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Formaldehyde-induced DNA adducts as biomarkers of in vitro human nasal epithelial cell exposure to formaldehyde

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Abstract

Formaldehyde (FA) is a mutagen that, at high concentrations and long durations, has been reported to cause nasal cancer in rats and in some humans. The level of FA-induced modified DNA in nasal cells should serve as a biomarker of FA exposure and effect. In the present study, a high-performance liquid chromatography (HPLC)-ultraviolet (UV) method at 254 nm was developed and optimized to detect and quantify hydroxymethyldeoxynucleosides after the isolated DNA in exposed human nasal epithelial cells (HNEC) was enzymically digested. Normal and modified deoxynucleosides were successfully resolved from one another and from tissue and enzyme blank interferences. The viability of HNEC exposed to FA in solution for 24 h decreased, and there was a linear dose response between % nonviability and FA dose from 10 to 500 μg/mL. Amounts of 18.0 ± 1.5 pmol N^6-dA and 12.0 ± 1.2 pmol N^2-dG derivatives were determined in a 10 μL injection after 1.4×10^7 HNEC (106 μg DNA) were exposed to 500 μg/mL in solution. The respective tissue concentrations in pmol hydroxymethyldeoxynucleoside/mg DNA were 170 ± 14 and 113 ± 11. The lower quantifiable limits were about 97 and 88 pmol/mg DNA, respectively. Diffusive exposure of HNEC to air FA up to 100 ppm (v/v) for 24 h did not produce quantifiable hydroxymethylnucleosides. FA-modified deoxynucleosides may be useful biomarkers for FA exposure in biological monitoring samples taken by nasal lavage or brush biopsy.

Keywords: DNA adducts; Formaldehyde; Hydroxymethyldeoxynucleosides; HPLC/UV; Human nasal epithelial cells; Mutation

1. Introduction

Formaldehyde (FA) is a colorless gas that is widely used in hospitals and industry as its aqueous solution, formalin [1,2]. FA is a mutagen that causes nasal squamous cell carcinomas in rats at high air concentrations beyond 6 ppm (v/v) in chronic exposures [3–5]. FA in air has also caused cancer in worker cohorts. Danish studies in 1986 [6] and 1995 [7] found relative risks of 3.0 for nasopharyngeal cancer in male workers exposed to ≥1 ppm FA for at least 10 years. In 2000 [8], occupational exposure to FA in the United States was reported to increase the risk for nasopharyngeal cancer at five cancer registries (odds ratio = 3.0 for people exposed to >1.1 ppm for over 10 years). IARC [9] considers FA to be a probable human nasal carcinogen (Group A2). More positive associations of FA exposure and cancers were reported in 2003 for hypopharynx/laryngeal cancer in Europe [10], for leukemia and

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Hodgkin's disease in a 25 619 worker cohort in the United States [11], and to lung cancer deaths in 14 014 male workers in Britain [12]. A common theme of all these studies is that only exposures to high FA concentrations (>1 ppm) for lengthy periods are likely to cause human cancer.

In 2002, FA was also found to be mutagenic in humans. Sister chromatid exchange SCE (number per chromosome and cells with more than eight SCEs) in the peripheral lymphocytes of 90 pathology staff exposed above 0.7 ppm for at least 15 min per working day over 15 years were significantly higher ($P < 0.01$) than for unexposed staff, or for staff exposed to lower doses and/or shorter durations [13]. The biological effects of FA arise from its interaction with proteins and nucleic acids [1,2,14].

Biological monitoring of human exposure to FA is more related to adverse health effects than its air concentration. No significant differences were found in pre- and post-exposure blood FA concentrations and urine formic acid for exposure of ppb (v/v) FA air concentrations to workers [15,16]. The amount of DNA–protein crosslinking (DPC) in peripheral lymphocytes has been proposed as a biomarker of exposure and as a biomarker of effect for genotoxic damage caused by FA inhalation [17]. DPC is not a unique biomarker for FA, and also occurs after exposure to agents like acetaldehyde, Cr(VI), and hydroxyl radical [18]. Peripheral lymphocytes are also not the major target cells for cancer, though the hematopoietic system may be affected after chronic exposure [11].

DNA adducts, the initial products of mutagenesis, are potential biomarkers of mutagen exposure, and may also be predictive markers of effect for mutagenic carcinogens in target organ cells [19]. ^{32}P-postlabeling can distinguish DNA adducts of high molecular weight compounds like aromatics from non-modified DNA, but not those of low molecular weight like FA [20]. The major analytical chemistry alternative is to detect the free bases, deoxynucleosides, and deoxynucleotides produced from FA-modified DNA by conventional enzymic hydrolysis. FA reacts with DNA to form hydroxymethyl derivatives [19,21–25]. Since the modified free bases and nucleotides had been reported to crosslink with themselves [21], modified deoxynucleosides have been analyzed after enzymic hydrolysis in the present study. FA-modified deoxynucleosides had been detected but not quantified in vitro in Chinese hamster ovary cells [22] and human lymphoblast cells [19], using a high-performance liquid chromatographic (HPLC) method that did not allow resolution of the major analytes from interfering compounds in the tissue and hydrolytic enzyme blanks. Our group recently solved the chromatographic problems for the analysis of these modified deoxynucleosides from hydrolyzed human placental DNA [25].

