Toxicological analysis of low-nicotine and nicotine-free cigarettes

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ABSTRACT

Low-nicotine and nicotine-free cigarettes are commercially available under the brand-name Quest®. Some consumers may believe that these are safer cigarettes, and they may smoke more cigarettes or inhale more smoke to compensate for low nicotine yields. Thus, we have studied the toxicological effects of these two cigarettes and compared them with the Kentucky reference cigarette 2R4F. Also, the availability of nicotine-free cigarettes allows for the assessing the role of nicotine in cigarette smoke. In addition to nicotine, some tobacco-specific nitrosamines, aldehydes, and volatile organic compounds were also reduced in the Quest® cigarettes compared to the 2R4F. However, aromatic amines were higher in the nicotine-free compared with low nicotine cigarettes. The Ames test revealed that cigarette smoke condensates from the nicotine-free (CSC-F), low nicotine (CSC-L) and 2R4F (CSC-R) cigarettes had a similar mutagenic potency. Exposure to any CSC caused a similar dose-dependent LDH leakage from normal human bronchial epithelial cells. However, CSC-F had more inhibitory effects on the cell growth than CSC-L and CSC-R. Adding nicotine to the CSC-F attenuated this inhibition. Both Quest® CSCs decreased gap junction intercellular communication and caused cell cycle arrest. CSC exposure increased cytoplasmic nucleosomes, sub-G1/G0 population and apoptotic comet tails. Proapoptotic protein Bax increased independent of p53 induction after exposure to CSC-F. In conclusion, these studies are not consistent with a perception that low-nicotine or nicotine-free cigarettes may have less toxicity in human cells. Nicotine, as it exists in CSC, attenuates cytotoxicity possibly in part through inhibition of apoptotic pathways.

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1. Introduction

Cigarette smoking causes approximately 80–90% of lung cancers in the US (Kabir et al., 2007). Currently, tobacco companies are developing and marketing cigarettes with the publicly stated intent to reduce health effects compared with conventional cigarette products. While there have been attempts in the past to manufacture safer cigarettes, the experience with "light" cigarettes, the best example of a failed attempt, has illustrated the dangers associated with such endeavors (Harris et al., 2004; Stratton et al., 2001). Newer technologies and promotion by the tobacco industry, therefore, require the development of a scientific basis to inform the current debates about whether the new products present hope or hazards. An example of novel tobacco products currently on the market are the cigarettes produced by Vector Tobacco under the trade names of Quest®-1, -2 and -3 which are low nicotine, extra-low nicotine and nicotine-free cigarettes yielding 0.6, 0.3 and <0.05 mg of nicotine, respectively. These products have been sold since 2003 and advertising leads the consumer to believe that they are less harmful, less carcinogenic and less addictive (O'Connor et al., 2005; Shadel et al., 2006). It is known that lower nicotine cigarettes can lead to compensatory smoking such that exposure to cigarette smoke may be increased (Strasser et al., 2007). Thus, a toxicological evaluation is needed to inform consumers and policy makers about actual similarities and differences of these products from conventional cigarettes.

Cigarette smoke (CS) is a complex chemical mixture containing more than 4000 different compounds, of which about 69 are known or probable human carcinogens (Fowles and Dybing, 2003; Hoffmann et al., 2001). While it is possible to study individual chemical constituent of tobacco smoke, this information does not provide a complete picture of the effects that these chemicals may have in the context of complex exposures. In laboratory animal

studies, the shape of the dose-response curve (Smith and Hansch, 2000) and time- and tissue-gene expression response (Gebel et al., 2004) differ with different tobacco smoke constituents, and so the potency of a tobacco smoke constituent complex mixture cannot be predicted. Mainstream smoke consists of particulate and vapor phases of which the former can be collected with the use of smoking machines passing the smoke through a Cambridge filter pad. The material collected is known as cigarette smoke condensate (CSC) and contains most of the chemicals, and particularly the carcinogens in the smoke. Furthermore, the preparation of CSC can be standardized using a smoking machines, and so CSC rather than whole smoke or mixtures of the key constituent chemicals is what is most often used in smoke research (Foy et al., 2004; Hellermann et al., 2002). CSC is composed of tar, nicotine and water, and though the tar has often been the focus of toxicological studies, nicotine is one of the most physiologically active components of cigarette smoke. In addition to its addictive properties, some evidences support the hypothesis that nicotine may contribute directly to carcinogenesis through stimulation of nicotinic acetylcholine receptors (nAchR) in non-neuronal cells (Carlisle et al., 2004; Minna, 2003), thereby affecting cell proliferation and apoptosis (Chu et al., 2005; Mai et al., 2003), and promoting angiogenesis (Heeschen et al., 2001). Nicotine is, however, not itself tumorigenic in experimental animal studies (Martin et al., 1979), but there have been limited studies of the role of nicotine in CSC (Gullihorn et al., 2005). The availability of the nicotine-free Quest® cigarette offers a novel opportunity for toxicological evaluation of the role of nicotine in cigarette smoke and allows the direct assessment of the tar fraction of cigarette smoke independent of nicotine effects.

Most cigarette carcinogens are also strong mutagens which can form DNA adducts, and thereby activate proto-oncogenes, inactivate tumor suppressor genes (DeMarini, 2004), or disrupt critical regulatory pathways predisposing cells to carcinogenesis. To evaluate the mutagenic potential of CSC from the various products we made use of the *Salmonella*/microsome plate incorporation Assay, also known as the Ames test, which is the most widely used method to evaluate the mutagenicity of cigarette smoke and other mutagenic agents (Mortelmans and Zeiger, 2000). In addition to genetic insults, smoke constituents also activate biochemical pathways that are associated with apoptosis, cell cycle progression and cell growth (Tsurutani et al., 2005; Yang and Liu, 2004). The cytotoxicity of the CSC samples and their ability to alter proliferation, cell cycle progression and apoptotic index have been evaluated using primary cultures of human bronchial epithelial cells and standard methodologies. Inhibition of gap junction intercellular communication (GJIC) has been found to be associated with tumor promotion in most if not all tumor cells (Mesnil et al., 2005). Cultured human bronchial epithelial cells and endothelial cells derived from human coronary arteries were used to evaluate the inhibition of GJIC by CSC. The inhibition of GJIC by CSC fractions was considerably lower than that by un-fractionated CSC (Vang et al., 1995), indicating that compounds in the CSC act strongly synergistically. Change of cigarette components may also change its inhibitory effect on GJIC.

In order to more fully characterize the toxicological effects of Quest® cigarettes and to evaluate the impact that nicotine had on the end-points under study, we conducted smoke chemistry analysis and a comparative evaluation of the genotoxicity and cytotoxicity of the mainstream CSC from Quest® low-nicotine and nicotine-free cigarettes and 2R4F Kentucky Reference research cigarettes. Our studies did not show that low-nicotine or nicotine-free cigarettes would have less toxicity in human primary bronchial epithelial cells. Nicotine, as it exists in CSC, maybe afforded some protection from cytotoxicity.

