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Original Contribution

Photogenotoxicity of folic acid

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ABSTRACT

Folic acid (FA), also named vitamin B9, is an essential cofactor for the synthesis of DNA bases and other biomolecules after bioactivation by dihydrofolate reductase (DHFR). FA is photoreactive and has been shown to generate DNA modifications when irradiated with UVA (360 nm) in the presence of DNA under cell-free conditions. To investigate the relevance of this reaction for cells and tissues, we irradiated three different cell lines (KB nasopharyngeal carcinoma cells, HaCaT keratinocytes, and a melanoma cell line) in the presence of FA and quantified cytotoxicity and DNA damage generation. The results indicate that FA is phototoxic and photogenotoxic by two different mechanisms. First, extracellular photodecomposition of FA gives rise to the generation of H_2O_2, which causes mostly DNA strand breaks. If this is prevented, e.g., by the presence of catalase, DNA damage generated by intracellular FA becomes evident. The damage spectrum in this case consists predominantly of oxidatively generated purine modifications sensitive to the repair glycosylase Fpg, as characteristic for type I photoreactions, and is associated with the formation of micronuclei. In KB cells, the DNA damage is strongly enhanced after pretreatment with the DHFR inhibitor methotrexate, which prevents the loss of the chromophore associated with the intracellular reduction of FA by DHFR. The results indicate that FA is photoreactive in cells and gives rise to nuclear DNA damage under irradiation.

Folic acid (FA) is a water-soluble vitamin and the precursor of cofactors required as one-carbon donors for the synthesis of DNA bases and other essential biomolecules. The bioactivation involves the reduction to tetrahydrofolic acid by dihydrofolate reductase (DHFR) and NADPH (Fig. 1A). The uptake of FA and its reduction products into cells requires active transport by one of the following membrane proteins: (i) the high-affinity folate receptors (FRs), which act by receptor-mediated endocytosis and subtypes of which (named FRα, FRβ, and FRδ) are expressed in epithelial, hematopoietic, and some types of T cells, respectively; (ii) the reduced folate carrier (RFC or SLC19A1), which is ubiquitously expressed and acts as an organic anion antiporter; and (iii) the proton-coupled folate transporter (SLC46A1), which is active predominantly in intestinal cells and has its optimum at low pH [1–3].

FA, which has an absorption maximum at 368 nm, decomposes under irradiation with solar light or UVA in the presence of oxygen, yielding 6-formylpterin and, by further oxidation, pterin-6-carboxylic acid (6-carboxypterin) (Fig. 1B) [4–6]. The photodegradation proceeds with high quantum yields and has been discussed as one of the reasons for the development of skin tanning in evolution, because the FA depletion by natural exposure to sunlight can become physiologically relevant [7–11]. Moreover, many pterins, including the fragmentation products of FA, are efficient photosensitizers. Under irradiation in the presence of DNA, they have been shown to result in the formation of 8-oxo-7,8-dihydroguanine (8-oxoG) and possibly other guanine modifications [12,13]. The relevance of this reactivity for mammalian cells has not yet been established. It may deserve special interest in view of the observation that the irradiation of various types of cells with visible light or UVA gives rise to oxidatively generated DNA modifications and induces micronuclei even if no xenobiotic photosensitizer is added [14–18]. The chemical nature of the endogenous photosensitizer involved has not yet been identified, and it can be hypothesized that endogenous pterins could play a role.

Here, we present an analysis of the DNA damage mediated by photoexcited FA under cell-free conditions and in various cultured cells. The results indicate that FA is indeed photogenotoxic. DNA damage mediated by extracellular generation of H_2O_2 on the one hand and by excitation of intracellular FA on the other can be distinguished. The damage formed by the latter mechanism consists mostly of modified purines and is more extensive if the intracellular reduction of FA by DHFR is inhibited by methotrexate (MTX).

Materials and methods

Materials

FA (> 99%) and MTX, superoxide dismutase (SOD), and catalase were obtained from Sigma–Aldrich. WST-1 reagent was purchased

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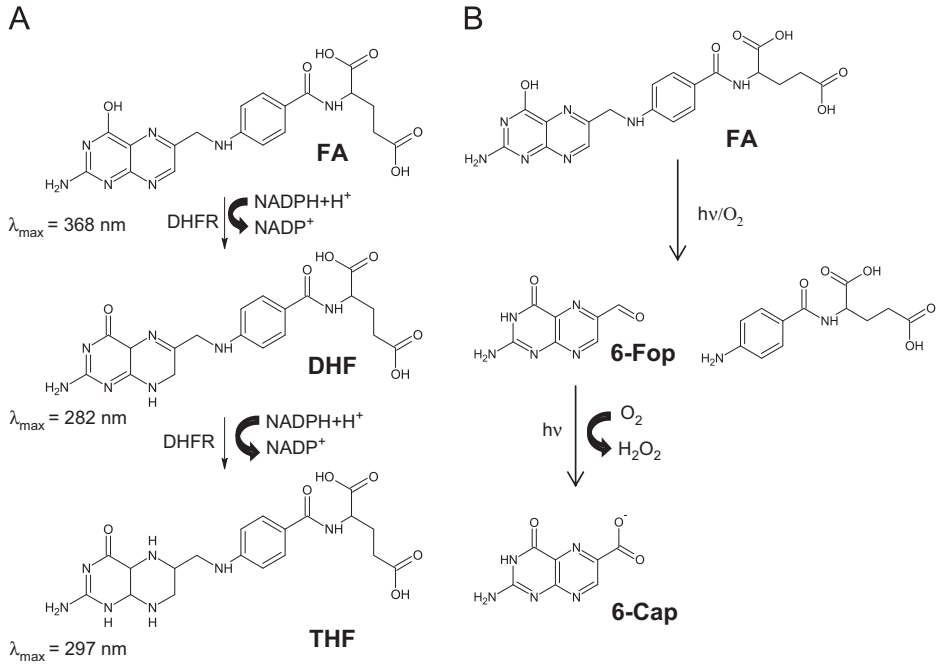

