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TRX-ASK1-JNK signaling regulation of cell density-dependent cytotoxicity in cigarette smoke-exposed human bronchial epithelial cells

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Lee YC, Chuang C-Y, Lee P-K, Lee J-S, Harper RW, Buckpitt AB, Wu R, Oslund K. TRX-ASK1-JNK signaling regulation of cell density-dependent cytotoxicity in cigarette smoke-exposed human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 294: L921–L931, 2008. First published February 15, 2008; doi:10.1152/ajplung.00250.2007.—Cigarette smoke is a major environmental air pollutant that injures airway epithelium and incites subsequent diseases including chronic obstructive pulmonary disease. The lesion that smoke induces in airway epithelium is still incompletely understood. Using a LIVE/DEAD cytotoxicity assay, we observed that subconfluent cultures of bronchial epithelial cells derived from both human and monkey airway tissues and an immortalized normal human bronchial epithelial cell line (HBE1) were more susceptible to injury by cigarette smoke extract (CSE) and by direct cigarette smoke exposure than cells in confluent cultures. Scraping confluent cultures also caused an enhanced cell injury predominantly in the leading edge of the scraped confluent cultures by CSE. Cellular ATP levels in both subconfluent and confluent cultures were drastically reduced after CSE exposure. In contrast, GSH levels were significantly reduced only in subconfluent cultures exposed to smoke and not in confluent cultures. Western blot analysis demonstrated ERK activation in both confluent and subconfluent cultures after CSE. However, activation of apoptosis signal-regulating kinase 1 (ASK1), JNK, and p38 were demonstrated only in subconfluent cultures and not in confluent cultures after CSE. Using short interfering RNA (siRNA) to JNK1 and JNK2 and a JNK inhibitor, we attenuated CSE-mediated cell death in subconfluent cultures but not with an inhibitor of the p38 pathway. Using the tetracycline (Tet)-on inducible approach, overexpression of thioredoxin (TRX) attenuated CSE-mediated cell death and JNK activation in subconfluent cultures. These results suggest that the TRX-ASK1-JNK pathway may play a critical role in mediating cell density-dependent CSE cytotoxicity.

airway epithelial cells; injury; thioredoxin; mitogen-activated protein kinase

CHRONIC EXPOSURE TO CIGARETTE smoke is known to cause or contribute to numerous and devastating human diseases including atherosclerosis, lung cancer, and chronic obstructive pulmonary disease (COPD). The nature of the inciting injuries and the pathogenesis of these diseases induced by cigarette smoke are incompletely understood. According to the International Agency for Research on Cancer (IARC), cigarette smoke contains over 4,000 diverse compounds, of which at least 60 are known carcinogens in animal studies (11). The presence of these carcinogens may explain why 90% of all lung cancer is due to cigarette smoke (24, 28). However, this does not explain

how cigarette smoke leads to the development of COPD, which is the fourth leading cause of death in the United States (1). In addition to the generation of carcinogens, cigarette smoke also produces numerous reactive oxygen species (ROS) that may be responsible for targeted airway epithelial cell injury (28). Reports have suggested that one puff of cigarette smoke contains 10^{14} free radicals (36) or an estimated 18.64–54.81 nanomoles of H_2O_2 per liter of total ROS (10) in mainstream cigarette smoke. These levels of free radicals could result in an oxidative/reductive imbalance that leads to cell injury and death of airway epithelial cells on smoke exposure.

Apoptosis and necrosis are two mechanisms by which ROS could induce airway epithelial cell death. Apoptosis is a programmed cell death system where the cell initiates a specific sequence of signals leading to morphological and biochemical changes including DNA condensation and fragmentation and cell shrinkage (13). Necrosis is considered an accidental cell death where extreme environmental insults lead to loss of the cell membrane integrity and cell lysis. Although the exact mechanisms of necrosis are not well-known, the mechanism of apoptosis has been well-characterized. ROS have been shown to cause apoptosis via a variety of signal transduction pathways. ROS through the oxidation of thioredoxin (TRX) can induce apoptosis by activation of apoptosis signal-regulating kinase 1 (ASK1), which, in turn, phosphorylates and activates the JNK and p38 MAPK, ultimately leading to apoptosis (16). The signaling events resulting from smoke exposure in airway epithelial cells are still incompletely understood.

Our laboratory has been interested in the effects of air pollutants, such as ozone and cigarette smoke, on conducting airway epithelial cells. In this study, we show that variations in cell density, specifically subconfluent compared with confluent cells, have a significant impact on smoke-induced cell injury and death. We have found that cells in confluent cultures are quite resistant to smoke-induced cell death, whereas cells in subconfluent conditions are much more susceptible to smoke-induced cell death. In this study, we show that this cell density-dependent cell death phenomenon is associated with activation of the JNK pathway and that cell death can be attenuated by inhibition of JNK pathway and the elevation of TRX expression.

MATERIALS AND METHODS

Cell culture. Human airway tissues were obtained from University of California Medical Center (Sacramento, CA) and National Disease Research Interchange (Philadelphia, PA) with consents. The protocol

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has been periodically reviewed and approved by the University Committee on the Protection of Human Subject for Research (University of California at Davis). Monkey airway tissues were obtained from the California Regional National Primate Research Center (Davis, CA). Primary tracheobronchial epithelial (TBE) cells were isolated from tissue as previously described (39, 40). Primary cells and the immortalized human bronchial epithelial cell lines, HBE1, with or without tetracycline (Tet)-on TRX and mutant TRX expression, were cultured in a serum-free DMEM/Ham's F-12 with insulin (5 μ g/ml), transferrin (5 μ g/ml), EGF (10 ng/ml), dexamethasone (0.1 μ M), cholera toxin (10 ng/ml), and bovine hypothalamus extract (15 μ g/ml). For subconfluent cultures, cells were plated either on 96- or 6-well plates at either 1.0×10^4 or 1.0×10^5 cells/well, respectively, 2–3 days before smoke extract exposure. For confluent cultures, the plating cell density was fourfold higher than that of subconfluent cultures. Within a day after plating, a confluent culture could be seen in these wells. Confluence was confirmed by measurement of trans-epithelial resistance at an air-liquid interface.