2. Materials and methods

2.1. Materials

Quest® nicotine-free (<0.05 mg nicotine) and low-nicotine yield (0.6 mg nicotine) cigarettes, manufactured by Vector Tobacco Inc. (Timberlake, NC), were purchased from local commercial sources. The 2R4F Kentucky Reference Research Cigarette (0.8 mg nicotine yield) was obtained from the Smoking and Health Institute of the University of Kentucky (Lexington, KY). Nicotine hydrogen tartrate salt was purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade or better and bought from standard suppliers unless otherwise stated.

2.2. Smoke chemistry analysis

Smoke emission testing was conducted by Arista Laboratories (Richmond, VA). Five cigarettes were randomly selected from each of 50 packages for analysis. Smoking machines (Tri-City, Cerulean, Borgwaldt) were used for sample collection to a glass fiber pad (total particulate matter, TPM), a solvent based impinger (volatile constituents), or a combination of the two (semi-volatile constituents). Cigarette samples were conditioned prior to smoking by exposure to a controlled environment (60% relative humidity, 23.5 °C) for 48 h. Using smoking conditions specified by the Federal Trade Commission (FTC; 35 ml puffs with 2 second duration every 60 s, smoked down to a specified length), we analyzed 60 constituents in the mainstream smoke of two Quest® cigarettes and 2R4F cigarettes. The analytes selected were those whose abundance was sufficient to allow quantification of the analytes in a composite sample of 5–40 cigarettes, and which are of general interest as either direct toxicants or indicators of a class of compounds that may be toxicants (Roemer et al., 2004; Smith and Hansch, 2000).

2.3. Cigarette smoke condensate (CSC)

CSC was prepared using a custom 30-port rotary smoking machine (Baumgarten; Belmont, Switzerland) smoking the cigarettes using the standard FTC puffing regimen, as above. The total particulate matter (TPM), herein also referred to as CSC was collected onto Cambridge filter pads (Whatman, Maidstone, UK), and extracted (40 min with constant shaking) with dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO). The condensates were coded, aliquot and stored at −80 °C until assayed within 3 months after preparation. The condensates from 2R4F cigarettes, Quest® low-nicotines cigarette or nicotine-free cigarettes were designated as CSC-R, CSC-L and CSC-F, respectively.

2.4. Salmonella/microsome plate incorporation assay (Ames test)

This assay was conducted as previously described (Mortelmans and Zeiger, 2000). Briefly, the *Salmonella* TA98 or TA100 strain was inoculated into DIFCO nutrient broth (Becton Inc., France) and cultured at 37 °C overnight to a density of about 10^6 cells/ml. Top agar supplemented with biotin-histidine was melted and maintained at 45 °C. Test samples along with positive (2-anthramine, 1.0 µg/plate) and vehicle controls were mixed sequentially with Arochlor-induced Sprague–Dawley rat S9 mix (Moltox Inc., Boone, NC), then top agar and bacteria, and poured directly onto pre-prepared VBMG agar plates (Presque Isle Cultures, Erie, PA). After the top agar set, the plates were incubated at 37 °C for 72 h in the dark. Bacterial colonies were counted and analyzed using a AccuCount™ 1000 automatic counter (Biologics, Inc.). Five or six dose levels (0–0.25 mg/plate) were assayed in triplicate along with negative controls and used to identify the linear portion of the dose-response curve. The primary outcome is the number of revertant colonies per plate, corrected for the number of colonies in control plates. A plot of average revertant colonies against dosage was carried out to determine the dose-response curve slope of each testing product by linear regression method. A result is considered to be positive when there is a significant dose-related increase, where the number of revertant colonies is twice the spontaneous background.

2.5. Isolation and culture of normal human bronchial epithelial (NHBE) cells

Fresh human bronchi were immersed in cold DMEM/Ham's F12 medium immediately after resection. The use of these tissues was approved by the Georgetown University Institutional Review Board and with the informed consent of each patient. Histologically normal bronchial tissues were trimmed, cut, and incubated at 4 °C overnight in 0.1% protease media (Sigma) as described (Blouquit et al., 2002). After adding 10% fetal bovine serum, the cells were detached by gentle scraping, harvested by centrifugation, and resuspended in Clonetics® BEGM medium (Cambrex, Walkersville, MD). The cells were then plated on a type I collagen-coated flask and incubated at 37 °C in 5% CO_2/95% air. The media was changed after 24 h and then every other day thereafter. The cells were passaged when they reached 80% confluence. Immunocytochemical staining showed that more than 95% of cells prepared by this method were cytokeratin positive (clone AE-1/AE-3 pan, Signet, Dedham, MA). The cells were used between passages 2 and 6.

2.6. LDH release assay, WST-8 conversion assay and cell growth assay

The acute cytotoxicity of CSC or nicotine was examined using lactate dehydrogenase (LDH) release method. NHBE cells were placed into a 96-well plate at a density of 5000 cells/well overnight, and then treated with CSC and/or nicotine at 37 °C for 3 h. The plates were centrifuged and the supernatants transferred to a fresh 96-well plate. The LDH activity in the supernatant was determined as per the manufacturer's instruction (TOX-7; Sigma) (Putman et al., 2002). All tests were performed in triplicate and the experiment was performed at least three times. Cell viability of treated NHBE cells in the plates was confirmed using the trypan blue dye exclusion method (Deng et al., 2000).

WST-8 was also used to determine cytotoxic activity of CSC or nicotine on NHBE cells. The detection sensitivity of CCK-8 is higher than other tetrazolium salts such as MTT, XTT, MTS or WST-1. NHBE cells were seeded into 96-well plate at a density of 1×10^4 cells/well and treated with CSC with or without nicotine overnight. WST-8 conversion was then assessed using a one-step Cell Counting Kit-8 (CCK-8), according to the manufacture's instructions (Dojindo Molecular Technologies Inc. Gaithersburg, MD). The WST-8 method only generates a signal from the viable cells. All tests were carried out in triplicate.

To determine the inhibitory effects of CSC and nicotine on cell growth, NHBE cells were seeded into 24-well plate at a density of 5×10^4 cells/well and treated with sub-lethal dosage of CSC (as determined by LDH release assay) in the absence or presence of nicotine for 3 days. The floating and adherent cells were then harvested with 10 mM EDTA and counted by a Coulter Counter. The cell growth inhibition was expressed as percentage of untreated control wells. All treatments are performed in triplicate. The experiment was performed at least three times.

