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γ H2AX: A potential DNA damage response biomarker for assessing toxicological risk of tobacco products

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Abstract

Differentiation among American cigarettes relies primarily on the use of proprietary tobacco blends, menthol, tobacco substitutes, paper porosity, paper additives, and filter ventilation. These characteristics substantially alter per cigarette yields of tar and nicotine in standardized protocols promulgated by government agencies. However, due to compensatory alterations in smoking behavior to sustain a preferred nicotine dose (e.g., by increasing puff frequency, inhaling more deeply, smoking more cigarettes per day, or blocking filter ventilation holes), smokers actually inhale similar amounts of tar and nicotine regardless of any cigarette variable, supporting epidemiological evidence that all brands have comparable disease risk. Consequently, it would be advantageous to develop assays that realistically compare cigarette smoke (CS)-induced genotoxicity regardless of differences in cigarette construction or smoking behavior. One significant indicator of potentially carcinogenic DNA damage is double strand breaks (DSBs), which can be monitored by measuring Ser 139 phosphorylation on histone H2AX. Previously we showed that phosphorylation of H2AX (defined as γ H2AX) in exposed lung cells is proportional to CS dose. Thus, we proposed that γ H2AX may be a viable biomarker for evaluating genotoxic risk of cigarettes in relation to actual nicotine/tar delivery. Here we tested this hypothesis by measuring γ H2AX levels in A549 human lung cells exposed to CS from a range of commercial cigarettes using various smoking regimens. Results show that γ H2AX induction, a critical event of the mammalian DNA damage response, provides an assessment of CS-induced DNA damage independent of smoking topography or cigarette type. We conclude that γ H2AX induction shows promise as a genotoxic bioassay offering specific advantages over the traditional assays for the evaluation of conventional and nonconventional tobacco products.

Keywords

Tobacco smoke; H2AX; Double strand breaks; DNA damage

1. Introduction

Although lung cancer is among the few malignancies for which we know the primary etiological agent (i.e., cigarette smoke, CS) [1], and despite high public awareness of the associated health risks, availability of prescription and nonprescription nicotine replacement aids, and access to community-based cessation programs, approximately 20% of adult Americans actively smoke due to a variety of causes [2–5]. Consequently, for those individuals who do not quit there is a pressing need to discover innovative approaches that not only increase cessation rates, but also reduce the health risks associated with smoking. One controversial possibility regarding the latter idea is to significantly reduce the toxicity and carcinogenicity of cigarettes [6,7]. However, before any potential benefits of reduced-risk cigarettes could be objectively promoted, an integrated genetic, cellular, and clinical model is needed to define the physiological processes disrupted in smokers, and the roles they play in lung carcinogenesis. One important element of this model will be to develop a straightforward CS exposure system that (i) is capable of directly comparing the DNA damage potential of various cigarettes regardless of differences in smoking behavior or cigarette construction [6], (ii) assesses a valid biomarker of cancer risk in humans, and (iii) uses whole CS similar to that which the smoker inhales to obviate the inherent deficiency of current *in vitro* tests that examine sub-fractions of CS which cannot accurately gauge complex synergistic interactions occurring upon exposure to nonfractionated CS [8–10].

Current models of CS-induced carcinogenesis conclude that polycyclic aromatic hydrocarbons, in particular benzo(a)pyrene (BaP), and tobacco specific nitrosamines such as NNK [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone] and NNN (N'-nitrosonornicotine) are the main CS components that cause lung cancer [11,12]. However, the multi-step process driving malignant conversion of lung cells is arguably multifaceted, requiring not only long-term exposure to these two classes of DNA-damaging agents, but also to the mix of tumor promoters, mutagens, organic compounds and free radicals in CS that collectively drive the accumulation of genetic defects at many loci [12–14]. These defects, observed in premalignant asymptomatic lung tissues as well as lung cancers, clearly point to genomic instability as an early pivotal effect of CS [15–17]. The tumorigenic relevance of this instability is revealed by studies indicating that smokers with less efficient DNA repair capacities are at higher risk for developing lung cancer [18,19]. Since maintaining DNA integrity is essential for the prevention of lung cancer, defining the various types of damage that disrupt DNA may provide additional viable biomarkers of cancer risk in CS-exposed individuals [20–22].

DNA double strand breaks (DSBs) are DNA lesions that can result in genetic abnormalities that are hallmarks of lung cancers and other malignancies [21–23]. A sensitive means to detect DSB formation is to assess histone H2AX phosphorylation, a variant of a family of at least eight protein species of the nucleosome core histone H2A [24,25]. Phosphorylation of H2AX on Ser 139 at sites flanking DSBs is mediated by the DNA damage checkpoint control genes ataxia telangiectasia mutated (ATM) [26–29], ataxia telangiectasia and Rad3 related (ATR) [30], and/or DNA-dependent protein kinases (DNA-PKs) [31]. However, most evidence concludes that ATM mediates H2AX phosphorylation in response to DSB formation [27,32–35]. The availability of antibodies to phosphorylated H2AX (denoted γ H2AX) allows for immunocytochemical detection of DSBs [36]. Upon DSB induction, γ H2AX in chromatin appears as discrete foci [28,36] with each focus representing a single DSB [28,37]. The intensity of γ H2AX immunofluorescence (IF) measured by cytometry strongly correlates with the number of DSBs [38] and has been proposed as a surrogate for cell killing in viability assays [39]. Multiparameter analysis of γ H2AX IF and cellular DNA

content make it possible to relate the abundance of DSBs and extent of DNA damage to the position of the cell in the cycle [40,41].