The HBE1 cell line was obtained from Dr. J. Yankaskas at University of North Carolina (41) and maintained in the culture medium similar to that for primary TBE cultures. This cell line maintained mucous cell differentiation in vitro in the presence of all-*trans*-retinoic acid (5).

Human TRX cDNA was PCR prepared and Flag tagged in a pCMV-3XFlag-tag vector according to the manufacturer's recommended protocol (Sigma, St Louis, MO) between the restriction sites, *Cla*I and *Bam*HI. After sequence verification, the Flag-TRX construct was then recloned into the pTRE2 vector between *Cla*I and *Sall*I sites. For the pTRE2-mutant TRX-Flag clone, the cloning strategy was the same, but mutations in the Cys residues of the active site, -Cys³²-Gly-Pro-Cys³⁵-, were introduced with Ser before the cloning (38). To generate the Tet-on-inducible Flag-tag TRX cell lines, we first generated a constituted Tet regulator expression cell line, HBE1-Tet. This cell line was further used to generate TRX- and mutant TRX-inducible cell lines, namely HBE1-Tet-TRX-Flag and HBE1-Tet-mutant TRX-Flag. For these Tet-on-inducible Flag-TRX expression HBE1 cell lines, cells were treated with doxycycline (5 μ g/ml) for induced TRX and mutant TRX expression 48 h before cigarette smoke extract (CSE) exposure.

For the inhibitor studies, 10–40 μ M SP-600125 (a specific JNK inhibitor), SB-203580 (a specific p38 inhibitor), and PD-98059 (a specific inhibitor of MEK; Calbiochem, San Diego, CA) were dissolved in DMSO. These doses were used based on the information obtained from various publications as well as our unpublished data that these doses cause no cell toxicity (Trypan blue dye exclusion) and the efficacy in the inhibition of the activities of these kinases but not other unrelated kinases (data not shown). Cells were treated with the inhibitors for 30 min before and during the CSE exposure. Control conditions were treated with DMSO.

CSE preparation and exposure. Fresh CSE was prepared according to the protocol described by Liu and colleagues (17). Briefly, mainstream smoke from seven cigarettes (2R4F; University of Kentucky) were withdrawn steadily via a speed-adjustable peristaltic pump at a rate of 50 ml/min and bubbled through a 27.5-cm height vessel containing 10-ml DMEM/Ham's F-12 (1:1) medium to obtain 100% CSE. To maintain the quality and the reproducibility of the daily prepared CSE, we used the absorbance at 305 nm for the measurement of CSE content in the preparation. This was based on the initial spectroscan of CSE medium by Beckman spectrophotometer for CSE-specific absorbance wavelength. The 100% CSE prepared under this specified protocol has 0.5 absorbance at 305-nm wavelength. Using this approach, different concentrations of CSE were prepared accordingly, and the media was used within 1 h after the preparation.

Mainstream smoke exposure. For mainstream smoke exposure, cells were plated on Transwell chambers (12 mm diameter, 0.4- μ m pore; Costar, Corning, Corning, NY) at cell densities of 5 and 20×10^4 cells/chamber for subconfluent and confluent cultures, respec-

tively. One day after plating, the cultures were placed in an air-liquid interface condition as previously described (40). Mainstream smoke exposure was prepared as previously described (42, 43). During the exposure, the chambers were placed on French square glass bottle (Fisher Scientific, Pittsburgh, PA) with the media fed basally.

Cell scraping experiments. Confluent monolayers of HBE1 cells were grown on one-chamber slides (Nalge Nunc, Naperville, IL) or 30-mm cell culture dishes. Using a cell scraper trimmed to fit in the slide, a 3- to 4-mm area of the confluent cells was scraped in the middle of the slide. The cultures were maintained for 48 h after scraping and then exposed to CSE for 24 h. Following the CSE, monolayers of cells were stained with the cell viability assay.

Cell viability assay. After the smoke exposure, cells were stained with a LIVE/DEAD assay (Invitrogen, Carlsbad, CA) according to the manufacturer's suggested protocol. In brief, 96-well plates of CSE-exposed cells were washed once with 200 μ l/well of PBS. Then, 100