2.7. Gap junction intercellular communication (GJIC)

GJIC was determined by a 'flow cytometry' technique adapted from the methods of Czyz et al. (2000) and Rudkin et al. (2002). Normal bronchial epithelial cells were grown to 70–80% confluence in T-25 flasks. Cells, designated as "donor cells" were exposed to 40 μg/ml of CSC or nicotine overnight and then sequentially labeled with the gap junction–permeable dye calcein AM (0.05 μM, Molecular Probes) for 30 min and PKH26 (2 μM, Sigma), a nontransferable membrane dye for 7 min. The double-stained donor cells were then plated onto monolayers of unstained "recipient" cells at ratio of 1:10 (donor:acceptor) and incubated for 2 h at 37 °C. The co-cultures were then harvested, washed, resuspended and subjected to FACS analysis (Becton Dickinson FACStar Plus dual laser system, Heidelberg, Germany). GJIC results in the transfer of calcein from donor cells to recipient cells. The results are presented as a coupling ratio C_r, which is given by the number of calcein-transferred recipient cells which are labeled green, per donor cell (labeled with red and green). Heptanol (Sigma), a rapid inhibitor of GJIC, was used as a positive control (3 mM) for the inhibition of GJIC.

2.8. Cytoplasmic nucleosome detection

NHBE cells were plated in 6-well plate at 50% confluence and treated with CSC and/or nicotine at 37 °C overnight. The cells were then harvested by trypsinization, washed, resuspended and assayed for apoptosis as indicated by the amount of cytoplasmic nucleosome which was determined with Cell Death Detection ELISA kit (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. As a positive control for the induction of apoptosis, etoposide (VP-16, Calbiochem, La Jolla, CA) was used.

2.9. Cell cycle analysis

NHBE cells were placed in T-25 flasks at 50% confluence and incubated with CSC and/or nicotine at 37 °C for different times. After treatment, $1–2 \times 10^6$ of floating and adherent cells were harvested by trypsinization, fixed in ice-cold 75% ethanol at −20 °C overnight. The cells were then treated with DNase-free RNase A (0.15 mg/ml, Sigma) at 37 °C for 30 min followed by incubation with propidium iodide (10 μg/ml, Sigma) at 37 °C for 10 min. The DNA content of individual cells was analyzed with a fluorescence-activated cell sorter. Cells with less DNA than that of G1/G0 cells (sub-diploid DNA peak) were considered to be apoptotic (Liu et al., 2005).

2.10. Neutral Comet assay

The Comet assay was conducted using a CometAssay kit (R&D Systems Inc., Minneapolis, MN) under neutral conditions, which detects mainly double strand DNA breaks and can be useful for assessing the DNA fragmentation associated with apoptosis. Briefly, NHBE cells were cultured in 6-well plates and treated with 50 μg/ml CSC or VP-16 (1 mM for 18 h). Attached and floating cells were then harvested and mixed with molten LMAgarose. The agarose/cells were pipetted over the sample area of CometSlides and after hardening the slides were immersed in Lysis solution for 30 min followed by horizontal electrophoresis in TBE buffer at 1 V/cm for 15 min. After fixing with 70% ethanol for 5 min, the slides were air-dried and stained with

SYBR Golden. The cells were viewed with an epifluorescence microscope and photographed. For scoring the comet pattern, 100 nuclei from each slide were counted. The comet was scored from 0 to 4 according to Collins criteria by measuring tail length, head size, tail intensity and head intensity (Collins, 2002; Yasuhara et al., 2003). Typical apoptotic cells are defined as those with diffuse fan-like tails and very small heads (Liu et al., 2005; Yasuhara et al., 2003). Scores more than 2 (i.e. relative tail intensity >50%) are attributed to apoptosis (Collins, 2002; Yasuhara et al., 2003).

2.11. Western blotting

Protein levels were assayed by Western blot analysis, as previously reported (Chen et al., 2005). NHBE cells were plated in 6-well plates at 80% confluence and treated with CSC and/or nicotine at 37 °C overnight. After treatment, the cells were harvested with lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.5 mg/ml leupeptin, 1 mM EDTA, 1 mg/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride) and the protein concentration determined by BCA assay (Pierce, Rockford, IL). Samples (30 μg) of protein were loaded onto a Novex® 10% tri-glycine gels (Invitrogen, Carlsbad, CA), separated by PAGE, and transferred to a sheets of hydrophilic polyvinylidene fluoride (PVDF). The membranes were blocked with 5% nonfat milk for 30 min and then immuno-stained with primary antibodies against either p53, PARP, Bcl2, Bax, or Caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight, followed by a peroxidase-conjugated secondary antibody. The immuno-positive bands were visualized with Super Signal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) system.

2.12. Statistical analysis

Evaluation was carried out using SAS software. Data were expressed as mean ± S.D. Each experiment was repeated at least two times. Concentration-dependent differences of testing products in Ames test and cell cycle with flow cytomery were analyzed by analysis of covariance (ANCOVA). The unpaired Student's t-test was used to evaluate the significance of difference in chemistry analysis, cell growth and proliferation assays and cytotoxic experiment at equal concentrations of CSC from different cigarettes. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Smoke chemistry analysis of Quest® and reference cigarettes

We compared the cigarette smoke from the nicotine-free (we confirmed as 0.032 ± 0.001 mg/cig), 0.6 mg for the low-nicotine cigarette (we confirmed as 0.53 ± 0.01 mg/cig), and the 2R4F cigarette (we confirmed as 0.80 mg/cig) generated using the FTC smoking protocol. Table 1 showed statistically significant differences and similarities for only 20 smoke constituents (because of space limit) which are well investigated. The tar yields were 7.87 ± 0.16, 8.99 ± 0.26 and 9.62 ± 0.38 mg/cig, respectively, although the puff counts were 30% lower for the Quest® cigarettes. Compared with reference 2R4F cigarette, the levels of tobacco-specific nitrosamines (TSNAs) were substantially lower for the two Quest® cigarettes. NNK [4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone] and NNN (N-nitrosonornicotine) are the two carcinogenic TSNAs primarily formed during the curing process from nicotine and nornicotine (Hayes et al., 2007). The levels of NNK were 10.3 ± 1.9, 24.8 ± 4.1 and 132.0 ± 4.0 ng/cig for the nicotine-free, low-nicotine and reference cigarettes, respectively ($p < 0.001$). Other TSNAs (NNN, NAT, NAB) were also lower, but not to the same degree, and the pattern was different, with the yield of NNN being higher in nicotine-free cigarettes (67.9 ng/cig) than in low-nicotine cigarettes (49.2 ng/cig) ($p < 0.01$). The yield of other carcinogens, such as benzo(a)pyrene were a little bit but significantly lower in the low-nicotine or nicotine-free cigarettes compared to 2R4F cigarettes. A similar reduction in volatile organics was seen compared to the reference cigarettes, in general about 50–60% for 1,3-butadiene and benzene ($p < 0.01$), although the semi-volatile constituents were not different. Formaldehyde levels were similar for the 2R4F and the low-nicotine Quest®, but reduced by almost half for the nicotine-free Quest®. Interestingly, though the levels for aromatic amines were similar for the low-nicotine Quest® and 2R4F,

Table 1
Yields of smoke constituents under FTC condition (mean ± S.D.) of reference cigarette, Quest® low-nicotine and nicotine-free cigarettes