In the present research, we sought to apply our chromatographic method to quantify the FA-modified deoxynucleosides released by enzymic hydrolysis from human nasal epithelial cells (HNEC) after exposure to FA in vitro, to determine the dose–effect relationships of modified deoxynucleosides to FA in solution and air, and to define the relationships of the modified deoxynucleosides with cell viability. Such analyses of HNEC from nose lavage or brush biopsy may be useful for epidemiology studies.

2. Materials and methods

2.1. Chemicals

Formalin (37% (w/w) FA in 10% methanolic aqueous solution) was purchased from Aldrich (Milwaukee, WI). Its actual FA content (34 ± 1%) was obtained by sodium sulfite titration [25]. Deoxyadenosine (dA), deoxyguanosine (dG), deoxycytidine (dC), thymidine (dT), human placental DNA (Type XIII), alkaline phosphatase (Type XVII from human placenta), Trypan blue solution (0.4%), Hanks® balanced salt solution, and 1× Trypsin-EDTA solution were purchased from Sigma (St. Louis, MO). DNase I (from bovine pancreas) and snake venom phosphodiesterase I (from *Crotalus adamanteus*) were obtained from ICN (Costa Mesa, CA). *O*-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) was obtained from Lancaster Laboratories (Lancaster, PA). Bis–Tris buffer, disodium EDTA, 10× phosphate-buffered saline (PBS), 1 M Tris–HCl, methanol (Optima), hexane (Optima), and ammonium acetate (HPLC grade) were purchased from Fisher Scientific (Tustin, CA). MEM medium was from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was supplied by Omega Scientific (Tarzana, CA). QIAmp DNA mini kits for isolation of genomic mitochondrial, bacterial, parasital, or viral DNA were obtained from QIAamp (Valencia, CA).

HNEC (catalog number 1210711) and HNEC growth medium (catalog number 2030701; bovine pituitary extract 0.4%, epidermal growth factor 0.5 ng/mL, insulin 5 μg/mL, hydrocortisone 0.5 μg/mL, epinephrine 0.5 μg/mL, triiodothyronine 6.7 ng/mL, transferrin 10 μg/mL, and retinoic acid 0.1 ng/mL) were purchased from Oligene (Berlin, Germany) via express air delivery on dry ice. The nasal epithelial cell growth medium is a sterile liquid culture medium without serum and of pH 7.6 and 285 mOsm/kg. Oligene obtained human nasal tissue (by "surgical operation" on a 20-year-old Caucasian donor), from which pure HNEC were isolated, and then primary HNEC cultures grown. After more culturing, cryovial samples were then produced and stored/transported at −80 °C. Each cryovial has at least 500 000 cells. At least five population doublings are guaranteed. The cells are not mutated or transformed under culture conditions.

2.2. Instrumentation

The HPLC system consisted of a Hewlett-Packard (HP) 1090 liquid chromatograph, a HP 1050 ultraviolet (UV) diode array variable wavelength detector, and a HP 3396 series II integrating recorder. A 10 μL manual injection loop was loaded with 100 μL. The C_{18} reversed-phase analytical column (Ultrasphere ODS 5 μm, 250 mm × 4.6 mm) was obtained from Beckman (Fullerton, CA). A Neubauer hemocytometer chamber, coverslips, and microscope (10×) were purchased from Fisher Scientific. Gas chromatography-^{63}Ni-electron capture detection (GC-ECD) was done on a HP5890 gas chromatograph with its injector at 250 °C and equipped with a 30 m × 0.32 mm i.d. 1 μm film DB-1701 chemically bonded fused-silica capillary column linked with ^{63}Ni-electron capture detector also at 250 °C.

2.3. Preparation of hydroxymethyldeoxynucleoside standards

No commercial hydroxymethyldeoxynucleoside standards are available. Standards were made by reacting deoxynucleosides with FA in 5 mM Bis–Tris buffer-0.1 mM disodium EDTA buffer [25]. Each deoxynucleoside and the mixture (0, 0.5, 1.0, 2.0, and 4.0 nmol/ml) were exposed to 100 μg/mL FA (1.0 μL 10% formalin diluted from concentrate with 10% methanolic water) at 37 °C for 6 h. The deoxynucleosides were resolved by a HPLC gradient elution program relative to 200 mM ammonium acetate: a linear gradient of 0–2% methanol for the first 40 min, then to 6% methanol from 40 min to 50 min. The total flow rate was 0.90 ± 0.01 mL/min. The absorbance was monitored at 254 nm.