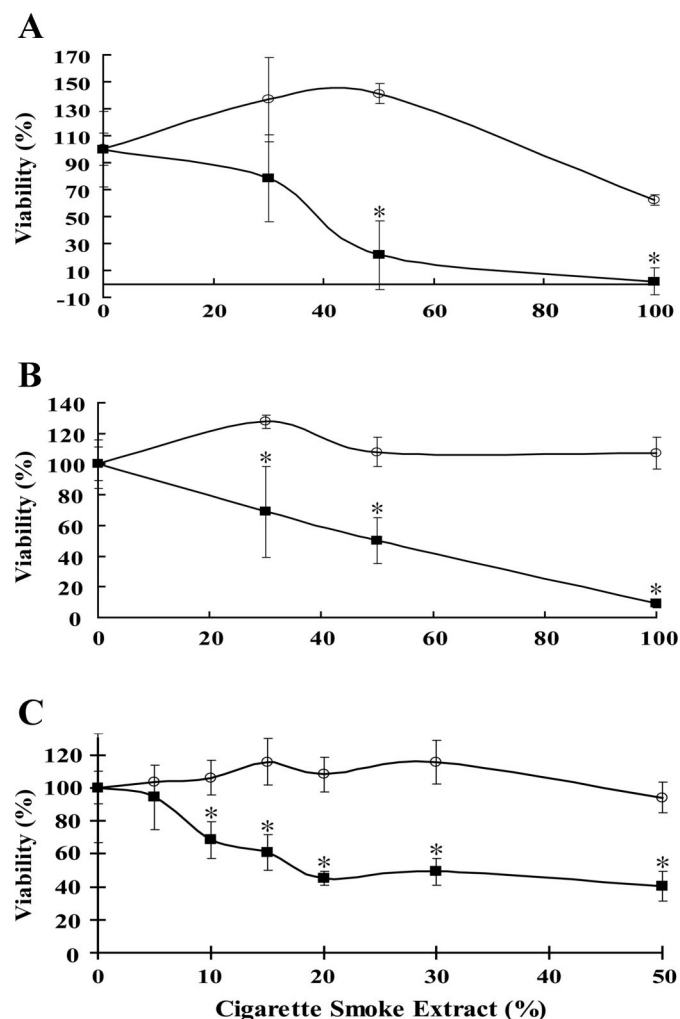


Fig. 1. Cell viability assay of various airway epithelial cells after 24-h cigarette smoke extract (CSE) exposure. Human primary bronchial epithelial cells (A), monkey primary bronchial epithelial cells (B), and immortalized human bronchial epithelial cell line (C) were exposed to various doses of CSE followed by a cell viability assay as described in the text. The subconfluent (closed square) cultures demonstrated a marked decrease in cell viability compared with the confluent (open circle) cultures in all the cell types. Values are expressed as percent viable cells and represent the means \pm SD of triplicate samples relative to the confluent culture. * $P < 0.05$. Experiments were repeated 3 times with primary cultures derived from different donors and different passages of the immortalized normal human bronchial epithelial cell line (HBE1) with similar results.

μl of a 2 μM calcein and 4 μM ethidium homodimer-1 (EthD-1) mixture was added to the cells in the dark. After a 30-min incubation at 37°C, the plates were read with a fluorometer at wavelengths 485/530 and 530/620 nm for calcein and ethidium homodimer, respectively. To normalize the cell density level, 20 μl of 1% saponin

(Fisher Scientific) was added to the wells, and the plate was read at 530/620 nm wavelength. For the fluorescent images, monolayers of cells were incubated with 20 μl of 1.6 mM ethidium homodimer and 5 μl of 2 μM calcein in 10-ml PBS for 45 min at room temperature. The slides were submitted to similar excitation/

