Overexpression of thioredoxin in Fanconi anemia fibroblasts prevents the cytotoxic and DNA damaging effect of mitomycin C and diepoxybutane

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Received 15 December 1997; revised version received 18 December 1997

Abstract Adult T cell leukemia derived factor (ADF)/thioredoxin (Trx) is known to be an important intracellular antioxidant involved in a number of redox reactions such as ribonucleotide reductase (RNR) as well as of tyrosinase. Since RNR is a key enzyme of nucleotide metabolism and DNA synthesis, a reduced Trx level would result in reduced enzymatic activity and cause DNA damage. Furthermore, Trx is considered to be an effective regulator of redox sensitive gene expression. The role of Trx in nucleotide metabolism and gene expression may be an explanation for increased chromosomal instability as well as hypersensitivity towards oxygen, ROI and ROI generating agents. The activity of tyrosinase, the key enzyme of melanin biosynthesis, is influenced by the thioredoxin level and by superoxide radicals. Low thioredoxin levels and high superoxide concentrations activate tyrosinase causing hyperpigmentation of the skin. In addition to the observed high superoxide concentration in Fanconi anemia (FA) patients, a low thioredoxin level might be responsible for the hyperpigmentation (café-au-lait spots) in this disease. We observed that overexpression of the thioredoxin cDNA in FA fibroblasts completely abolished the DNA damaging effects of mitomycin C and diepoxybutane and inhibited the constitutive activity of the nuclear factor κB (NF-κB) in SV40 transformed FA fibroblasts. However, spontaneous chromosomal breakage was not affected.

Key words: Fanconi anemia; DNA repair; Oxidative stress; Thioredoxin; Nuclear factor kappa B

1. Introduction

Fanconi anemia (FA) is an autosomal recessive disorder characterized by progressive pancytopenia, multiple congenital abnormalities, a high risk of cancer, hyperpigmentation of the skin and chromosomal instability [1,2]. Chromosomal instability and hypersensitivity towards the DNA crosslinking agents mitomycin C and diepoxybutane, substances that increase the number of chromosomal breaks in FA cells but not in cells from healthy individuals, suggest a molecular defect in DNA repair [3]. However, no such defect in DNA repair has been found [4–7].

Moreover, the recently cloned FA-A and FA-C proteins have been localized to the cytoplasm. In addition, hypersensitivity towards oxygen and increased ROI levels have been shown in FA cells [8–11]. Furthermore, involvement of the cytochrome P450 enzyme system in ROI generation and mediation of mitomycin C and diepoxybutane toxicity in FA cells imply that a deficient DNA repair is not the primary defect of this disease [7,12].

The abnormal oxygen metabolism of FA cells points to a defective antioxidative mechanism. In FA fibroblasts, however, antioxidative enzymes like catalase, superoxide dismutase (SOD), glutathione reductase, and phospholipid hydroperoxide glutathione peroxidase (PHGPx) have been shown to be normal [7,13]. On the other hand, antioxidants have been shown to be beneficial for DNA stability and survival of FA cells [7,14].

Thioredoxin is an intracellular antioxidant [15] and regulator of redox sensitive gene expression [16] that may have implications in FA cells. As a cofactor of the ribonucleotide reductase it is necessary for DNA synthesis and DNA stability [17,18]. Furthermore, it regulates the activity of tyrosinase [19].

We investigated the effect of the antioxidant thioredoxin on FA cells. We hypothesized that reduced thioredoxin levels would disturb redox dependent gene expression, influence nucleotide metabolism resulting in increased chromosomal instability and would cause activation of tyrosinase resulting in hyperpigmentation of the skin, features well documented in FA [16,20–23].

2. Materials and methods

2.1. Cell lines and culture conditions

The FA cell line 1424 originates from a male patient and was established and classified as FA-A by K. Sperling, Berlin. The control cell line CoHe was established in our laboratory. Both derived from lower arm punch biopsies. Cells were grown in minimum essential medium (MEM-Eagle with Earle salts, Seromed) supplemented with 10% fetal calf serum (FCS; Life Technologies) without antibiotics at 37°C in a 5% CO_2 atmosphere and 90% humidity. Both strains were routinely checked for mycoplasma infections.

2.2. Chemicals

All chemicals were purchased from Sigma or Roth unless otherwise indicated. Electroporation cuvettes were from Bio-Rad laboratories. Oligonucleotides were synthesized by TIB MolBiol, Berlin. Poly dI-dC was from Pharmacia, Freiburg. Restriction enzymes were from Life Technologies. Mitomycin C was from Boehringer Mannheim.

