells were washed with PBS and incubated with PBS and 0.5% Triton (Sigma-Aldrich) for 15 min, subsequently rinsed with a washing buffer (WB) consisting of 3% bovine albumin (Sigma-Aldrich) and 0.05% Tween 20 (Sigma-Aldrich) in PBS, incubated with corresponding primary antibody in WB overnight at 4°C, and washed for 15 min in WB with gentle agitation. In addition to the antibodies used for Western blotting, cleaved PARP (Asp214) antibody (human specific) #9541 (Cell Signaling, Inc.) has been used. Incubation with secondary anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 568 antibodies (both from Molecular Probes) diluted in WB was done at 4°C for 80 min followed by a 15-min washing step in WB with gentle agitation. After the last antibody labeling step, the preparations were mounted in anti-fading medium containing 4′,6′-diamidino-2-phenylindole as a DNA counter stain (Vector Laboratories). Microscopic analysis and quantification were done using an Olympus BX-50 fluorescence microscope. Images were captured with a Leica DMRB epifluorescence microscope equipped with a DC 200 digital camera and the Leica DC Viewer capturing software. Images were finally computer edited using Photodeluxe (Adobe version 2.0).

**Statistical Analysis**

Statistical analyses of data from four replicate trials were carried out by ANOVA and Fisher’s protected least significant difference test using the STATVIEW program (Abacus Concepts, Inc.). All percentage values were subjected to arcsine transformation before statistical analysis. All values are expressed as mean ± SEM. A probability of $P < 0.05$ was considered to be statistically significant.

**Results**

**PARP Cleavage in FA Cells**

Our proteomics data suggest that PARP family proteins, including caspase substrates are differentially expressed in FA cells and associated the FANCD2 immunoprecipitated pellets (Table 1). Because PARP plays a role in apoptosis, we studied the PARP level in various FA complementation groups. We reinvestigated the previously reported lack of PARP activity in fibroblasts from FA patients (25) by determining the level of PARP in the cells from a FA-D2 complementation group that usually represents a more severe phenotype compared with upstream (core) FA subgroups (26). Our Western blot data suggest that the PARP is cleaved in FA-D2–deficient (PD20) but not in the corrected (PD20cor) cells. This event seems to be independent from FANCD2 monoubiquitination as only moderate PARP cleavage was observed in a nonmonoubiquitinatable mutant (K561R) counterpart (Fig. 1A). Pretreatment of cells with PARP inhibitor, 3AB, led to reduction of PARP cleavage (Fig. 1B). To further examine whether PARP cleavage can occur in normal cells converted by siRNA to FA-phenotype, we performed FANCD2 knockdown experiments by transfecting primary human fibroblasts with FANCD2 siRNA. We observed the appearance of cleaved forms of PARP 1 day after transfection (Fig. 1C). Interestingly, the upper band corresponding to the uncleaved 116-kDa PARP seemed to be 8 to 10 kDa higher in the FANCD2-depleted samples, suggesting possible PARP modification. Because PARP can be activated by phosphorylation (27), we tested such possibility by treatment of the cell lysates with λ-phosphatase. Reversal of the mobility shift suggested PARP phosphorylation on depletion of FANCD2 (Fig. 1C). Similar reversal was obtained by the pretreatment of FANCD2 siRNA–depleted cells with GF109203X, the inhibitor of PKC that evokes PARP phosphorylation in vitro. This led to the decrease in intensity of the upper PARP band.

5 Our unpublished data.
To confirm this event, we immunoprecipitated cell fractions after siRNA FANCD2 depletion with a PARP antibody followed by Western blotting and probing with anti-phospho-specific PARP antibodies. We recognized increased signals of multiple bands of phosphorylated PARP in the FANCD2-depleted samples and partial decrease of phospho-PARP in the λ-phosphatase–treated samples, whereas total PARP level remained unchanged (Fig. 1D). These data confirm that conversion of normal cells into FA phenotype is accompanied by PARP cleavage.

