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TOXICITY OF TOBACCO-RELATED ALDEHYDES IN CULTURED HUMAN BRONCHIAL EPITHELIAL CELLS

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INTRODUCTION

One approach to the problem of extrapolating experimental animal data to humans is the development of in vitro model systems using human tissues and cells. Remarkable progress has been made during the last few years in establishing conditions for culturing normal human epithelial tissues and cells. Isolated epithelial cells can now be transferred at clonal density for three or more times and grown for more than 30 cell generations in serum-free media (Lechner et al., 1984). Pathobiological effects of many environmentally and endogenously occurring agents that may be cytotoxic or carcinogenic to the lung are presently being studied in epithelial cells cultured from human bronchus. Parameters measured include cell survival, growth and differentiation, metabolism of chemical carcinogens, DNA damage and repair, and malignant transformation (Harris et al., 1984).

Among more than 6,000 identified components in tobacco smoke, several reactive and volatile aldehydes are found in the gaseous phase and are of interest because of their potential carcinogenicity in the human respiratory tract. In particular, acetaldehyde, acrolein, and formaldehyde are present in amounts ranging from ~~ng~~ to mg per cigarette (Wynder and Hoffman, 1979).

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Such aldehydes possess a carbonyl-related high affinity for nucleophilic sites (i.e., sulfhydryl- and amino-groups) in cellular macromolecules. These aldehydes are metabolites of xenobiotics, e.g., N-nitrosodimethylamine, cyclophosphamide, and ethanol, and are also formed endogenously as products of normal intermediary metabolism.

In this study we have investigated effects of these aldehydes on different biological parameters including colony survival, clonal growth, cross-linked envelope formation, content of cellular thiols, and DNA damage, i.e., DNA-single strand breaks (SSB) and DNA-protein crosslinks (DPC), in cultured human bronchial epithelial cells. The potency of these aldehydes to cause different cyto- and genotoxic effects is compared and discussed.

MATERIALS AND METHODS

Cells and Cell Culture Conditions

Normal human tracheo-bronchial tissues were obtained from donors at the time of autopsy or surgery and maintained in culture according to methods described by Lechner et al., 1984. Primary epithelial cell cultures were enzymatically dissociated with calcium-free HEPES buffered saline solution containing EDTA, 0.02%; polyvinylpyrrolidine, 1%; and trypsin, 0.02%, and subcultured into surface-treated (fibronectin, 10 µg/mL; bovine serum albumin, 10 µg/mL; and Vitrogen collagen, 30 µg/mL) Petri dishes containing LHC-medium. Normal bronchial fibroblast lines were also developed from explanted bronchial tissues (Lechner et al., 1984).

Survival and Clonal Growth Assay

Epithelial cells (5×10^3) or fibroblasts (8×10^2) were seeded in 60 mm dishes. After 24 to 48 hrs in culture, cells were exposed to the indicated doses of aldehyde in serum-free LHC medium for various times at 37°C. Subsequently, fresh aldehyde-free growth medium was added and after 7-9 days of incubation, the cultures were fixed and stained. The mean colony forming efficiency (CFE) was determined from duplicate experiments and based on colonies each containing at least 16 cells. The clonal growth rate (CGR), i.e. cells per colony, was counted using an ARTEK 800 image analyzer. To obtain the mean clonal growth rate (PD/D), the number of cells in 9 randomly selected clones from each of 2 dishes was counted and the $\log^2$ of the average number

of cells per clone was divided by the number of days of incubation. Variance-weighted Student's t-test was used to evaluate significant differences between control and treated groups.

Morphologic Quantitation

Cultures were fixed with a phosphate buffered 1% formaldehyde and 4% glutaraldehyde solution for light and electron microscopy. For light microscopic studies, cultures were stained with May-Grünwald Geimsa stain. Cell planimetry was performed with the aid of the image analyzer and 250 cells analyzed for each condition.

