Effects of 10 Cigarette Smoke Condensates on Primary Human Airway Epithelial Cells by Comparative Gene and Cytokine Expression Studies

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Cigarettes vary in tobacco blend, filter ventilation, additives, and other physical and chemical properties, but little is known regarding potential differences in toxicity to a smoker's airway epithelia. We compared changes in gene expression and cytokine production in primary normal human bronchial epithelial cells following treatment for 18 h with cigarette smoke condensates (CSCs) prepared from five commercial and four research cigarettes, at doses of ~4 µg/ml nicotine. Nine of the CSCs were produced under a standard International Organization for Standardization smoking machine regimen and one was produced by a more intense smoking machine regimen. Isolated messenger RNA (mRNA) was analyzed by microarray hybridization, and media was analyzed for secreted cytokines and chemokines. Twenty-one genes were differentially expressed by at least 9 of the 10 CSCs by more than twofold, including genes encoding detoxifying and antioxidant proteins. Cytochrome P450, family 1, subfamily A, polypeptide 1 (*CYP1A1*) and NAD(P)H dehydrogenase, quinone 1 (*NQO-1*) were selected for validation with quantitative real-time PCR (qRT-PCR) and Western blot analyses. *NQO-1* expression determined with microarrays, qRT-PCR, and Western blotting differed among the CSC types, with good correlation among the different assays. *CYP1A1* mRNA levels varied substantially, but there was little correlation with the protein levels. For each CSC, the three most induced and three most repressed genes were identified. These genes may be useful as markers of exposure to that particular cigarette type. Furthermore, differences in interleukin-8 secretion were observed. These studies lay the foundation for future investigations to analyze differences in the responses of *in vivo* systems to tobacco products marketed with claims of reduced exposure or reduced harm.

Key Words: cigarette smoke condensates; primary human lung epithelial cells; gene expression; toxicity; cytokine.

and reducing the health of smokers in general (Das, 2003; Department of Health and Human Services [U.S.], 2004). Cigarette smoke contains more than 4000 chemicals, including more than 60 carcinogens (Chen *et al.*, 2008). Comparisons of different cigarette types have clearly demonstrated differences in the chemical composition of the smoke as a function of additives (Baker *et al.*, 2004a), paper type (Baker *et al.*, 2004b), tobacco processing (Martin *et al.*, 2003), and other manufacturer-specific features (Harris, 2001).

Some studies have suggested that reduction of nicotine, the addictive component of cigarette smoke, may permit a gradual reduction of smoking (Djordjevic *et al.*, 2000). However, there is also evidence for compensatory behavioral changes (increased puff volume and frequency and increased numbers of cigarettes smoked) (Djordjevic *et al.*, 2000; Kabat, 2003) that result in altered smoke composition (Counts *et al.*, 2005) and increased exposure to harmful components of cigarette smoke. Of potentially greater concern is evidence that cigarette smoke condensates (CSC) from low-nicotine cigarettes are more toxic to normal human bronchial epithelial (NHBE) cells than CSC from high-nicotine cigarettes, possibly due to nicotine-mediated suppression of apoptotic pathways (Chen *et al.*, 2008). Therefore, understanding the consequences of differences in smoke composition as a function of cigarette design and smoking behavior is important in reducing the health burden of cigarette smoking.

Previous studies have successfully demonstrated differences in toxicity among different cigarette types or cigarette smoke fractions. Several studies (Fukano *et al.*, 2006a,b; Hoshino *et al.*, 2001; Lannan *et al.*, 1994) have investigated the effects of smoking machine-generated CSC or whole smoke on cultured cell lines, particularly A549 cells, a transformed cell line with some characteristics of alveolar type II cells. Comparison of the effects of whole smoke and filtered smoke from "light" cigarettes sold in different countries with that of

The adverse health effects from cigarette smoking account for 440,000 deaths in the United States each year. Smoking harms nearly every organ in the body, causing many diseases

a research cigarette blended to be representative of a U.S. light cigarette in A549 cells showed differences in a dose-dependent depletion of reduced glutathione among the cigarette types (Ritter *et al.*, 2004).

There have also been efforts to understand the metabolic targets of cigarette smoke exposure. Differences were observed between the effects of whole smoke and the gas/vapor phase from a single cigarette type on the metabolic pathways of human lung epithelial cells (Vulimiri *et al.*, 2009), suggesting that cigarette smoke fractions can affect these pathways differentially. Exposure of NCI-H292 cells, a human lung epithelial cell line, to whole smoke demonstrated increases in expression of Muc5AC, an important form of mucin, and release of interleukin (IL)-6, IL-8, and MMP1 (Phillips *et al.*, 2005). Another study suggested changes in genes regulating DNA damage, cell cycle, inflammation, and oxidative defense in cigarette smoke-exposed NHBE cells (Fields *et al.*, 2005).