Constituents	Unit per cig	Reference 2R4F	Quest® Low-nicotine	Quest® Nicotine-free
FTC parameters				
TPM	mg	11.13 ± 0.41	10.32 ± 0.34	8.50 ± 0.22[*]
Tar	mg	9.62 ± 0.38	8.99 ± 0.26	7.87 ± 0.16[*]
Nicotine	mg	0.80 ± 0.03	0.53 ± 0.01[*]	0.032 ± 0.001[*]
Water	mg	0.71 ± 0.00	0.80 ± 0.13[*]	0.60 ± 0.06[*]
Carbon-monoxide	mg	12.05 ± 0.17	10.69 ± 0.21[*]	9.97 ± 0.57[*]
Puff count	puff	9.2 ± 0.1	6.6 ± 0.1[*]	6.0 ± 0.0[*]
Aldehydes(carbonyls)				
Acrolein	μg	57.2 ± 6.6	22.0 ± 3.5[*]	19.8 ± 3.0[*]
Acetaldehyde	μg	617 ± 75	337 ± 45[*]	363 ± 53[*]
Formaldehyde	μg	20.3 ± 0.6	21.0 ± 1.7	9.5 ± 2.1[*]
Aromatic amines				
2-Naphthylamine	ng	6.30 ± 0.07	5.51 ± 0.71	8.32 ± 0.86[*]
4-Aminobiphenyl	ng	1.25 ± 0.01	1.11 ± 0.08	1.93 ± 0.14[*]
N-Nitrosamines				
NNK	ng	132.0 ± 4.0	24.8 ± 4.1[*]	10.3 ± 1.9[*]
NNN	ng	140.0 ± 12	49.2 ± 2.4[*]	67.9 ± 3.3[*]
Phenols				
Catechol	μg	43.7 ± 2.7	35.3 ± 1.9[*]	20.0 ± 0.6[*]
Phenol	μg	8.33 ± 0.62	11.6 ± 0.7[*]	9.47 ± 0.43
Resorcinol	μg	0.67 ± 0.01	0.74 ± 0.05	0.37 ± 0.02[*]
PAH				
Benzo(a)anthracene	ng	13.9 ± 0.8	10.5 ± 0.4[*]	8.66 ± 0.29[*]
Benzo(a)pyrene	ng	5.82 ± 0.25	4.51 ± 0.27[*]	3.75 ± 0.21[*]
Volatiles				
Benzene	μg	49.4 ± 1.6	15.0 ± 1.0[*]	14.8 ± 1.5[*]
Isoprene	μg	371 ± 3	149 ± 8[*]	130 ± 7[*]
1,3-Butadiene	μg	32.7 ± 1.1	18.1 ± 1.5[*]	15.7 ± 0.9[*]

[*] $p < 0.05$ compared with Reference 2R4F.

it was higher for the nicotine-free Quest®. For example, the levels of 4-aminobiphenyl, were increased 42% over the low-nicotine Quest® ($p < 0.05$) and 35% over the 2R4F ($p < 0.05$).

### 3.2. In vitro mutagenicity of CSC and nicotine

The mutagenic activity of CSC and nicotine was measured using the Ames test. CSC from 2R4F reference (CSC-R), Quest® low nicotine (CSC-L) and nicotine-free (CSC-F) cigarettes tested against *Salmonella* TA98 with activation by S9 all exhibited a dose-dependent revertant response ($p < 0.01$) with slopes of 0.388, 0.356 and 0.364, respectively. The revertant colonies at a dose of 0.15 mg CSC/plate were more than 10 times the spontaneous background for the three CSC ($p < 0.001$). When compared on a revertants per mg TPM basis, there was no statistical difference ($p > 0.05$) in the dose response slopes of the three CSCs (Fig. 1) by ANCOVA test. The Ames test with TA100 bacteria produced similar data (not shown). Nicotine alone had no mutagenic activity and when nicotine was added back to Quest® CSC-F at levels similar to conventional "low tar" yield cigarettes, the slope of the dose response curve was not altered.

3.3. In vitro cytotoxic and anti-proliferative effects of CSC and nicotine in NHBE cells

To determine the cytotoxic and antiproliferative effects of the CSCs, four *in vitro* assays with different endpoints were selected and performed using NHBE cells according to methods previously published (Matsuoka et al., 2000; Putnam et al., 2002). Acute toxicity of the CSCs was assessed using an LDH release assay (Fig. 2A) and trypan blue exclusion test, the toxic effects of an overnight exposure to the condensates was determined by evaluating total cell viability with a CCK-8 assay (Fig. 2B), and effects on cell proliferation and

growth were measured by determining the change in cell number after three days of exposure (Fig. 2C).

LDL release reflects direct damage to the cell plasma membrane and is the most sensitive cytotoxic method for short-time exposures (Putnam et al., 2002). Quest® and 2R4F CSCs all caused a dose-dependent elevation in LDH release at doses > 60–80 μg/ml, but there was no significant difference among them at any dose level ($p > 0.05$) (Fig. 2A), indicating they produce similar acute toxicity. The studies assessing cell viability by trypan blue dye exclusion produced similar data again showing dose-dependent toxicity, but no difference among the three CSCs (data not sown). A viability > 90%
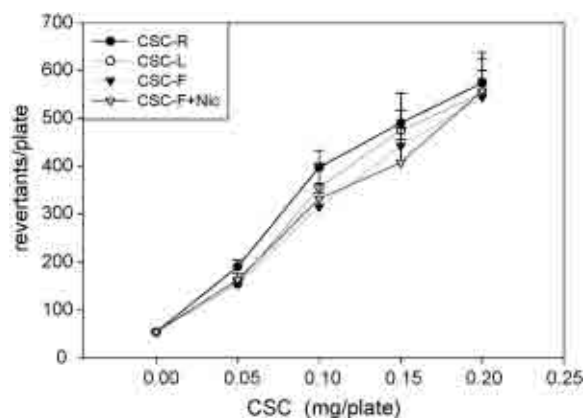
Fig. 1. Mutagenic activity of different CSCs by Ames test. Evaluation was performed in bacterial strain TA98 in the presence of S9 solution. Mutagenicity was expressed as revertants/plate/mg CSC. All testing was conducted with triplicate plates at each concentration. Ames test in TA100 (data not shown here) revealed a similar pattern. Shown is one example of three or more independent experiments, all with similar results.
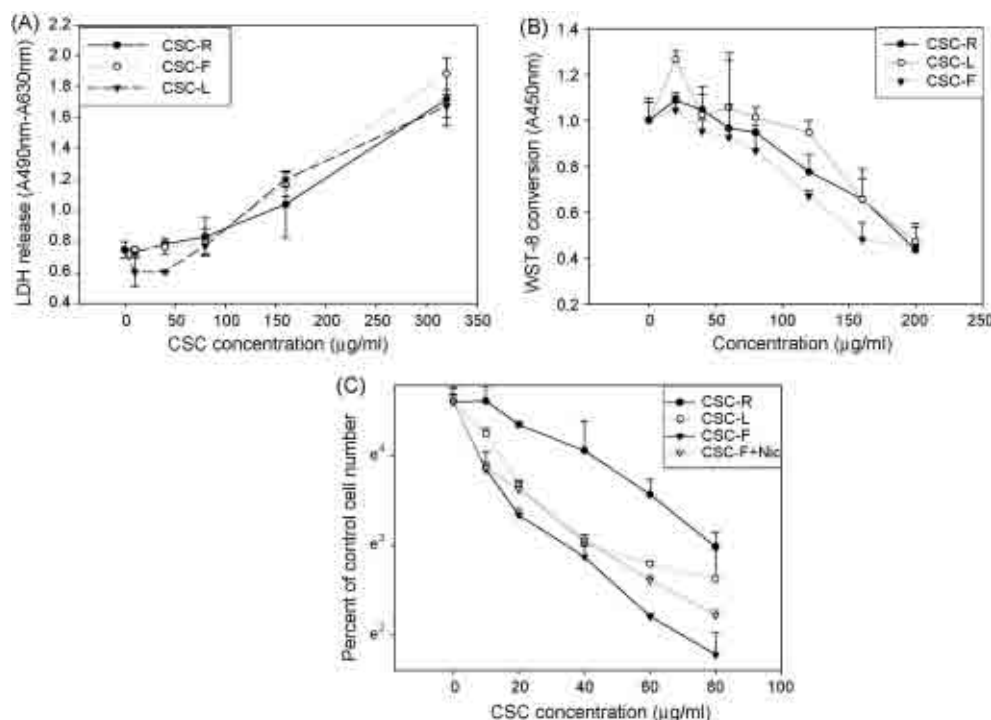
Fig. 2. Cytotoxic and anti-proliferative effects of CSC and nicotine in normal human bronchial epithelial (NHBE) cells. NHBE cells were exposed to the indicated concentration of CSC from Kentucky 2R4F reference cigarettes (CSC-R), or Quest® low-nicotine cigarette (CSC-L) or nicotine-free cigarette (CSC-F) in the absence or presence of nicotine (10 μM): (A) the level of LDH released from cytoplasm into the culture media after exposure for 3 h was determined with Sigma TOX-7 kit; (B) after overnight exposure, cell viability was assayed with a CCK-8 kit, which measures mitochondrial dehydrogenase activities of viable cells by reducing WST-8 to produce a yellow color formazan dye; (C) cell number after exposure for 3 days. The inhibition of cell growth was expressed as percentage of untreated control wells. All tests were performed in triplicate. Shown is one example of three or more independent experiments, all with similar results.