Fig. 1. (A) Metabolism of FA to dihydrofolic acid (DHF) and tetrahydrofolic acid (THF). (B) Photodegradation of FA to 6-formylpterin (6-Fop) and 6-carboxypterin (6-Cap).

from Roche Applied Science (Mannheim, Germany). Monoclonal antibodies were purchased from Enzo Life Science (Lörrach, Germany). DNA from bacteriophage PM2 was prepared according to the method of Salditt et al. [19]. Formamidopyrimidine-DNA glycosylase (Fpg) and T4 endonuclease V (T4endoV) were partially purified from inducible overproducing *Escherichia coli* strains (Fpg, JM105 carrying the plasmid pFPG239; T4endoV, A32480 carrying the plasmid ptac-denV) [20]. Endonuclease III was kindly provided by S. Boiteux (Fontenay aux Roses, France). The repair endonucleases were tested for their incision at various substrate and nonsubstrate modifications (i.e., reference modifications such as oxidized purines induced by methylene blue plus light, thymine glycols induced by OsO_4, AP sites induced by low pH, and pyrimidine dimers induced by UV_{254}) under the applied assay conditions (see below) to confirm that the correct substrate modifications are fully recognized and no incision at nonsubstrate modifications takes place.

KB cells (nasopharyngeal carcinoma cells) were purchased from Cell Lines Service (Eppelheim, Germany), HaCaT cells (immortalized human keratinocytes) were obtained from N.E. Fusenig (Heidelberg, Germany), and M2IV/04 cells (melanoma cell line) were kindly provided by B. Volkmer (Dermatology Center, Buxtehude, Germany). KB and HaCaT cells were cultured in high-glucose Dulbecco's modified Eagle's medium (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% glutamine. M2IV/04 cells were cultured in RPMI medium (PAA) supplemented with 10% FCS and 1% penicillin/streptomycin.

Analysis of cell-free DNA damage

The quantification of DNA modifications induced in cell-free DNA was carried out by means of a relaxation assay [21,22]. Briefly, PM2 DNA (10,000 bp) was irradiated at a concentration of 10 μg/ml in the presence of increasing FA concentrations in phosphate buffer (5 mM KH_2PO_4, 50 mM NaCl, pH 7.4) on ice. For the experiments with UVA, the reaction mixtures were irradiated on ice in 96-well plates for 20 min with a Philips HPW 125-W mercury lamp emitting at 365 nm, placed at a distance of 10 cm, equivalent to 30 kJ/m^2. For the irradiations with an Osram halogen lamp (1000 W), the samples were placed at a distance of 33 cm, corresponding to a dose rate of 375 W/m^2, measured between 400 and 800 nm. To test for the involvement of superoxide anion radicals ($O_2^{\bullet -}$) and H_2O_2 in the DNA damage generation, the PM2 DNA was irradiated with the halogen lamp for 15 min in the presence of 20 μM FA and either 315 U/ml catalase or 200 μg/ml SOD or both. To test for the participation of singlet oxygen, the irradiation was done in buffer containing D_2O instead of H_2O. Subsequently, an aliquot of 0.2 μg DNA in 20 μl BE_1 buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) was incubated for 30 min at 37 °C with 10 μl of BE_1 buffer (for the determination of directly produced single-strand breaks (SSBs)) or 10 μl of one of the following repair endonuclease preparations: (i) Fpg protein, 3 μg/ml in BE_1 buffer, or (ii) T4 endonuclease V, 3 μg/ml in BE_{15} (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 15 mM EDTA) buffer. The reactions were terminated by addition of 10 μl of 1% sodium dodecyl sulfate and the DNA was applied to an agarose electrophoresis gel. Fluorescence scanning of the relaxed and supercoiled forms of the DNA after staining with ethidium bromide allowed us to calculate the number of SSBs or—if an incubation with a repair endonuclease preceded the gel electrophoresis—the number of SSBs plus endonuclease-sensitive modifications (ESSs). The number of ESSs was obtained by subtraction of the number of SSBs.

Analysis of cell proliferation

KB cells were harvested and plated in six-well plates (150,000 per well). They were allowed to grow for 48 h before the medium was removed. For the experiments with MTX, cells were preincubated with 100 μM MTX in phosphate-buffered saline (PBS) for 1 h at 37 °C. Subsequently, PBS with or without catalase (315 U/ml) and increasing amounts of FA were added to the cells and the incubation was continued for 15 min. Plates were irradiated (in the absence of MTX) with the halogen lamp at 33 cm distance for 15 min on ice (equivalent to 338 kJ/m^2 between 400 and 800 nm). Irradiation solutions were removed, medium was added, and cells were allowed to proliferate under culture conditions for 72 h. The proliferation factor was calculated as the ratio of cell numbers counted after 72 h and directly after irradiation.