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SHORT COMMUNICATION

Inhibition of intercellular communication in human keratinocytes by fractionated asphalt fume condensates

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Asphalt fume condensate (AFC) and chromatographically separated fractions have been shown to cause cancer in mouse skin. The levels of known carcinogenic initiators in these complex mixtures, however, are considered too low to account for their carcinogenic potency. It has been proposed that AFC may contain co-carcinogenic or tumor-promoting agents in addition to carcinogenic initiators. Modulation of gap junctional intercellular communication (GJIC) has been implicated as an important effect of tumor promoters. In this study, we examined the effect of five chromatographically generated fractions of AFC on GJIC in cultured human epidermal keratinocytes (HEK). HEK cells were exposed overnight to medium containing DMSO extracts of AFC fractions. GJIC was evaluated by dye-coupling of micro-injected Lucifer Yellow CH. All AFC fractions produced a concentration-dependent inhibition of GJIC. The apparent potency of each fraction correlated with its relative polarity based on HPLC elution characteristics. Cells with reduced GJIC as a result of AFC fraction exposure were found to exclude propidium iodide, suggesting that inhibition of GJIC occurred in the absence of cell killing. However, significantly reduced culture DNA content was found following the overnight exposure to the highest concentrations of AFC fractions C, D and E.

Asphalt, which is obtained from the refining of petroleum, has generally been considered non-tumorigenic due to the low temperature involved in its production (1,2). However, fumes arising from the heating of asphalt during application may pose a potential carcinogenic hazard. Results of early animal studies on inhalation exposure of rats and guinea-pigs (3) and of mice (4) to asphalt fumes did not reveal the presence of carcinogenic activity. These studies did, however, demonstrate a chronic inflammatory response in lungs due to the inhalation of asphalt fumes. In a more recent animal study with asphalt fume condensate (AFC*) prepared from roofing asphalt, Neimeier *et al.* (5) demonstrated carcinogenicity following application on mouse skin. An important finding of this study was that AFC did not contain high enough concentration of any specific class of known carcinogens (e.g. 5–6 ring polyaromatic hydrocarbons) that might account for its carcinogenic activity. In a later study (6), AFC condensate was chromatographically separated into five fractions (labeled A, B, C, D, E) for testing in a mouse skin bioassay. Only fractions B and C exhibited carcinogenic activity under the conditions of the bioassay. An evaluation of fractions A, D and E at a single dose indicated the absence of tumor promoting and co-carcinogenic activity. However, fractions B and C were not tested for tumor-promoting activity. Other complex mixtures such as cigarette smoke condensate (7,8) and dewaxed heavy paraffinic distillate (9,10) have been shown to possess both initiating and promoting capability, when tested in the classic two-stage mouse skin model. Many known tumor promoters including complex mixtures such as cigarette smoke condensate (11,12) have been found to inhibit gap junctional intercellular communication (GJIC), and bioassays based on this effect have been developed (13,14). A previous investigation using the Chinese hamster V79 cell metabolic co-operation assay (15) found that the AFC fractions were able to inhibit GJIC. However, the V79 cell assay is of limited use because it is an indirect measure of GJIC, it provides no information on species and organ specificity, and the cytotoxicity of a test agent is assessed in a separate assay at a much lower cell density. The fluorescent dye microinjection assay provides a system for direct observation and quantification of GJIC in a relevant cell system. The purpose of this study was to evaluate further all five AFC fractions for the ability to inhibit GJIC using fluorescent dye microinjection in a human epidermal keratinocyte (HEK) cell culture model.

Production of AFC, chromatographic separation into five fractions and identification of chemical constituents have been previously described (16). Briefly, the parent AFC was fractionated by preparative HPLC on an (aminopropyl) silica column by elution with increasingly polar solvents from 100% hexane to methanol/water/triethylamine (90:10:0.05, v/v/v). Fraction A eluted off the column first, and contained the least polar compounds, followed by fractions B, C, D and E, with fraction E containing the most polar compounds. The eluting solvents were flash evaporated and the residue for each fraction diluted to 50% (w/v) with acetone/cyclohexane (1:1, v/v). This material was used for the *in vitro* studies described herein.

Preliminary studies indicated that, due to cytotoxicity, cyclohexane had to be removed prior to exposure of cells. Dosing solutions were prepared by adding an aliquot of AFC fraction to DMSO and removing the acetone and cyclohexane by solvent exchange under a gentle stream of nitrogen gas at room temperature. An equal volume of acetone was added to aid in dissolving precipitate that formed in some of the fraction dosing solutions. An aliquot of dosing solution was then added to culture medium to obtain the desired final concentration and maintain carrier solvent concentrations of 0.1% DMSO and 0.1% acetone.