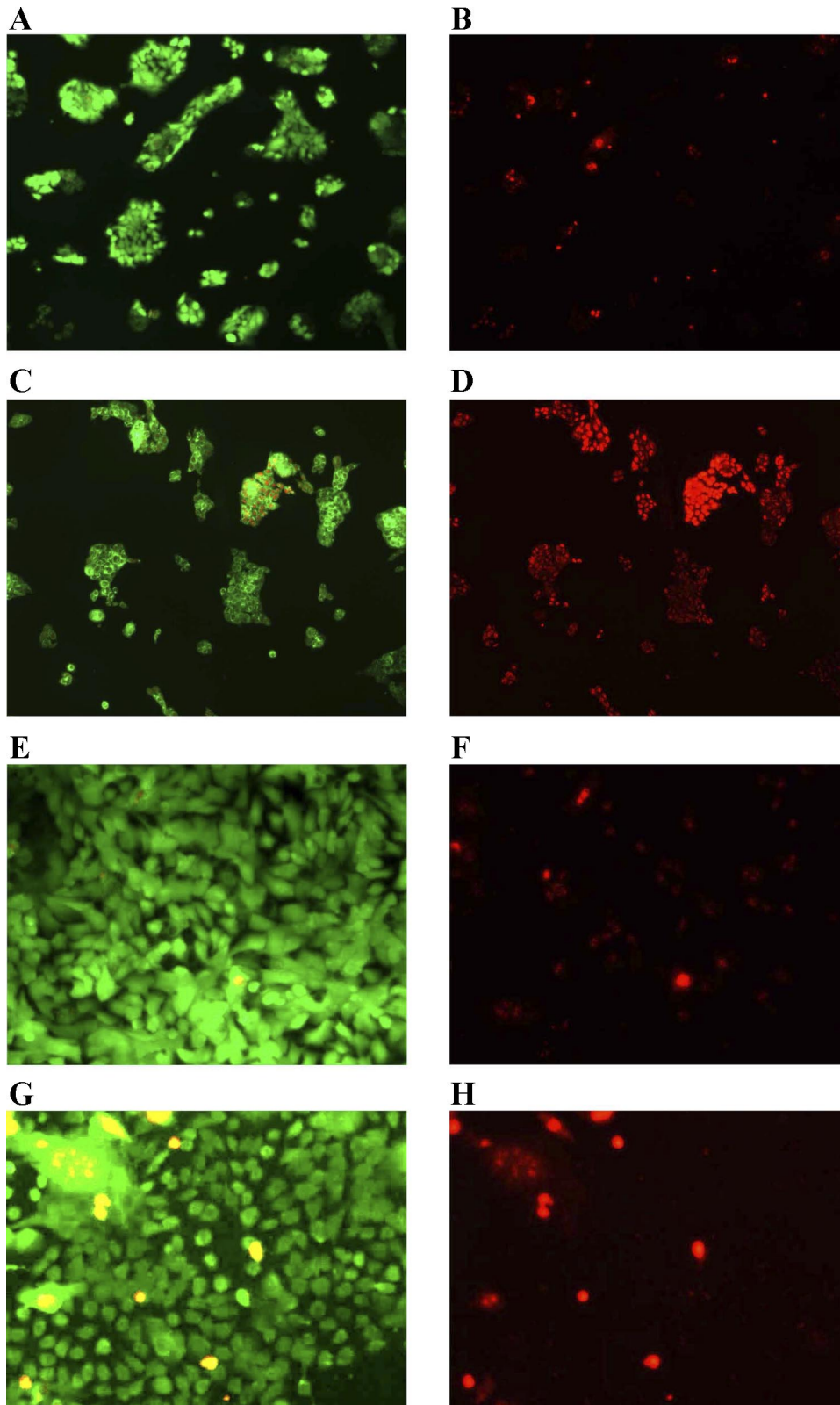


Fig. 2. Immunofluorescence of subconfluent and confluent HBE1 cells after 24-h exposure to CSE. HBE1 cells were grown on slide chambers. After 48 h, the cells were treated with 50% CSE for 24 h, and a LIVE/DEAD assay was performed as described in the text. The green fluorescence represents live cells stained with calcein, and the red fluorescence represents dead cells stained with ethidium homodimer. *A* and *B*: 0% CSE in subconfluent cultures shows the majority of cells are live (green staining) with very few dead cells (red staining). *C* and *D*: 50% CSE in subconfluent cultures demonstrates more clusters of dead (red staining) cells. *E* and *F*: 0% CSE in confluent cultures again demonstrates a majority of live cells (green staining) with few dead cells (red staining). *G* and *H*: 50% CSE in confluent cells shows a majority of live cells (green staining) with very few dead cells (red staining) in contrast to the subconfluent cultures exposed to CSE in *C* and *D*. The original magnification was $\times 100$.

emission wavelengths and examined under an Olympus or Zeiss fluorescence microscope.

Western blot analysis. After CSE exposure, proteins were collected and measured in a cell lysis buffer. Cell extract proteins (50 $\mu\text{g}/\text{lane}$) were run on a 12.5% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane for Western blot analysis as previously described (8). Monoclonal antibodies for anti-JNK, -p38, -ERK, and their phosphorylated products were obtained from BD Bioscience (San Jose, CA). Anti-Flag and anti- β -tubulin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). A polyclonal antibody of anti-c-Jun was purchased from Cell Signaling (Beverly, MA), and anti-ASK1 was a generous donation from Dr. Hidenori Ichijo (University of Tokyo, Tokyo, Japan). Densitometer was measured using multi-gauge software (Fujifilm). These experiments were repeated a minimum of three times.

HPLC analysis of GSH. GSH was measured as previously described (14). Briefly, acidified cell lysis was subjected to reverse-phase HPLC coupled with electrochemical detection to directly measure reduced GSH. The response was linear from 0.25 to 1000 ng of GSH ($R^2 = 0.995$). The amounts of GSH in cultured cells were normalized with the protein levels.

ATP analysis. An ATP assay (PerkinElmer, Wellesley, MA) was performed according to the manufacturer's suggested protocol. In brief, CSE-exposed cultures were washed once with 200 $\mu\text{l}/\text{well}$ PBS in 96-well plates, and then each well was added with 100 μl of PBS and 50 μl of mammalian cell lysis buffer. After 5 min on an orbital shaker, 50 μl of the substrate solution was added to each well, and the plate was placed back on the orbital shaker for another 5 min. Finally, the plate was dark-adapted for 10 min, and luminescence was measured by a luminometer.

siRNA knockdown of JNK1 and JNK2. Subconfluent monolayers of HBE1 cells were transfected with 100 nM short interfering RNA (siRNA) sequences for JNK1, JNK2, or a negative control sequence (Ambion, Austin, TX) using Oligofectamine (Invitrogen) in serum-free media, Opti-MEM (Invitrogen). The media was replaced with growth media the next day. On *day 3*, cells were treated with either 0% or 30% CSE while the cells were still subconfluent for the LIVE/DEAD assay. Alternatively, RNA or protein was harvested at

time 0 to demonstrate knockdown of JNK1 and JNK2. On *day 4*, either protein was harvested (24 h following CSE treatment) or a cell viability assay was performed as previously described.

Statistics. Statistical evaluation of data was performed with a two-tailed Student's *t*-test for unpaired values. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Cell density-dependent cytotoxicity in response to CSE. There have been several studies investigating the toxicity of cigarette smoke in various cell systems. A majority of these studies demonstrated cell type-specific sensitivity or a dose-dependent effect to CSE. In this study, we demonstrate that cell density at the time of smoke exposure is critical to the airway epithelial response. CSE caused cell injury in subconfluent cultures with a dose-dependent phenomenon with three different airway epithelial cell types including primary TBE cells from humans (Fig. 1A), nonhuman primate HBE cells (Fig. 1B), and HBE1 cells (Fig. 1C). In contrast, less cell death to CSE was observed in confluent cultures in the same cell types. These experiments have been independently repeated three times with primary cells from three different donors. A similar cell density-dependent cell death was seen in primary human HBE cultures that were maintained under an air-liquid interface condition in response to mainstream smoke exposure (data not shown). Since CSE and mainstream smoke have a similar cell density-dependent cytotoxicity, CSE was used for the subsequent studies.

To confirm the results of the LIVE/DEAD assay, cultures were visualized with a Zeiss fluorescence microscope. As shown in Fig. 2, subconfluent HBE1 cells exposed to CSE have close to 100% positive staining with EthD-1, a marker of necrotic cells (Fig. 2D) compared with the control cells not exposed to CSE (Fig. 2B). In contrast, confluent HBE cultures