of NHBC was observed when exposure to CSC at dose < 60 μg/ml which was used as a sublethal dose threshold for other assays.

The CCK-8 assay (Fig. 2B), quantifies total cell number and viability by assaying mitochondrial dehydrogenase activity and revealed that although all three CSC treatments suppressed WST-8 conversion, CSC-F at doses of 80, 120 and 160 μg/ml was apparently more toxic than CSC-L or CSC-R ($p < 0.05$). That meant that besides the similar acute cell killing mechanism evidenced by LDH release assay, other toxic mechanisms existed and they were different for Quest® and reference cigarette smokes.

In addition to their cytotoxicity, all three CSCs exhibited a dose-dependent suppression of NHBE cell proliferation and growth (Fig. 2C) even the concentration was as low as 10 μg/ml which did not kill the cells (Fig. 2A and B). However, the reference cigarette CSC produced less inhibition than the two Quest® cigarette CSCs, in spite of possessing higher levels of some of the carcinogens (Table 1). Furthermore, the CSC-F inhibited the cell growth to a greater extent than CSC-L ($p < 0.05$). When 10 μM of nicotine (equivalent to the nicotine content in 80 μg/ml of CSC-L) was added to the CSC-F, growth suppression was significantly reduced ($p < 0.05$) yielding cell numbers close to those with CSC-L treatment, supporting a possible role for nicotine in cell survival.

3.4. Effects of CSC on gap junction intercellular communication (GJIC)

To evaluate the effects on gap junction formation, a GJIC was conducted at noncytotoxic concentrations of CSC, as determined by the LDH release assay. Calcein, which can travel from cell to cell through gap junctions, was transferred from donor bronchial epithelial cells to unstained recipient cells after co-culture for 2 h with a coupling ratio (C_r) of 2.05 (Fig. 3). Treatment of donor cells with 40 μg/ml

of CSC-R, or Quest® CSC-L or CSC-F overnight did not affect the staining of the donor cells with PKH26 or calcein the retention of which is dependent on cell viability (data not shown), but remarkably impaired the ability of the cells to transfer the calcein to the recipient cells (Fig. 3). The C_r decreased to 1.21, 0.85 and 0.62 for CSC-R, or Quest® CSC-L or CSC-F, respectively. This indicates that the Quest® CSCs inhibited GJIC significantly more than CSC from the reference cigarettes. There was, however, no statistical difference between the two Quest® CSCs according to three independent experiments ($p > 0.05$). The addition of nicotine at 10–50 μM did not significantly affect the GJIC (data not shown).

3.5. Effects of CSC and nicotine on apoptosis

To completely elucidate the mechanisms responsible for the NHBE cell loss by exposure to CSCs, we investigated their effects on apoptosis in addition to acute necrotic effect and antiproliferative effect. NHBE cells were exposed overnight to the apoptosis-inducer VP-16 or CSCs at either low dose (50 μg/ml) or high dose (100 μg/ml). Cytoplasmic nucleosome levels were then determined using the sensitive ELISA method. CSC-R and both Quest® CSCs exposure at sub-lethal doses of 50 μg/ml did not trigger a significant apoptotic response (Fig. 4A). In contrast, at higher doses, the cytoplasmic nucleosome level was significantly increased ($p < 0.05$) after exposure to CSC-F (100 μg/ml), but not to CSC-L or CSC-R, indicating that the nicotine-free CSC could induce an apoptotic effect. When the equivalent amount of nicotine (10 μM) was added to the CSC-F, this increasing effect was abolished, indicating that nicotine in the CSC suppressed the CSC-induced apoptosis.

