# Involvement of the Fanconi anemia protein FA-C in repair processes of oxidative DNA damages

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**Abstract Fanconi anemia (FA) is an autosomal recessive disorder characterized by skeletal abnormalities, pancytopenia and a marked predisposition to cancer. FA cells exhibit chromosomal instability and hypersensitivity towards oxygen and cross-linking agents such as diepoxybutane and mitomycin C. An increased level of reactive oxygen intermediates and an elevation of 8-oxoguanine in FA cells point to a defective oxygen metabolism in FA cells. We investigated the repair activity of oxidatively damaged DNA in lymphoblastoid cells from FA patients of complementation groups A–E. The repair activity for oxidatively damaged DNA was significantly reduced in lymphoblastoid cell lines of complementation groups B–E. Complementation of the FA-C cell line with the wild type FA-C gene restored the repair activity to normal. This indicates that the FA-C protein participates in the repair of oxidatively damaged DNA.**


*Key words:* Fanconi anemia; FA-C protein; Oxidative DNA damage; DNA repair

## 1. Introduction

Fanconi anemia (FA) is an autosomal recessive disorder characterized by progressive pancytopenia, hyperpigmentation of the skin and multiple congenital abnormalities [1,2]. In addition, FA patients have a considerably increased risk of developing cancer, mainly acute myeloid leukemia and squamous cell carcinoma. FA cells exhibit an increased rate of spontaneous chromosomal breaks and show hypersensitivity to cross-linking agents such as mitomycin C, diepoxybutane and cisplatin as well as hypersensitivity towards oxygen [3–5]. These features suggested a molecular defect in the DNA repair system of FA patients [6–10], detection of which has led to results which are discussed ambiguously [11–14].

Genes have been cloned for the complementation groups FA-A and FA-C [15,16]. No homologies to other genes have been found and the functions of the resultant proteins, probably of cytoplasmic origin, are unknown [17,18]. Recently, an increased level of reactive oxygen intermediates (ROI) [19] and an increased level of 8-hydroxy-2′-deoxyguanosine in FA cells have been reported [20,21], both of which could potentially generate oxidatively damaged DNA. Whilst overproduction of ROI and oxygen and mitomycin C hypersensitivity may be due to defective redox cycling [14,22], chromosomal instability indicates improper handling of damaged DNA.

We investigated the capacity of FA cells to repair oxidatively damaged DNA. We made use of the chloramphenicol acetyltransferase (CAT) assay [23] and generated oxidative DNA damage in vitro with the aid of potassium permanganate, which creates a broad spectrum of oxidative DNA lesions.

## 2. Materials and methods

### 2.1. Chemicals

Chemicals were purchased from Sigma, Roth or Merck unless otherwise indicated. [$^{14}$C]Chloramphenicol was purchased from ICN.

### 2.2. Plasmids

For CAT experiments the pCAT$_{control}$ (Promega) and pcDNA3CAT (Invitrogen) plasmids were used.

### 2.3. Cell lines and culture conditions

The lymphoblastoid cells used in this study (Table 1) were cultured in RPMI 1640 medium (Biochrom, Berlin) containing 10% fetal calf serum (Biochrom, Berlin). All cells were cultured at 37°C under an atmosphere of 5% $CO_2$ in air. The stably transfected cells were grown in the same medium supplemented with hygromycin (final concentration, 200 μg/ml; Boehringer Mannheim, Germany).

### 2.4. Transient transfections

Lymphoblastoid cells were transfected by electroporation. Briefly, cells were washed with 1×HeBS buffer (20 mmol/l HEPES, pH 7.1; 137 mmol/l NaCl; 5 mmol KCl; 0.7 mmol $Na_2HPO_4{\cdot}2H_2O$; 6 mmol/l D-glucose), resuspended in 1×HeBS buffer to a cell density of 2–4×10$^6$ cells/50 μl, transferred to a 4-mm cuvette containing 10 μg plasmid (damaged or undamaged) and incubated for 10 min at room temperature. The volume was adjusted to 100 μl with 1×HeBS. Electroporation was performed using the Bio-Rad Gene Pulser (Bio-Rad Laboratories, CA) with a pulse-setting at 250 V/200 Ω/250 μFd. After 10 min at room temperature cells were resuspended in RPMI 1640 supplemented with 15% fetal calf serum and antibiotics. 48 h after transfection, cells were used for CAT assays.