Exposure of A549 pulmonary adenocarcinoma cells to whole CS induces H2AX phosphorylation in a dose-dependent manner and, since ATM activation precedes or occurs coincident with H2AX phosphorylation, the increase in γ H2AX expression reflects induction of DSBs [42,43]. Interestingly, CS-induced DNA damage was more pronounced in S phase cells than in other cell cycle compartments [42–45]. Since CS depresses antioxidant potential, provokes oxidative stress, and induces lung cell proliferation in smokers [46–48], the increased sensitivity of replicating cells to DSB formation may play a central role in the generation of genotoxic events that contribute to pulmonary carcinogenesis. Support for this idea comes from recent studies showing that induction of a DNA damage response involving γ H2AX expression occurs in precursor lung lesions, and may be a dominant barrier preventing genetic instability and malignant conversion [49,50].

Different methods are being used to assess mutagenic/ carcinogenic properties of CS. Most common are the mutagenicity Ames test, DNA comets, micronuclei and sister chromatid exchange assays. The H2AX phosphorylation assay offers several advantages over these traditional tests. One advantage is its high sensitivity, which allows one to detect the induction of even a single DSB [28,36]. Other advantages include the rapidity of the DNA damage detection and the fact that since measurements are made at rates of hundreds of cells per second, data can easily be obtained from many thousands of individual cells [40–43]. This assay also allows one to correlate DNA damage with the cell cycle phase or if the treatment is cytotoxic, with induction of apoptosis [38–41]. In the present study the γ H2AX assay have been used to measure the genotoxic potential of CS in relation to the tar content. The data show that regardless of the cigarette style (i.e., full-flavor, light, or ultra-light), DNA damage, as detected by this assay, strongly correlates with the amount of tar to which the cells were exposed.

2. Materials and methods

2.1. Cells and cigarette smoke treatment

Whole cigarette smoke (CS) treatment and culturing of A549 human lung adenocarcinoma cells was performed as described previously [42,45]. Briefly, cells were seeded into dual-chambered slides (Nunc Lab-Tek II, VWR International, West Chester, PA) such that they were typically at 70% confluency at the time of exposure to CS. The cell culture medium was replaced with 37 °C Dulbecco's PBS (D-PBS) containing calcium and magnesium (BioSource, Rockville, MD) and the slides were placed in a smoke exposure chamber designed to deliver smoke uniformly diluted with 5% CO₂ in air at a constant flow rate of 500 cm³/min. Whole CS was generated under Federal Trade Commission (FTC)/ International Organization for Standardization (ISO) [51] smoking conditions (35 ± 0.3 cm³ puff, one puff every 60 s, 2-s puff duration with none of the ventilation holes blocked) or under Massachusetts Department of Public Health (MDPH) smoking conditions (45 ± 0.5 cm³ puff, one puff every 30 s, 2-s puff duration with 50% of the ventilation holes blocked) unless otherwise noted using a KC 5 Port Smoker (KC Automation, Richmond, VA). For the specific experiments described in Fig. 4 where the effects of changing puff volume and number of cigarettes were under investigation, the puff interval and puff duration were maintained under the FTC/ISO protocol, i.e., 60 s puff interval and 2 s puff duration, with no ventilation hole blocking. CS was delivered to the smoke exposure chamber at a constant flow rate of 500 cm³/min except under MDPH conditions, when the CS was delivered at a constant flow rate of 1000 cm³/min. A549 cells were exposed to CS for 20 min, an exposure time that we have previously shown to generate significant levels of γ H2AX in a dose dependent manner [42,43,45]. Mock-exposed cells were treated under identical conditions as

the CS-exposed cells except for the absence of a cigarette in the smoking port. Following treatment or mock treatment, the D-PBS covering the cells was aspirated and replaced with 1ml per chamber of fresh culture medium at 37°C. The cells were placed in the 37°C, 5% CO₂ incubator and incubated for various times (i.e., 15 min to 4 h) after which the medium was aspirated and the cells fixed with 1% paraformaldehyde by gently rocking the slides at room temperature for 15 min. Following aspiration of the fixative, the chamber slides were disassembled and the slides submerged in 50 ml conical tubes filled with 70% ethanol for storage prior to γ H2AX analysis.

Immunocytochemical detection of γ H2AX was carried out as previously described [52]. CS treated, fixed cells were rinsed twice in PBS and incubated in 0.1% Triton X-100 (Sigma) in PBS for 15 min at room temperature, followed by incubation in a solution of 1% (w/v) bovine serum albumin (BSA; Sigma) in PBS for 30 min to suppress non-specific antibody binding. The cells were then incubated in 100 μ l volume of 1% BSA containing 1:200 dilution of phospho-specific (Ser 139) histone H2AX mouse monoclonal antibody (mAb) (Millipore, Temecula, CA). After overnight incubation at 4°C, the slides were washed twice with PBS and then incubated in 100 μ l of 1:100 dilution of Alexa Fluor 488 goat anti-mouse IgG (H + L) (Invitrogen/Molecular Probes, Eugene, OR) for 45 min at room temperature in the dark. The cells were then counterstained with 1 μ g/ml 4,6-diamidino-2-phenylindole (DAPI; Invitrogen/Molecular Probes) in PBS for 10min. Each experiment was performed with an IgG control in which cells were labeled only with secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (H + L) without primary antibody incubation to estimate the extent of non-specific binding of the secondary antibody to the cells.