The hydroxymethyldeoxynucleosides identified were: N^6-hydroxymethyldeoxyadenosine (N^6-dA), N^2-hydroxymethyldeoxyguanosine (N^2-dG), and N^4-hydroxymethyldeoxycytidine (N^4-dC). The amount of hydroxymethyldeoxynucleoside was determined by subtracting the normal deoxynucleoside after reaction from the original deoxynucleoside, and assuming one reaction product for each hydroxymethyldeoxynucleoside. The standard curves were then utilized for the deoxynucleoside mixture and DNA hydrolysate determinations. All concentrations were evaluated in triplicate. Our developmental work [25] showed the results for pure N^6-dA and N^2-dG collected from the column were equivalent with those from the indirect method.

2.4. Cell culture

The cryovial from Oligene was transferred on receipt to a water bath at 37 °C to achieve rapid thawing whilst stirring. Within a laminar flow hood, the completely thawed cell suspension was transferred aseptically to a 15 mL centrifuge tube and 4 mL of HNEC growth medium was added. The solutions were centrifuged at 300 × g for 5 min and the supernatant was removed aseptically. The cell pellet was resuspended in 2 mL of HNEC growth medium using a serological pipet. The cell suspension was then transferred into a 15 cm plastic petri culture dish (Corning) for cell culture. All incubations were carried out at 37 °C under an atmosphere of 5% CO_2 in a humidified incubator for the monolayers to reach confluence (usually within 1–2 weeks). Culture dish tops were always on. The culture medium was removed by aspiration every 2 days, and replaced with fresh medium.

At 80–90% confluence, the culture medium was removed by aspiration, the attached cells were washed three times with 1× phosphate-buffered saline, the cells treated with 2 mL 1× trypsin-EDTA at 37 °C for 10 min, and the trypsinization stopped with 2 mL 10% FBS/MEM medium. After centrifugation at 300 × *g* for 5 min, the supernatant was removed by pipet to provide cellular pellets. The pellets were washed three times again with 2 mL PBS buffer, and then suspended in 2 mL HNEC growth medium for subculturing.

This process was repeated in multiple subculturing steps with initially 0.3×10^6 cells/plate in 20 mL (18 mL growth medium, and 2 mL growth medium cell suspension) until enough cells were available for exposure experiments (2.5×10^6 initial cell density/plate).

2.5. Cell counting and cell viability

The HNEC cellular pellet was resuspended in PBS buffer and passed three times through a 26-gauge stainless steel needle to disperse cell aggregates.

Cell counting of each PBS suspension from each dish was done with a Neubauer hemocytometer chamber by light microscopy using Trypan blue dye exclusion [26]. The cells were first 1:1 diluted in 0.1% Trypan blue solution. The cells in the four corner squares and the middle square in both chambers were counted. Each square of the hemocytometer, with cover slip in place, represented a total volume of $0.1\ \mathrm{mm}^3$. Cell concentration was found by calculating the mean and its standard deviation of the 10 squares.

2.6. Incubation of HNEC with FA in solution

HNEC were treated in HNEC growth medium with FA (0, 10, 25, 50, 100, 250, 400, and 500 μg/mL) at 37 °C for 24 h, in triplicate, at a cell density of 2.5×10^6 cells in 20 mL growth solution (18 mL growth medium plus 2 mL growth medium cell suspension). The method blank sample had 10% methanolic water instead of FA as did each diluted FA solution. HNEC were harvested as in Section 2.4. HNEC were resuspended in PBS buffer and washed again at 300 × *g* for 5 min to remove FA. After cell counting, 1.4×10^7 HNEC in 200 μL PBS buffer obtained by amalgamating cell cultures of the same FA concentration were transferred to a 1.5 mL microcentrifuge tube.

DNA was isolated from the HNECs by using a QIAgen DNA mini kit according to the supplier's protocol. The amount of DNA was determined assuming that $A_{260} = 1$ was 50 μg DNA/mL [22]. Triplicate samples of DNA and reagent blanks were also processed and analyzed.

2.7. Incubation of HNEC exposed to FA in air

The HNEC culture medium was plated into culture dishes and exposed to FA in air (0, 10, 25, 50, and 100 ppm) at 37 °C for 24 h. Each appropriate 20-fold higher FA concentration was generated in a 100 L Tedlar gas bag (0, 61, 153, 305, and 610 μL 34% FA (w/w), respectively, were injected separately into the gas bag and then heated with a hairdryer to ensure water evaporation and gas mixing [27]). A personal portable pump fed the FA in the gas bag at 5.0 mL/min to mix with 95 mL/min 5% CO_2 in air to comprise the 100 mL/min exposing flow entering the incubator. A dynamic sampling solid sorbent method was used to confirm FA air concentration [27]. FA in growth medium with no HNEC present was also determined by hexane extraction after addition of PFBHA [28].

