Quantitative Morphometry of Respiratory Tract Epithelial Cells as a Tool for Testing Chemopreventive Agent Efficacy

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Abstract. *Previously, we developed a 30-day transformation assay (focus inhibition, FIA) of rat tracheal epithelial (RTE) cells to identify cancer preventive agents. This study reports nuclear density (ND) as a morphometric biomarker for efficacy evaluation of at an early stage before transformed foci appear. Positive (oltipraz, D-carvone, fumaric acid, and 2-amino-4-methylpyridine or 2-A-MPR) and negative agents (myristoleic acid, anethole trithione, hydrocortisone, and 3'-hydroxyflavanone), identified from FIA, were tested for their effect on ND. RTE cells plated for 24 h were treated with a carcinogen, benzo[a] pyrene (B[a]P) or plus a test agent. The data based on the number of nuclei in agent-treated and control cells at day 14 showed that all FIA-positive agents inhibited ND from 23-66% at 0.3-1000.0 μM and except for myristoleic acid, all of the FIA-negative compounds were also negative in the morphometry assay. As there is strong correlation between the FIA and morphometry data, morphometry analysis is useful for rapid screening of potential chemopreventive agents.*

In the past, our laboratory had developed a primary cell culture model of rat tracheal epithelial (RTE) cells to analyze and quantitate the process of transformation in the respiratory system (1, 2) and subsequently adapted this model for studying the inhibition of chemically induced transformation (3). Later this protocol had been expanded to test >400 potential chemopreventive compounds (4, 5) and an evaluation of the collected data to predict the efficacy in animal models revealed that the RTE assay had an excellent correlation to hamster lung model, with a predictive value of 76% for positive agents and 100% for negative agents (6). Analysis of the mechanism of action of a variety of positive agents identified in the RTE assay showed that there is specificity towards antioxidants/free radical scavengers including retinoic acids and their derivatives (5), tea compounds and other polyphenols (7, 8) and iNOS inhibitors (9).

In the standard 30-day transformation (focus inhibition) assay (FIA), RTE cells were exposed to B[*a*]P, present in tobacco smoke and the environment, for 24 hours in the presence and absence of a test agent and cultured for 30 days with only the agent. Normal cells differentiated after 2 weeks, whereas the B[*a*]P-treated cells continue to grow and form colonies of different morphological types of cells or foci. At 30 days, these foci of preneoplastic cells were scored and foci inhibition was expressed as a decrease in their number compared to the B[a]P-alone exposed cultures. Targeting intermediate biomarkers in cancer pathways is an ideal strategy for chemoprevention modalities. In the current study, in order to test the feasibility of morphometry as an intermediate biomarker in respiratory cell cancer progression, 4 positive and 4 negative compounds identified from the standard FIA assay were evaluated for their effect on progressive morphological changes based on nuclear density (ND), by nuclear image morphometry). The data from this assay indicates the potential of these agents to inhibit nuclear changes during oncogenic transformation at an earlier stage in epithelial tissues, which is indicative of their anticarcinogenic effect at a later stage in the respiratory cell transformation process.

Materials and Methods

Chemicals. Benzo[*a*]pyrene, 2-amino-4-methylpyrine (2-A-MPR), D-carvone, fumaric acid, myristoleic acid, hydrocortisone, Giemsa and May-Grunwald were purchased from Sigma Chemical Co. (St Louis, MO). 3'-hydroxyflavanone was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Anethole trithione and oltipraz were obtained from NCI repository, Rockville, MD, USA. The agents were solubilized in either EtOH or DMSO (final solvent concentration <0.2%).