2.2. Spectrophotometric determination of smoke concentration in aqueous media

Whole CS from a reference cigarette was collected under FTC conditions onto a 44mm Cambridge pad. The amount of smoke collected on the pad was determined by weight of the total particulate matter (TPM), which is directly related to nicotine-free dry solids, more commonly known as tar. The TPM on the Cambridge pad was dissolved in a known amount of methanol. Aliquots of this solution were added to PBS and the resulting solutions were placed into a quartz cuvette and the absorbance at 300 nm was measured using an Agilent Technologies 8453 Ultraviolet–Visible (UV–vis) spectrophotometer. These measurements were used to construct a linear calibration curve according to the method described by Sloan et al. [53] for the determination of cigarette filter extracts. For all samples, the PBS solution was removed from the dual-chambered slides containing the cell cultures post-CS exposure and placed into a quartz cuvette and the absorbance was measured at 300 nm. The concentration of CS in each sample was determined by comparing the absorbance of that sample to that of the linear calibration curve. The concentration of all samples was reported as μ g TPM/ml. In all cases the UV–vis measurements gave values that were consistent with the number of cigarettes smoked and the estimated tar yield from each cigarette. As a further confirmation, the concentration of nicotine in the culture media was also measured (data not reported) and was found to be consistent with the UV–vis data. This method proved to be particularly valuable at comparing varying tar yield cigarettes in our cell exposure system, which does not deposit the TPM onto a pad for weight measurement but rather deposits the TPM from the cigarette directly onto cultured cells throughout the exposure procedure.

2.3. Measurement of cell fluorescence by laser scanning cytometry

Cellular green γ H2AX and blue (DAPI) fluorescence emission was measured using a Laser Scanning Cytometer (LSC; iCys; CompuCyte, Cambridge, MA), utilizing standard filter settings; fluorescence was excited with 488-nm argon ion and violet diode lasers, respectively [54]. The intensities of maximal pixel and integrated fluorescence were measured and recorded for each cell. At least 3000 cells were measured per sample.

2.4. Statistical analysis

To compare the changes in γ H2AX immunofluorescence intensity (IF), the mean fluorescence intensity (integral values of individual cells) was calculated for cells in each phase of the cycle by gating G₁, S and G₂M cells based on differences in DNA content (DNA index, DI). The mean of the fluorescence values for G₁, S and G₂M populations of cells in the IgG control groups were then subtracted from the respective means of the CS treated cells. Since histone and DNA content double as cells proceed from G₁ to G₂ phase, the mean values of H2AX for the S and G₂ M cell populations were divided by 1.5 and 2.0, respectively, in order to express the degree of change in γ H2AX IF, i.e., the increase in phosphorylated protein per unit of DNA. All experiments were run under identical instrument settings. Data are presented as mean γ H2AX IF of each cell cycle compartment or, where not indicated, of the entire population (G₁, S and G₂M). The standard deviation was estimated based on Poisson distribution of the measured cell populations. Each experiment was run at least in triplicate, and some experiments were additionally repeated. The intersample variation in the triplicates did not exceed the value of one standard deviation of individual samples. Other statistical details are given in the figure legends.

3. Results

3.1. Bivariate analysis of γ H2AX IF and DNA content in A549 cells exposed to CS

Most cells, including A549 lung adenocarcinoma cells, have a low constitutive level of expression of γ H2AX IF as seen in Panel A of Fig. 1. However, analysis of A549 cells 1 or 2 h following 20 min of exposure to whole CS (generated under the FTC/ISO protocol as described in Section 2) from 2R4F cigarettes demonstrated increased levels of γ H2AX IF in cell cycle phase-dependent fashion. Invariably, S phase cells appear to be most sensitive to CS exposure (Fig. 1, Panel B). Eventually, all cells increase in γ H2AX IF (Fig. 1, Panel C) relative to mock-treated control cells. As can be seen in the plot of mean γ H2AX IF versus time since CS exposure, there is a constant increase in γ H2AX IF that remains cell cycle phase-specific and tends to saturate at approximately 2 h and then decreases (see Fig. 1, Panel D).

We next assessed the induction of γ H2AX in A549 lung cells upon exposure to whole CS generated from the three main styles of commercially available American cigarettes that primarily differ in terms of their FTC/ISO reported delivery levels of tar and nicotine: i.e., full-flavor, light and ultra-light cigarettes which deliver by the FTC/ISO smoking regimen 15, 10, and 6mg of tar, respectively. We assessed the same brand of cigarettes available in these three styles from the leading U.S. manufacturer of tobacco products. Fig. 2 (Panels A and B) shows that upon exposing A549 cells for 20 min to CS generated from these three cigarette styles using the FTC/ISO smoking protocol, there is a corresponding linear increase in γ H2AX levels (and thus of DSBs) as the FTC/ISO associated tar deliveries increase from ultra-light style (6mg tar) to the full-flavor style (15mg tar). However, it was of interest to determine if these data resulted from actual differences in tar toxicities of these three cigarette styles or simply reflected a dose-response increase as tar yields increased from 6 to 15mg per cigarette. Therefore, we plotted γ H2AX values measured for these cigarettes against the estimated tar delivery for each style of cigarette during a 20min smoke exposure. Fig. 3 clearly shows that the total γ H2AX induced by each of the three types of cigarettes was essentially equivalent when expressed as a function of milligram of delivered tar. Therefore, these data support the conclusions of various epidemiological studies that cigarette toxicity of most commercial U.S. cigarettes, at least as measured by the γ H2AX-DNA damage assay, is essentially identical per weight unit of tar regardless of differences in tar delivery as measured by the FTC/ISO protocol.