The temperature program for GC-ECD to determine formaldehyde PFBHA *O*-oxime was: 80 °C for 10 min and then 25 °C/min to 250 °C with holding there for 10 min. The column flow rate was 3.0 ± 0.3 mL/min. The signal was visualized with a HP 3396 integrator. The amount injected was quantified by the method of external standards using the pure formaldehyde derivative of PFBHA [28]. The concentration of FA in solution was calculated by assuming 1:1 stoichiometry, correcting for molecular weight and fraction injected, and knowing the volume of the growth medium. The concentration of FA in air was calculated similarly except the air volume drawn through the solid sorbent tube was used instead.

2.8. Detection of hydroxymethyldeoxynucleosides

The 0.5 ml DNA solutions were hydrolyzed by incubation with DNase I (10 U) and 10 mM magnesium chloride at 37 °C for 30 min. This was followed by addition of 75 μl 1 M Tris–HCl (pH 8.3), alkaline phosphatase (0.05 U) and snake venom phosphodiesterase (0.05 U) at 37 °C for 30 min to liberate the deoxynucleosides as published elsewhere [25]. The solution was centrifuged at 22 000 × *g* at 14 °C for 15 min to remove precipitated material.

Aliquots (10 μL) of the supernatant were injected into the HPLC after manual loop loading with 100 μL.

The same gradient elution program was used as for the deoxynucleosides above. However, peaks related to the enzyme blank continued to elute after 60 min, and column cleaning was necessary with 25% methanol at 1.5 mL/min for 15 min. Triplicate samples of DNA and reagent blanks were processed and analyzed.

2.9. Statistical analyses

Average area versus moles injected plots were generated for each treatment from the appropriate triplicates data. Linear regression analysis involved calculation of the slope, intercept, their standard deviations, correlation coefficient, and *P*-value. Detection limits (DL) were defined as three times the noise and lowest quantifiable limits (LQL) were defined as 10 times the noise.

Average and standard deviation comparisons were facilitated by one-way ANOVA and the Student *t*-test. *P*-values of 0.05 were considered to be significant.

3. Results

3.1. FA solution cytotoxicity

HNEC were successfully grown in 15 cm culture dishes by 144 h after seeding. Cultures exposed to 0, 10, 25, 50, 100, 250, and 500 μg/mL FA for 24 h showed a dose-dependent reduction in cell viability as expressed in terms of trypan blue exclusion (Fig. 1). The toxicity threshold for ≤90% viability appeared to be between 100 and 250 μg/mL initial FA culture medium concentration. An initial concentration of 500 μg/mL resulted in approximately 70% viability.

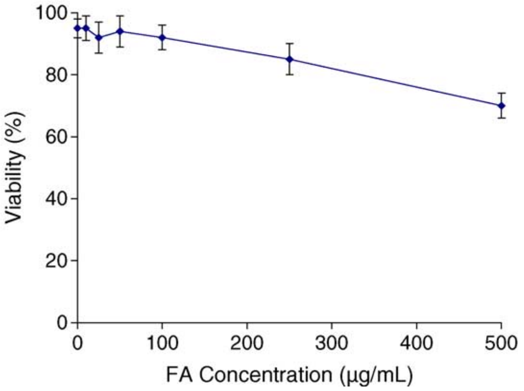

Fig. 1. Viability and standard deviation ($n = 3$) in HNEC cultures incubated 24 h with different initial solution FA concentrations up to 500 μg/mL.

When % HNEC nonviability was plotted against FA concentration in μg/mL, linearity was observed for FA solution concentrations of 10–500 μg/mL obeying Eq. (1):

$$\%\,\text{nonviability} = 0.0517\text{FA} + 3.38, \quad r = 0.9956; \quad n = 5; \quad P \leq 0.05 \qquad (1)$$

3.2. Optimization of the deoxynucleoside HPLC analytical method

All deoxynucleosides and modified deoxynucleosides eluted with baseline resolution for the optimized procedure except for a small interference on dC (Fig. 2). This dC interference was characteristic of HNEC as the enzyme blank did not contain this peak.

In the preceding developmental work [25], the effects of ammonium acetate concentration, flow rate, and methanol concentration on the elution of deoxynucleosides generated from DNA and enzyme reagent blank peaks were investigated starting with the conditions of Beland et al. [22]. The results with the method optimized for human placental DNA [25] initially showed dC and N^2-dG had interferences from unexposed HNEC using 5 mM ammonium acetate and the flow gradient. Setting the flow at 0.3 mL/min and increasing the ammonium acetate concentration resolved the N^2-dG interference but caused more interference on dC and N^4-dC beyond 200 mM. Use of 200 mM ammonium acetate produced resolution for N^2-dG, dC, and N^4-dC. Since the whole gradient elution program still exceeded 2 h, the flow-rate of the mobile phase was optimized to 0.9 mL/min so that separations occurred in less than 1 h and allowed minimum dC interference (Fig. 2). Under these conditions, the three modified deoxynucleosides from HNEC had baseline separation. The retention times were 14.3, 43.1, 54.3 min for N^4-dC, N^2-dG, and N^6-dA, respectively (Fig. 2A and B). Determination of hydroxymethylnucleosides from digested HNEC required cleaning of the column before the next injection but this was not required for standardizations. Only one extra peak resulted from the reaction of