Gene expression arrays have considerable potential for identification of potential biomarkers of exposure and effect (Sen *et al.*, 2007). To better define the effect of a wide range of CSCs on human respiratory tract cells, the present study was designed to analyze CSC-induced patterns of gene expression in NHBE cells. A previous study using a similar set of CSCs showed some differences in their mutagenicity as a function of total tar content but a much larger range based on nicotine content (DeMarini *et al.*, 2008). Therefore, in the current study, the CSCs were compared at doses of nicotine concentrations that were minimally toxic.

MATERIALS AND METHODS

Cigarette smoke condensates. The cigarette types used in the study and the range in nicotine content are illustrated in Table 1. Cigarette smoke was generated at the CSC under the standard Federal Trade Commission (FTC) or Massachusetts intense (MI) method using a single port smoking machine (CH Technologies, Westwood, NJ) to a butt length of 23 mm or filter overwrap + 3 mm, whichever was greater. Samples 1–9 (CSC1–CSC9) were smoked according to FTC conditions (35-ml puff volume, 2-s puff, and 1 min between puffs), and CSC10 was smoked according to the MI conditions (45 ml puff volume, 2-s puff, 30-s interval, and ventilation holes 50% blocked). CSC were prepared by bubbling the mainstream smoke through 10 ml of PBS in a 30-ml midjet bubbler (Kontes, Vineland, NJ); this solution, defined as “100% CSC,” was filtered through a 0.22- μ m filter, aliquoted, and kept frozen. To ensure consistency of exposures, separate aliquots of the frozen stock were used for each experiment.

Cells. Primary human bronchial/tracheal epithelial cells were purchased from Clonetics (now Lonza, East Rutherford, NJ). A single male, nonsmoking Caucasian donor was used for all experiments. The cells were cultured in 100-mm dishes in Bronchial Epithelial Cell Growth Medium (with retinoic acid) as recommended by the supplier. Approximately 8–10 doublings after purchase, cells were plated at a density of 2.8×10^5 cells per 100-mm dish for the experiments. When cells were 70% confluent, at 6 days after plating, they were treated with CSC or with PBS for the control cultures. NHBE cells were treated with CSC from the various cigarettes at doses that represent a final nicotine concentration of ~ 4 μ g/ml for 18 h or for the viability measurements, a range of concentrations of the CSCs on a volume basis. All dosing was performed in duplicate, including the nontreated controls.

TABLE 1
Smoke Types and Nicotine Content Provided by the CDC

Smoke type code	Cigarette	Abbreviation	Smoking machine conditions	Nicotine (mg/cigarettes)
CSC 1	2R4F Kentucky reference cigarette	2R4F	FTC	0.75
CSC 2	Commercial “low nitrosamine” cigarette	LNIT	FTC	0.8
CSC 3	Experimental 100% reconstituted tobacco cigarette	REC	FTC	0.8
CSC 4	Commercial “low ignition propensity” cigarette	LIP	FTC	0.6
CSC 5	Experimental 100% burley tobacco cigarette	BUR	FTC	7.5
CSC 6	Experimental 100% flue-cured tobacco cigarette	BRI	FTC	5
CSC 7	Commercial ultra low-tar nonmenthol cigarette	UL	FTC	0.5
CSC 8	Commercial full flavor nonmenthol cigarette	FF	FTC	0.9
CSC 9	Commercial light nonmenthol cigarette	LT	FTC	0.8
CSC 10	Commercial light nonmenthol cigarette	LTMAS	MI	1.6

Viability using water-soluble tetrazolium assay. Cells were cultured in 96-well tissue culture plates and treated in triplicate with a range of concentrations of the CSCs from 0.1 to 25% in culture medium. Following 18 h of incubation, the culture medium was removed and replaced with a 1:10 dilution of water-soluble tetrazolium (WST; Roche). The color change resulting from the conversion to the formazan was measured in the medium using a Versamax plate reader (Molecular Devices, Inc.).

Preparation of RNA. Total RNA was extracted and purified with Qiagen RNeasy kit from each sample (Qiagen, Valencia, CA). The total RNA concentrations were measured using the NanoDrop ND-1000 (ThermoScientific, Wilmington, DE), and RNA integrity was checked using Agilent 2100 Bioanalyzer to assure sufficient quality before proceeding with microarray hybridization.

Microarray hybridization. Affymetrix Human U133 2.0 Plus GeneChips were used for gene expression analysis. All procedures were performed according to the manufacture’s instructions (Affymetrix, Santa Clara, CA). Briefly, 1.0 μ g of total RNA was used to generate double-stranded complementary DNA (cDNA) using an oligo dT-primer containing the T7 RNA polymerase promoter site and the One-Cycle Target Labeling Kit (Affymetrix). cDNA was purified via column purification using the GeneChip Sample Cleanup Module (Affymetrix), and biotinylated complementary RNA (cRNA) was synthesized by *in vitro* transcription using the GeneChip IVT Labeling Kit (Affymetrix). Biotin-labeled cRNA was purified and the absorbance measured at 260 nm to determine yield (NanoDrop). Twenty micrograms of the labeled cRNA was fragmented and hybridized to the

Affymetrix GeneChip HG U133 2.0 Plus arrays for 16 h at 45°C following the Affymetrix protocol specific to this array type. Washing and staining were performed on the Affymetrix Fluidics 450 station according to the antibody amplification protocol (EukGE-Ws2v5; Fluidics script). The GeneChips were scanned using the Affymetrix GeneChip Scanner 3000.