Cross-linked Envelope (CLE) Formation

The percentage of cells capable of forming CLE was ascertained by a modification of the method of Sun and Green, 1976, as described by Willey et al., 1984. Cells were inoculated at 50,000 cells/well onto coated 12-well plates (24 mm wells) containing LHC-4 medium. Twenty-four hrs later, the number of cells per well was calculated using a grid and the medium was replaced with medium containing the test compound plus 0.8% agar (LGT agarose, Miles Biochemicals, Elkhorn, IN). After a 6 hr incubation, 2 mL of sodium dodecyl-sulfate (SDS) (4%) and dithiothreitol (20 mM) in distilled H_2O was added over the agar. After a further 4 hr incubation at 37°, the number of SDS-insoluble CLEs per well and percentage of CLE per cell population were then calculated.

Total Thiol and Glutathione (GSH) Content

Bronchial cells were plated at 5-10 x 10^4 cells per 60 mm dish 48 to 72 hrs before assay of total thiol content. Briefly, cells (2-3 x 10^5) were exposed to the indicated aldehydes for 1 hr in serum-free LHC medium. After washing twice with phosphate buffered saline (PBS), cells were lysed and protein-precipitated by addition of 0.7 mL 6.5% trichloroacetic acid to the dish. The cellular precipitate was removed from the dish by scraping with a rubber policeman. The resulting suspension containing protein and released cellular thiols was centrifuged at 1,500 xg for 8 min and 0.5 mL of supernatant analyzed for total thiol content according to Saville, 1958. GSH was specifically analyzed by fluorometry or by high performance liquid chromatography as described by Grafstrom et al., 1980.

Alkaline Elution Assay of DNA Damage

The procedure used was developed and reviewed by Kohn et al., 1981. The cells were removed at 4°C in a solution of Dulbecco's Ca^{2+}/Mg^{2+}, - free phosphate buffered saline containing 15 nm Na_2EDTA with a rubber policeman and collected onto a 2-μm polycarbonate filter (Nuclepore, Pleasanton, CA) to measure DNA SSB. The cells were lysed with 5 mL of 2% SDS/0.1 M glycine/0.02 M Na_2EDTA (pH 9.6), and 2 mL of the same solution containing 0.5 mg/mL of proteinase K was then pumped through the filter at 0.04 mL/min. This solution was followed by 0.02 M EDTA (acid form)/0.1% SDS plus tetrapropylammonium hydroxide (pH 12.2). Eluted fractions were collected and assayed for radioactivity. The combination of the polycarbonate filtration, proteinase K digestion, and the addition of SDS in the eluting solution completely removed DPC caused by aldehydes at concentrations up to 1 mM. An internal standard, [^{3}H]thymidine-labeled L1210 cells that had received 300 rads at 0°, was included in each assay. To measure the extent of DPC, the assay was modified by omission of the proteinase digestion of the cell lysates and SDS was deleted from the eluting solution. The cells were exposed to 3 krads of ionizing radiation (to shear cellular DNA) before these cells were collected on polyvinyl chloride filters (Millipore Corp., Bedford, MA). The SSB and DPC frequencies were estimated and calculated as described by Kohn et al., 1981.

Sources of Test Agents

Acetaldehyde and acrolein were obtained from Aldrich Chemical Company and formaldehyde from Fisher Scientific Company.

RESULTS

Cytotoxicity of acrolein, formaldehyde, or acetaldehyde was investigated in bronchial epithelial cells by measurement of the CFE of secondary cultures at clonal density. Clearly, acrolein was substantially more cytotoxic than formaldehyde which in turn was markedly more toxic than acetaldehyde. The dose required to reduce CFE to 50% of control from 1 hr exposure, was about 2 μM for acrolein, 400 μM for formaldehyde, and 10 mM for acetaldehyde (Table 1). Similar effects on survival were observed in exponentially growing human fibroblasts (data not shown). However, mass cultures of quiescent fibroblasts maintained at confluency were less sensitive to aldehyde-induced cytotoxicity. When confluent cells were subcultured 48 hrs after exposure to either

formaldehyde or acrolein, approximately 5-fold higher concentrations were required to inhibit CFE to a similar extent as in exponentially growing cells (data not shown).