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Cells and media. Rat tracheal epithelial (RTE) cells were isolated from 8- to 10-week-old male Fisher 344 rat (Charles River, Raleigh, NC) trachea after excising it surgically. The trachea was filled with Ham's F12 medium plus 1% protease and incubated overnight at 4˚C. The lumens were rinsed with F12 medium. The cells were centrifuged, counted and re-suspended in serum-free media (Ham's F12 supplemented with 3 mg bovine serum albumin/ml, 0.8 mM of calcium chloride, 0.1 μg chorela toxin/ml, 5 ng epidermal growth factor/ml, 80 μM ethanolamine, 3 μg fungizone/ml, 0.1 μg hydrocortisone/ml, 10 μg insulin/ml, 50 μM phosphoethanolamine, 5 μg human transferrin/ml and 1% bovine pituitary extract).

RTE cell focus inhibition assay (FIA). Based on an initial cytotoxicity test, a test agent concentration that showed 20% reduction in growth (compared to solvent control) plus four half-log dilutions were used for the FIA. The RTE cells were plated onto 60 mm culture dishes at 1×10^4 cells/dish. After 24 hours' plating, cells were treated with B[*a*]P only, B[a]p plus five half log doses of test agent or B[*a*]p plus positive control (13-*cis*-retinoic acid at 1 nM). Media and DMSO control groups were also included. At day 7, a set of replicate from each group was taken for determining the toxicity of the dose and the culture media was shifted to selection medium consisting of Ham's F12 plus 5% FBS, 0.1 μg hydrocortisone/ml, 1 μg insulin/ml and 3 μg fungizone/ml and changed weekly thereafter. At the end of day 30, the dishes were fixed with 70% ethanol, stained with Geimsa and scored for transformed colonies (10).

Experimental evaluation. Three types (type I with <1300 cell/mm^2, type II with 1300-2500 cell/mm^2 and type III with >2500 cell/mm^2) of transformation colonies (foci) were scored. Only type II and III foci were used for the evaluation of inhibition. Numbers of foci from the test agent group were averaged, the solvent background was subtracted and the results were compared to the group treated with B[*a*]P alone to calculate the percentage of inhibition (10).

Morphometric analysis of RTE cells.

Cell culture for time point selection and testing of agents. Freshly isolated RTE cells (3×10^3) were plated in chamber slides with 2 ml of serum-free media. The same experimental groups were set up as for FIA with four replicate slides per group. After 7 days in culture, 2 slides were fixed, stained and scored for CFE counts and the other 2 slides were cultured in selection media for 14 or 21 days, followed by fixing cells with 70% EtOH and staining the nuclei with May-Grunwald-Giemsa. The morphological changes of RTE cells were determined by measuring nuclear density in each group. In addition, nuclei scoring of selected areas containing cells with type I, II and III morphology in B[*a*]P group at 14 days was carried out and compared with the data from the 30-day cultures.

Computer-aided image analysis. Twenty-four images from random areas in each stained slide were taken using a high resolution color video camera (DXC-970 MD, Sony, Japan) and Nikon Optiphot fluorescent microscope. The entire area in each captured image was measured using a customized image analysis software program (OPTIMAS 6.5 software program; Vashaw Scientific, Norcross, GA, USA). The total number of nuclei in this area was scored automatically by using the same software program. Differences between treated and control samples were evaluated by the Student's *t*-test. An agent was considered positive if two or more concentrations inhibited B[*a*]P-induced morphological changes by 20% or more.

Evaluation of morphometry. The evaluation of morphometry of morphometry was carried out in three steps. First, the ND in each image of solvent control, B[*a*]P and B[*a*]P plus test agent (5 doses) group was calculated.

$$ND = \frac{Total\ \ number\ \ of\ \ nuclei}{Total\ \ area}$$

Next, the mean ND of each group was calculated.