3.2. DSB formation is proportional to actual tar delivery across varying machine smoking parameters

Since Fig. 3 shows that the levels of γ H2AX are essentially equivalent across styles of cigarettes on a per milligram tar basis, we speculated that γ H2AX levels (when corrected for tar delivery) would also be equivalent regardless of government-defined smoking machine parameters. To test this prediction, we used the γ H2AX assay to test two industry standard research reference cigarettes manufactured by the University of Kentucky to be representative of tar and nicotine deliveries of two major styles of commercially available cigarettes: a representative light style cigarette (2R4F which delivers an FTC/ISO determined yield of 9.70mg tar and 0.85mg nicotine, and has 28% filter ventilation), and a representative ultralight low yield style cigarette (1R5F which delivers an FTC/ISO determined yield of 1.67mg tar and 0.16mg nicotine, and has 70% filter ventilation). Since no standardized machine smoking protocol accurately reflects the myriad habits of consumers [55–58], we chose to increase tar delivery from machine-smoked cigarettes by manipulating two compensatory mechanisms observed to occur in smokers of low yield cigarettes, i.e., increasing puff volume or the number of cigarettes smoked. Additionally, in order to plot the measured γ H2AX levels versus TPM delivery for cigarettes using different smoking parameters other than those stipulated in the FTC/ISO protocol, we incorporated a standard methodology for determining TPM levels in solution based on UV–vis absorbance (as described in Section 2). It is important to note here that tar is directly proportional to nicotine content as shown by the 1999 Massachusetts Benchmark Study of 26 commercial brands which demonstrated that under FTC and MDPH conditions tar to nicotine yields across all cigarettes were linear with a correlation coefficient of 0.99 and 0.98, respectively [59,60]. These 26 brands represented all common brand styles sold in the USA and covered a FTC tar yield range from 1 to 26mg per cigarette. This linear relationship between tar and nicotine in cigarette smoke allowed us to estimate nicotine delivery to the cell exposure system by measuring tar delivery.

Fig. 4 (Panel A) shows a linear relationship of γ H2AX induction with actual TPM delivery from the 1R5F ultra-light cigarette as smoking intensity rises either by increasing the number of cigarettes or increasing the puff volume. For example, in Panel A, when four 1R5F cigarettes were machine smoked at the lowest puff volume of 35 cm³ the UV-vis absorption of the PBS solution was 0.32 absorption units corresponding to 38.4 μ g/ml of TPM delivered and a γ H2AX fluorescence value of 98. However, similar TPM deliveries and γ H2AX fluorescence values were observed upon machine smoking only two 1R5F cigarettes with an increased puff volume of 75 cm³, e.g., 0.31 absorption units corresponding to 37.3 μ g/ml TPM and 93 γ H2AX fluorescence units. The effect of increasing puff volume was again clearly demonstrated upon machine smoking three 1R5F cigarettes with 55cm³ puffs (0.39 absorption units corresponding to 46.5 μ g/ml TPM delivery and 125 γ H2AX fluorescence units). Finally, when three 1R5F cigarettes were smoked at the largest puff volume studied (i.e., 75cm³), the UV–vis absorption increased to 0.52 absorption units corresponding to 62.8 μ g/ml TPM delivery and 210 γ H2AX fluorescence units. The relationship between UV–vis absorption and $\Delta\gamma$ H2AX IF was highly correlated with an R^2 value of 0.991. Comparable data were derived from a similar assessment of the 2R4F light cigarettes (see Fig. 4, Panel B).

Fig. 4 Panel C depicts the data points collected for both experiments graphed on the same chart. This graph makes two important points: (i) the amount of DNA damage induced by TPM delivery as measured by γ H2AX is linear for both styles of cigarettes and increases with increasing smoking intensities (R^2 value of 0.92), and (ii) there is relatively little difference in amount of damage between a light style of cigarette when equivalent tar deliveries for each cigarette are measured, despite the fact that the light cigarettes deliver

nearly six times the amount of tar as the ultra-light low tar cigarettes according to FTC/ISO reported values. For example, Fig. 4, Panel C shows similar DSBs as measured by γ H2AX when two light style cigarettes are smoked at 55cm³ puff volume versus three ultra-light style cigarettes smoked at more intense puffing parameters of 75cm³ puff volume (i.e., ~200 relative γ H2AX fluorescence units for the light and ~210 γ H2AX fluorescence units for the ultra-light cigarettes). These data suggest that a smoker may sustain equivalent amounts of DNA damage in the form of DSBs from a putative low yield cigarette as from a higher tar cigarette simply by increasing smoking intensity (i.e., increased number of cigarettes and/or increased puff volume), and further support the fact that the FTC/ISO method does not reliably predict the variations in actual constituent uptake and, thus, cannot effectively compare the toxicological potential of different cigarette brands and types [57,61–64].