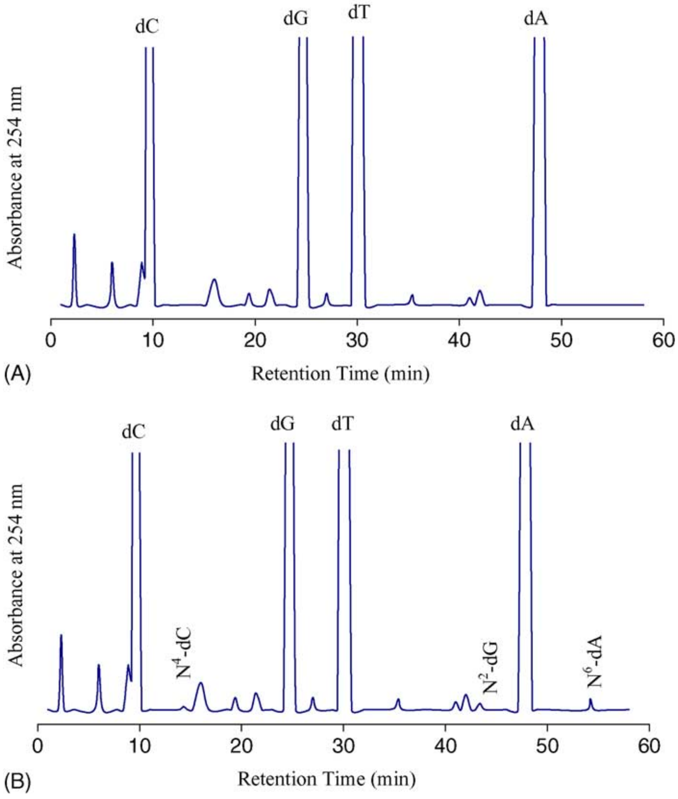

Fig. 2. Chromatograms of the reverse-phase C_{18} column HPLC separation of normal deoxynucleosides, hydroxymethyldeoxynucleosides, and enzyme interferences. (A) Normal deoxynucleosides and enzyme interferences from HNEC. (B) Normal deoxynucleosides, modified deoxynucleosides, and enzyme interferences from HNEC exposed to 500 μg/mL FA (24 h, 37 °C). The HPLC program: 200 mM ammonium acetate and a linear gradient of 0–2% methanol for the first 40 min, then to 6% methanol from 40 min to 50 min, at 0.9 mL/min.

FA with each pure deoxynucleoside under these synthesis conditions, as reported elsewhere [25]. Longer reaction times and different pH conditions lead to crosslinked products as impurities [20,25].

3.3. *Hydroxymethyldeoxynucleoside standard curves*

Fig. 3 illustrates the linearity of the standard curves of the normal deoxynucleosides. The standard curves for the hydroxymethyldeoxynucleosides are shown in Fig. 4. The slopes for N^6-dA and N^2-dG are not different at $P \leq 0.05$, but both slopes are significantly bigger than the slope for N^4-dC. The slopes of the normal deoxynucleoside and its hydroxymethyldeoxynucleoside are not significantly different.

Table 1 shows the DL and LQL of three hydroxymethyldeoxynucleosides for this HPLC program. The LQLs for N^6-dA, N^2-dG, and N^4-dC are 10, 9.3, and 20 pmol/10 μL, respectively, with N^4-dC being the least sensitive.

Table 1
The DL and LQL of unmodified- and hydroxymethyl-deoxynucleosides for analysis of DNA from HNEC

	Retention time (min)	DL[a] (pmol/10 μL)	LQL[b] (pmol/10 μL)
dC	9.6	5	18
N^4-dC	14.3	6	20
dG	24.7	2	8
dT	30.1	5	16
N^2-dG	43.1	3	9
dA	47.8	3	11
N^6-dA	54.3	3	10

[a] DL: three times S.D.

[b] LQL: 10 times S.D.

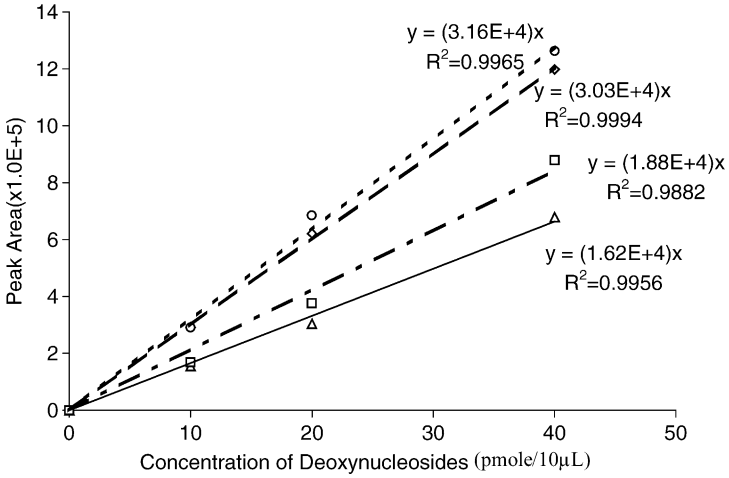

Fig. 3. Calibration curves and regression equations describing the relationship between its peak area and the amount of deoxynucleoside (dA, ◇; dG, ○; dC, △; dT, □) in an injection volume of 10 μL. The slope units are in area units/pmol.