**Table 1. Caspase-related proteins co-immunoprecipitated with FANCD2 protein**

<table>
<thead>
<tr>
<th>Protein name*</th>
<th>Protein ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PACAP</td>
<td>A5A3E0</td>
<td>Proapoptotic caspase adaptor protein that interacts and inhibits caspase-3 activity</td>
</tr>
<tr>
<td>PKN1</td>
<td>NP_002732.3</td>
<td>PKN1 is a fatty acid and Rho-activated serine/threonine protein kinase</td>
</tr>
<tr>
<td>MAP1A</td>
<td>NP_002364.5</td>
<td>Microtubule-associated protein 1A, in which proteolysis results from the Aβ-mediated activation of caspase-3 and calpain</td>
</tr>
<tr>
<td>Calpastatin</td>
<td>NP_00113954.0</td>
<td>Calpastatin-type II is a highly specific inhibitor of calpain, in which activation is essential for the enzymatic activation of the critical effector caspase-3</td>
</tr>
<tr>
<td>NAP1L3</td>
<td>NP_004529.2</td>
<td>Nucleosome assembly protein 1-like 3 is a substrate for caspase-3</td>
</tr>
<tr>
<td>CARD10 (CARMA3)</td>
<td>ENSG00000100065</td>
<td>Caspase recruitment domain family, member 10</td>
</tr>
</tbody>
</table>

*Proteins identified by matrix-assisted laser desorption/ionization–time-of-flight technique in immunopellets of FANCD2-immunoprecipitated cell extracts.

(Fig. 1C, right). To confirm this event, we immunoprecipitated cell fractions after siRNA FANCD2 depletion with a PARP antibody followed by Western blotting and probing with anti-phospho-specific PARP antibodies. We recognized increased signals of multiple bands of phosphorylated PARP in the FANCD2-depleted samples and partial decrease of phospho-PARP in the λ-phosphatase–treated samples, whereas total PARP level remained unchanged (Fig. 1D). These data confirm that conversion of normal cells into FA phenotype is accompanied by PARP cleavage.

**FIGURE 1.** PARP cleavage and upregulation in FA cells. A. Immunoblot showing PARP cleavage in FANCD2-deficient (PD20), FANCD2-proficient (PD20cor), or nonmonoubiquitinable mutant (K561R) cells. B. Immunoblot showing PARP processing in PD20 and PD20-corrected cells treated (+) or untreated (−) with PARP inhibitor 3AB. C. PARP cleavage is increased in primary fibroblasts transfected with FANCD2 siRNA for the corresponding time intervals. Arrows, mobility shift representing PARP phosphorylation. Note the reduction of PARP signal in the sample treated with λ-phosphatase or in the siRNA FANCD2–depleted cells pretreated with PKC inhibitor. D. Immunoblot of the above FANCD2 knockdown samples immunoprecipitated with PARP antibody followed by probing with phospho-PARP or total PARP antibodies. E. Immunoblots showing PARP cleavage in different FA subgroups treated or untreated with the PARP inhibitor, 3AB.
and phosphorylation. Importantly, the appearance of PARP in the upper band was specific to phosphorylated PARP as pretreatment of cell samples with phosphatase inhibitors restored the appearance of the upper band (Supplementary Fig. S1A). Cleaved PARP was not due to degradation processes as no difference in the band intensities (either upper or the lower) were shown in the lane with the phosphatase inhibitors (Supplementary Fig. S1B). In order to confirm that no IFN response occurs after FANCD2 siRNA transfection, we analyzed siRNA-transfected primary cells or cells stimulated with Poly-I-poly-C (positive control) by Western blotting with eIF2α/P-eIF2α antibodies, or by performing reverse transcription-PCR experiments to test the expression of the IFN pathway–related gene STAT1 (Supplementary Fig. S2). To further expand our finding for other FA subgroups, we performed Western blot analyses of the cell extracts isolated from FANCG fibroblasts, FANCL (EUFA 868), FANCJ (LFA 177), and FANCA (LFA 82)–transformed lymphoblastoid cells to show an increased PARP cleavage compared with the FA-corrected counterparts or with non-FA lymphoblastoid cells. This cleavage was partially reduced by pretreating the cells with N-acetylcystein (NAC; Fig. 1E). FA cells of I and M subgroups revealed similar cleavage of PARP protein (data not shown). Immunofluorescence staining of the siRNA FANCD2–depleted cells (Supplementary Fig. S3); PD20, K561R, and PD20cor (Supplementary Fig. S4); or FANCG, FANCL, and FANCA cells (supplementary Fig. S5) against cleaved anti-PARP antibody revealed an increased intensity in PARP signaling. Interestingly, pretreatment of cells with NAC decreased the