After scanning, the microarrays were normalized using the Affymetrix Microarray Suite 5.0 (MAS 5.0). Signal values were scaled by global methods to a target value of 500. A pivot table was generated from MAS 5.0 and imported into GeneSpring GX (version 7.3; Agilent Technologies). The mean signal value for the two replicates for each of the samples was compared to the mean signal value of the two undosed control samples. For each of the pairwise comparisons, the 54,681 transcripts were first filtered to retain transcripts that had signal values above the background (> 150 RFU in one of two samples, either treated or control). The background/noise level is ~ 75 RFU for these arrays. Next, filtering was performed to keep transcripts that change at least twofold (up or down) compared to the control. The resulting gene lists, for each of the 10 samples, were then used to identify genes that were common to the 10 samples and to select genes that were unique to the 10 samples. The Affymetrix microarray data for this experiment have been submitted to the gene expression omnibus (GEO) repository and can be accessed under GEO record GSE18235.

Quantitative expression analysis by quantitative real-time PCR. RNA from each treatment that was not used for microarray hybridization was kept frozen at -80°C in a dried state. After microarray analysis was completed, RNA was reconstituted in water and the concentration determined using the NanoDrop ND-1000 spectrophotometer. The 260:280 and 260:230 nm ratios were calculated by the NanoDrop spectrophotometer and used to evaluate the RNA purity. All samples had sufficient RNA for quantitative real-time PCR (qRT-PCR) except for the sample from NHBE cells treated with REC.

The primers and probes for growth differentiation factor 2 (*BMP-9*) (Hs00211913_m1), NAD(P)H dehydrogenase, quinone 1 (*NQO-1*) (Hs00168547_m1), cytochrome P450, family 1, subfamily A, polypeptide 1 (*CYP1A1*) (Hs00153120_m1), matrix metalloproteinase 1 (interstitial collagenase) (*MMP-1*) (Hs00233958_m1), and the housekeeping gene *CDKN1B* (hs00153277_m1) were from Applied Biosystems Inc.

The TaqMan Universal PCR Master Mix was distributed into the 96-well plate and each RNA (200 ng) sample in triplicates was added to the reaction mix. The primer/probe sets were added to each well and target messenger RNAs (mRNAs) were amplified by real-time PCR in 20- μl reactions in the ABI PRISM 7900HT real-time PCR system. For all reactions, CT values > 37 were eliminated for evaluation of preamplification efficiency. A standard linear model was applied to the qRT-PCR data to determine whether expression levels in CSC-treated NHBE cells showed differences from the control group. Because all results were derived from the linear amplification curve, the use of $\Delta\Delta\text{CT}$ method ensures that only mRNA amplification within the linear range was compared.

Analysis of protein expression. Lysates of the cultures were prepared, and protein concentration was determined using a Bradford protocol (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Samples

were denatured in Laemmli sample buffer at 95°C for 5 min, and aliquots consisting of 15 μg of protein along with Cruz-compatible standards (Santa Cruz Biotechnology, Santa Cruz, CA) and prestained molecular weight standards were loaded on 10% SDS-polyacrylamide gels with a 3% stacking gel using a MiniProtein III electrophoresis system (Bio-Rad). Proteins were electroblotted onto polyvinylidene fluoride membranes. The blots were stained with Ponceau S and then blocked with 5% nonfat dry milk + 3% BSA and 0.2% Tween-20 in Tris-buffered saline (TBS). Primary antibodies (rabbit anti-NQO1, Santa Cruz 25591 or anti-CYP1A1, Santa Cruz 20772) diluted 1:200 and/or mouse anti- β -actin (Santa Cruz 47778) diluted 1:1000 were added in TBS containing 1% BSA and 0.2% Tween-20 (TBS-wash) and incubated 2 h at room temperature. The blots were washed with the diluent and secondary antibodies (horseradish peroxidase [HRP]-conjugated goat anti-rabbit, Santa Cruz 2030) and/or alkaline phosphatase-conjugated goat anti-mouse (Sigma) at 1:1000 in TBS-wash were added. Blots were washed and developed with Lumigo (KPL HRP substrate) or CDP-Star (APase substrate; Sigma), and images were acquired using a Fuji imaging system (Woodbridge, CT). Bands corresponding to the target proteins were identified by molecular weight and quantified using MultiGauge software. Data are reported as the ratio of the target protein intensity to β -actin.