TABLE 1. EFFECTS OF TOBACCO-RELATED ALDEHYDES ON THE COLONY FORMING EFFICIENCY OF HUMAN BRONCHIAL EPITHELIAL CELLS[a]

	Colony Forming Efficiency (%)		
Concentration (mM)	Acrolein	Formaldehyde	Acetaldehyde
0.001	92	-	-
0.003	30	-	-
0.010	2	100	-
0.030	<1	95	-
0.100	-	70	-
0.300	-	58	100
1.00	-	4	77
3.00	-	<1	72
10.0	-	-	50
30.0	-	-	22
100	-	-	<1

[a] Cells were exposed for 1 hr to the indicated doses of respective aldehyde in serum-free LHC medium. "-", means not tested.

Differentiation of cultured epithelial cells often results in decreased clonal growth rate, formation of cross-linked envelopes, and flattening of cells leading to an increased surface area (Willey et al., 1984). As assayed by CGR the aldehydes were not found to be mitogenic, and all were inhibitory in a dose-dependent fashion. Inhibition of CGR to approximately 50% was obtained at 10 μM acrolein, 200 μM formaldehyde or 30 mM acetaldehyde (Table 2). At these doses all aldehydes markedly increased the formation of cross-linked envelopes, considered as one of the late events in terminal squamous differentiation. Median cell planar surface area was increased approximately three-fold by exposure to acetaldehyde or acrolein, but insignificantly changed by formaldehyde.

The cellular content of thiols, mainly GSH, protects the cell against cytotoxic effects by reacting with electrophilic compounds. The content of total intracellular thiols in epithelial cells was decreased to less than 50% after 10 μM of acrolein

and reduced to 6% at 100 μM (Table 3). A similar and dose-dependent depletion of thiol content was observed in both fibroblasts and epithelial cells. In comparison, formaldehyde and acetaldehyde were each much less effective than acrolein because at 100- or 300-fold higher concentrations, total thiols were only decreased with 20-30 percent in both cell types. GSH was determined to constitute 85-90 percent of the total thiols in both epithelial cells and fibroblasts. The content of GSH was decreased to a similar extent as the total thiol levels by all aldehydes (data not shown).

TABLE 2. EFFECTS OF TOBACCO-RELATED ALDEHYDES ON GROWTH AND DIFFERENTIATION OF HUMAN BRONCHIAL EPITHELIAL CELLS[a]

Aldehyde (mM)	Clonal growth rate (PD/D)	Median cell size (sq μ)	Cross-linked envelope formation (%)
Control	1.02 ± 0.10	930 ± 270	2 ± 1
Acrolein (0.01)	0.58 ± 0.06	2550 ± 840	23 ± 3
Formaldehyde (0.20)	0.52 ± 0.08	983 ± 400	12 ± 2
Acetaldehyde (30.0)	0.50 ± 0.07	3200 ± 680	7 ± 2

[a] Assays were performed as described in Materials and Methods and results expressed as mean ± S.D.

TABLE 3. EFFECTS OF TOBACCO-RELATED ALDEHYDES ON THIOL LEVELS IN HUMAN BRONCHIAL FIBROBLASTS AND EPITHELIAL CELLS[a]

Aldehyde (mM)	% of Control	
	Fibroblasts	Epithelial cells
Acrolein		
0.003	91	97
0.010	46	39
0.030	11	18
0.100	6	5
Formaldehyde		
0.1	97	-
0.3	82	-
1.0	71	-
3.0	71	68
Acetaldehyde		
1.0	108	-
3.0	99	-
10.0	78	80

[a] Cells were exposed to the various concentrations of the respective aldehyde for 1 hr in serum-free LHC-medium. From 5 different determinations, fibroblasts contained 59 ± 11 and epithelial cells 73 ± 15 nmol total thiols per 10^6 cells. "-", means not tested.