HEK and growth medium was obtained from Clonetics Corp. (San Diego, CA). The HEK growth medium was a modified MCDB-153 supplemented with bovine pituitary extract (0.4%, v/v), insulin (5 μg/ml), epidermal growth factor (0.1 ng/ml), hydrocortisone (0.5 μg/ml) and gentamicin/amphotericin-B. Cells were grown in 35 mm × 10 mm dishes (8 cm^2) for micro-injection studies and 24 well plates (2 cm^2) for DNA content studies. Subconfluent cultures of HEK were exposed overnight to medium containing an AFC fraction, 0.1% DMSO/0.1% acetone or nothing.

GJIC was assessed by the dye-coupling method in which the

*Abbreviations: AFC, asphalt fume condensate; GJIC, gap junctional intercellular communication; HEK, human epidermal keratinocytes; PI, propidium rodide.

transfer of microinjected fluorescent dye to adjacent cells occurs through intercellular gap junctions (17). Micropipets were made from glass capillary tubes (World Precision Instruments, New Haven, CT) by a gravity pipet puller (Narishige, Tokyo, Japan) and backfilled with 5% (w/v) Lucifer Yellow CH (Sigma Chemical Co., St Louis, MO) in 0.1 M lithium chloride (Sigma). At the time of microinjection cell densities of control cultures were $0.8-1.2 \times 10^5$ cells/cm^2. Although the cultures were subconfluent, fields of view (at 200×) that were confluent could easily be found using inverted phase-contrast microscopy on a Nikon Diaphot microscope (Nikon, Inc., Garden City, NJ). Prior to microinjection the dishes were randomized, and injections and quantitation of dye-coupling were performed blind. A cell was iontophoretically injected with dye for 10–20 s, and the spreading of injected dye to adjacent cells was complete within 5 min. Typically, in control cultures, the dye would spread two or three rows of cells from the injected cell. The number of neighboring cells containing fluorescent dye were counted 5–10 min after injection using inverted epifluorescence microscopy (Nikon filter cube B-2A). The total number of dye-positive adjacent cells was used as an index of GJIC. These microinjection conditions provided a consistent spreading of fluorescent dye. Each AFC fraction was examined in a separate experiment, and cultures for an experiment were derived from the same subculture (not greater than the fourth passage). The variation among the controls for each fraction (Table I) was due to the use of different lots of frozen HEK for establishment of cultures.

The ability to exlude propidium iodide (PI, Sigma) was used to assess cell killing by AFC fractions. Cultures in 35 mm × 10 mm dishes were incubated in medium containing 10 μg/ml PI for 10 min at 37°C. The cultures were rinsed twice and 2 ml medium added. Cells were viewed (at 200×) using inverted epifluorescence microscopy (Nikon filter cube G-2A) for fluorescent staining of nuclei that would indicate disruption of membrane integrity resulting from cell death. Culture DNA content was measured using the fluorescent dye Hoechst 33258 (Sigma) by the method of Downs and Wilfinger (18).

All fractions of AFC possessed a limited solubility in aqueous culture medium. This was evident by the appearance of insoluble droplets dispersed in the medium and a thin film on the surface of the medium. None the less, exposure of cultured human keratinocytes to AFC fractions produced biological effects that were dependent on the total mass of the added fraction. Following an overnight exposure (20–24 h), all the fractions exhibited a concentration-dependent inhibition of dye-coupling (Table I). At exposure concentrations of ≥10 μg/ml, the inhibition of dye-coupling increased from fraction to fraction according to the order A < B≤ C < D < E. This order corresponds to the expected polarity of the components of each fraction based on their HPLC elution characteristics.

Toxicity was assessed following overnight exposure to AFC fractions at cell densities that were comparable to those used in the intercellular communication studies. For all AFC fraction exposure concentrations, membrane damage was absent to the extent that cells were able to exclude PI (data not shown), suggesting that live cells were microinjected. However, exposure of HEK to some AFC fractions and concentrations was not without gross effects since culture DNA content was significantly reduced following an overnight exposure to 25 to 50 μg/ml of fractions C, D or E (Table II).

It is increasingly apparent that many of the chemicals identified

Table I. Effect of AFC fractions on dye-coupling in keratinocytes[a]

Fraction concentration (μg/ml)	AFC (no. of dye-coupled cells[c])				
	A	B	C	D	E
0	13 ± 1	23 ± 2	17 ± 1	25 ± 2	24 ± 2
5	ND	13 ± 2	10 ± 2	17 ± 2	18 ± 1
10	10 ± 1	10 ± 1[b]	8 ± 2	8 ± 1[b]	4 ± 1[b]
25	6 ± 1[b]	7 ± 1[b]	2 ± 1[b]	0.1 ± 0.1[b]	0.1 ± 0.1[b]
50	6 ± 1[b]	1 ± 0.4[b]	0.1 ± 0.1[b]	0.1 ± 0.1[b]	0 1 ± 0.1[b]
100	3 ± 2[b]	ND	ND	ND	ND

ND, not determined.
[a]Dye-coupling was assessed by microinjection of a single cell with 5% Lucifer Yellow CH and counting the dye-positive neighboring cells. There were five injections per dish and three dishes per group. For statistical analysis, dishes were considered the experimental replicate.
[b]Statistically significant from control values at $P < 0.05$ by analysis of variance and subsequent comparisons of means using the Tukey range test.
[c]Values are mean ± SEM.