An additional important observation to note from the data presented in Fig. 4 is that the linear regression line intercepts the 'y' (or TPM) axis at 0.15 absorbance at 300 nm for the 1R5F (Panel A) and 0.19 absorbance for the 2R4F (Panel B) corresponding to 17.8 and 22.4 μ g/ml of TPM delivery to the dual chamber slide, respectively. This observation indicates that there is a threshold level of tar delivery to the cells that is required for detecting tar-induced DNA damage by the γ H2AX assay. Moreover, the data show that despite the large difference in FTC/ISO tar yields, ultra-light cigarettes induce similar amounts of DSBs as do light cigarettes (when compared on a mg/tar basis). This tar delivery/DNA damage detection threshold (or initiation point at which an increase in γ H2AX is detectable above mock-treatment levels) provides a reliably convenient method for comparing various styles of cigarettes directly when utilizing the same smoking protocol. However, it should be noted that while this threshold of DNA damage response detection can be defined using any machine smoking protocol, the same protocol should be used when doing direct comparisons of different styles and/or types of cigarettes.

3.3. Market survey shows varying γ H2AX induction thresholds across commercially available brands and styles of cigarettes

Upon establishing the linear relationship between γ H2AX values, tar deliveries, and machine smoking parameters, and confirming the utility of assessing the DNA damage detection threshold, we performed a market survey on nine commercially available products of different cigarette styles, i.e., light and ultralight, from different manufacturers and compared them using the γ H2AX assay in order to test the hypothesis that the level of DNA damage does not correspond to the amount of FTC/ISO reported tar values. We chose to machine smoke the market survey cigarettes by the MDPH smoking protocol since it more closely approximates the highly variable smoking behavior of humans by increasing puff volume and frequency and, most importantly, restricting filter ventilation of machine-smoked cigarettes [58,65,66]. Representative results are shown in Fig. 5, which plots γ H2AX IF versus TPM in order to determine the threshold of DSB detection for three different commercial brands of cigarettes representing two different styles (Marlboro Light, Marlboro Ultra-Light, and Camel Ultra-Light). For the three different brands of cigarettes tested in Fig. 5 the regression plot shows a linear relationship between TPM and γ H2AX, and when extrapolated to the 'y' intercept, reveals that the minimal tar deposition needed to reach the threshold of DSBs detection varies from approximately 26 to 30 μ g/ml TPM. Thus, the use of more intense smoking parameters for CS generation resulted in no substantive difference in the level of γ H2AX between light and ultra-light styles of cigarettes.

Table 1 lists the complete survey of the nine samples tested. The majority of the cigarettes tested fall between 25 and 40 μ g/ml TPM deliveries for the γ H2AX induction threshold with one cigarette brand (Doral) requiring as little as 12 μ g/ml of TPM to induce γ H2AX. The

results with Doral ultra-light cigarettes could be anticipated from the data shown in Fig. 3, which argue that even very low tar yield cigarettes can generate significant DSBs when the CS is generated under more realistic intense smoking parameters such as with the MDPH protocol. While a precise mechanism explaining the result with the Doral cigarettes is unclear at present, one possibility is that the proprietary tobacco blend used to manufacture this brand results in increased DNA damage upon combustion.

3.4. Filter additives can significantly modulate the extent of tar deposition required to induce γ H2AX

High activity carbon is a selective vapor phase removal filter component that can reduce the level of free radicals as well as other hazardous vapor phase volatile organic compounds (VOCs) [13,67]. Thus, since we have previously shown that the vapor phase of CS causes DNA damage and the induction of H2AX phosphorylation primarily via a free radical mechanism [42,45], we were interested in determining if specific modifications to the modern standard cellulose acetate (CA) filter, which dominates the American cigarette market, could decrease the level of DSBs regardless of the smoking parameter used. We constructed prototype cigarettes using a proprietary blend of natural tobaccos similar in composition to commercially available American blended cigarettes that deliver tar and nicotine amounts typical of the light and ultra-light cigarette styles used in the market survey. The prototypes under investigation also contain filters consisting of cellulose acetate with one or more selective removal filtration additives (i.e., high activity carbon and/or polystyrene divinylbenzene primary amine containing resin) in various ratios. The MDPH smoking protocol was used during the exposure experiments in order to extrapolate the threshold γ H2AX initiation points (i.e., the point at which there is a measurable increase in γ H2AX levels compared to mock treatment) of the control 1R5F cigarette and the three prototypes chosen for filter additive analysis.

In order to provide a direct comparison with the commercial samples from the market study described in Fig. 5, the FTC/ISO tar and nicotine values for the control 1R5F and prototype cigarettes are provided in Table 2. This table also lists the type of cigarette, additive loading ratio and TPM amount required to initiate DSBs as determined by the γ H2AX assay. We note in Table 2 that despite the fact that the prototype cigarettes delivered substantially more tar than the 1R5F reference cigarette as determined by FTC/ISO smoking regimen, it still required ~4–6 times the amount of TPM delivery to initiate DSBs by the prototype cigarettes than by the reference cigarette or the majority of commercial cigarette brands and styles (e.g., see Table 1). These data suggest that the prototype cigarettes may cause considerably less DNA damage, at least in the form of DSBs per mg of TPM even under a more intense compensatory smoking regimen, and that the level of DNA damage can be directly impacted by filtration additives that selectively remove classes of toxic CS constituents. However, considering the variability in smoking patterns due to the phenomenon of compensation, these results should not be construed as implying that one type of cigarette is safer than another.