The potency of aldehydes to cause DNA damage was measured by the sensitive alkaline elution technique. Exposure of cells to 100 μM of either acrolein or formaldehyde for 1 hr caused substantial DNA damage, whereas 10-fold higher concentrations of acetaldehyde caused no detectable DNA damage (Table 4). Formaldehyde caused substantially higher levels of DPC than SSB, whereas the level of both types of lesions was similar to exposure to acrolein. Nonsignificant levels of DNA-DNA cross-links were induced by these aldehydes at the tested concentrations.

TABLE 4. DNA SINGLE STRAND BREAKS (SSB) AND DNA PROTEIN CROSS-LINKS (DCP) CAUSED BY DIFFERENT ALDEHYDES IN HUMAN BRONCHIAL EPITHELIAL CELLS[a]

Agents	Dose (μM)	SSB/10^{10}Daltons	DPC/10^{10}Daltons
Formaldehyde	100	1.4	10.8
Acrolein	100	6.9	6.8
Acetaldehyde	1000	<1.0	<2.0

[a] Cells were exposed to the indicated aldehyde for 1 hr in serum-free LHC medium and subsequently assayed for DNA damage by the alkaline elution technique.

DISCUSSION

Human bronchial epithelial cells in serial culture provide an useful in vitro model system for studying pathobiological effects of environmental chemicals. As a broad endpoint, cytotoxicity in terms of reproductive sterilization can be quantitated by measuring colony forming ability of cells passaged at low density. Exposure of epithelial cells to any of the three tobacco-related aldehydes caused a different pattern of pathobiological response. Acrolein markedly reduced the CFE at 3 μM whereas one or two orders of magnitude higher concentrations of either formaldehyde or acetaldehyde were required to significantly decrease CFE. As compared to the effective dose required to inhibit colony survival, three-fold higher concentrations of acrolein were required to significantly decrease clonal growth rate of a small number of surviving cells. In contrast, formaldehyde significantly decreased the clonal growth rate at concentrations that only slightly affected colony survival. Acetaldehyde caused a response pattern of decreasing both colony survival and clonal growth rates only at very high concentrations, i.e. at least 10 to 30 mM.

Terminal squamous differentiation of epithelial cells is an important response from several aspects. Terminal differentiation, ultimately leading to cell death, causes a requirement for epithelial cell renewal in vivo. Similarly, dividing bronchial epithelial cells in culture with time become committed to undergo terminal squamous differentiation, ultimately leading to cell death and shedding from the tissue culture dishes. Compounds that specifically induce terminal differentiation could be confused as cytotoxic in the CFE assay. Another aspect of differentiation involves mechanisms of importance in multistep carcinogenesis. Resistance to inducers of terminal differentiation is regarded as one of several potential clonal expansion advantages that could lead to selective survival and growth of carcinogen-"initiated" preneoplastic or neoplastic cells (Yuspa and Morgan, 1981; Harris et al., 1985). Selective growth of these cells may be facilitated from an enhanced induction of differentiation of the normal "non-initiated" cells. Because epidemiological and laboratory studies suggest that tobacco smoke has tumor promoting activity (Wynder and Hoffman, 1979), it is of interest to note that tobacco-related aldehydes induced terminal differentiation as judged by the formation of cross-linked envelopes. The concept of enhanced terminal differentiation of normal cells as a selection mechanism for preneoplastic cells is strongly supported by the fact that agents with tumor-promoting activity in the mouse skin carcinogenesis model such as aplysiatoxin, 12-O-tetradecanoylphorbol-13-acetate or teleocidin B, are the most potent inducers of terminal differentiation of normal human bronchial epithelial cells currently known (Harris et al., 1985).