3.4. Quantitation of deoxynucleosides in digested HNEC after exposure to solution FA

The time to quantify modified DNA in HNEC by HPLC with column cleaning was 65 min and the sampling protocol is finished within 3 h. About 106 μg DNA was obtained from 1.4 × 10^7 HNEC. The absorbance A_{260}/A_{280} ratio of the isolated DNA was 1.73 ± 0.30, which indicated that there was very little contaminating RNA or proteins.

Fig. 5 shows amounts of hydroxymethyldeoxynucleosides in 10 μL injections after 1.4 × 10^7 HNEC were exposed to different FA concentrations at 37 °C for 24 h. N^4-dC was below the LQL. Amounts of 18.3 ± 1.5 pmol N^6-dA and 12.4 ± 1.2 pmol N^2-dG were found in 10 μL after 500 μg/mL FA exposure.

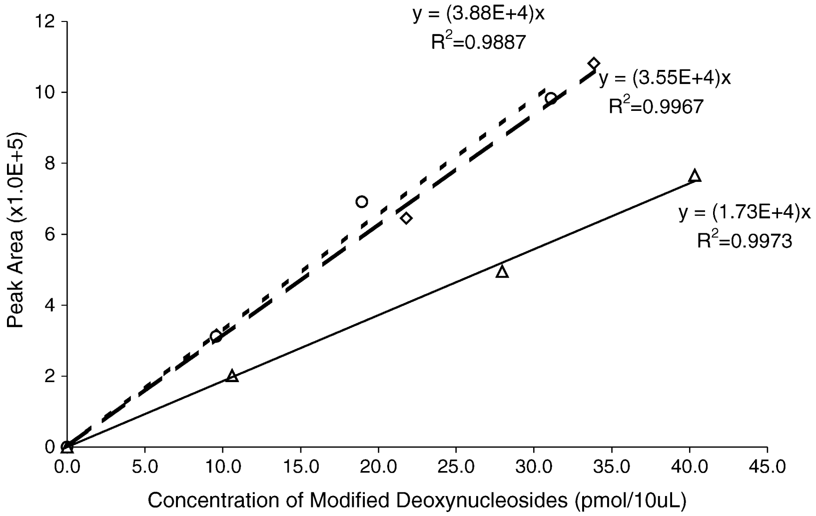

Fig. 4. Calibration curves and regression equations describing the relationship between its peak area and the amount of hydroxymethyldeoxynucleoside (N^6-dA, ◇; N^2-G, ○; N^4-dC, △) in an injection volume of 10 μL. The slope units are in area units/pmol.

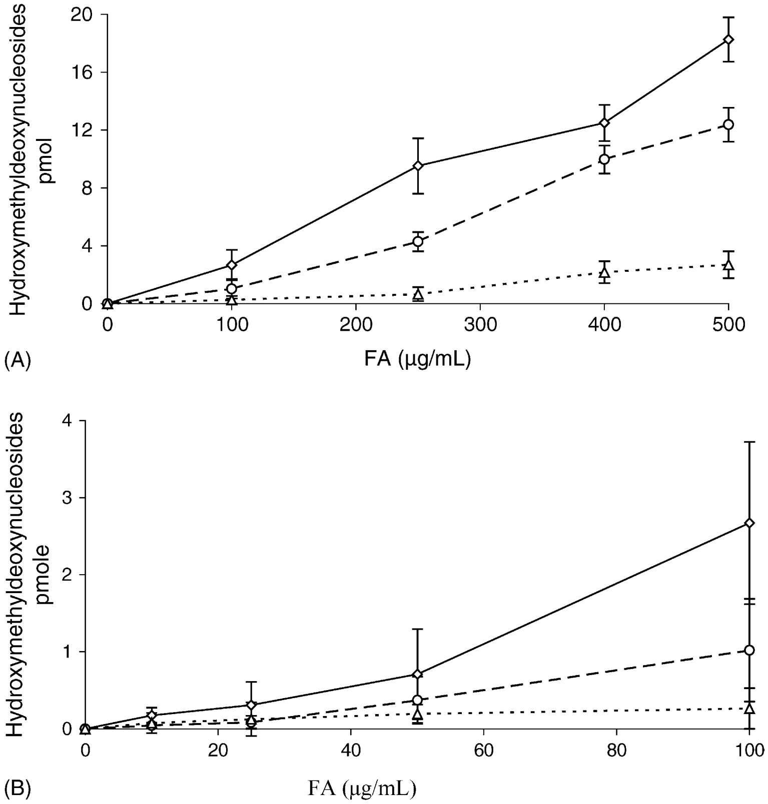

Fig. 5. Dose effect curves for hydroxymethyldeoxynucleosides (N^6-dA, ◇; N^2-dG, ○; N^4-dC, △) in 10 μL injections after 1.4×10^7 HNEC were exposed to different FA concentrations in solution at 37 °C for 24 h. (A) 100–500 μg/mL FA; (B) 10–100 μg/mL FA.