Measurement of released cytokines by Luminex. The following proteins were assayed in the conditioned medium from cells treated for 18 h with CSC: granulocyte-macrophage colony stimulating factor (GM-CSF), interferon γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and tumor necrosis factor α were measured using a bead-based multiplex system (Biosource 10-plex human cytokine), using a Bioplex 100 instrument (Bio-Rad) according to the supplier's recommendations. CSC preparations were tested at constant dilutions of the CSC (0.1 and 5%) as well as at constant low-nicotine (0.08 $\mu\text{g}/\text{ml}$) and high-nicotine (4 $\mu\text{g}/\text{ml}$) concentrations.

Statistical analysis. Results are presented as percent of control or fold change. Correlations between the various measures of altered gene expression or protein content were determined by plotting pairs of parameters for each sample (4 $\mu\text{g}/\text{ml}$ doses only). Linear regression was performed using GraphPad Prism version 5.0, and coefficients of determination (r^2) and p values, calculated from an F -test, are reported.

RESULTS

Cell Viability

None of the CSCs were overtly toxic at concentrations of $< 5\%$. In fact, there was a substantial increase in the WST conversion rate for all CSC types at the 2.5 and 5% levels (Fig. 1). However, all CSC types were toxic at concentrations of 25%. 2R4F, LIP, and LTMAS produced less than 50% of

FIG. 1. CSC toxicity. CSC was diluted in culture medium to the indicated concentrations, and cells were exposed for 18 h prior to analysis of mitochondrial function using WST. Data indicate the percent of the control values (no CSC) and the SE. Horizontal lines show the range of the controls (mean and SEM).

the control signal at 20%. Only LTMAS produced < 50% of the control signal at the 10% concentration. For subsequent experiments, the exposures were normalized to similar nicotine content, ~4 µg/ml (3.75–4.5 µg/ml) nicotine that corresponds to 5% 2R4F, 5% LNIT, 5% REC, 6.6% LIP, 0.5% BUR, 0.8% BRI, 8% UL, 5% FF, 5% LT, and 2.5% LTMAS.

Identification of Similarly Affected Genes

After combining the genes from all 10 treatments with high signal values that were altered by at least twofold compared to controls, the genes were further sorted to identify those with differential expression found in at least 9 of the 10 treatment samples. Twenty-one genes that were found to meet these criteria are displayed as a heat map (Fig. 2A) and are listed in Table 2 along with their fold change relative to the control samples.

To validate the findings observed by microarray analysis, we selected 3 genes, *CYP1A1*, *NQO-1*, and *BMP-9*, from the list of 21 commonly changed in expression by 9 of the 10 cigarettes and that have been shown to have biological significance in responses to toxic exposures and to cigarette smoke. *CYP1A1* is a gene that encodes for an enzyme that metabolizes toxic compounds (DeMarini *et al.*, 2008; Wardlaw *et al.*, 1998). *NQO-1* is an antioxidant gene (Ahn *et al.*, 2006). Both of these genes were upregulated. In contrast, the gene that encodes for the secretory protein BMP-9, recently shown to act as a tumor suppressor (Ye *et al.*, 2008) and anti-angiogenic factor (David *et al.*, 2008), was strongly downregulated by CSC exposure. Because MMP-1 has a potential involvement in cigarette smoke-induced emphysema (Selman *et al.*, 2003) and was previously shown to be elevated in a lung epithelial cell line following exposure to whole cigarette smoke (Phillips *et al.*, 2005), it was also chosen for further analysis of its expression using qRT-PCR, although it was not among the top 21 genes as described above.

BMP-9 was not detected in any of the mRNA samples by qRT-PCR. Of the other three genes, *CYP1A1* was most strongly induced, followed by *NQO-1* (Fig. 2B). *MMP-1* was induced least. However, 2R4F induced *CYP1A1* ~35-fold, while the other CSCs induced the expression by 10- to 20-fold except for BUR, FF, and LT, which induced expression < 10-fold. *NQO-1* and *MMP-1* mRNA levels were increased between 5- to 10-fold by all cigarettes, except by BRI, BUR, and LT, which induced expression less than 5-fold. These results suggest some differences in the effect the CSCs have on expression of *CYP1A1*, *NQO1*, and *MMP-1*.