3.5. Tobacco type can also impact γ H2AX induction

The components of commercial American Blended Cigarettes (ABC) is generally uniform in that they include primarily flue-cured and burley varieties of tobacco with lesser amounts of oriental type tobacco, stem from the tobacco plants and reconstituted tobacco filler material at various percentages. Upon observing the significant decrease of γ H2AX induction by the addition of carbon and/or resin in the cigarette filter (Table 2), we decided to perform a preliminary γ H2AX assay investigation on laboratory cigarettes constructed from either flue-cured only or burley only tobacco components in order to assess the potential differences in DNA damage capacity between two of the most prevalent tobacco

components that make up a traditional ABC blend. The TPM delivery was measured via the spectrophotometric analysis as described previously in order to normalize any potential tar delivery discrepancies from the laboratory prepared samples. Three flue-cured variety cigarettes and three burley variety cigarettes were compared on the γ H2AX assay in the same fashion as was performed for the market study, i.e., each tobacco variety was measured for γ H2AX damage from one, two and three cigarettes followed by a linear regression plot to determine the TPM amount required to initiate DNA damage. Analysis of the results provided an average intercept/DNA damage threshold point from the three different flue-cured variety cigarettes of $43.2 \mu\text{g TPM/ml} \pm 11.5$ and $53.1 \mu\text{g TPM/ml} \pm 5.0$ for the three different burley variety cigarettes.

4. Discussion

The observed increase in cell expression of γ H2AX following exposure to CS reports the induction of DNA damage response by CS-generated genotoxic components and appear not to be due to overt cytotoxicity or induction of apoptosis in A549 cells for the following reasons. First, as judged by trypan blue incorporation, the colorimetric measurement of the activity of lactate dehydrogenase which is rapidly released into the supernatant upon damage of the plasma membrane, and/or direct cell counting, the A549 cells exposed to CS for 20 min retain a viability of >95%, a result we have consistently observed [see [42,43,45]]. Second, we have previously demonstrated [45] that exposure of A549 cells to CS from an industry standard cigarette containing 15.7mg 'tar' and 1.01mg nicotine for up to 20min followed by up to 4 h growth in fresh medium failed to increase the number of cells undergoing apoptosis as (assayed by examination of caspase 3 activation) over that seen in mock-treated cultures. In addition, the present results are not unique to A549 cells in that similar kinetics of appearance and extent of DNA damage in the form of γ H2AX IF was observed in normal human bronchial epithelial (NHBE) cells [45] and in another human lung cancer cell line, H1299 (data not shown), exposed to CS under similar conditions as A549.

We have previously described the γ H2AX assay and shown it to be a robust and reliable in vitro method to measure the levels of DSBs induced in cultured human lung cells after exposure to CS [42]. Based on these previous data, we proposed that this assay could provide a novel biological risk assessment method for cigarettes that is independent of brand or type of cigarette, and which is proportional to CS dose and nicotine/tar delivery regardless of smoking behavior. In the current report, we provide further evidence to support this thesis and show that the induction of DSBs by freshly prepared and delivered whole CS is linear with increasing tar delivery, the latter modulated by time of exposure and puff volume. The linear responsiveness of H2AX assay provides a simple and straight-forward method to extrapolate and predict the extent of DNA DSBs caused by CS along a continuum of possible human smoking patterns. Moreover, it can yield a direct measurement of one type of DNA damage on a milligram of tar or nicotine delivered basis. The data also indicate that specific modifications to the cigarette filter, such as inclusion of gas phase filtration agents like high activity carbon, can reduce the induction of DSBs by CS. In addition to tar, a case could be made that nicotine itself may also play a contributory role in DSB formation. We think this unlikely since, although accumulating data to date suggest that nicotine may have an impact on cancer development (e.g., by inducing angiogenesis or cell growth, by inhibiting apoptosis, or by altering gene expression), there is no published literature suggesting that nicotine causes significant DNA damage at physiological relevant concentrations [68–70]. Furthermore, in preliminary experiments, we have observed that cells exposed to exogenous nicotine at a dose up to 1.2mg per cigarette in combination with whole cigarette smoke from nicotine-free cigarettes did not increase the expression of γ H2AX (unpublished data).

It is well known that many complex issues arise when attempting to compare the relative toxicities of different cigarettes [10]. In principle, one collects smoke from a cigarette by various methods and performs assorted chemical, biological or toxicological tests with the collected CS. The initial challenge which must be addressed is to define a set of parameters to be used for smoke collection that best reflects actual human smoking behavior, which has been shown to be highly variable not only between individuals, but even for an individual smoker during a typical day of consuming cigarettes [58,66,71]. Thus, it has become clear that no machine smoking procedure can accurately mimic the wide range of human smoking characteristics. Therefore, standardized machine generated smoking regimens [e.g., FTC/ISO, MDPH or Health Canada (HC)] merely reveal the relative yield of tar and nicotine contents of different cigarettes according to a convention of analytical standards, but are not indicative of actual human smoking behavior. Several factors that determine per cigarette yields of tar and nicotine delivery to smokers include the number and size of puffs as well as filter ventilation hole blocking that can occur with lower-yield cigarette products [51]. In particular, it has been noted that the single most effective cigarette modification that reduces FTC/ISO-measured CS emissions is filter ventilation, a process whereby small perforations around the filter introduce fresh air during puffs that dilutes the amount of tar, nicotine, CO, and other hazardous CS constituents delivered to the smoker [9,72]. However, smokers tend to cover the holes when inhaling, resulting in significantly more exposure to CS constituents than predicted by the FTC/ISO machine smoking regimen which does not include blocking of filter ventilation holes as part of its protocol. There is consensus among public health advocates that filter vent hole obstruction by smokers is the primary factor that equalizes the toxicity of all conventional cigarettes [57,63,64,73]. Consequently, when performing research in the area of cigarette-driven toxicity, the cigarette should be viewed solely as a reservoir capable of delivering as much tar and nicotine as the consumer desires regardless of the style of cigarette or FTC/ISO-determined yields of these components.