2.3. Transfection experiments

Cells were grown to confluence, harvested by trypsinization, washed twice with 5 ml HeBS buffer (20 mM HEPES pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D-glucose) and finally resuspended in HeBS to a density of 1×10^6 cells/50 μl and incubated for 10 min at room temperature. Cells were transferred to a 0.4 cm electroporation cuvette, 10 μg plasmid DNA was added and cells were electroporated (200 V, 200 Ω and 125 μFd) in a gene pulser from Bio-Rad. Following electroporation cells were incubated for 10 min at room temperature and finally resuspended in MEM-Eagle with 15% FCS and antibiotics. 200 ng/ml G418 was added to the growth me-

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Abbreviations: ADF, adult T cell leukemia derived factor; Trx, thioredoxin; NF-κB, nuclear factor kappa B; FA, Fanconi anemia; ROI, reactive oxygen intermediates; MMC, mitomycin C; DEB, diepoxybutane

PII S0014-5793(97)01608-6

dium 24 h after electroporation. For the NF-κB experiments cells were collected 60 h after transfection. For stable integration of the ADF gene the plasmid DNA was restricted with *Bgl*II. The cells were grown with 200 ng/ml G418 for the whole culture period.

2.4. Micronucleus assay

For chromosomal instability determination the micronucleus technique was used [24]. Non-transfected and ADF transfected cells seeded on glass slides were immediately treated with 20 ng/ml DEB or 100 ng/ml MMC for 48 h. The cells were washed twice with PBS, fixed in ice-cold methanol, stained for 30 min with bis-benzimide (1 μg/ml in PBS). Coverslips were mounted with a PBS/glycerol/distilled water solution (3/1/1). 1000 cells per slide were scored for micronuclei using a fluorescence microscope with phase contrast optics (Zeiss). Mitotic cells were counted and the mitotic index was determined simultaneously.

2.5. Determination of cell viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to determine cell viability according to Green et al. (1984) with minor modifications. 5000 cells/well were seeded in 24 multiwell plates and incubated for 24 h. After 24 h cells were treated with DEB for 2 h or MMC for 1 h. Cells were washed twice with PBS and incubated in growth medium for another 24 h. For the last 4 h 100 μl MTT (5 mg/ml stock solution PBS, sterile filtered) per 1 ml growth medium was added. The medium was decanted, cells were washed twice with PBS and the formed formazan dye was dissolved in 500 μl ethanol (100%). The absorption of the solution was measured at λ_{570}–λ_{630} in a spectrophotometer (Beckman, DU 650).

2.6. NF-κB detection

For NF-κB experiments 1×10^6 cells were transfected with the pCR/CMV plasmid containing the ADF gene or an empty pCR/CMV plasmid and finally were seeded on plates (Costar, 10 cm diameter). 48 h after seeding, cells were harvested for nuclear extracts.

2.7. Preparation of nuclear extracts

Nuclear extracts were prepared as described [25,26]. Briefly, monolayers were washed twice with TBS (Tris buffered saline) pH 7.4, cells collected by scraping and spinning in a microfuge. Pellets were resuspended in 400 μl buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF; DTT and PMSF were added just before use from a stock solution). Tubes were placed on ice for 15 min, 25 μl of 10% Nonidet NP 40 (Boehringer Mannheim) was added and the tubes were vortexed for 10 s. Cell homogenates were centrifuged for 30 s. Supernatants were removed and pellets were resuspended in 30–50 μl ice-cold buffer C (20 mM HEPES pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF), followed by vigorous shaking on an Eppendorf mixer at 4°C for 15 min. Extracts were centrifuged for 5 min in a microfuge at 4°C. Supernatants were frozen in liquid nitrogen and stored at −80°C. 5 μg protein of the nuclear extract was used for band shift assays. Protein content was determined as described (Bradford, 1976), the reagent was obtained from Bio-Rad Laboratories GmbH.

2.8. Electrophoretic mobility shift assay

EMSAs (electrophoretic mobility shift assays) were performed as described [27]. Briefly, 20 μl binding reaction contained: 1.5 μg poly dI-dC; 1–2 ng ^{32}P-labelled Igκ oligo-probe (5000–10000 cpm Cerenkov); 10 μl 2×shift buffer (20 mM HEPES pH 8.4; 60 mM KCl; 8% Ficoll; 0.03% Nonidet NP 40); 2 mg BSA; 5 μg nuclear extract. Binding reactions were started by adding the nuclear extracts and performed for 30 min at 30°C. Samples were analyzed on native 4% polyacrylamide gels.