To further investigate the microarray and qRT-PCR results, we analyzed expression of CYP1A1 and NQO-1 proteins by Western blotting (Figs. 3A and 3B). Two of the proteins (MMP-1 and BMP-9) selected for analysis were not detected in any of the protein lysates by Western blot analysis. While NQO1 was detected at moderately low levels, CYP1A1 was detected at very low levels. Interestingly, expression levels of NQO-1 protein when normalized for β-actin was increased by

some of the CSCs except for BUR, BRI, UL, and FF (Fig. 3C). While there were some trends for increases in CYP1A1 protein levels for several of the CSCs, the high variability precluded any of these reaching statistical significance (Fig. 3D). Linear regression was used to assess the r^2 and p values for each comparison to determine whether the relative change in expression of NQO-1 and CYP1A1 correlated when measured by Affymetrix and Western analyses (Fig. 3E). A statistically significant correlation was observed for NQO-1 mRNA levels as quantified by microarray and the protein concentration quantified by Western blots (Fig. 3E) ($r^2 = 0.50$, $p = 0.01$). A similar correlation was observed between the qRT-PCR data and the Western analyses (data not shown). However, no correlation was found for *CYP1A1* mRNA levels quantified by microarray and protein concentrations by Western analysis ($r^2 = 0.03$, $p = 0.63$).

Identification of Uniquely Affected Genes

The genes uniquely modified in expression by each of the 10 CSCs were selected using a series of Venn diagrams to exclude common genes found similarly modified in expression by the other 9 CSCs. This approach produced a list of genes that were differentially expressed by each CSC. For each CSC, the three most highly induced and three most repressed genes were selected and presented as a heat map (Fig. 4). In addition, the list of these genes along with their respective fold change compared to control samples is shown in Table 3.

Effect of Various CSCs on Cytokine Secretion

For additional characterization of the relative proinflammatory effects of the various CSCs, the secretion of 10 chemokines and cytokines were determined 18 h after treatment. GM-CSF, IL-1β, and IL-6 were released at low levels in only one experiment and were therefore not considered reliable (data not shown), while IL-8 was released consistently in three independent experiments. All other cytokines tested were not detected in any of the conditioned medium. Treatment with REC, BRI, and LTMAS produced significant increases in IL-8 secretion (Fig. 5A). Of note, while the mRNA levels for IL-8 showed an increase by several CSCs (data not shown), this chemokine was not on the list of either commonly or uniquely induced genes. Of particular interest is the greater stimulation of IL-8 release by LTMAS compared to LT because these condensates were generated from the same cigarette under different machine-smoking conditions.

To observe whether there was a dose-dependent effect on IL-8 secretion, we generated dose-response curves for selected smoke types. CSCs that were generated from the same cigarette by different methods, LT (ISO method) and LTMAS (MI method), showed that the intense smoking machine method produced a greater proinflammatory response compared to smoke generated using the ISO method when diluted to the same nicotine concentration (Fig. 5B). For cigarettes that were

FIG. 2. (A) Heat map of genes that are modified more than twofold by 9 of the 10 cigarette type in NHBE cells. Gene transcripts that are induced by CSC treatment are colored red and genes that are downregulated are colored blue. Absolute signal intensity is shown with darker colors indicating higher and paler shades indicating lower values. The color indicates the fold induction for each independent gene and the intensity indicates the absolute signal values for that specific gene. (B) Validation of the microarray results for three selected genes using qRT-PCR. All samples contained sufficient RNA to conduct qRT-PCR except for NHBE cells treated with REC. Data show fold change from untreated controls (mean and SEM from three repeats).