Table 1
Lymphoblastoid cell lines used in the experiments

| Cell line | Genotype/phenotype |
|---|---|
| HSC92 | Wild type |
| HSC72 | FA-A |
| HSC230 | FA-B |
| HSC536 | FA-C |
| HSC536pDR | FA-C, stably transfected with the empty vector |
| HSC536pDR/FA-C | FA-C, stably transfected with the wild type FA-C gene |
| HSC62 | FA-D |
| VU130L | FA-E |

All lymphoblastoid cells were donated by H. Joenje, Amsterdam.

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*2.5. CAT assay*

CAT assays were performed as described [22]. Briefly, transfected cells were harvested after 48 h, resuspended in 0.3 mol/l Tris-HCl, pH 7.0 and lysed by three times freezing and thawing. 50 μl of the lysate were incubated with 25 μl CAT mix (4 mg/ml acetyl coenzyme A, 1 μCi [$^{14}$C]chloramphenicol) for 4 h at 56°C. Chloramphenicol and acetylated derivatives were extracted with 0.9 ml ethyl acetate. After evaporation of the ethyl acetate the products were separated by thin layer chromatography on silica gel sheets (Kieselgel 60 $F_{254}$, Merck, Darmstadt, Germany) with chloroform/methanol (95/5) as solvent. Spots were either identified by autoradiography, cut out and counted by liquid scintillation, or they were quantified by means of the Fujifilm BAS-1500 Phosphoimager.

*2.6. Oxidative damage of plasmid DNA*

0.02–0.04 μg/μl CAT plasmid solutions were treated with 0–3 mM potassium permanganate for exactly 2 min at room temperature. Plasmids were precipitated with ethanol/sodium acetate and placed on ice immediately after treatment. Resulting $MnO_2$ was completely removed by precipitation and washing with 70% ethanol for several times.

## 3. Results

To investigate the repair capacity of cells from FA patients and unaffected controls, we chose a set of well defined lymphoblastoid cells and transfected them with extracellularly oxidatively damaged CAT plasmid DNA. As damaging agent we used potassium permanganate ($KMnO_4$) to create oxidative DNA injuries [24–26]. The CAT plasmid carries the bacterial gene for chloramphenicol acetyltransferase behind the eukaryotic SV40 promoter. CAT activity, determined in cell extracts, depends on the amount of damage and the repair capacity of the tested cells. The result for each sample represents the percentage of conversion of chloramphenicol to its acetylated derivatives, related to the CAT activity of cells transfected with untreated plasmid. To determine the state of $KMnO_4$ treated plasmid DNA, the plasmids were analyzed by agarose gel electrophoresis (Fig. 1a,b). A $KMnO_4$ concentration of more than 2.5 mM led to strand breaks as visible by the reduction of the supercoiled form (Fig. 1a). $KMnO_4$ concentrations of more than 25 mM resulted in the total destruction of plasmid DNA (Fig. 1b). Because of these results the plasmid DNA used for the CAT experiments was treated with less than 5 mM $KMnO_4$.