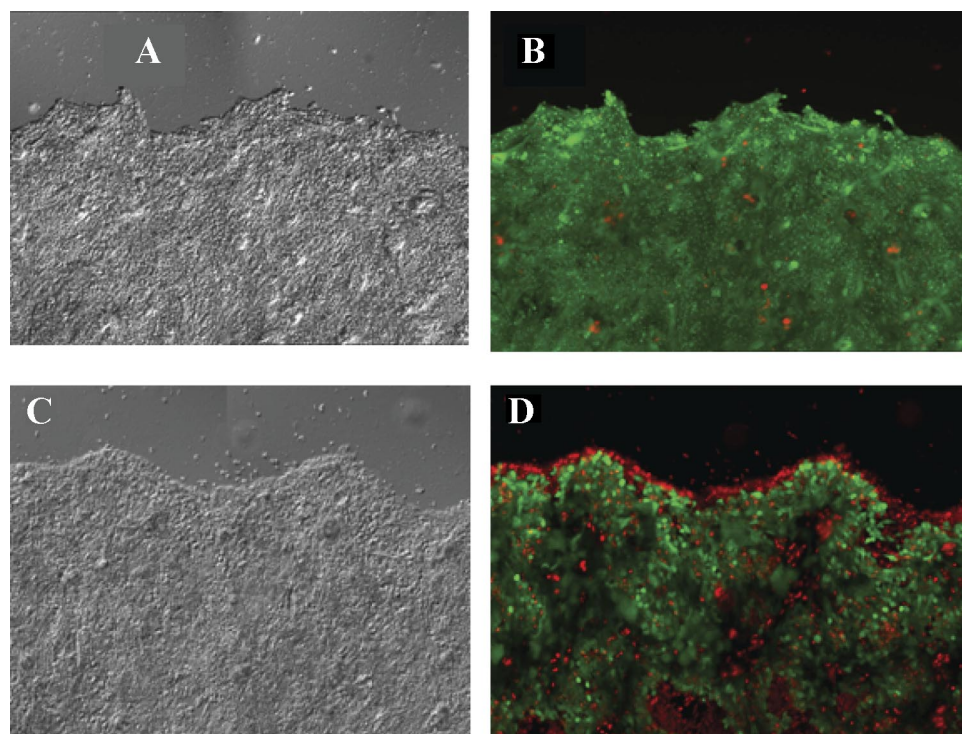


Fig. 3. LIVE/DEAD assay of CSE-induced cytotoxicity in subconfluent region of injured HBE monolayers. Monolayers of HBE1 cells were scraped, and cells were allowed to grow another 48 h with cells migrating from the site of injury. A: phase contrast image of scraped HBE1 cells exposed to 0% CSE. B: LIVE/DEAD assay of the scraped HBE1 cells exposed to 0% CSE. Note few injured (red) cells. C: phase contrast image of scraped HBE1 cells exposed to 15% CSE for 24 h. D: LIVE/DEAD assay of the scraped HBE1 cells exposed to 15% CSE for 24 h. Note many injured (red) cells especially prominent along the scraped subconfluent margin of the culture.

(Fig. 2H) show no significant uptake of EthD-1 after CSE exposure. These results confirmed the cell density-dependence of CSE toxicity.

To control for the variability between separate cultures, we verified these results in the same culture condition by scraping an area of a confluent monolayer and allowing the cells to grow from the injured margin, creating a subconfluent condition within the confluent monolayer. After 48 h of cell growth at the site of scraping, the cultures were exposed to 15% CSE for 24 h and then stained for cell death and viability. As shown in Fig. 3, cells in the leading edge along the injured margin are predominately necrotic, whereas cells in the confluent monolayer remain more viable. These results confirm the results of a cell density-dependent cytotoxicity to CSE within the same culture condition.

Reduction of ATP and GSH in CSE-exposed cells. Many studies have shown that ROS is one component in cigarette smoke that initiates metabolic cytotoxicity including inhibition of cellular ATP and GSH levels (25, 27, 29, 32). As shown in

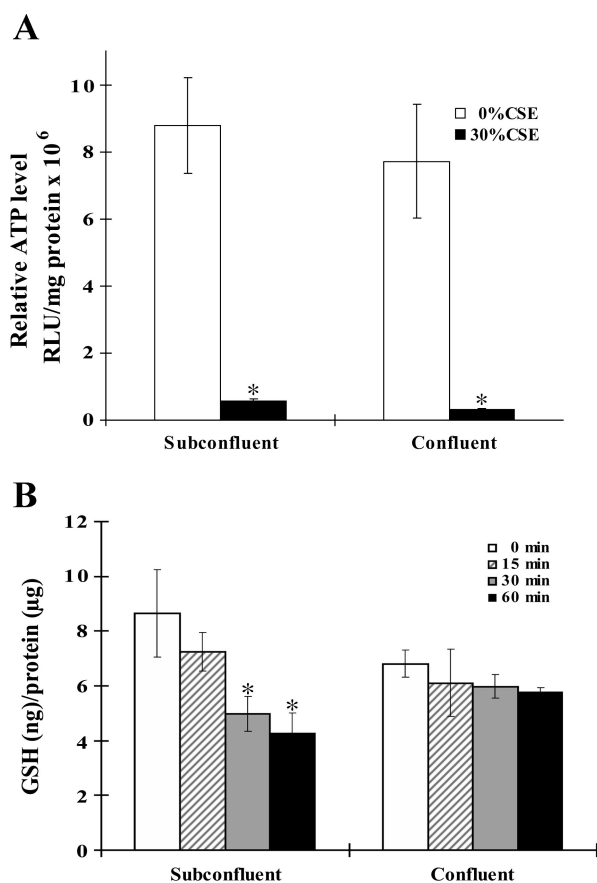


Fig. 4. CSE induced ATP and glutathione depletion in HBE1 cells. **A:** ATP level measurement of HBE1 cells that was exposed to 30% CSE for 24 h. In both cell densities, the 30% CSE (closed bar) significantly decrease the ATP level of the cells compared with the control (open bar). Values are expressed as means \pm SD of quadruplicate samples from 1 representative experiment. **B:** GSH level measurement of HBE1 cells that were exposed to 30% CSE for 15 min (hatched bar), 30 min (gray bar), and 1 h (closed bar) using HPLC. The subconfluent cultures demonstrate significant decrease in glutathione level within 1 h of CSE exposure, whereas confluent culture does not show significant change in glutathione level compared with the control (open bar). Values are expressed as means \pm SD of duplicate samples from 1 representative experiment. * $P < 0.05$ compared with control. RLU, relative light units.