TABLE 2
Genes Changing in At Least 9 of 10 CSC Samples

Gene symbol	Gene title	Fold changes										
		2R4F	LNIT	REC	LIP	BUR	BRI	UL	FF	LT	LTMAS	Average
SAMD5	Sterile alpha motif domain containing 5	5.99	3.27	3.37	7.67	-1.11	2.49	3.14	4.09	2.48	5.07	3.65
AKR1C1/C2	Aldo-keto reductase family 1, member C1	5.94	3.02	4.05	8.23	-1.20	2.24	3.98	3.83	2.29	5.94	3.83
GPX2	Glutathione peroxidase 2 (gastrointestinal)	6.88	4.12	4.09	6.89	1.00	2.22	4.19	5.13	2.90	6.30	4.37
NQO1	NAD(P)H dehydrogenase, quinone 1	5.73	4.14	4.37	6.31	1.21	2.09	3.81	4.80	2.88	5.44	4.08
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	9.65	5.05	6.56	18.14	1.76	2.58	8.17	5.21	4.41	13.02	7.45
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	6.27	3.69	4.44	8.56	1.89	2.67	5.37	3.80	3.42	6.48	4.66
MAG1(AGPAT9)	1-Acylglycerol-3-phosphate O-acyltransferase 9	4.50	2.72	5.17	5.66	1.72	2.67	4.07	4.07	2.20	4.12	3.69
TFRC	Transferrin receptor (p90, CD71)	5.73	5.03	5.99	6.90	1.85	3.16	4.47	6.25	3.04	5.65	4.81
RAVER1	Ribonucleoprotein, PTB-binding 1	2.18	2.12	2.57	2.42	2.22	1.65	2.16	2.01	2.03	2.56	2.19
HTF9C	HpaII tiny fragments locus 9C	5.70	4.15	5.34	4.16	4.43	3.91	3.97	4.29	5.54	1.85	4.33
PGAR (ANGPTL4)	Angiopoietin-like 4	2.24	3.69	3.39	-2.14	1.64	2.59	2.62	4.37	3.21	2.11	2.37
BE673671	—	-4.63	-4.41	-9.80	-4.07	-3.61	-6.10	-6.49	-4.74	-4.90	-4.61	-5.34
Unknown	hCG2007354	-6.58	-5.49	-5.38	-6.76	-3.13	-5.24	-13.02	-2.85	-3.33	-5.08	-5.69
FBX032	—	-2.13	-4.31	-3.30	-2.04	-2.79	-2.51	-2.66	-2.38	-3.58	-2.11	-2.78
LYPD6B	LY6/PLAUR domain containing 6B	-2.17	-2.34	-3.64	-2.09	-3.48	-2.02	-2.60	-8.93	-1.95	-2.79	-3.20
MXRA5	Matrix-remodeling associated 5	-2.51	-2.52	-2.78	-2.10	-2.00	-1.64	-2.26	-2.94	-2.72	-2.15	-2.36
WNT4	Wingless-type MMTV integration site family, member 4	-3.85	-4.41	-5.32	-2.98	-2.86	-2.31	-2.51	-8.77	-3.73	-2.69	-3.94
BMP9 (GDF2)	Growth differentiation factor 2	-3.61	-1.65	-3.95	-5.41	-3.08	-4.03	-4.17	-4.95	-2.53	-5.52	-3.89
BRAK (CXCL14)	Chemokine (C-X-C motif) ligand14	-10.22	-4.24	-5.56	-12.67	-1.83	-2.48	-3.04	-5.92	-2.82	-3.76	-5.25
METTL7A	Methyltransferase like 7A	-2.44	-2.31	-3.45	-3.69	-1.98	-2.11	-2.02	-2.96	-2.62	-2.29	-2.59
PIG11(TP53I11)	Tumor protein p53 inducible protein 11	-2.65	-2.72	-2.82	-5.08	-5.99	-5.05	-2.08	-3.31	-2.27	-1.75	-3.37

representative for the lowest (UL) and highest (BUR) nicotine levels, the dose-response curve showed very little effect at any dose of the high-nicotine cigarette, while a significantly increased IL-8 release was observed at the highest concentration of the low-nicotine commercial “ultralight” cigarette (Fig. 5C).

DISCUSSION

The present study investigated whether a wide range of commercial and experimental cigarettes affect NHBE cells by causing changes in gene expression and cytokine secretions in common or unique ways. All the CSCs similarly demonstrated a strong increase in expression of genes that coded for xenobiotic and detoxifying functions such as CYP1A1 and CYP1B1 and antioxidants such as GPX2 and NQO-1. The concordance among these responses increases confidence that these effects on gene expression are important biological events. These findings are consistent with previous reports showing gene changes in NHBE cells following exposure to cigarette smoke *in vitro* (Maunder *et al.*, 2007) or in airway cells obtained by brushings from smokers (Spira *et al.*, 2004) and in animal studies (Gebel *et al.*, 2004). It has been proposed

that respiratory epithelial cells respond to cigarette smoke by enhancing the activity of the antioxidant system to prevent damage by lung toxicants (Lannan *et al.*, 1994). This response by airway cells can be important for disease development because an inadequate response to oxidative stress is implicated as a major risk factor for chronic obstructive pulmonary disease (Bowler *et al.*, 2004; MacNee, 2000; Rangasamy *et al.*, 2009).

While the protein levels for CYP1A1 did not correlate with the fold changes determined by the microarray or qRT-PCR, a good correlation was found for changes in NQO-1 mRNA and protein levels by the various cigarette types. It is possible that the low protein levels detected for CYP1A1 by Western blot analysis did not allow for reliable quantitation due to poor signal-to-noise ratio. Therefore, lack of reliable quantitation could have contributed to the lack of correlation with the mRNA measurements. However, it is also possible that protein levels are regulated by protein stability or other mechanisms that are independent of mRNA levels. Future studies need to clarify this discrepancy.

MMP-1, while detected by qRT-PCR, was not detected by immunoblotting, suggesting that this protein is expressed at very low levels. However, previous reports have also shown that MMP-1 is induced in NHBE cells exposed to cigarette

FIG. 3. Analysis of expression for NQO1 (A) and CYP1A1 (B) by Western blot analysis. Protein levels were normalized using β -actin levels (C and D) from three representative Western blots. (E) Relative concentrations of NQO1 (left) and CYP1A1 (right) as measured by Affymetrix and Western blots (normalized to β -actin) were compared. Linear regression was used to assess the correlation coefficient (R^2) and the p values for each comparison.

smoke (Imai *et al.*, 2001; Maunders *et al.*, 2007; Mercer *et al.*, 2004). It is possible that proteins such as MMP-1 are expressed at levels that are sufficient to cause destruction to the lung by acting in their immediate surroundings but will be difficult to detect by conventional methods, such as Western blotting.