FIGURE 2. FA cells show higher caspase-3 activity and apoptotic levels versus corrected counterparts. A. Immunoblot shows the levels of cleaved caspases-3 in FA cells treated or untreated with NAC. B. Immunofluorescent image shows the levels of cleaved caspase-3 (red channel) in PD20 (A and A*), K561R (B and B*), or PD20-correction (C and C*) cells. C. Colorimetric measurement of caspase-3 activity in FA cells (P* < 0.05). D. Apoptotic index of FA cells as measured by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–based assay. Example of Typhoon laser–scanned PD20cor cells stained either with propidium iodide to define apoptotic cells (scanned in red channel at 620 nm) or with fluorescein 12-dUTP to define total number of cells (green channel at 520 nm; left). The percentage of apoptosis was determined by comparing two scanned images using 2D-Bio-Rad software applied for three independent measurements (right). P* < 0.01.
intensity of PARP signaling, suggesting that PARP cleavage might be ROS mediated. Overall, these data show activation and cleavage of PARP enzyme in FA cells without exogenous damage. Although endogenous stress is probably a primary cause of caspase-3/PARP activation, the possibility of autodamage of FA cells due to exogenous stimuli cannot be completely disregarded.

Caspase-3 Is Upregulated in FA Cells

Caspase-3 plays a central role in the execution of the apoptotic program and is primarily responsible for the cleavage of PARP during cell death (28-30). At the same time, caspase-3 is a mediator of ROS accumulation within the cell. To correlate PARP cleavage with caspase-3 activity, we tested the levels of active (cleaved) caspase-3 in FA cells. Western blot analyses revealed significant upregulation of cleaved caspase-3 in the cell extracts of several FA complementation groups, but not in the corrected counterparts (Fig. 2A). Cleaved caspase-8 and caspase-6 levels were also upregulated in some FA cell lines (data not shown). Increased caspase-3 signaling was confirmed by immunofluorescence staining of PD20, PD20-corrected cells, K561R cells (Fig. 2B), or primary fibroblasts transfected with siRNA or scRNA FANCD2 (Supplementary Fig. S6). Moreover, we detected that the total level of PAR polymer synthesis, a proapoptotic marker, was also elevated in...

FIGURE 3. Increased level of oxidized proteins and glutathione peroxidase activity in FA cells. A. Protein oxidation detection by the OxyBlotTM. B. Levels of oxidatively modified proteins quantified by scanning densitometry and expressed as fold increase versus normal controls using the TotalLab2.0 image program. Columns, mean (P < 0.02); bars, SD. C. Glutathione peroxidase activity in FA cells nontreated or pretreated with NAC was determined by NADPH oxidation at 340 nm. Columns, mean (n = 5 in each group; *, P < 0.01); bars, SD.
FANCD2-deficient cells (Supplementary Fig. S7) or on FANCD2 depletion (data not shown). To test caspase-3 enzymatic activity in various FA complementation groups, we performed a colorimetric activity assay. In all FA cells, compared with the corrected counterparts, caspase-3 activity was higher (Fig. 2C). Moreover, the difference in the FA-D2 group or in the primary cells depleted from FANCD2 by siRNA were much higher than that of the FA-upstream groups. Interestingly, only cells from the FA-D2 group showed significant reduction of caspases-3 activity on treatment with NAC. This caspase assay was alternatively validated by a chemiluminescence approach in which relative units of active caspase-3, measured after 30 minutes of incubation with the corresponding substrate, were comparable with of colorimetric assay data (data not shown). Thus, our results provide the first evidence that caspase-3 activity is upregulated in FA cells without exogenous DNA damage.

Although caspase-3 and PARP cleavage are known markers of apoptosis, no confirmed data showing apoptosis of intact FA cells have been described thus far. To compare levels of apoptosis for different FA complementation groups, we performed a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling–based apoptosis assay (Fig. 2D). Our data show that FA cells had higher apoptotic levels when compared with corrected counterparts in the absence of DNA-damaging treatments. These findings were in parallel to the levels of caspase-3 activity. Because pretreatment with ROS scavenger decreased the number of apoptotic cells (Fig. 2D), PARP cleavage (Fig. 1E), and caspase-3 activity (Fig. 2C), we concluded that moderate oxidative stress is a common property of FA cells growing in the absence of damaging factors.