As mentioned above, every cigarette tested in the market study is an ABC style and is not composed entirely of one type of tobacco but rather tobacco blends made up of various percentages of tobacco and filler material. The preliminary data from the flue-cured and burley only cigarettes suggest the possibility that CS derived from the combustion of blended tobaccos and filler material is a complex process that is not simply modeled by test cigarettes containing only one of the two most prevalent tobacco components in an ABC style product. These complex blending phenomena could explain the results observed in Table 1 for the range of TPM amounts required to initiate DNA DSBs for the various cigarette brands; however since the market study cigarette blends are proprietary, further investigation into this question is warranted. The preliminary H2AX results obtained from test cigarettes constructed from only one type of tobacco suggest that trend data could be generated and possibly related to distinct types of tobaccos. Future tests should not only include additional tobacco types, e.g., oriental, stem and reconstituted tobacco filler material, but a comparison of leaf position from different tobacco types may also provide useful information concerning the effect on the H2AX assay of CS generated from the combustion of individual types of tobaccos and filler material. It is important to note that since we observed such a significant change in damage initiation potential with both the introduction of selective filtration agents in the cigarette filter as well as with modification of the traditional tobacco blend, it is clear that this assay is responding to both the vapor and tar phase components in whole CS.

Although CS, as well as various constituents of CS, can cause disruptions to the genome [74,75], transcriptome [76,77], and proteome [78], DSB formation is a valid biomarker of potential cancer risk since these lesions are one type of DNA damage that, if unfaithfully repaired, can lead to translocations and chromosomal instability, two mechanisms with contributory roles in the etiology of multiple types of malignancies, including lung cancers

[20,21–23,79,80]. Thus, the rapid induction of high levels of DSBs by CS may present an independent risk factor to the smoker, especially since one recent study indicated that the probability of a DSB being inaccurately rejoined is relatively low when DSBs are spatially separated, but increased considerably when multiple breaks coincide [81], while another study showed that heavily clustered DSBs could lead to complex genetic changes similar to those seen in human cancers [82]. DSBs have not yet been sufficiently assessed in premalignant lung lesions or in asymptomatic bronchial epithelium of chronic smokers [21], but recent studies observed an activated DNA damage response in precursor lung lesions that included the expression of phosphorylated ATM and H2AX suggesting that DSB formation is a likely early genomic event. The presence of an active DNA damage response is hypothesized to be a dominant anti-cancer barrier preventing the cell from undergoing genetic instability and malignant conversion [49]. Cells may breach this barrier and undergo progression towards a more disordered state if there are complementary mutations or other defects in key genes within this pathway (e.g., p53, Chk2, ATM, etc.) [49]. Evidence to support the plausibility of this model in lung cancer comes from at least three observations: (i) p53 mutations are among the most common genetic defects in lung cancer [83,84]; (ii) Chk2 kinase expression is down-regulated in non-small cell lung cancers due to promoter methylation [85]; and (iii) a significant reduction in DSB repair capacity is directly associated with increased promoter methylation of specific genes in this damage-induced pathway which, in turn, is associated with an elevated risk of lung cancer [86]. Collectively, these data suggest that one of the earliest and potentially pivotal carcinogenic DNA defects caused by CS is the induction of large numbers of DSBs.

Consequently, reducing formation of DSBs by any toxic stimulus could potentially mitigate long-term risk. However, it remains to be rigorously proven whether charcoal-containing filters, or other modification involving cigarette design or tobacco type, can attenuate to any significant degree the deleterious effects that CS has on the respiratory system of long-term smokers since there are other types of direct and indirect genomic [87] and genotoxic insults caused by CS [88–90]. A major limitation to assessing any beneficial effects of activated carbon-containing filters is the dearth of reliable in vitro bioassays or in vivo dose-responsive biomarkers that correlate with future disease risk [10,90–93]. The data presented here indicate that the γ H2AX assay can discriminate a reduction in γ H2AX foci (and presumably DSBs) when using prototype cigarettes with activated carbon-containing filters despite delivering substantially more tar than conventional cigarettes, suggesting that prototype cigarettes which cause considerably less DNA damage per mg of TPM can be quantitatively assessed. The relevance of this assay stems from the fact that the habitual U.S. smoker consumes an average of 16.6 cigarettes/day [94], and probably inhales somewhere between 100 and 125 puffs/day of a highly complex mixture of reactive gases and suspended particulate matter with carcinogenic and toxic potential that can cause direct and indirect damage to lung cells in a repeating cycle of tissue injury and repair that contributes to tumor induction. Yet despite a range of studies indicating that the chemical composition [57] and overall disease risk of CS is relatively constant across different brands and styles of cigarettes [57,95], most long-term smokers do not develop lung cancer. Thus, understanding the nature of DNA-damaging events induced by CS may clarify the molecular basis of differential sensitivities among smokers. Since many CS constituents manifest their carcinogenic potential by attacking DNA, it is possible that the nexus of chronic CS-induced DSBs in smokers coupled with specific haplotypes that mute prompt and efficient DNA repair can result in a cycle of amplification and progression of genomic defects that instigate a range of pulmonary diseases including lung cancer [79,96]. The ability to directly relate DSB formation to CS exposure using γ H2AX as a surrogate biomarker may be an important addition to the battery of available DNA damage tests used to assess the genotoxic properties of tobacco products [42] since it potentially reflects the formation of a type of

DNA damage responsible for chromosomal abnormalities linked to the genesis of lung cancers and other human malignancies.