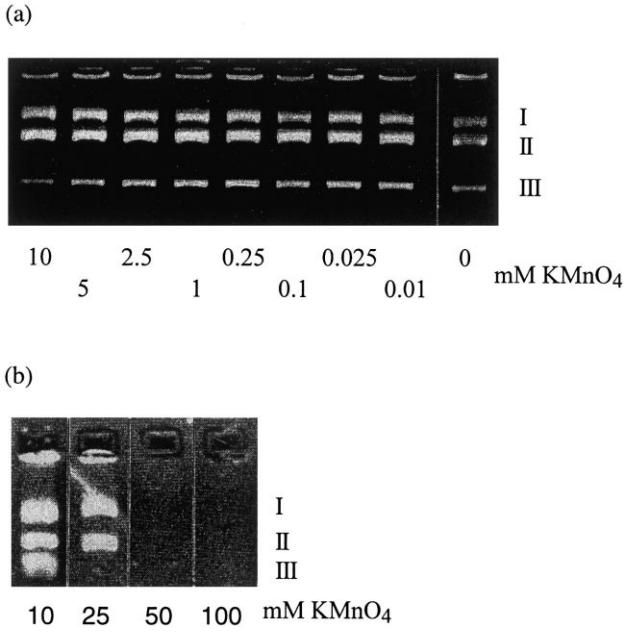


Fig. 1. Analysis of $KMnO_4$ damaged plasmid DNA on 1% agarose gel. I, relaxed form; II, linearized form; III, supercoiled form. a: 0–10 mM $KMnO_4$. b: 10–100 mM $KMnO_4$. The plasmid DNA was treated as described in Section 2. Equal amounts of plasmid DNA were applied in each lane.

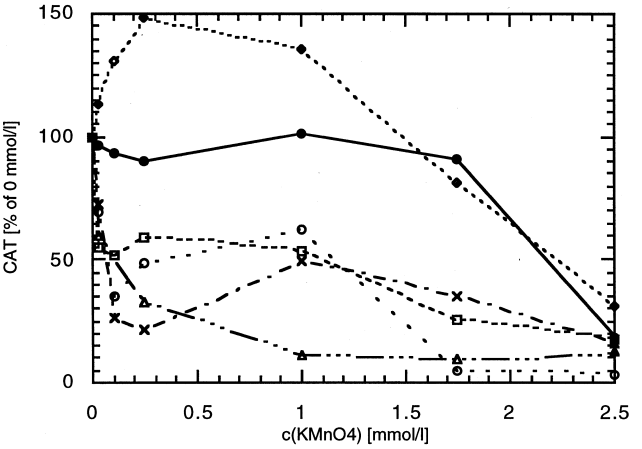


Fig. 2. Reactivation of $KMnO_4$ damaged CAT plasmid by lymphoblastoid cells. The vector pcDNA3 CAT was damaged with various concentrations of $KMnO_4$. The result for each sample represents the percentage of conversion of chloramphenicol to its acetylated derivatives, related to the CAT activity of cells transfected with the untreated plasmid. ●, HSC92 (control); ◇, HSC72 (FA-A); △, HSC230 (FA-B); ○, HSC 536 (FA-C); ×, HSC62 (FA-D); □, VU130L (FA-E).

Fig. 2 shows CAT reactivation curves for FA cells of complementation groups A–E in comparison to a normal control cell. With the exception of strain HSC72 (FA-A), all FA lymphoblastoid cells (FA-B–E) revealed a considerably less efficient repair of oxidative DNA damages than did the healthy control cells (Fig. 2). The FA-A cell line (HSC72) repaired the CAT plasmid more efficiently than did the healthy control cells.

To explore whether the repair deficiency manifested in lymphoblastoid cells reflected an inborn feature of FA, repair activity for oxidatively damaged CAT plasmid was measured in the FA-C cell line HSC536 that had been stably transfected with the wild type FA-C gene. Indeed, the complemented HSC536 cell line exhibited an almost normal repair capacity. Transfection of the empty vector, however, had no beneficial effect on DNA repair (Fig. 3). This experiment clearly demonstrated the involvement of FA-C in a distinct cellular process.

## 4. Discussion

Increased amounts of ROI and high oxygen sensitivity are exerted by FA cells. Consequent oxidative damage to DNA and insufficient DNA repair might result, which may be responsible for the elevated level of 8-oxoguanine and chromosomal instability characteristic of FA cells.

