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Investigations on potential co-mutagenic effects of formaldehyde

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ABSTRACT

The genotoxicity and mutagenicity of formaldehyde (FA) has been well-characterized during the last years. Besides its known direct DNA-damaging and mutagenic activity in sufficiently exposed cells, FA at low concentrations might also enhance the mutagenic and carcinogenic effects of other environmental mutagens by interfering with the repair of DNA lesions induced by these mutagens. To further assess potential co-mutagenic effects of FA, we exposed A549 human lung cells to FA in combination with various mutagens and measured the induction and removal of DNA damage by the comet assay and the production of chromosomal mutations by the cytokinesis-block micronucleus assay (CBMN assay). The mutagens tested were ionizing radiation (IR), (±)-anti-B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE), N-nitroso-N-methylurea (methyl nitrosourea; MNU) and methyl methanesulfonate (MMS). FA (10–75 μM) did not enhance the genotoxic and mutagenic activity of these mutagens under the test conditions applied. FA alone and in combination with MNU or MMS did not affect the expression (mRNA level) of the gene of the O^6-methylguanine-DNA methyltransferase (MGMT) in A549 cells. The results of these experiments do not support the assumption that low FA concentrations might interfere with the repair of DNA damage induced by other mutagens.

1. Introduction

The genotoxicity and mutagenicity of formaldehyde (FA) has been well characterized during the last years. It has been shown that FA induces various DNA adducts and it is generally accepted that the most important DNA alterations induced by FA are DNA–protein crosslinks (DPX). In proliferating cells, unrepaired DPX can arrest DNA replication and lead to the induction of mutations in which chromosomal effects such as chromosome aberrations and micronuclei (MN) seem to be most efficiently induced whereas FA is a poor inducer of true gene mutations [1,2]. It is likely that a direct genotoxic effect of FA does not occur at low concentrations because of the high reactivity of FA and efficient cellular defence mechanisms [3]. However, concerns have been raised that low FA concentrations might enhance the mutagenic and carcinogenic activity of other environmental mutagens by interfering with the repair of DNA-lesions induced by other mutagens and thus causing co-mutagenic effects [4,5]. Co-mutagenic effects of FA *in vitro* were already reported several years ago [6–9]. The "International Agency for Research on Cancer" has classified FA as a known animal and human carcinogen and discussed that FA, besides its direct genotoxic activity, might interfere with the process of DNA repair by three different mechanisms, namely by inhibiting DNA repair enzymes, by inhibiting the removal of DNA lesions or by altering gene expression [5]. The "Final Report on Carcinogens Background Document for Formaldehyde" of the National Toxicology Program (NTP) reviewed studies which investigated co-carcinogenic effects of FA when administered after initiation with carcinogens. The report came to the conclusion that FA might act as a co-carcinogen in combination with other substances because some of the studies did show an enhanced tumor response [4]. A more recent publication [10] suggested that the co-mutagenic effects of FA reported in the earlier studies [7,8] may be explained by a deficiency in FDH (formaldehyde dehydrogenase) which leads to elevated cellular FA levels, inactivation of repair enzymes and increased sensitivity toward mutagens/carcinogens. In fact, there is experimental evidence that deletion of *FDH* in mice causes inactivation of the repair enzyme O^6-alkylguanine-DNA alkyltransferase and increases sensitivity toward genotoxic alkylating agents [11].

FA is a ubiquitous pollutant in the environment from many outdoor an indoor sources. Major sources of outdoor exposure include power plants, manufacturing facilities, automobile exhaust emissions, forest fires and other natural sources of combustion. Other than in occupational settings (production and use of aqueous solutions of FA, synthesis of various resins, use as a preservative and disinfectant), the highest levels of airborne FA have been measured indoors where it is released from various building materials, consumer products and tobacco smoke [5]. Because of its ubiquitous presence in the environment, co-exposures with other mutagens/carcinogens and combination effects are likely.

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Therefore, we investigated potential co-mutagenic effects of FA in human A549 cells, which have previously been used for characterizing the genotoxicity and mutagenicity of FA [12–14] and for measuring gene expression after FA exposure [14]. We used the comet assay for measuring DNA damage and repair induced by various mutagens in A549 cells in the absence and presence of FA exposure. We also tested whether mutagen-induced MN frequencies are influenced by pre-treatment of A549 cells with FA. Because published date suggested that FA exposure might influence the O^6-methyl-guanine-DNA methyltransferase (MGMT) and enhance the mutagenic and carcinogenic action of alkylating agents [5,10], we also measured the mRNA expression of the MGMT in A549 cells exposed to FA in combination with methylating mutagens. Our study aimed to assess potential co-mutagenic effects of FA in mammalian cells and to find out whether such effects should be considered in the context of risk assessment.