However, 0.17 ± 0.10 pmol N^6-dA, 0.042 ± 0.038 pmol N^2-dG, and 0.08 ± 0.13 pmol N^4-dC were found in 10 μL after 1.4×10^7 HNEC were exposed to 10 μg/mL, clearly near DLs. The order of yields of hydroxymethyldeoxynucleosides in HNEC exposed to a specific FA concentration was N^6-dA > N^2-dG > N^4-dC. The actual corresponding respective LQLs in pmol/mg DNA were therefore 97, 88, and 190.

3.5. Incubation of HNEC exposed to air FA

The hydroxymethyldeoxynucleosides in digested HNEC exposed up to 100 ppm in air were not detected above the LQL even though the HNEC were viable. Fig. 6 shows the FA concentration in culture medium without HNEC and in water alone after exposing the culture dishes to air FA concentrations in the CO_2 incubator. The water FA concentration shows

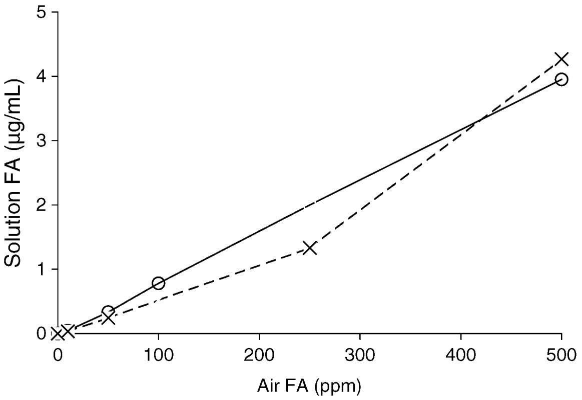

Fig. 6. FA concentration absorbed by culture medium (×) without HNEC compared with by water (○) alone after FA air exposure from 10 ppm to 500 ppm for 24 h (v/v).

a direct linear relationship with air FA concentration. The medium FA concentrations were generally lower than for water alone except at 500 ppm FA where there was no statistical difference at $P \leq 0.05$. The slope for water was 0.0079 μg/mL solution/ppm air ($r = 0.9997$; $P \leq 0.05$); and that for the medium up to 250 ppm FA was 0.0053 ($r = 0.9951$; $P \leq 0.05$). Therefore, the medium at or below 250 ppm FA in air contained 67% of the FA of water at the same FA exposure conditions.

4. Discussion

The background HPLC blank for isolated DNA from HNEC differed from that from human placental DNA in vitro, necessitating some changes to the analytical method of Zhong and Que Hee [25]. The chromatograms exhibited well-resolved peaks for all three modified deoxynucleosides at retention times <60 min (Fig. 2). However, the separation of dC was still incomplete due to a small interference from HNEC. The present study for the first time achieved chromatographic separation of analytic peaks from tissue interferences to allow accurate quantitation of hydroxymethyldeoxynucleosides of hydrolyzed DNA of HNEC after exposure to known concentrations of FA in solution (Fig. 2). The results demonstrate that hydroxymethyl deoxynucleosides are formed in HNEC after exposure to FA in solution just as they are in vitro with free deoxynucleosides and with placental DNA [25].

Since more N^6-dA is formed than N^2-dG of the two modified purine deoxynucleosides, N^6-dA is the most sensitive potential marker of FA dose. N^6-dA is also more stable than N^2-dG [25]. The best potential marker of effect is most likely to be N^2-dG since three hydrogen bonds are involved rather than just the two for dA, and N^6-dG is more sensitive than N^4-dC.

Although HNEC have been difficult to maintain in cell culture [29] and may form domes [30], this study has also confirmed that HNEC can be cultured successfully in vitro. The use of HNEC for method development before harvesting of nasal cells from live humans is therefore feasible.

The degree of HNEC cytotoxicity caused by FA in solution depended on the exposure concentration, with a threshold for ≤90% viability between 100 and 250 μg/mL FA. FA did cause a dose-dependent decrease in viability that was linear between 10 and 500 μg/mL FA (Fig. 5; Eq. (1)). The production of each hydroxymethylnucleoside was also linear at $P \leq 0.05$ with solution FA concentration and % HNEC nonviability. Huber et al. [31] have reported that microvascular endothelial cells exposed to 250 μg/mL formalin suffered 34% decrease in viability. In comparison at this concentration, we observed about 15% viability decrease for HNEC. FA cytotoxicity also has been investigated in cultured human lung A549 epithelial cells [32]. Induction of DNA double strand breaks occurred only after cell viability was less than about 60%. The FA threshold adverse concentration at 8, 24, and 72 h of exposure was above 100 μM (3 μg/mL). Such results show that effects on DNA can be observed at low FA concentrations in solution, and that breaching cell homeostasis allows more FA to attack DNA.