3. Results

An altered antioxidant status despite an intact antioxidant defense system has been described as a possible cause of Fanconi anemia [13]. Antioxidants have been shown to have a beneficial effect on FA cells [7,14]. Thioredoxin is a small but important cellular antioxidant whose effect on FA cells

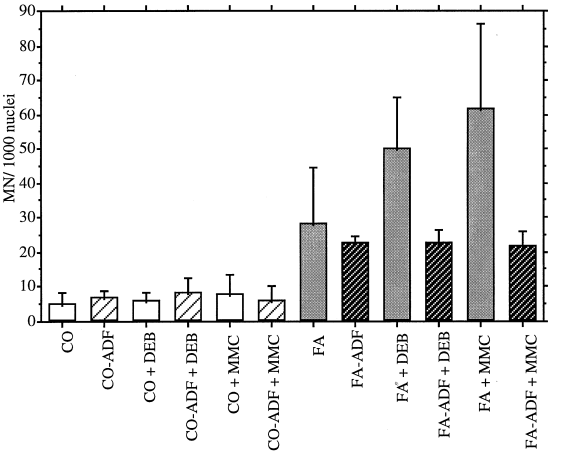

Fig. 1. Effect of mitomycin C (200 ng/ml) and diepoxybutane (20 ng/ml) on micronucleus production of untransfected and ADF transfected FA cells and control cells. Bars: means and standard deviations of 6–18 independent experiments.

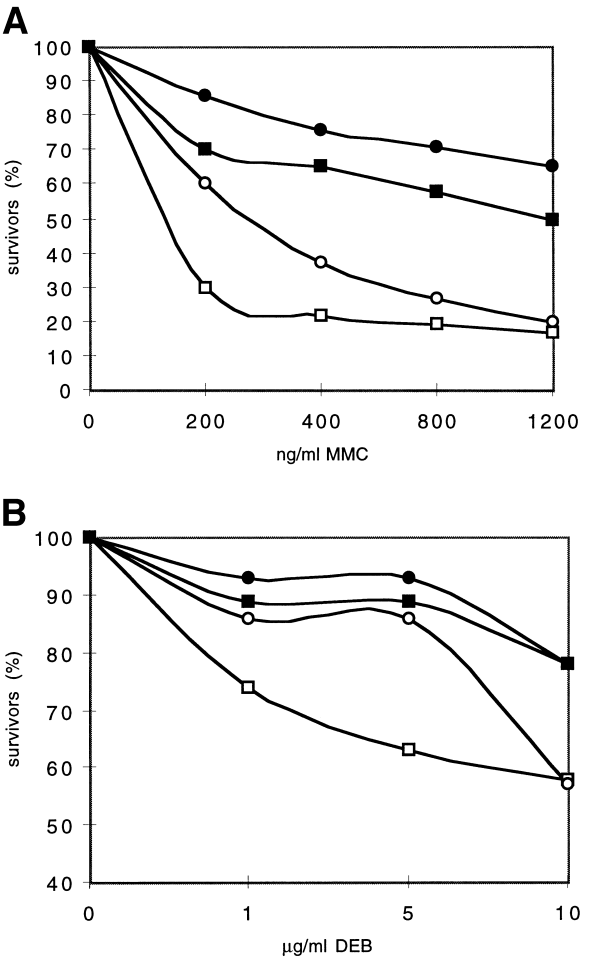

Fig. 2. A: Survival rates of non-transfected and ADF transfected FA and control cell lines treated with increasing amounts of mitomycin C (A) and diepoxybutane (B). Control cells: (○) not transfected; (●) ADF transfected. FA cells: (□) not transfected; (■) ADF transfected. Symbols: means of three separate experiments.

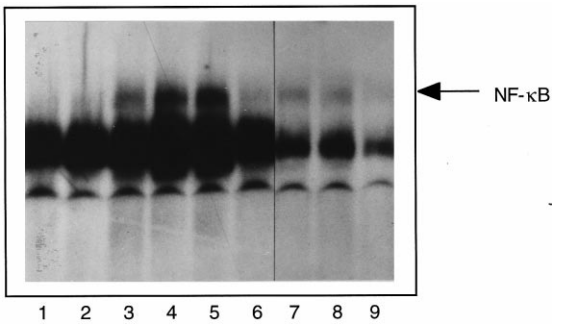

Fig. 3. Reduction of constitutive NF-κB activity in SV40 transformed FA cells by the transfection of cells with ADF. (1) SV40 transformed control cells; (2) HeLa uninduced; (3) HeLa induced with 200 μM hydrogen peroxide; (4) FA-6963; (5) FA-6963 transfected with pCR/CMV plasmid DNA; (6) FA-6963 transfected with pCR/CMV carrying the ADF gene; (7) FA-6914; (8) FA-6914 transfected with pCR/CMV plasmid DNA; (9) FA-6963 transfected with pCR/CMV carrying the ADF gene.

has not been previously investigated. We investigated the effect of thioredoxin on chromosomal breakage and cell viability in FA cells by overexpressing the thioredoxin protein by means of the stably transfected thioredoxin cDNA carrying plasmid pCR/CMV-ADF.