BMP-9 showed a strong downregulation by microarray analysis but was not detected either by qRT-PCR or by

Western blotting. These findings suggest that *BMP-9* expression levels are low in control cells; therefore, further down-regulation cannot be verified by these detection techniques. The reason for the discrepancy between the two mRNA quantitation methods is not entirely clear, but it could simply be the result of a greater sensitivity of the array. Alternatives include the possibility that the oligo used by the array detected

FIG. 4. Heat map of genes that were uniquely modified by the 10 cigarette types. For each cigarette, three upregulated and three downregulated genes were selected that were modified in expression at least twofold. Gene transcripts that are induced by CSC treatment are colored red and genes that are downregulated are colored blue. Thus, the color indicates the fold induction for each independent gene and the intensity indicates the absolute signal values for that specific gene.

a similar gene product, not similar in the same regions used for the primers/probe for the qRT-PCR. Pathway mapping analyses of the expression data using Systems Biology and Pathway Analysis (GeneGo Software, Encinitas, CA) suggested that the Wnt and BMP-9 signaling pathways were strongly downregulated by all cigarette types (data not shown). Downregulation of the Wnt receptor and the TGF- β signaling pathways have been found for NHBEs exposed to cigarette smoke (Mauders *et al.*, 2007).

TABLE 3
List of Genes Uniquely Affected by Cigarette Smoke Type

Cigarette type	Probeset ID	Gene name	Fold change
2R4F	230756_at	ZNF683	11.22
2R4F	237274_at		7.23
2R4F	1569095_at	LOC731424	5.50
2R4F	1555536_at	ANTXR2	-3.05
2R4F	209460_at	ABAT	-3.27
2R4F	209940_at	PARP3	-3.51
LNIT	204815_s_at	DHX34	8.87
LNIT	1554602_at	RBM8A	5.88
LNIT	1554430_at	FAM165B	5.46
LNIT	215907_at		-2.83
LNIT	207114_at	LY6G6C	-2.84
LNIT	216589_at	LOC390998	-3.02
REC	1554042_s_at	C20orf141	7.57
REC	209812_x_at	CASP2	4.12
REC	224795_x_at	IGK/IGKC	3.85
REC	229350_x_at	PARP10	-2.69
REC	223551_at	PKIB	-2.94
REC	222749_at	SUFU	-4.83
LIP	209374_s_at	IGHM	9.54
LIP	205830_at	CLGN	7.02
LIP	206515_at	CYP4F3	5.45
LIP	209083_at	CORO1A	-4.25
LIP	225728_at	SORBS2	-6.41
LIP	232475_at	C15orf42	-7.81
BUR	1565617_at		3.27
BUR	212873_at	HMHA1	3.04
BUR	221715_at		2.78
BUR	228331_at	C11orf31	-3.26
BUR	244411_at		-3.99
BUR	239203_at	C7orf53	-4.30
BRI	216600_x_at	ALDOB	3.90
BRI	239959_x_at		3.04
BRI	243409_at	FOXL1	2.69
BRI	214692_s_at	JRK	-2.52
BRI	223915_at	BCOR	-2.53
BRI	1563919_a_at	CCDC123	-3.24
UL	241904_at	LOC732229	2.76
UL	212555_at	PRKAR1B	2.68
UL	1553540_a_at	SLC29A2	2.59
UL	216624_s_at	MLL	-2.68
UL	215492_x_at	PTCRA	-2.84
UL	214795_at	ZMYND8	-3.10
FF	1555952_at	SLC19A1	9.91
FF	230525_at	LOC283875	3.61
FF	220292_at	ZNF434	2.93
FF	227443_at	C9orf150	-3.18
FF	208583_x_at	HIST1H2AG	-3.30
FF	227155_at	LMO4	-3.44
LT	226470_at	GGT7	5.19
LT	220476_s_at	C1orf183	3.02
LT	219846_at	GON4L	2.78
LT	225185_at	MRAS	-2.54
LT	205965_at	BATF	-2.55
LT	239991_at		-2.66

TABLE 3—Continued

Cigarette type	Probeset ID	Gene name	Fold change
LTMAS	226216_at	INSR	4.38
LTMAS	239067_s_at	PANX2	4.13
LTMAS	205784_x_at	ARVCF	2.91
LTMAS	205208_at	ALDH1L1	-2.69
LTMAS	1556336_at	CCBL2	-2.72
LTMAS	235834_at	CALD1	-3.04

While these oxidant and metabolism-related genes were induced by all cigarette types, certain genes were uniquely affected by the different cigarettes. These genes are difficult to group into certain pathways at this time, but pathway analysis may help to interpret their expression. Further research and analysis are needed to determine if these genes are appropriate as biomarkers for exposure to cigarettes that vary in tobacco blend, machine-generated smoke yield, or other design aspects. As previously mentioned, tobacco type can influence the mainstream smoke delivery of nicotine and other chemicals. For example, nitrate and nitrogen-derived chemicals such as the tobacco-specific nitrosamines and ammonia are higher in burley tobacco (e.g., BUR) (Ding *et al.*, 2008; Rodgman and Perfetti, 2008), while flue-cured tobacco (e.g., BRI) has higher levels of polyaromatic hydrocarbon compounds, hydroquinones and catechol (Ding *et al.*, 2008; Wooten *et al.*, 2006). These observations may be relevant in the observation that responses to BUR differed from the other types within the group of upregulated genes.