**Protein Oxidation and ROS Accumulation in FA Cells**

ROS have been implicated in PCD, cancer development, and aging (31) but can also be produced in normal cells by mitochondrial electron transport, cellular redox system, or immune responses (32). Proteins oxidized by ROS often result in the incorporation of carbonyl groups into corresponding side chains. Therefore, we assessed the efficacy of oxidation processes in FA cells by measuring the amount of ROS-modified proteins. Our data on OxyBlot analyses show that the FA cells undergo oxidation more easily than corresponding corrected counterparts (Fig. 3A and B). The same is true for primary cells converted to FANCD2-like phenotype by siRNA. Moreover, because NAC-pretreated samples partially reduce the level of oxidized proteins, accumulation of ROS seems to play a role in FA cells. We then tested the activity of cellular glutathione peroxidase, a member of the GPx enzymes that serves as a quencher to protect cells from ROS damage. We showed that the GPx activity in FA cells is impaired comparatively to the corrected counterparts and that pretreatment with NAC only slightly affects this activity (Fig. 3C). Similar effects were found by measuring catalase activity in FA cells (data not shown). These facts suggest that FA cells have impaired protective properties against oxidative stress, which may enhance the protein oxidative profile.

**TNF-α Overproduction and MAPKs Upregulation in FA Cells**

Recently, overproduction of TNF-α in FA has been reported both in vivo and in vitro (33–35). However, previous experimental approaches did not establish whether TNF-α overproduction was directly linked to accumulation of ROS in FA cells. To address this point, we compared the TNF-α level in the supernatants of exponentially growing FA cells treated or untreated with ROS inhibitor. We observed that the TNF-α level was increased in all tested supernatants of FA cells and that NAC significantly inhibited secretion of TNF-α (Fig. 4A). This observation suggests that TNF-α secretion can be an early event of FA etiology and that the accumulation of this factor may eventually lead to ROS accumulation and alter caspase-3/PARP levels. At the same time, MAPKs may potentially activate PARP cleavage and caspase-3 activity. Recently, aberrant activation of MAPKs was implicated in FANC-C pathway loss-of-function and was also observed in FANCA-depleted HeLa cells (33, 36). At the same time, ROS have been shown to activate several MAPKs and affect a wide array of cellular processes, including proliferation, differentiation, and apoptosis (37). Because phosphorylation of ERK1/2 and P38 is a common event during oxidative stress, we tested the activation of these kinases in FA cells by Western blotting. We found that in all tested FA cells, the levels of p-P38 and p-ERK1/2 were higher than in the corrected counterparts (Fig. 4B). Pretreatment with ROS inhibitor significantly reduced MAPK phosphorylation. Similar observations were obtained by the immunofluorescent staining of FANCD2-deficient and FANCD2-proficient cells (Supplementary Fig. S7A). Importantly, increased upregulation of both pERK1/2 and p-P38 on FANCD2 depletion, and partial reduction of ERK1/2 phosphorylation after NAC treatment was observed in primary fibroblasts as shown by immunofluorescence staining (Supplementary Fig. S8). Thus, we suggest ROS-dependent upregulation of pERK1/2 in FA cells. It may be possible that the loss of function in FA genes results in TNF-α oversecretion, which, in turn, activates MAPK by a ROS-dependent feedback loop and then triggers caspases-3/PARP cascade. Therefore, blocking TNF-α production may affect the expression of active caspase-3. To test this hypothesis, we pretreated FA cells with an ERK inhibitor PD98059, which was earlier proved to reduce TNF-α production in FA-C cells (33). We showed that inhibition of pERK1/2, on exposure to PD98059, was accompanied by the reduction of cleaved caspase-3 level in FA cells (Fig. 4C). Because ERK1/2 inhibition suppresses TNF-α production, it may also reduce ROS level and, consequently, decrease caspase-3 activity. Thus, it is unclear if endogenously accumulated ROS increase the production of TNF-α followed by triggering TNF receptors, accumulating additional ROS, and increasing apoptotic markers, or whether oversecretion of TNF-α–mediated ROS...
accumulation precedes these events. To rule out this possibility, we tested whether caspase-3 and ROS accumulation are dependent on TNFR1 by blocking TNFR1 with a specific peptidomimetic WP9QY. FANCD2-deficient or corrected cells treated or untreated with ROS inhibitor were incubated with WP9QY, and the levels of cleaved caspase-3 and intracellular ROS have been measured (Fig. 4D). Our results show that blocking TNF receptor does affect caspase-3 activity in D2−/−, but not in D2−/−-corrected cells, suggesting that caspase-3 response is TNFα/TNFRI mediated. Intracellular ROS level is low in both cell lines and is TNFα/TNFRI dependent for D2−/− but not for D2−/−-corrected cells. Therefore, at least for the D2−/− subgroup, it is unlikely that ROS accumulation plays a primary role in apoptotic events. Rather, it is the overproduction of TNFα that causes caspase-3/PARP response.