Overexpression of thioredoxin resulted in a decrease of chromosomal breaks in MMC and DEB treated FA cells (Fig. 1). And as shown by the MTT viability test the cytotoxic effect of these substances was also reduced (Fig. 2A,B). In contrast, non-transfected FA cells and control cells showed no such beneficial effect in their survival ratio (Fig. 2A,B). Also the rate of spontaneous chromosomal breaks was not significantly reduced. The overexpression of thioredoxin was measured by determination of NF-κB activity. NF-κB activity becomes diminished at an increased thioredoxin level. Thus, the measurement of NF-κB activity is a rather sensitive method to determine the overexpression of thioredoxin [28].

As shown previously by our laboratory, NF-κB, which is induced by ROI, is constitutively activated in SV40 transformed FA cell lines. This constitutive NF-κB activity could be reduced by the addition of antioxidants [7]. To check whether thioredoxin also reduces this NF-κB activity, we overexpressed thioredoxin transiently in SV40 transformed FA cell lines. An almost complete reduction of this constitutive NF-κB activity was observed (Fig. 3).

4. Discussion

A defective antioxidative defense system has been discussed as possible cause of Fanconi anemia. However, no differences in the activities of the antioxidative enzymes superoxide dismutase, glutathione peroxidase, PHGPx and catalase were found in FA. Likewise, the level of the intracellular antioxidant glutathione was unaffected [7,13]. However, extracellularly added antioxidants like DTT or desferrioxamine can reduce the spontaneously raised level of chromosomal breaks in FA cells [7,14,29]. This suggests a disturbed oxygen metabolism in FA cells may be operative.

The intracellular antioxidant thioredoxin is responsible for optimal activity of ribonucleotide reductase and therefore essential for DNA synthesis [17]. A defective thioredoxin molecule would therefore lead to a decreased activity of ribonucleotide reductase and result in increased chromosomal breakage, a characteristic feature of FA cells.

Thioredoxin is also considered to be an effective regulator of redox sensitive gene expression. Thus, a reduced thioredoxin level may be responsible for increased DNA damage and hypersensitivity of FA cells towards oxygen, because of the inability to induce gene expression.

The activity of tyrosinase, an enzyme catalyzing the first step in melanin biosynthesis, is thioredoxin dependent. Thioredoxin inhibits the activity of tyrosinase [20]. The absence of thioredoxin causes a hyperactive tyrosinase activity that results in increased melanin biosynthesis and hyperpigmentation of the skin, a typical feature of FA patients.

Thioredoxin is an important general antioxidative molecule in the cell. A defective thioredoxin would theoretically lead to an increase of the intracellular ROI level as found in FA cells.

The plasmid generated overexpression of the antioxidant thioredoxin prevents the cytotoxic and genotoxic effect of these agents, but does not reduce the level of spontaneous chromosomal breaks. Since antioxidants like DTT can reduce induced as well as spontaneous chromosomal breaks [7,29], we assume that the antioxidant thioredoxin is not able to enter the nucleus in FA cells. The cytoplasmic action further becomes obvious by the inhibition of the ROI dependent constitutive NF-κB activity in SV40 transformed FA fibroblasts. Thus, thioredoxin detoxifies ROI in the cytoplasm, for example ROI generated by redox cycling of MMC or DEB, but not in the nucleus where ROI damages the DNA. The non-cellular antioxidant DTT, enabled to enter the nucleus, decreases the amount of spontaneous chromosomal breaks. This fact suggests that in consequence of the primary defect thioredoxin may not enter the nucleus in FA. A disturbed physiological oxidant-antioxidant homeostasis would follow, leading to increased oxidative DNA damage and an altered gene expression.

Further experiments will show if translocation of thioredoxin into the nucleus of FA cells influences the spontaneous chromosomal instability.

Acknowledgements: We thank Prof. P.A. Baeuerle, Tularik Inc., San Francisco, CA, for his kind gift of the CR/CMV-ADF plasmid. We are indebted to the Thyssen-Stiftung and to the Fonds der Chemischen Industrie for financial support. The work was further supported by the Deutsche Forschungsgemeinschaft SCHW 532/2-1 and by the Österreichische Nationalbank, Jubiläumsfonds 4842. We are also grateful to Dr. D.H. Kauffmann Jockl, New York, NY, for critically reading and improving the manuscript.

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