Mean $$ND = \frac{Total\ \ ND\ of\ \ individual\ images}{n}$$

where *n* is the total number of images taken for each group.
Finally, the percentage inhibition at each concentration of the test agent (TA) was calculated.

$$\%\ Inhibition\ by\ TA = (1 - \frac{Mean\ \ nuclear\ \ density\ in\ B[a]P + TA}{Mean\ \ nuclear\ \ density\ in\ B[a]P}) \times 100$$

Results

The data on the ND at 14 and 21 days showed that, there was a substantial increase in ND in the B[a]P-treated group (3.0 and 3.5 fold, respectively), when compared with the DMSO group (Figure 1). However, in order to develop morphometry as an early marker for RTE cell transformation, the early time point (14-day) was selected for testing agents. Figure 2 shows data from the 14-day experiment using a chemopreventive agent, 13-*cis*-retinoic acid that is routinely used as a positive inhibitor control in the FIA. This agent inhibited the nuclear density by 56% when compared with B[*a*]P-treated group.

The characteristics of sequential changes in RTE cell morphology and density after the cells were treated with B[*a*]P and allowed to progress for 14 days in culture is shown in Figure 3. The morphology, staining intensity and the number of nuclei per unit area show striking similarities to the cells in the three types of foci that appear in the 30-day culture (10). Comparing the nuclear density at 14 days with cell density at 30 days, it appears that there is a very close correlation in the number of nuclei/cells in Type I foci in both 14- and 30-day cultures. Interestingly, there was a substantial increase in the number of nuclei/mm^2 in both Type II and III foci at 14-day cultures (Table I), when compared to 30-day cultures.

The data on the effect of all agents on RTE cell nuclear morphometry are shown in Table II and the data on comparing the morphometry data with FIA data are shown in Table III. All agents were nontoxic at all concentrations. 2-A-MPR, a 5-lipoxygenase inhibitor, tested at 0.3 to 30.0 μM, inhibited nuclear density in a dose-dependent manner by 31-41%. Correspondingly, it inhibited RTE cell transformation

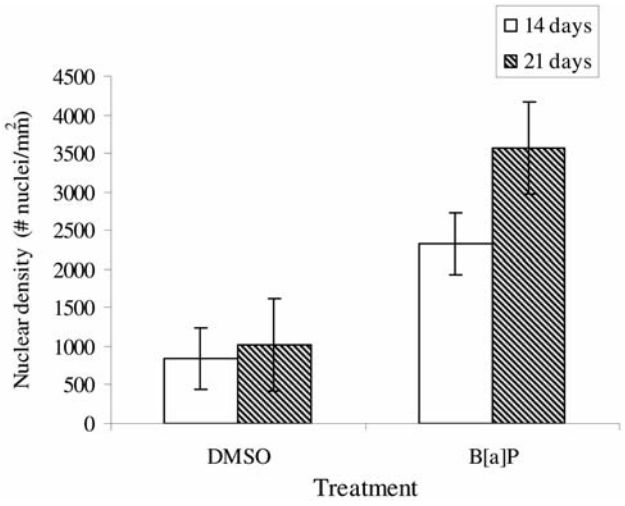


Figure 1. *Changes in nuclear density in DMSO control and B[a]P-treated cultures of RTE cells at 14 and 21 days (data generated from three experiments).*