Fig. 4A, ATP levels were drastically reduced to 10–20% in both subconfluent and confluent cultures after CSE treatment. In contrast, GSH was only reduced in subconfluent cultures and not in confluent cells (Fig. 4B).

Differential CSE-induced cytotoxicity is related to differential activation of MAPK pathways. Activation of the MAPK pathway has been shown to be essential in cell injury and repair (4, 19). To elucidate the differential activation of ERK, JNK, and p38 pathways, we treated subconfluent and confluent cultures of HBE1 cells with various concentrations of CSE for 3 h and examined levels of phosphorylated ERK, JNK, and p38 by Western blots. As shown in Fig. 5, ERK pathway activation was readily demonstrated in subconfluent and confluent cultures. This activation was CSE dose-dependent (Fig. 5B) and time-dependent (Fig. 5D) in both culture conditions. Both JNK and p38 pathways were activated in subconfluent cultures by CSE but not in confluent cultures (Fig. 5A, CSE dose study; Fig. 5C, time course study). To further confirm the differential activation of JNK, we detected phosphorylation of c-Jun and found that c-Jun was also phosphorylated in subconfluent cultures in a dose-dependent manner (Fig. 5E). In contrast, c-Jun phosphorylation was not seen in CSE-exposed confluent cultures.

JNK inhibitor alone attenuates the CSE cytotoxicity in subconfluent cultures. Since JNK and p38 were differentially activated in CSE-exposed cells, we investigated the involvement of these pathways in the cell density-dependent CSE cytotoxicity by using inhibitors of the MAPK pathway. The inhibitor doses were based on the manufacturer's specifications, published information, and our preliminary dose studies showing no cell cytotoxicity evaluated by Trypan blue dye exclusion and lactate dehydrogenase release as well as the specificity on the inhibition of the corresponding kinase activity. The p38 (SB-203580) and ERK (PD-98059) inhibitors had no effect on CSE-mediated cytotoxicity at a range of doses (data not shown). However, when subconfluent cultures were treated with the JNK inhibitor before CSE exposure, CSE-related cell death was reduced (Fig. 6). These results suggest the involvement of JNK activation in CSE-induced cell death in subconfluent cultures.

Differential activation of ASK1 by CSE. To determine the signaling events that are upstream of activated JNK, phosphorylation of ASK1 was investigated by Western blot in cultures after CSE exposure. As shown in Fig. 7, phosphorylation of ASK1 occurred in cells of subconfluent cultures after CSE exposure. The phosphorylation was time-dependent and consistent with the activation of the JNK pathway. In contrast, there was either no or very low levels of ASK1 phosphorylation in confluent cultures after CSE treatment. This result suggests that, like JNK, ASK1 is differentially activated by CSE in cells of subconfluent cultures.

Overexpression of TRX attenuates the CSE cytotoxicity and JNK signaling. Reduced TRX is known to inhibit activation of ASK1. However, when TRX is oxidized, it releases ASK1, permitting ASK1 to be phosphorylated and exerting downstream signaling activation. To determine if TRX is one of the key players in regulating CSE cytotoxicity, we performed the LIVE/DEAD assay and Western blot analysis on Tet-on inducible Flag-tagged TRX HBE1 cell lines. As shown in the Western blot in Fig. 8, doxycycline induced an overexpression of Flag-TRX in this cell line (lanes 3 and 4) with less consti-

tutive expression in the absence of doxycycline (*lanes 1 and 2*). Using these inducible cell lines, we examined if CSE-induced cell death could be reduced. As shown in Fig. 9, CSE-induced cell death was significantly attenuated with an overexpression of Flag-TRX, while, there was no attenuation in subconfluent cultures that overexpressed Flag-mutant TRX. Additionally, overexpression of Flag-TRX attenuated CSE-induced JNK phosphorylation in CSE-exposed subconfluent cultures (Fig. 8), whereas JNK phosphorylation was not attenuated with an overexpression of Flag-mutant TRX. These results further suggest the significant role of the TRX-ASK1 axis in the modulation of CSE-induced cell death and the activation of JNK signaling.

Knockdown of JNK1 and JNK2 protects subconfluent HBE1 cells from CSE cytotoxicity. Knockdown of JNK1 and JNK2 was confirmed by quantitative real-time PCR and Western blot for total JNK. As shown in Fig. 10A, the average change in cycle threshold ($\Delta\Delta Ct$) value of JNK1 was 0.014 compared with 0.035 for the random oligomer (negative control) and

0.023 for JNK2 in HBE1 cells transfected with siRNA for JNK1. In comparison, HBE1 cells transfected with siRNA for JNK2 had an average $\Delta\Delta Ct$ value of 0.011 for JNK2 compared with 0.023 for the random oligomer and 0.026 for JNK1 as shown in Fig. 10B. These results demonstrate relatively selective knockdown of the JNK mRNA with their respective siRNA. Western blot data confirmed the knockdown of JNK2 compared with the negative control siRNA transfected cells both before (*time 0*) and 24 h after 0% and 30% CSE treatment as shown in Fig. 10C. Because of the low expression of JNK1 in our experimental conditions, we were unable to confirm knockdown of JNK1 by Western blot.