In previous experiments it was shown that the CAT-based assay is a powerful tool to analyze DNA repair of human cells [12,14,23]. Using the CAT assay procedure, we analyzed oxidative DNA damage repair in cells of FA patients and in a

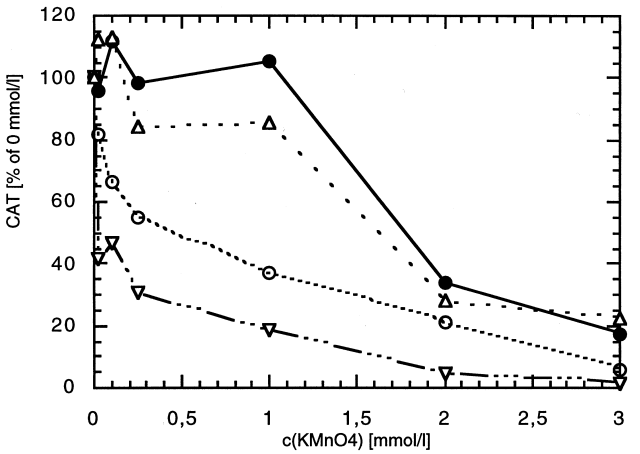


Fig. 3. Restoration of repair capacity of oxidative damages in HSC536 (FA-C) cells by transfection of the wild type FA-C gene. ●, HSC92 (control); ○, HSC536; ▽, HSC536 pDR (stably transfected with the empty vector); △, HSC536 pDR/FA-C (stably transfected with the wild type FA-C cDNA).

healthy control. A decreased repair efficiency became evident in all lymphoblastoid FA cell lines except FA-A (Fig. 2).

That this oxidative DNA damage repair deficiency is directly related to the basic molecular defect of FA could be shown for FA-C, one of the two FA complementation groups with a cloned gene defect. Expression of FA-C protein in FA-C lymphoblastoid cells by stable transfection of the wild type FA-C cDNA normalized the repair capacity of this cell line (Fig. 3) and disclosed the involvement of the FA-C protein in repair processes of oxidatively damaged DNA. Unrepaired DNA lesions signal delay to the cell cycle. The recently published involvement of the FA-C protein in a pathway that signals to the cyclin B/cdc2 kinase [27] may be operative in this signal delay. Since FA-C is localized to the cytoplasm [17,18] and binds to a number of cytosolic proteins [28], the protein may be a member of a multifunctional complex that triggers redox-dependent cellular functions not only in the cytoplasm but also in the nucleus. Recently, the FA-C and FA-A proteins have been shown to form a complex that is also found in the nucleus [29]. Thus, uncoupled FA-A and FA-C may protect the cell against cytosolic ROI overproduction. In order to regulate ROI concentration in the nucleus and to repair ROI damaged DNA, FA-C binds to FA-A, and the complex is finally translocated to the nucleus via the nuclear localization signal of the FA-A protein. There the complex may combine probably with additional FA proteins, to form an active repair complex.

The fact that in our experiments the FA-A lymphoblastoid cell line (HSC72) displayed an excess of oxidative DNA damage repair capability is of special interest. Explanations might be: the defective FA-A protein is not able to protect the cell against cytosolic ROI overproduction (see above). An increased ROI level results ([19], own unpublished results) that would lead to an increase in oxidative DNA lesions. FA-C not efficiently transported by the defective FA-A becomes superinduced and binds to transport proteins with lower binding affinity than FA-A. Subsequently, most potent DNA repair results in FA-A cells as demonstrated by our experiments. Alternatively, the FA-A protein may serve special functions in unravelling eukaryotic chromatin not required for CAT plasmid. Third, the effect could be unique to the special cell line HSC72.

The existence of at least eight FA genes [30] and the fact of a reduced repair capacity of oxidatively damaged DNA in complementation groups B–E indicate that the FA proteins function in a complex molecular pathway or are part of a multiprotein complex that is involved in ROI detoxification, DNA repair or maintenance of DNA stability.

For the complementation groups B, D and E the defective genes have not yet been cloned. A likely possibility for a FA defect might be a mutation in an enzyme specialized for oxidized DNA repair. Thus, further investigations on DNA repair enzymes that remove oxidative lesions from DNA in these complementation groups are of great interest.

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