### Discussion

Evidence of FA sensitivity to oxygen was first provided by Joenje et al. (38), who showed that the chromosomal instability of primary FA cells could be reduced if grown at lowered oxygen tension (39). Data obtained from mouse models provided strong genetic evidence that FANCC−/− cells are hypersensitive to endogenously generated oxidants, although it was unknown whether the molecular mechanism responsible for this hypersensitivity was due to altered redox signaling (40, 41). In turn, our data confirm that ROS are playing a role in FA cells and that caspase-3/PARP enzymes are activated along with MAPKs without exposure of FA cells to exogenous damaging factors. Recent studies of different human cDNA libraries, using a yeast two-hybrid system, identified interactors of...
FANCA, FANCC, and FANCG that involve proteins of oxidative metabolism groups (42). Moreover, works of Paganò et al. (13, 15) suggested that the effects of commonly used damaging agents, e.g., mitomycin C or cis-platin, may equally activate both DNA repair/damage and oxidative-damage response in FA cells. Potential sources of endogenous stresses may involve interstand cross-links formed by products of lipid peroxidation and other forms of oxidative DNA damage (43).

Some other factors playing an important role in aging and anemia include inflammatory ROS that are linked with DNA damage–induced premature senescence in hematopoietic stem and progenitor cells. This seems to be mediated through TNF-α–induced senescence that correlates with the accumulation of ROS and oxidative DNA damage (44). Interestingly, FANCC seems to be involved both in the protection of cells against oxidative damage and in the control of TNF-α activity (45). Cytoplasmic FANCC interacts with heat shock protein 70 to protect cells from IFN-γ/TNF-α–induced apoptosis (46). Such activation may involve transforming growth factor β1, which induces concomitant activation of a caspase signaling cascade, in response to oxidative damage, followed by PARP cleavage and p53-independent apoptosis (47). All these facts may potentially bridge the activity of caspases in FA cells throughout oxidative stress and, more importantly, provide some platform for explaining proapoptotic nature of FA in the absence of mutagenic stress. Observations by us and others of MAPKs activation in FA cells, along with the previous findings of TNF-α release, may provide an explanation for caspase-3 activity and PARP cleavage. One possible scenario that explains this phenomenon goes to the recent findings of Rosselli’s group (33) where they showed that ERK inhibition reduced TNF-α oversecretion in FA-C cells. This was in parallel with the decreased matrix metalloproteinase-7 promoter activity. In our study, we reduced the level of cleaved caspases-3 by inhibiting ERK in both FANCD2-deficient and FANCD2-proficient cells. If this is true for other FA cells, then the disruption of ERK (and possibly other MAPKs), followed by the reduction of TNF-α, may regulate caspases triggering. We also show that blocking TNFR is accompanied by a decrease of cleaved caspase-3 level in D2–/– cells, but not in D2–/––corrected cells, and that intracellular ROS level is TNFα/TNFFR1 dependent for D2–/– but not for D2–/––corrected cells. This suggests that caspase-3 response is TNFα/TNFFR1 mediated and overproduction of TNFα precedes ROS accumulation. Yet it is still unclear why the increase of such apoptotic markers as caspases-3, caspase-8, PARP, and PAR does not initiate apoptosis in FA cells. It should be noticed, however, that most the cells used in our study were SV40-transformed cell lines. Therefore, using primary mice or human FA cells should rule out the possibility of nonspecific effects due to immortalization. It is also possible that FA genetic background possess some compensatory factors that prevent either ROS increase or caspase-3–driven apoptosis or both. Therefore, the challenge for future studies is to find out the relationship between these processes and the different FA intermediates. On a practical side, deeper understanding of why FA cells encounter oxidative stress in the absence of DNA-damaging agents provide a strong link to a possible therapy for FA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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