Studies of FA cytotoxicity have also been reported for in vitro mammalian cell culture, mostly concerning mutagenesis [33–38]. Speit and Merk [38] showed mutagenicity in lymphoma L5178Y cells above an initial FA solution concentration of 62.5 μM (1.86 μg/mL) after a 2 h exposure. The effect was predominantly in the small colony mutants, suggestive of mutations being caused by chromosomal aberrations rather than from DNA cross-linking [36,37]. In the γ-ray-induced DNA migration in the Comet assay of V79 Chinese hamster cells, relative cloning efficiency decreased above 62.5 μM (1.86 μg/mL), and induction of sister chromatid exchange and increased HPRT mutant frequency occurred at 250 μM (7.5 μg/mL) [37]. This suggested that cytotoxicity events preceded observable mutagenic phenomena. The importance of cytotoxicity in tumor formation is implied by the better fit of the cytolethality-regenerative cellular proliferation model relative to the DPC model to explain the dose response of nasal cell tumors and preneoplastic lesions caused by FA above 6 ppm in F344 rats [39], and also for the need of cytotoxicity to start DNA double strand breaking for human lung A549 epithelial cells [32].

Hester et al. [40] also instilled 40 μL of 400 mM (12 000 μg/mL) FA into each nostril of F344 rats and after 24 h removed nasal epithelia for microarray hybridization analysis. Dysregulation of multiple cellular pathways occurred, not surprising at this very high toxic FA concentration. Acute and subchronic inhalation exposures of male F344 rats in vivo have shown that air concentrations above 6 ppm for 6 h/day, 5 days/week over 9 days and 6 weeks cause damage (cuboidal-transitional and respiratory epithelia) and hyperplasia to specific sites in the nose that were similar to those in the nose of the rhesus monkey [41,42]. After 1 day of exposure to rats, mild damage was caused at 6.0 ppm, moderate damage at 10 ppm, and marked damage at 15 ppm; there was no discernable damage below 6 ppm [41]. The relevance of these in vivo animal findings to humans is unclear, but there is a concentration threshold for the adverse effects. The dose thresholds for human cancers from epidemiology studies have already been discussed [6–12]. Pantropic and mutant p53 proteins in human peripheral lymphocytes also increase above a FA air exposure concentration threshold of 0.113 ppm [17]. *p53* is implicated in apoptosis, cancer protection, metastasis, DNA repair, and necrosis [43,44].

The FA concentration in our HNEC growth medium blank in vitro is low after air FA exposure. For example, only 4–5 μg/mL was detected in the medium blank and water after 500 ppm air exposure (Fig. 6). Using the data of Fig. 5B, amounts of 4–5 μg/mL of FA in solution produce DNA hydroxymethyldeoxy-nucleosides well below their LQLs (Table 1), accounting for our actual observations of hydroxymethyldeoxynucleosides. Decreasing the culture medium solution layer thickness will also allow more FA to be concentrated. Higher air concentrations for 24 h exposure or longer exposures may be also required to produce quantifiable hydroxymethyldeoxynucleosides under these diffusive conditions of FA exposure. Having the cultures open may work. An alternative is to bubble FA into the culture medium rather than relying on diffusion, but this will require many changes in the cell-culturing method. The HNEC cell culture exposure format may not simulate exposure conditions in the nose where the protective liquid film is much thinner than for our HNEC in vitro culture conditions, and air flow rates are much higher than diffusion, allowing more FA to be absorbed. Since the FA exposures were only over 24 h, this does not simulate the in vivo exposure conditions in rats, and in any case, only about five cellular doublings are expected from the cultured HNEC.

Another strategem is use a more sensitive technique like liquid chromatography–mass spectrometry–mass spectrometry (LC–MS–MS) multiple reaction monitoring (MRM) where fmol sensitivity has been achieved for the deoxynucleoside 8-oxo-7,8-dihydro-2′-deoxyguanosine [45]. Since our solution results indicate cytotoxicity begins between solution formaldehyde concentrations of 100–250 μg/mL, extreme sensitivity is not essential to study the effects of cytotoxicity.

Another research question relates to the effect of hydroxymethylation relative to hyper- and hypo-methylation, the latter being known to be important in the cancer progression phase [46].

The development of an HPLC assay that allows analysis of modified deoxynucleotides of FA now may also allow a decision as to whether single base alterations are more important than crosslinking in the genesis of mutagenesis and nasal cancer in HNEC caused by FA, since now both assays can be done simultaneously. The HPLC assay may also be useful for compounds that generate FA in situ like aspartame, bis(chloromethyl) ether, dichloromethane, hexamethylphosphoramide, diazolindinyl urea, and dimethylol urea. The technique may be applicable in biological monitoring of nasal cells sampled by lavage or brush biopsy [47,48].

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