We detected increases in the mRNA and protein levels of proinflammatory chemokine IL-8, which is not unexpected given the considerable literature indicating responses of this mediator to exposure to a range of environmental agents (Baeza-Squiban *et al.*, 1999) and CSCs (Fields *et al.*, 2005; Kabat, 2003). In addition, the data from the microarray analyses in general corroborated with the cytokine secretion results (data not shown). The effect does not correlate directly with nicotine dose because at similar nicotine levels, LIP and UL (both low nicotine), BRI (high nicotine), and LTMAS (same cigarette as LT) caused the largest increases in IL-8 secretion. However, after correction for multiple comparisons, the effects of LIP and UL were not statistically significant, although the smaller increase induced by REC was significant. Importantly, toxicity as determined by the WST assay did not correlate with the ability to induce IL-8 secretion. Clearly different cigarettes, and even the same cigarette smoked under different conditions, vary in the ratio of tar and other potentially toxic components; and the ratio between these and the nicotine content may have contributed to such findings. It is particularly noteworthy that the effect of the LTMAS was considerably stronger than that of LT, at the same nicotine dose. If there is validity to the concept that smokers use different smoking patterns to achieve the equivalent nicotine

FIG. 5. (A) IL-8 secretion following 18 h of exposure to 4 µg/ml nicotine. Significant increases (* $p < 0.05$) in IL-8 were found for REC, BRI, and LTMAS. The value for LIP, while elevated, did not reach statistical significance. This 4 µg/ml dose of nicotine was achieved with the following concentrations of the CSC: 2R4F, 5%; LNT, 5%; REC, 5%; LIP, 6.6%; BUR, 0.5%; BRI, 0.8%; UL, 8%; FF, 5%; LT, 5%; LTMAS, 2.5%. Dose-response for IL-8 production from two samples from the same cigarette smoked under different conditions (B) and from cells treated with the CSCs with the highest and lowest nicotine content (C).

dose, the reduced-nicotine content cigarettes may provide greater loads of these other toxins.

The strength of this study is that gene expression changes across 9 cigarette types and 10 CSCs were investigated and a

subset of these genes was validated by qRT-PCR and Western blotting. The results show that 9 cigarette types and 10 CSCs, including one that has been referred to as a “reduced exposure cigarette,” affected gene expression associated with xenobiotic metabolism and detoxifying functions. These findings suggest that humans with deficiencies in these protective mechanisms will experience a higher level of negative health effects. Because the exposures were performed at the same nicotine content, the results indicate that the probability of adverse health effects is increased regardless of the type of cigarette smoked. While this message has been suggested by others (e.g., Chen *et al.*, 2008), our study shows evidence for the detrimental effect of cigarettes based on expression profiling studies over a much larger number of cigarette smoke types. In addition, we identified unique gene expression response patterns. These biomarkers could potentially be used in future research to study CSCs that differ in machine-generated smoke yield or other design characteristics and in identifying the specific components that induce particular responses.

One limitation of the present study is that NHBEs were exposed to smoke condensates and not whole smoke from the various cigarettes. The use of CSC for treatment implies that certain smoke components, in particular the vapor phase, are excluded due to differences in volatility, solubility, or stability. The effect of these components could have significant effects on gene expression, which may be responsible for significant toxicological effects. A second limitation is the use of a single time point. Previous studies (reviewed Sen *et al.*, 2007) have suggested transient effects following exposures.

In our studies, various cigarette types were tested in NHBE cells from one individual. This approach removes the problem associated with interpretation of interindividual variability but also cannot provide information on how NHBE cells from various subjects may respond due to genetic variation. In addition, the cells were used under conditions where they are not fully differentiated by culture at air-liquid interface, which could affect their responses. Future studies should involve NHBEs from various individuals to appreciate the range of effect or similar responses and should ideally be performed with fully differentiated cells exposed to fresh whole smoke.

Because inflammation is part of the effect on the airway epithelial cells, future studies should also include exposure of rodents to fully evaluate differences in effects various cigarettes may have on the lung and health in general. Therefore, cigarettes should be tested in *in vivo* models of cigarette smoke-induced inflammation, chronic bronchitis, and emphysema, as has been done previously (Fields *et al.*, 2005; March *et al.*, 2006; Nikula *et al.*, 2000; Seagrave *et al.*, 2004).

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