Subconfluent monolayers of HBE1 cells with knockdown of JNK1 and JNK2 had more viable cells after 24 h of 30% CSE compared with random oligomer siRNA transfected cells as shown in Fig. 10D. In fact, both knockdown of both JNK1 and JNK2 increased cell survival with a slight increase in survival with knockdown of JNK1 (91.5% cell survival with JNK1 and 86% cell survival with JNK2 compared with 56.4% cell sur-

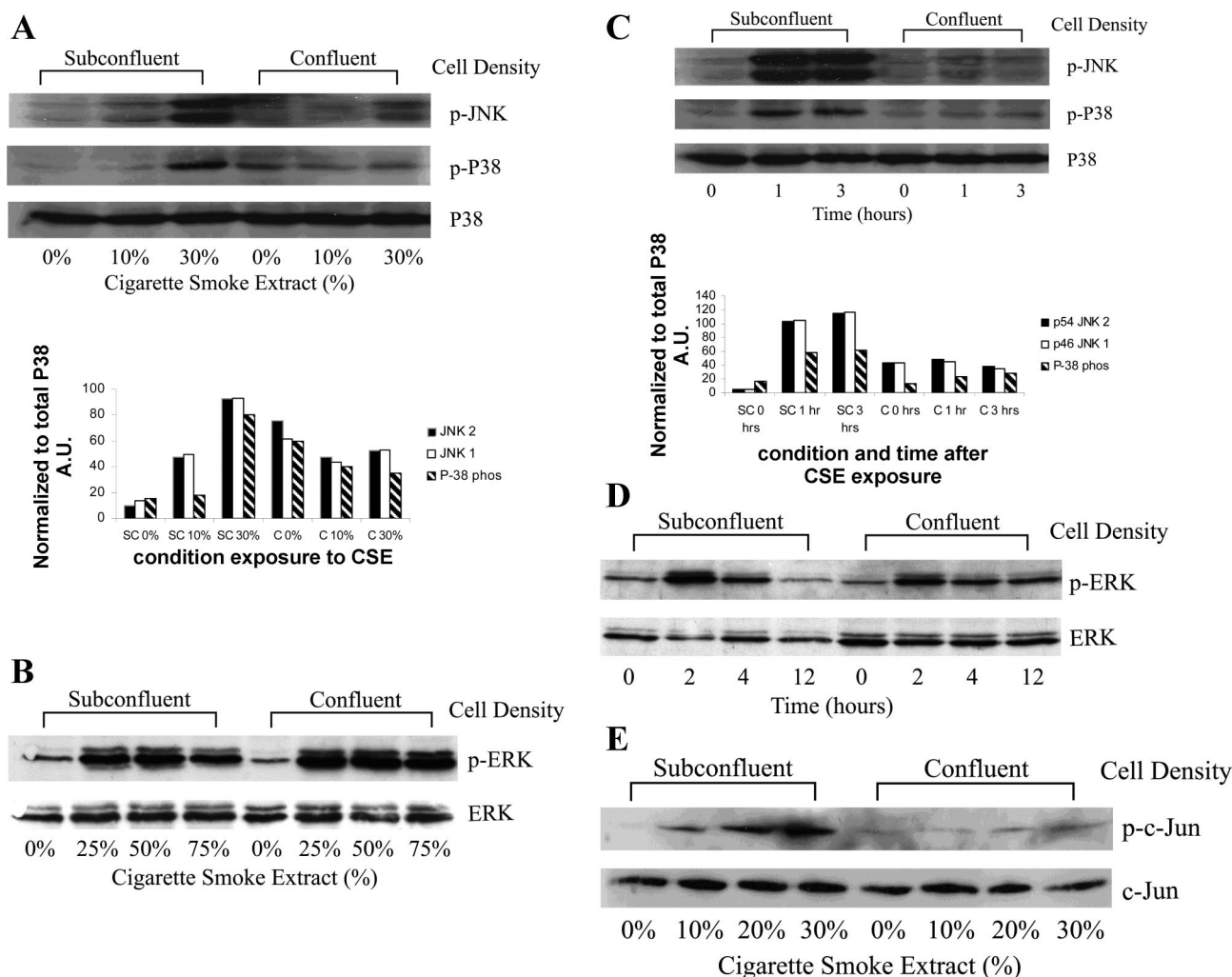


Fig. 5. Western blot analysis of CSE-exposed HBE1 cells in subconfluent (SC) and confluent (C) culture with anti-MAPK pathways. Dose-dependent exposure of 0%, 10%, and 30% of CSE (A) or 0%, 25%, 50%, and 75% of CSE (B) or 0%, 10%, 20%, and 30% of CSE (E) were exposed to HBE1 cell line for 3 h. Time-dependent exposure of 0, 1, and 3 h of 30% CSE (C) or 0, 2, 4, and 12 h of 50% CSE (D) were exposed to HBE1 cell line, and expression of anti-phospho-JNK, anti-phospho-c-Jun, anti-phospho-p38, and anti-phospho-ERK were determined in either subconfluent or confluent culture as indicated. Total anti-p38, anti-ERK, or anti-c-Jun was also determined to assure equal loading in each lane. Expression of p-JNK and p-p38 (A and C) were detected with multi-gauge software and normalized to total p-p38 in each lane. A.U., arbitrary units.

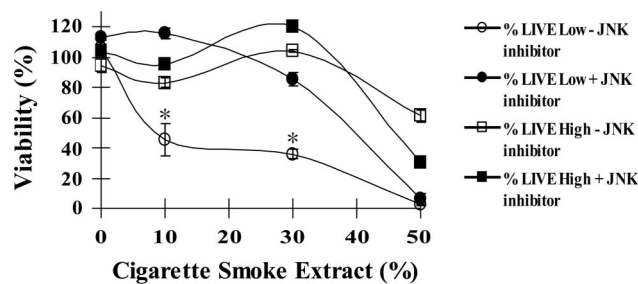


Fig. 6. Attenuation of CSE-induced cell injury with the JNK inhibitor SP-600125. Subconfluent and confluent cultures of HBE1 cells were exposed to different doses of CSE followed by a cell viability assay in the presence or absence of a JNK inhibitor. The subconfluent culture without the JNK inhibitor (open circle) demonstrates a marked decrease in cell viability compared with the subconfluent culture in the presence of the JNK inhibitor (closed circle). The confluent culture with (closed square) and without (open square) the JNK inhibitor demonstrates similar cellular viability at each dose of CSE. The values are expressed as percent viable (% LIVE) cells, and each point represents the mean \pm SD of triplicate samples. * $P < 0.05$ relative to confluent culture.

vival with negative control siRNA). These results substantiate the role of both JNK1 and JNK2 in CSE mediated cytotoxicity in subconfluent conditions.