To further explore if CSC induces apoptosis, the DNA content of populated cells was analyzed by flow cytometry, and DNA fragmentation and chromosomal condensation in individual cell was
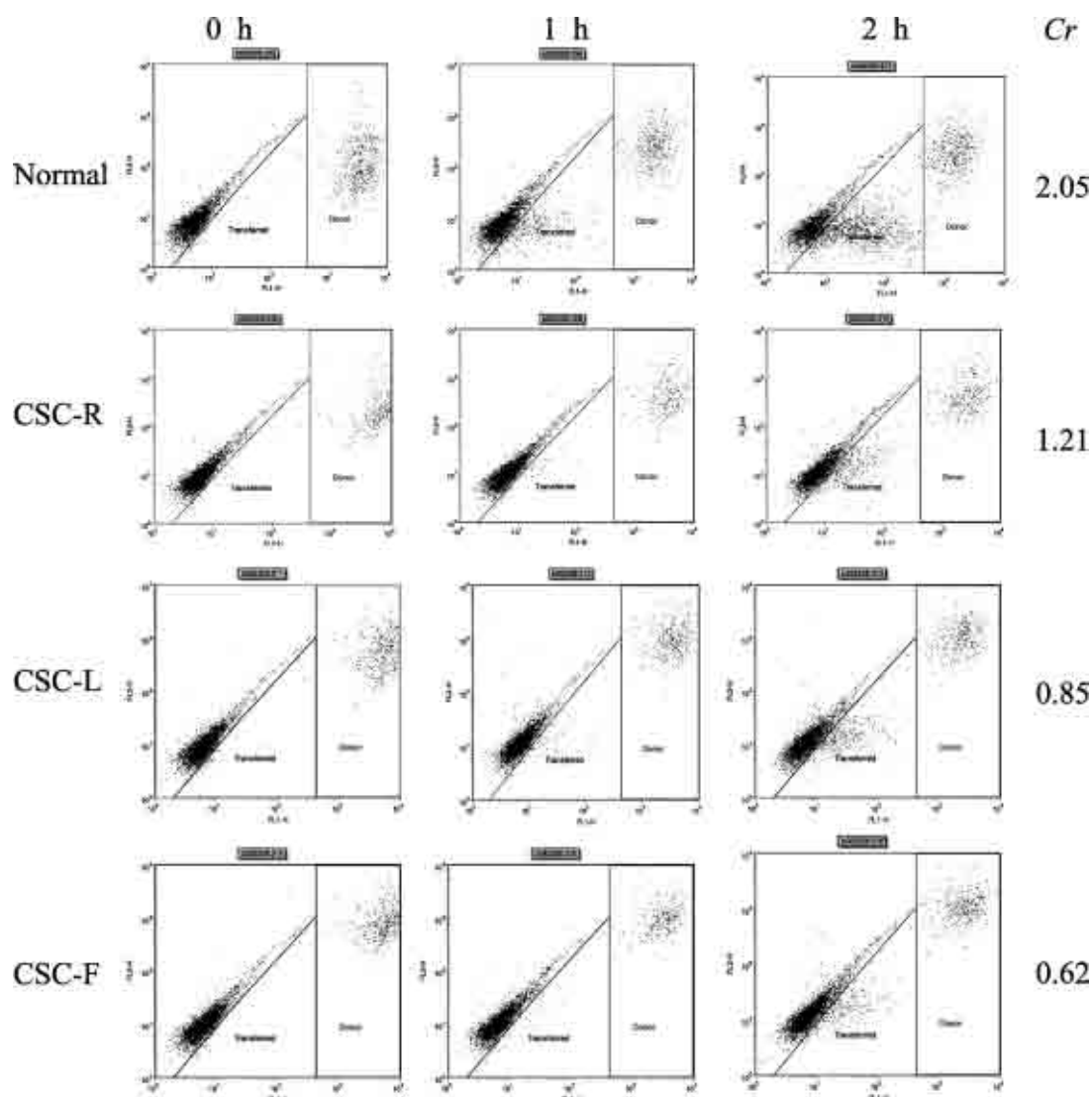
Fig. 3. Effects of CSC on gap junction intercellular communication (GJIC) analyzed by fluorescent dye transfer between NHBE cells. Double-stained (Calcein AM and PKH26) normal or CSC treated donor cells were mixed with non-dyed recipient cells at a ratio of 1:10. Calcein transfer occurred after contact between donor and recipient cells. The number of coupled recipient (calcein-dyed) cells per donor cell (coupling ratio C_r) was determined by flow cytometry after co-culture for 0, 1 and 2 h. Shown is one example of two or more independent experiments, all with similar results.

evaluated by the single-cell electrophoresis assay (neutral Comet assay). After exposure of NHBE cells to CSC-L or CSC-F, there was a gradual increase of the number of cells with hypodiploid DNA content with concentrations of up to 60 µg/ml (Fig. 4B and Table 2). The cell numbers in sub-G1/G0 peaks were significantly increased (>5%) after exposure to 40–80 µg/ml of CSC-F or CSC-L for 48 h (Table 2) compared with background numbers (spontaneous apoptosis-related), it was even higher for CSC-F compared to CSC-L ($p < 0.05$). When NHBE cells exposed to 50 µg/ml of CSCs for 18 h were electrophoresed under neutral conditions, there were more frequent and longer comet tails (oligonucleosomal DNA fragmentation) with small comet heads (chromosomal condensation) compared with control cells (Fig. 4C). The percentage of nuclei that yielded comet scores >2 were 1%, 43%, 15%, 12%, and 17% for medium control, VP-16, CSC-R, CSC-L, and CSC-F, respectively.

To investigate what molecules were involved in the CSC-induced apoptosis, we first tested if p53 can be induced in NHBE cells. As showed in Fig. 4D, VP-16 induces p53 expression. This was confirmed in four NHBE cell strains from four subjects. But none of three CSC treatments upregulated the expression of p53 protein

or its phosphorylation at Ser 15 residue in NHBE cells. The PARP protein was not detected in three NHBE cell strains before or after exposure to CSC-F (data not shown). Apoptosis can be divided into three interdependent phases: induction, decision and execution (Xin and Deng, 2005). The decision phases is largely regulated by the Bcl-2 family of apoptotic regulators, which includes Bcl-2 and Bax (Oltvai et al., 1993). CSC-F increased the Bax expression even at the low dosage (the band intensity at 40 µg/ml increased 3.26-fold compared to control), but it did not change the Bcl-2 expression (Fig 4D). Therefore the ratio of Bax/Bcl-2 increased after exposure to CSC-F. We did not find the expression of caspase-3 in NHBE cells before and after CSC treatment (data not shown).