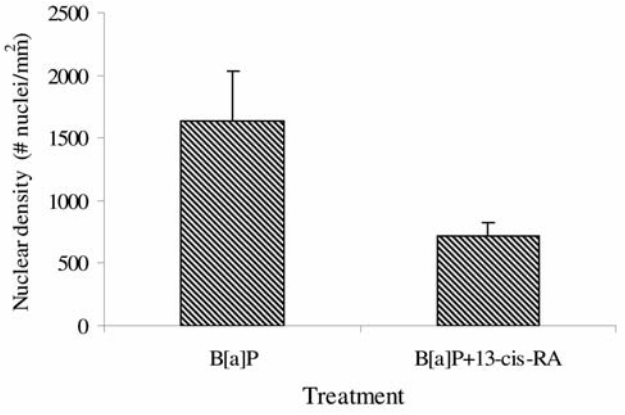
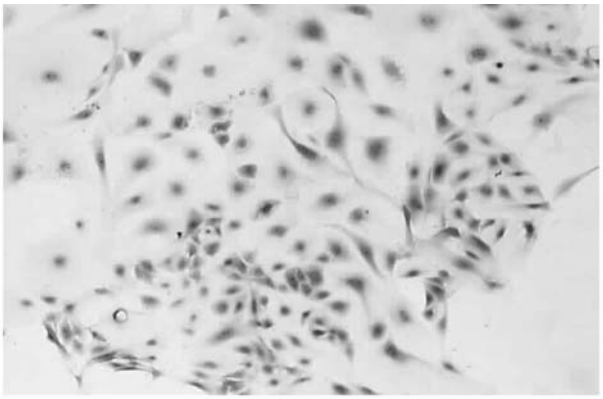
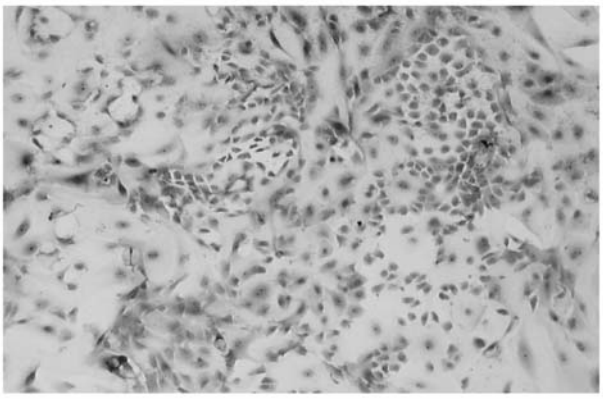


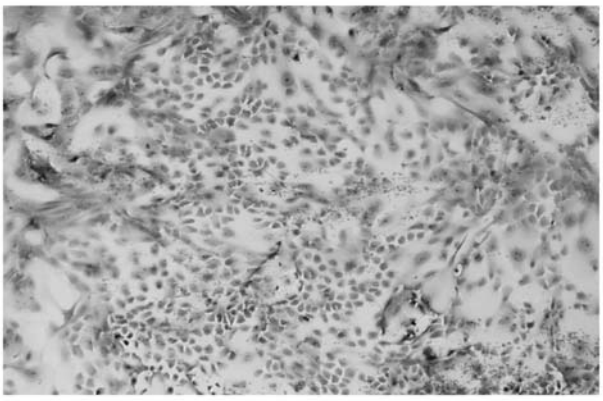
Figure 2. *Nuclear density of RTE cells treated with B[a]P and B[a]P+13-cis-retinoic acid (13-cis-RA) (positive control) (data generated from three experiments).*



A. Cells with Type I Morphology



B. Cells with Type II Morphology



C. Cells with Type III Morphology

Figure 3. *Progressive changes in cell morphology and density after B[a]P treatment (14 days). A: Cells with very small nuclear-to-cytoplasmic ratio and fibroblastic appearance; B: packed cuboidal cells; C: cells with tight swirling pattern and high nuclear-to-cytoplasmic ratio.*

at the same concentrations by 25-92% (IC_{50}=0.82 μM). D-Carvone, a monoterpene, tested from 3.0-300 μM, showed a dose-dependent response by inhibiting ND by 25-66% from 10-300 μM. In the FIA, it inhibited B[a]P-induced transformation ranging from 28-92% (IC_{50}=100 μM). Other studies have demonstrated antitumor activity by inhibiting *N*-nitrosodiethylamine-induced forestomach tumors and pulmonary adenomas in female A/J mice by 60 and 35%, respectively (11). However, it was not effective against metastatic tumor growth, while the precursors of D-carvone, D-limonene and perillic acid, were effective (12).