2. Materials and methods

2.1. Materials

FA (CAS No. 50-00-0; 16% aqueous solution, ultrapure, methanol free) was supplied from Polysciences Inc. (Warrington, PA, USA) and diluted in distilled water immediately before use. BPDE (Benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide) was purchased from BIU (Grosshansdorf, Germany). If not specifically indicated, all other chemicals used in these experiments were purchased from Sigma (Munich, Germany). Cell culture medium and ingredients were obtained from Invitrogen (Karlsruhe, Germany). Agarose (NEEO) was supplied by Roth (Karlsruhe, Germany) and low melting agarose (LMA, SeaPlaque, "GTG") was from Biozym (Hameln, Germany).

2.2. Cell culture

The A549 cell line (American Type Culture Collection, Rockville, MD) is an epithelial-like human lung cell line derived from lung tissue of a male Caucasian. Adherent cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% glutamine and 0.5% gentamicin. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2 and harvested with 0.15% trypsin and 0.08% EDTA. For the experiments, cells were seeded into plastic flasks about 24 h prior to mutagen exposure.

2.3. Comet assay

The comet assay was performed according to our standard protocol [15]. Aliquots of 10 μl cell suspension (about 15,000 cells) were mixed with 120 μl low melting point agarose (0.5% in PBS) and added to microscope slides (with frosted ends), which had been covered with a bottom layer of 1.5% agarose. Slides were lysed (pH 10; 4 °C) for at least 1 h and processed using a time of alkali denaturation of 20 min and electrophoresis (0.86 V/cm) of 20 min at a pH > 13. Slides were coded and images of at least 50 randomly selected cells stained with ethidium bromide were analyzed from each slide. Measurements were made by image analysis (Comet Assay IV, Perceptive Instruments, Haverhill, UK) and DNA migration was determined by measuring the tail moment to enable a direct comparison with previously published results [13,14]. Tail moment (TM) is calculated according to the formula: TM = (tail intensity/total comet intensity) × (tail center of gravity − peak position). Negative controls (untreated and non-irradiated) cultures were always processed concurrently in the comet assay.

2.4. Micronucleus test

The cytokinesis-block micronucleus assay (CBMN assay) was performed with A549 cells as described earlier [16]. A549 cells were cultivated for about 24 h, then exposed to mutagens and further cultivated in the presence of CytB (3 μg/ml) for 48 h. The frequency of micronucleated cells was measured in 1000 binuclear cells stained with acridine orange (60 μg/ml in phosphate buffer). All slides were coded before scoring. Toxicity (cytostatic effect) was measured using the nuclear division index (NDI) which was calculated from 500 cells according to the formula: $NDI = (M1 + 2M2 + 3M3 + 4M4 + 5M5)/N$, where $M1$–$M5$ indicates the number of cells with one to five nuclei and N the total number of cells scored.

2.5. Quantitative real-time RT-PCR using TaqMan probes

RNA was isolated from A549 cells by use of the QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany). Quantity and purity of the RNA were measured with an Epoch Microplate Spectrophotometer (BioTek, Bad Friedrichshall, Germany).

Real-time RT-PCR was performed using TaqMan gene expression assays for *MGMT* and *GAPDH* (endogenous control) from Applied Biosystems (Darmstadt, Germany). The reaction mix with a final volume of 10 μl contained 2 μl of the RNA (2 ng/μl, diluted with H_2O), 5 μl 2× TaqMan RT-PCR Mix, 0.5 μl of each primer (0.4 μM), 0.25 μl enzyme mix (40×) and 2.25 μl RNase-free water. After reverse transcription at 48 °C for 15 min and an initial activation step at 95 °C for 10 min, 50 cycles of 95 °C for 15 s and 60 °C for 60 s followed (ABI 7900HT Fast Real-Time PCR System (Applied Biosystems)). Each RNA sample was analyzed in triplicate from two or three independently repeated experiments (as indicated in Section 3). Data were analyzed using the ABI 7900 HT Sequence Detection Systems version 2.3 software (SDS 2.3). Quantification of the *MGMT* expression was performed *via* ΔCt value determination, which represents the difference in threshold cycles between the target and the reference gene (*GAPDH*).

2.6. Statistical analysis

Pre-experiments were performed to define the appropriate mutagen concentrations for the comet assay and the CBMN assay. FA was used in concentrations that did not lead to genotoxic effects in these assays with A549 cells in previous studies. If not specifically indicated, experiments were independently performed three times under the same conditions. Treatments were compared in a two-step procedure: first, a one-factor ANOVA allowing for unequal dispersion of the measurements under different treatments was applied to compare the mean values of all the treatments of the experiment simultaneously. In case of statistical significance (*i.e.*, at least two of the treatments are different) at level 0.05, pair wise comparisons of treatment means were carried out, with adjustment of *p*-values according to the method of Tukey. Adjustments for multiple testing were not performed between experiments.

3. Results

Fig. 1 shows the induction of DNA migration in the comet assay after exposure of A549 cells toward 1 Gy (A) and 2 Gy (B) gamma irradiation. Pre-treatment of the cell cultures with FA (20 or 50 μM) for 1 h did not significantly influence the DNA-damaging effect of gamma irradiation (data not shown). Cultivation of the cells under standard conditions for 1 h after irradiation led to a nearly complete removal of irradiation-induced DNA damage. The removal of irradiation-induced DNA damage was not significantly altered in FA pre-treated cultures.