Conjugation with GSH constitutes a major cellular defense mechanism against toxic compounds as well as many electrophilic products of metabolic activation. As an indicator of chemical reactivity and to what extent the tobacco-related aldehydes pose a cellular challenge, the effects on intracellular total thiols and GSH were investigated in bronchial cells. Exposure to acrolein markedly depleted cells of total thiols and GSH in a dose-dependent manner, whereas exposure to formaldehyde or acetaldehyde only caused a minor decrease in the thiol content. Significant depletion of thiols was induced by acrolein only at doses that had markedly reduced the colony survival. These data suggest that either mechanisms other than thiol depletion are of importance to cause reproductive sterilization of cells or that only small, experimentally undetectable changes in total thiols may be causative. The sulfhydryl group of GSH reacts with acrolein at the unsaturated β-carbon, and in vivo the corresponding mercapturic acid derivative is excreted in urine. Although formaldehyde dehydrogenase-catalyzed oxidation of formaldehyde to

formate also require reversible binding to GSH, formaldehyde is known to readily and irreversibly react with cysteine to give the thiazolidine-carboxylic acid (Schauenstein et al., 1977).

When the aldehydes were investigated for their ability to induce DNA damage, formaldehyde or acrolein both caused SSB and DPC but at different ratios. DNA damage by formaldehyde was induced at doses of comparatively high colony survival whereas the acrolein induced damage was obtained at markedly cytotoxic concentrations.

Several carcinogenic N-nitrosamines, including those found in tobacco smoke, e.g. N-nitrosodimethylamine, during metabolism lead to one-to-one stoichiometric generation of alkyldiazonium ion and aldehyde. Whereas the alkyldiazonium ion formed in this reaction is thought to be responsible for the carcinogenicity of N-nitrosamines, a possible contribution of aldehyde has been largely neglected. In addition to directly damaging DNA, formaldehyde inhibits repair of DNA damage caused by different chemical and physical carcinogens, including ionizing radiation, UV-radiation, benzo(a)pyrene diol epoxide, or N-methyl-N'-nitrosourea (Grafstrom et al., 1983, 1984). The DNA repair of O^6-methylguanine and the resealing of ionizing radiation-induced SSB seem to be preferentially sensitive. A number of mechanisms may be involved in the inhibition of DNA repair by formaldehyde. The high reactivity of the chemical probably causes methylation of chromatin or other proteins, including enzymes critical to DNA repair processes. Such interaction with DNA repair by formaldehyde occurs concommitantly with potentiation of the cytotoxicity of ionizing radiation or N-methyl-N'-nitrosourea, and the mutagenicity of N-methyl-N'-nitrosourea in normal human fibroblasts (Grafstrom et al., 1984).

In this study, different biological endpoints, i.e. cell reproductive sterilization, depletion of GSH, and induction of DNA damage, in human bronchial epithelial cells were induced by the aldehydes to a similar extent in cultured bronchial fibroblasts. These findings are of importance in light of the extensive use of cultured fibroblasts in toxicological evaluations during the past two decades. Acrolein, acetaldehyde, or formaldehyde all cause a distinguishable pattern of pathobiological consequences in bronchial epithelial cells. The cytotoxicity decreased in the order of: acrolein, formaldehyde and acetaldehyde by orders of magnitude. Aldehydes cause terminal squamous differentiation of epithelial cells as judged from decreased clonal growth rates and increased formation of cross-linked envelopes. Of several cyto- and genotoxic endpoints investigated, marked effects were induced by exposure to acrolein and

formaldehyde at concentrations less than 100 μM but higher concentrations of acetaldehyde were required. The many biological effects induced by formaldehyde, including differentiation, DNA damage, mutation and inhibition of DNA repair in human cells seem particularly relevant since these effects occur at moderately low levels of cytotoxicity.

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