Table II. Effect of AFC fractions on keratinocyte culture DNA content[a]

AFC concentration (μg/ml)	AFC (μg DNA/well[b])				
	A	B	C	D	E
0	1.26 ± 0.08	1.12 ± 0.02	1.44 ± 0.03	1.44 ± 0.04	1.45 ± 0.09
5	ND	1.08 ± 0.02	1.40 ± 0.04	1.38 ± 0.05	1.44 ± 0.06
10	1.35 ± 0.05	1.10 ± 0.01	1.38 ± 0.01	1.23 ± 0.07	1.32 ± 0.07
25	1.39 ± 0.05	1.04 ± 0.03	1.26 ± 0.03[c]	1.08 ± 0.05[c]	1.09 ± 0.07[c]
50	1.49 ± 0.07	1.05 ± 0.06	1.25 ± 0.02[c]	1.01 ± 0.05[c]	0.87 ± 0.08[c]
100	1.39 ± 0.08	ND	ND	ND	ND

ND, not determined.
[a]Culture DNA content was determined immediately following exposure of cells to AFC fractions for 20–24 h.
[b]Values are mean ± SEM of four wells.
[c]Satistically significant from control values at $P < 0.05$ by analysis of variance and subsequent comparisons of means using the Tukey range test

in animal bioassays as potential human carcinogens possess low or non-detectable genotoxic activity. These chemicals have been classified as non-genotoxic carcinogens and include many structurally diverse chemicals that probably exert their action through multiple mechanisms. Tumor promoters are non-genotoxic carcinogens that enhance tumor formation in well-defined experimental protocols using specific initiation−promotion animal bioassays. Human exposures to carcinogens often involve mixtures of chemicals that may contain both genotoxic and non-genotoxic contributions to carcinogenic activity. For a complete understanding of the relationship between exposure and cancer risk it is important to test for both genotoxic and non-genotoxic activities in chemical mixtures.

Inhibition of GJIC between cultured cells has been used to assess the potential tumor promoting activity of chemicals (14,19). GJIC has been hypothesized to be an important pathway for growth and differentiation regulatory signals (20). Inhibition of GJIC between transformed and normal cells may contribute to clonal expansion and consequently tumorigenesis (21,14). Cigarette smoke condensate and its fractions are the only other complex chemical mixtures that have been investigated for their effects on GJIC (11,12). Cigarette smoke condensate was found to inhibit GJIC, but by a mechanism that appeared to be different from the model tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (12).

In the present study, we have investigated the ability of fractionated AFC to inhibit GJIC in cultured HEK. All five fractions were found to inhibit GJIC in a concentration-dependent manner. Although inhibition of GJIC took place following exposures to AFC fractions that did not result in membrane damage sufficient to allow PI uptake, more subtle forms of cell toxicity may have been present. Indeed, inhibition of GJIC could itself be considered a form of cytotoxicity, and be used as a sensitive indicator of compromised cell function. At any single exposure concentration of ≥ 10 μg/ml, the apparent relative potency of each fraction for inhibition of GJIC was $A < B \leq C < D < E$. This result is consistent with the limited aqueous solubility for many components of the fractions and the relative contribution of these non-polar components to the total mass of each fraction. This is an especially important consideration when exposures take place in a serum-free medium which lacks the proteins and lipoproteins that facilitate transport and cellular absorption of lipophilic compounds. Therefore, in the absence of knowledge of the actual mass of AFC fraction to which the cells were exposed, the effective concentrations are best viewed as approximations.

Two of the AFC fractions, A and E, contain known mouse skin tumor promoters, but yielded negative responses in the mouse skin bioassay (6). Fraction A contains dodecane (22), and fraction E contains phenol (23). In the animal bioassays conducted by Sivak *et al.* (6), the fractions were applied at a mass proportionate to their amount in the dose of unfractionated AFC that was tumorigenic to mouse skin (5). The mass of dodecane in fraction A was determined to be 3.4 mg/g fraction A. The total mass of fraction A per application was ~12 mg. From this value the calculated amount of dodecane per application was 40 μg. This is much less than the 25−50 mg dodecane per application used in mouse skin tumor promotion bioassays (22). Since dose−response data for tumor promotion of AFC fractions is not available, no conclusion can be made regarding their potential activity. The present study suggests that the AFC fractions may contain tumor-promoting activity. Considering the mouse skin bioassay results with pure dodecane (22), such activity may require unrealistically high doses in the cases of AFC fractions A, D and E. The tumor-promoting activity of AFC fractions B and C remains to be determined and may make a significant contribution to the total carcinogenic potency of AFC.

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