Oltipraz, tested at 0.3 to 30.0 μM, weakly inhibited ND by 23.3% at both 10 and 30 μM, however, it inhibited foci formation by 38-79%. Oltipraz, a dithiolethione structurally related to chemical groups found in cruciferous vegetables, has been reported to be chemopreventive in a broad spectrum of animal models, including rat liver (aflatoxin-induced), colon, mammary gland, mouse skin, bladder, and colon; and

Table I. *Measurement of nuclear density in different types of foci in 14- and 30-day cultures.*

Foci	Area (mm^2)	No. of nuclei	14-Day nuclear density ($nuclei/mm^2$)	30-Day cell density ($cells/mm^2$)
Type I	0.188	234	1244.68	<1300
Type II	0.188	674	3585.11	1300-2500
Type III	0.188	830	4414.89	>2500

*Data taken from reference 10.

hamster trachea and lung (13-16). It has also been shown to possess antiangiogenic activity, as tumor growth was inhibited by 81% following oltipraz administration to athymic mice bearing SVR angiosarcoma xenografts (17). Oltipraz is believed to elicit its chemopreventive effects through induction of phase II enzymes, in particular glutathione *S*-transferases, quinone and glutathione reductases, glucose 6-phosphate and 6 phosphogluconate dehydrogenases (18). Fumaric acid (FA), another phase II enzyme inducer tested in this study, inhibited ND at concentrations of 10-300 μM by 38-66%. It exhibited similar inhibition of FIA (10-1000 μM) by 61-84%. FA has chemopreventive effects in several tissues. It suppressed the development of 3'-Me-DAB-induced hepatic carcinomas in rats (19), completely eliminated the formation of thioacetamide-induced hepatic carcinomas in both mice and rats (20, 21), suppressed nitrofuran-induced stomach and lung tumors (22), reduced the yield of AOM-induced foci in rat colon (23) and inhibited the development of esophageal papillomas, brain gliomas and mesenchymal tumors of the kidney (24). Mechanistically, FA has been shown to induce hepatic and intestinal UDP-glucuronosyltransferases (25) while dimethyl fumarate, a metabolite of fumaric acid, has been shown to be a potent inducer of quinine reductase and cytosolic glutathione *S*-transferases (26).

Among the negative compounds identified from FIA, Myristoleic acid, a monounsaturated fatty acid, however, showed a positive response by inhibiting the nuclear density (32 and 44%) at two high doses (Table II). It has been shown to have anticancer effects in prostate and pancreas by inducing apoptosis in prostate and pancreatic cancer cells and markedly inhibiting growth of pancreatic tumors xenografted into athymic mice (27-28). Anethole trithione, another phase II inducer, was tested at 0.03-3 μM and similar to the FIA data, it did not inhibit nuclear density at any test doses. Studies show that anethole trithione, a substituted dithiolthione and an analog of the oltipraz, administered in the diet, significantly inhibited the mammary cancer multiplicity induced by dimethylbenzanthracene (29). It also significantly inhibited the incidence and multiplicity of both invasive or noninvasive colon adennocarcinoma of F344 rat and elevated the activity of phase II enzymes (GST, NADPH dependent quinone reductase, UDP-glucuronosyl transferase) of colon mucosa colon tumor and liver (30).

Table II. *Effect of agents on RTE cell morphometry at 14 days.*

Agent	Test. conc. (μM)	Inhibitory conc. (μM)	% Inhibition	Results
2-A-4-MPR	0.3-30	0.3-30	31-41	+
D-Carvone	3-3000	10-300	25-66	+
Fumaric acid	10-1000	10-300	38-66	+
Oltipraz	0.3-30	10 & 30	23	+
Anethole trithione	0.03-3	None	NE	–
Hydrocortisone	0.3-30	30	24	–
3-Hydroflavanone	0.03-3	None	NE	–
Myristoleic acid	1-100	30 & 100	32 & 44	+

NE: Not effective.