3.6. Effects of CSC and nicotine on cell cycle

To further explore how CSC inhibited the cell proliferation and growth (Fig. 2C), CSC effect on the cell cycle was tested using FACS analysis of the NHBE cells. As showed in Table 2, both Quest® CSCs caused a dose-dependent cell cycle arrest early (16 h) at G1 checkpoint and later (48 h) at the G2/M checkpoint when CSC
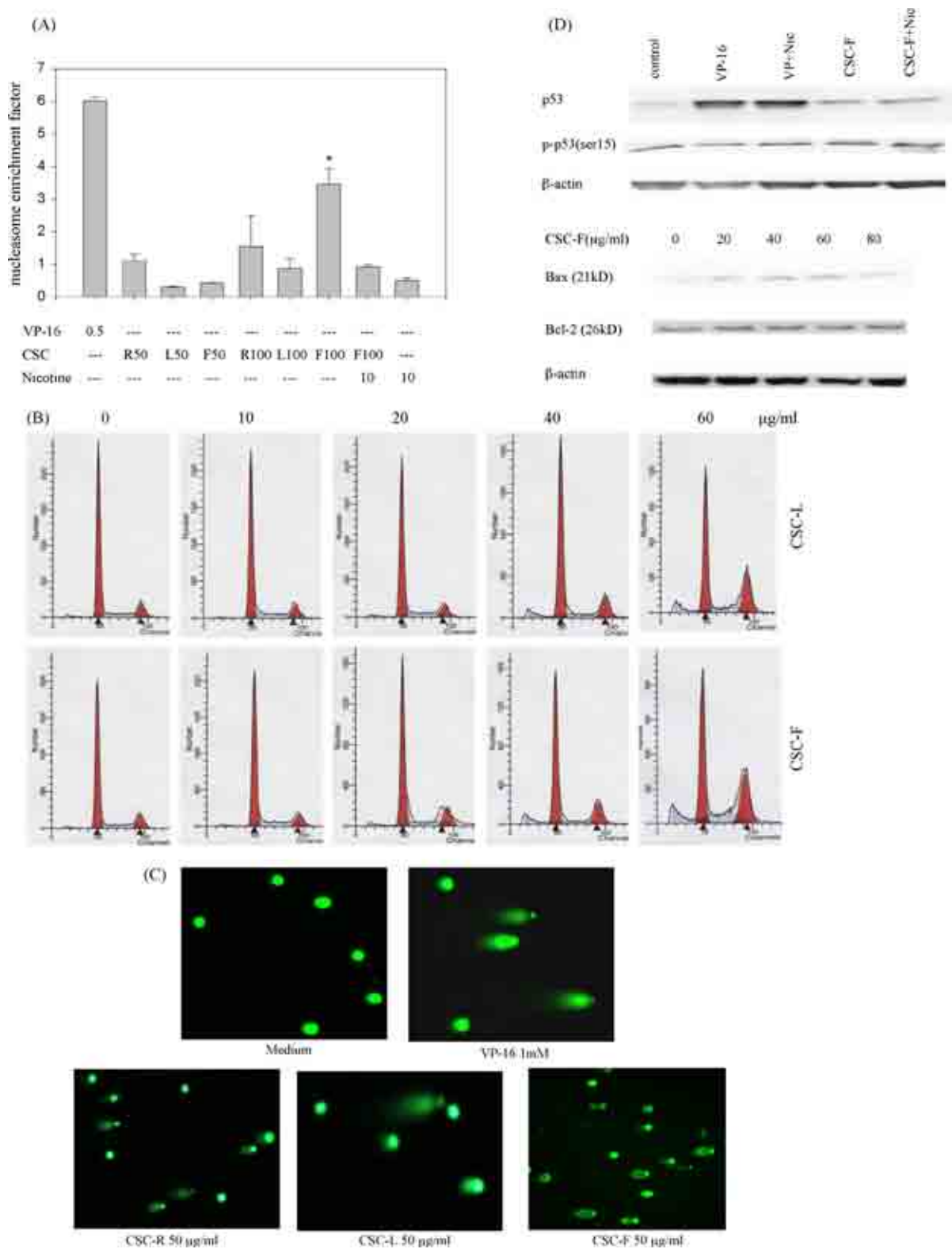
Fig. 4. Effects of CSC and nicotine on the induction of apoptosis. For all assays for apoptosis, NHBE cells were placed in 6-well plate and treated with different dose of CSC and/or nicotine or apoptosis-inducers at 37 °C overnight. The attached and floating cells were then harvested, washed and resuspended. For DNA fragmentation, the amount of cytoplasmic nucleosome (A) was determined with Cell Death Detection ELISA kit. For DNA content (B), the cells were collected and fixed with 75% ethanol. After stained with propidium iodine, cell cycle analysis was performed by flow cytometry. Sub-diploid DNA peaks were considered apoptotic cells. For neutral Comet assay (C), the cells were mixed with molten LMAgarose and placed in CometSlide. Cells in the slides were then lyzed and subjected to electrophoresis in TBE buffer horizontally at 1 V/cm for 15 min. After fixing with 70% ethanol and staining with SYBR Green I, cells were observed and photographed under an epifluorescence microscopy. The comet was scored from 0 to 4 according to Collins criteria by measuring tail length, head size, tail intensity and head intensity. (D) Effects of CSC and nicotine on the induction of apoptosis-related protein expression. NHBE cells were exposed to CSC-F and/or nicotine (10 μM), or apoptosis-inducer VP-16 (0.5 mM) at 37 °C overnight. Cell lysates (30 μg) were then immunoblotted with indicated antibodies.

Table 2
Effects of Quest® CSCs on the cell cycle progress in NHBE cells

CSC	Concentration (µg/ml)	G1/G0	S	G2/M	Sub G1/G0
CSC-F 16 h	0	74.7	11.7	13.6	6.60
	20	77.6	12.2	10.2	5.62
	40	80.8	9.5	9.7	4.29
	60	81.4	7.6	11.0	6.09
	80	78.6	9.7	11.7	10.35
CSC-L 16 h	0	71.5	13.6	14.9	3.12
	20	76.9	12.5	10.6	4.17
	40	79.8	10.3	9.9	7.81
	60	81.5	7.6	10.9	8.68
	80	77.8	8.4	13.8	10.83
CSC-F 48 h	0	70.8	16.1	13.1	1.33
	20	66.8	17.3	15.9	3.88
	40	68.0	11.6	20.5	9.75
	60	46.3	21.4	32.3	13.15
	80	34.5	28.9	36.6	22.95
CSC-L 48 h	0	72.4	15.7	11.8	1.65
	20	70.9	18.4	10.6	2.20
	40	70.5	12.6	16.9	6.86
	60	53.0	16.0	31.0	9.77
	80	40.4	29.6	30.1	13.01

concentrations were greater than 40 µg/ml, and the CSC-F did not show significantly different effects from CSC-L ($t = 2.14$, $P > 0.05$). When the nicotine (10 µM) was added to the CSC-F, it did not change the cell cycle progression. Furthermore, nicotine itself at the range from 1 to 100 µM did not affect the cell cycle (data not shown). This indicates that components other than nicotine from Quest® CSC resulted in cell cycle arrest effects. The data also showed that both CSC-F and CSC-L treatment resulted in more sub-G1/G0 cells than control (Table 2), which is usually recognized as apoptotic cells. This is consistent with the previous apoptosis data in Fig. 4.

4. Discussion

Quest® cigarettes are commercially available as low nicotine and denicotinized products. Some smokers might have the perception that lower nicotine yields equate with a safer cigarette, particularly in light of the marketing that has been done around these products. However, the data described herein does not support the perception that low-nicotine and nicotine-free Quest® cigarettes might be less harmful than conventional cigarettes. While the TSNAs and some volatile organics in Quest® cigarette smoke were variably decreased, aromatic amines were increased. Smokers using Quest® might reduce their exposure to some toxicants, their exposure to others would be increased. This situation would likely be exacerbated by the users compensating for the lower nicotine yield and inhaling more (Strasser et al., 2007), thereby further altering the smoke chemistry. In addition, Quest® CSCs were shown to have a similar mutagenic potential in Ames assay to 2R4F, and were slightly more potent with respect to the tumor promotion-relevant GJIC effect than 2R4F. Quest® CSCs as well as 2R4F CSC had strong cytotoxic and anti-proliferative effects on the primary NHBE cell cultures while CSC-F exhibited more anti-proliferative activity. Nicotine in cigarette smokes played some role in increased cell survival, possibly in part by anti-apoptosis. To our knowledge, this is the first demonstration that nicotine would have these effects in CSC, rather than experimental models using only nicotine.