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Conflict of interest

This study was funded by Vector Tobacco Inc. Drs. Albino, Jorgensen, Rainey, Gillman, Clark, and Ms. Gietl are employees of Vector Tobacco Inc. or Liggett Group LLC. Drs. Zhao, Traganos and Darzynkiewicz have received research funding from Vector Tobacco Inc.

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Fig. 1.

A549 cells were either mock-exposed (A) or exposed to whole CS from 2R4F cigarettes for 20 min, replated with fresh medium and harvested at 1 h (B) or 2 h (C) later. Each dot represents a single cell. The dotplots are divided into cell cycle phases based on the DNA index (DI = 1 for G₁, DI=2 for G₂M and the intervening cells in S). The red lines indicate the boundary of mock-treated cells that includes >97% of unexposed cells. The mean values of γ H2AX for each cell cycle compartment, collected at various times up to 4 h after exposure, are plotted in (D).

Fig. 2.

(Panel A) Comparisons of whole CS exposures in terms of γ H2AX induction using FTC/ISO protocol based on style of cigarette: IM16 (industry reference cigarette), full (commercially available full-flavor king size), light (commercially available light king size), and ultra (commercially available ultra-light king size). Note that the constitutive level of γ H2AX IF observed in mock-treated samples was subtracted from the mean IF of CS-treated samples ($\Delta \gamma$ H2AX IF). (Panel B) A comparison of γ H2AX levels in A549 cells exposed to whole CS generated by the FTC/ISO smoking regimen: IM16 (16 mg tar), full (15 mg tar), light (9.5 mg tar), ultra (6mg tar). The dashed line represents the continuation of the line through the data to 0 tar.

Fig. 3.

Induction of γ H2AX normalized for the estimated tar delivery. Measured γ H2AX values were plotted against the estimated tar delivery for each style of cigarette during a 20 min smoke exposure, and clearly show that the total γ H2AX induced by each of the three types of cigarettes was essentially equivalent when expressed as a function of milligram of delivered tar.

Fig. 4.

(Panel A) Plot of absorbance at 300 nm versus total γH2AX IF of PBS media post exposure to whole CS from two, three and four 1R5F industry reference ultralight cigarettes with 35, 55 and 75 ml puff volumes. (Panel B) Plot of absorbance at 300 nm versus γH2AX IF of PBS media post exposure to whole CS from two and three 2R4F industry reference light cigarettes with 35 and 55 ml puff volumes. (Panel C) Combined plot of Panels A and B. The constitutive level of γH2AX IF observed in mock-treated samples was subtracted from the mean IF of CS-treated samples ($\Delta\gamma\text{H2AX}$ IF).

Fig. 5.

Linear regression plot of measured total particulate matter (TPM) versus γ H2AX immunofluorescence obtained from representative market survey cigarettes: Marlboro Light, Marlboro Ultra-Light and Camel Ultra-Light. The y-intercept of the regression line represents the amount of TPM required from each cigarette to initiate DSBs detectable by the γ H2AX assay. The constitutive level of γ H2AX IF observed in mock-treated samples was subtracted from the mean IF of CS-treated samples ($\Delta \gamma$ H2AX IF).

Table 1

Amount of TPM from market survey cigarettes required to initiate measurable DNA damage compared to mock treatment.

Damage initiation $\mu\text{g/ml}$ (TPM)		
	Average	Error
Marlboro Light	30.95	5.02
Marlboro Ult	26.28	1.53
Camel Ult	29.66	0.37
Doral	12.39	0.87
Basic Ult	25.33	0.13
USA Gold Ult	33.56	4.96
Kent Ult	30.68	4.00
Winston Light	40.10	4.53
Winston Ult	34.81	2.96

DNA damage threshold. Threshold TPM level, estimated from linear regression analysis, required for DNA damage initiation (in the form of H2AX IF) above the low constitutive levels observed with mock treatment for nine commercially available cigarettes from the market survey. All survey samples were smoked according to MDPH protocol. The error reflects the range of TPM required for damage initiation between two separate measurements.

Table 2
Comparison of physical characteristics and DNA damage initiation points of 1R5F and prototype cigarettes A, B, and C.

Cigarette type	FTC/ISO nicotine (mg)	FTC/ISO tar (mg)	Additive loading (mg)		Damage initiation (µg/ml (TPM))	R ²
			Carbon	Resin		
1R5F	0.16	1.67	0	0	10.7	0.99
A	0.45	5.4	100	0	52.9	0.96
B	0.48	5.9	70	30	65.8	0.89
C	0.64	8.3	100	30	83.5	0.98

Comparison of DNA damage threshold for cigarettes. Comparison of the abundance of TPM constituents, as determined by linear regression analysis, required for DNA damage initiation (in the form of H2AX IF) above the low constitutive levels observed with mock treatment between industry standard 1R5F ultra-light cigarette and three prototype cigarettes, which contain varying amounts of carbon and/or resin additives in the filter. The 1R5F and prototypes listed in table were smoked according to MDPH protocol. FTC/ISO nicotine and tar deliveries are provided for comparison purposes to the market survey described in Table 1.