Table III. *Comparison of RTE cell FIA data and morphometry data.*

Agent	FIA		Morphometry		
	Results	Inhibitory conc. (μM)	Inhibitory conc. (μM)	% inhibition	Results
2-A-4-MPR	++	0.3-30	0.3-30	31-41	+
D-Carvone	+++	3-300	10-300	25-66	+
Fumaric acid	+++	10-1000	10-300	38-66	+
Oltipraz	+++	0.3-3.0	10 & 30	23	+
Anethole trithione	–	NE	0.03-3	NE	–
Hydrocortisone	–	NE	30	24	–
3-Hydroflavanone	–	NE	0.03-3	NE	–
Myristoleic acid	–	NE	30-100	32-44	+

NE: Not effective.

Hydrocortisone, another negative compound from the FIA assay, was tested at 0.3-30 μM and since it inhibited ND only at the highest dose by 24%, it was considered negative in the morphometry assay also. 3'-hydroxyflavanone was tested at 0.03-3.0 μM and similar to the FIA data, it did not inhibit nuclear density at any of the concentrations tested. In order to understand whether there is a correlation between morphometry and foci inhibition, data from the two studies were assembled and is shown in Table III.

Discussion

The use of intermediate biomarkers for cancer prevention trials, based on the morphological properties of precancerous lesions, has been proposed and is being tested in a series of clinical trials (30). Our earlier studies have focused on the development of a respiratory epithelial cell model by treating primary RTE cells with a chemical carcinogen, B[*a*]P, and using cell morphology and density to classify three types of foci associated with transformation in a 30 day culture (1, 10). However, the identification and quantitation of foci requires extensive training and experience of individuals involved in the study. In addition, as the scoring is done manually, there is always a potential for subjectivity in identifying the different types of foci representing various stages of neoplastic progression. Furthermore, as the standard FIA takes a minimum of 30 days to complete, we were interested to see whether the well defined morphological changes in foci cells can be observed at earlier time points and, if so, whether these changes can be quantitated and developed as an intermediate biomarker. In order to test this, the current study utilized a quantitative morphometric scoring of tracheal epithelial cells based on the number of nuclei/unit area and using a customized image analysis program (CAIA). Our study has shown that there is very good correlation between the standard FIA data and morphometric results. The average inhibition of nuclear density by 13-*cis*-retinoic acid from multiple experiments was 56% when compared with B[*a*]P-treated group. The four positive test agents in standard FIA were also positive in morphometric study. The exception is a negative agent in FIA (myristoleic acid), which showed positive result in the morphometric study, however, only at two high doses (Table II). It is possible that if given sufficient time to develop the characteristic foci at 30 days, these two doses may also become negative. Another two negative agents, anethole trithione and 3'- hydroxyflavanone did not inhibit nuclear density at any of the doses tested. As hydrocortisone exhibited a very weak inhibition (24%) only at one dose (30 μM), it is considered as negative in the morphometric study because our criteria for positive agents is to have at least two doses exhibiting 20% inhibition.

There are several advantages of using morphometric analysis to determine the progressive changes in RTE cells. The morphometric measurements should be able to identify the modulation of specific cellular changes by chemopreventive agents at an early time point, which in turn should correlate with the inhibition of Type II and III foci detected at the end of 30 days in the standard RTE FIA assay.

In addition, morphometric analysis of slide cultures can be used as a rapid prescreen for a significant number of compounds without the use of large number of replicate (~16-20) dishes for statistical significance. As morphological changes can be quantitated in a population of cells rather than a sufficient number of foci, the number of cells to be plated or animals to be used for cell isolation can be substantially reduced. Positive agents in the morphometry assay with no toxicity and other desirable biological activity can be tested subsequently in the 30-day standard assay for analyzing their effect on Type II and III foci (which are analogous to tumors in animals). Since the morphometry method of identifying the preinvasive changes using slide cultures is found to be sensitive, quantitative and reliable in the B[a]P transformation assay, it can be considered as a short-term biomarker for rapid testing of potential chemopreventive agents.

Acknowledgements

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