CSC contains a variety of chemical constituents that exhibit a wide range of toxicities. For the Ames test conducted with the TA98/TA100 *Salmonella* strains, the primary sources of mutagenic activity in the CSC are aromatic amines and heterocyclic amine

protein pyrolysate products (DeMarini, 2004), and mutagenicity is closely linked to the TPM yield of the cigarette (Roemer et al., 2004). Our data confirms that TA98/TA100 responsiveness is not limited to these chemical constituents since although there were differing levels of aromatic amines in the CSCs, there was no significant difference in the TPM and mutagenic activity between the nicotine-free and low nicotine Quest® CSCs. This study also showed that nicotine alone had no mutagenic activity and did not affect the slope of CSC-F-induced mutation in the Ames test when added back to mirror the levels found in CSC-L. This is in agreement with prior reports (Mizusaki et al., 1977).

Airway epithelial cells are directly exposed to cigarette smoke, and are susceptible to its toxic and carcinogenic properties. To investigate the cytotoxicity of Quest® cigarette smoke, we established a primary cell culture system for normal human bronchial epithelial cells derived from large airways, which are the precursor cells for squamous cell carcinomas. NHBE cells have been used in modeling the effect of chemicals on cell growth and morphology (Fields et al., 2005). For example, CSC has been proven to alter NHBE cells growth, the induction of terminal squamous differentiation, gap junction intercellular communication, and result in the formation of single strand DNA breaks (McKarns et al., 2000). Interestingly, compared to primary NHBE cell cultures, the immortalized NHBE cell line BEAS-2B exhibited different responses to CSC in some experiments. For example, BEAS-2B cells have high background expression levels of p53 (although the SV40 T antigen in these cells can bind to p53 and block its function) and PARP, compared with NHBE cells, and were sensitive to gamma irradiation or CSC stimulation (data not shown). In addition, overnight CSC treatment caused a G1cell cycle arrest in NHBE cells, but stimulated cell cycle transition through G1 in the BEAS-2B cells (data not shown). The BEAS-2B cell line has been studied extensively to elucidate carcinogenic mechanisms (Fields et al., 2005; Liu et al., 2005; Minna, 2003; West et al., 2003). However, it is different from NHBE cell in some ways, and only the NHBE cells are closer to physiological conditions.

The presence of functional gap junctions has been hypothesized to play a role in the normal physiological regulation of cell growth, differentiation and function, and it has been known for many years that loss of functional gap junctions is a common feature of cancer. It has been postulated that the loss of gap junctions may play a role in carcinogenesis and tumor progression (Mesnil et al., 2005). The GJIC assay tests the ability of agents to disrupt gap junction function in short term exposures since this ability has been suggested to relate to the tumor promotional effects of an agent (Tai et al., 2007). Our data showed that Quest® cigarette CSCs impaired the GJIC in NHBE cells, and that this inhibition was even slightly greater than that produced by CSC from the "low tar" reference cigarette. The tar yield of the cigarettes did not closely reflect the effects seen in the GJIC assay and nicotine had essentially no effect with CSC from the two Quest® cigarettes having similar GJIC disrupting activity independent of the nicotine content. The CSC constituents responsible for this effect are unknown but may relate to components such as anthracene and methylated anthracenes (Tai et al., 2007).

Our data showed that Quest® CSCs killed the primary NHBE cells through both acute necrotic cell death and apoptosis mechanisms. The former is consistent with the literatures that cigarette smokes can induce necrosis in a variety of cells, including NHBE cells (Liu et al., 2005) and the alveolar epithelial cell line A549 (Wickenden et al., 2003). However, there is conflicting data about whether CS damage leads to apoptosis. While some previous studies indicated that CSC induced apoptosis (Hellermann et al., 2002; Yang and Liu, 2004), Liu et al. (2005) cannot confirm this in NHBE cells. Wickenden et al. (2003) even found that CSC inhibited apoptosis induced by staurosporine or Fas ligation and the activation

of caspase 9 and 3, an effect that they postulated was caused by CSC mediated inhibition of caspase activation. There are three possible limitations of these studies that may account for their disparities. First, some detection techniques alone used for apoptosis may be providing misleading results. Intrinsic changes in the time courses of the apoptosis make it possible that an apoptotic signal are missed at detection time point, or the apoptotic effect is somewhat obscured by other more acute toxic death mechanisms. For example, the assays for DNA-strand breaks, such as TUNEL and ISEL (Hellermann et al., 2002), may have inadvertently identified cells with CSC-induced DNA strand breaks (Liu et al., 2005) as apoptotic. In our study, three assays (cytoplasmic nucleosome, DNA content and neutral Comet assays) were used to make it less likely that CSC is affecting the techniques themselves producing artifactual data. Another possibility for the discrepant results in the literature may relate to the preparation or handling of the CSC, resulting in different chemical constituent yields. While some investigators used gravimetric methods for tar collection onto Cambridge filters (Fields et al., 2005; Hellermann et al., 2002; Putnam et al., 2002) (the method used in this study), others have used "cold trap" methods for collecting compounds from the vapor phase as well as the particulate phase (Liu et al., 2005; Wickenden et al., 2003; Yang and Liu, 2004). These different methods might lead to collection of different chemical constituents, for example more free radicals and oxidants in vapor phase (Lee and Shacter, 1999). The final reason is that the preparations used in the literature used conventional CSC that contains high levels of nicotine. Nicotine and its nitrosated carcinogenic derivative NNK play an important role in the attenuation of apoptosis (West et al., 2003). This study is the first to demonstrate that nicotine-free CSC triggered a significant apoptotic response in NHBE cells and nicotine protected NHBE from CSC-induced injury at least in part by inhibition of apoptosis.

The ratio of Bcl-2 to Bax is thought to determine survival or death after apoptotic stimulation (Oltvai et al., 1993; Xin and Deng, 2005). Our data showed that CSC-F up-regulated the expression of Bax protein, but did not alter the Bcl-2. The decline in the Bcl-2/Bax ratio prompts cells to undergo apoptosis. This may be the important mechanism of CSC-F-induced apoptosis. On the other hand, nicotine can inactivate the Bax function through phosphorylation at ser-184 site (Xin and Deng, 2005). Bax is possibly the common target for CSC and nicotine for inducing apoptosis. Nicotine attenuated CSC-F-induced apoptosis maybe by inactivating the function of increased Bax proteins. This is worth further investigating because lung cancer cells express high levels of endogenous Bax not the Bcl-2. The increase of Bax protein in lung cancer cells may be caused by CSC and inactivated by nicotine.

In summary, a toxicological analysis does not indicate that low-nicotine and nicotine-free Quest® cigarettes have less adverse toxicological effects in the laboratory than conventional cigarettes. This should draw the attention of the consumers and policy makers. A more comprehensive evaluation in smokers is expected for these new products.

Acknowledgements

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