DISCUSSION

In this study, we demonstrated that cell density is a critical factor involved in CSE toxicity. At a high cell density such as confluence, cells are more resistant to CSE toxicity than at a subconfluent density. This phenomenon was consistently observed in both primary TBE cells and an immortalized human TBE cell line. This cell density-dependent response is also observed in the same culture and when cells are directly exposed to mainstream smoke. Consistent with the cell density-dependent cell death phenomenon, we also demonstrated that cells at the leading edge of a scraped confluent culture were more sensitive to CSE-induced cell death. This is especially relevant as this culture system serves as an *in vitro* model of airway epithelial injury and repair with smoke exposure. This same phenomenon of increased susceptibility of subconfluent regenerating airway epithelial cells to injury could be occurring in many diverse pulmonary diseases including asthma and oxidant injury in which the epithelium is repeatedly damaged with subsequent regeneration. In other experimental models using a hydrogen peroxide-induced injury of mouse fibroblast cells (18) and a nitrogen dioxide-induced injury of rat alveolar lung epithelial cells (23), cell density has also been shown to be a critical factor in cell death. Importantly, both H_2O_2 and nitrogen dioxide are components of cigarette smoke. Our data emphasize the importance of documenting the cell density and confluence at the time of exposure to various toxicants in a study.

The mechanisms of cell density-dependent toxicity are not known. There are two possibilities to explain the cell density-dependent cytotoxicity of this study. One possible explanation is that cells in the subconfluent cultures are receiving a greater exposure to CSE compared with cells in the confluent cultures. It is possible, especially for cells that are actively engaged in cell proliferation, that they may have larger surface areas than those that are confluent. Therefore, we cannot exclude the possibility that subconfluent cells are effectively receiving a

higher dose of CSE compared with confluent cells. However, our data are supportive of both the cell cultures may have received a similar level of CSE exposure because reduced ATP levels and ERK activation were found in confluent cultures as well as subconfluent ones. A reduction in ATP levels is an indication of DNA strand breaks and leads to the activation of poly(ADP-ribose) polymerase, an enzyme important in DNA repair and apoptosis (27). Possible explanations for these results are that the ATP reduction and ERK activation do not lead to cell death in confluent cultures or that cells in a confluent monolayer maintain the capacity to rapidly repair damage thus preventing cell death. The second explanation for

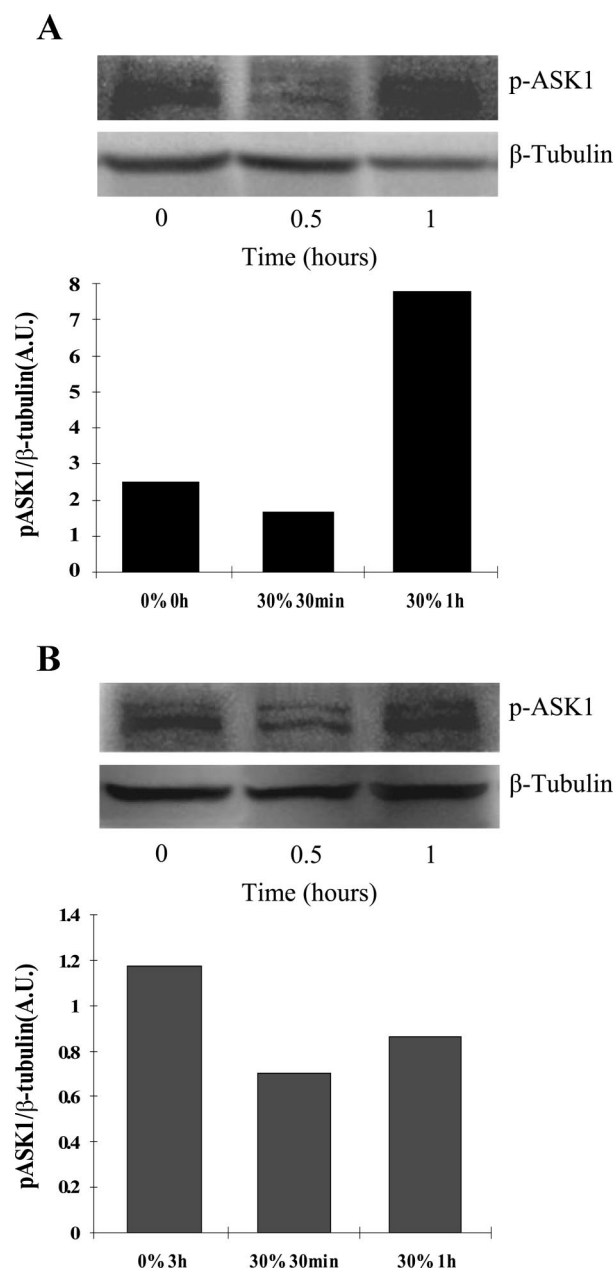


Fig. 7. Western blot analysis of apoptosis signal-regulating kinase 1 (ASK1) activation in CSE-exposed subconfluent and confluent HBE1 cells. Subconfluent (A) and confluent (B) HBE1 cells were exposed to 30% CSE for 0, 0.5, and 1 h. Then, the expression of phosphorylated ASK1 was detected with multi-gauge software and normalized to β -tubulin in each lane.

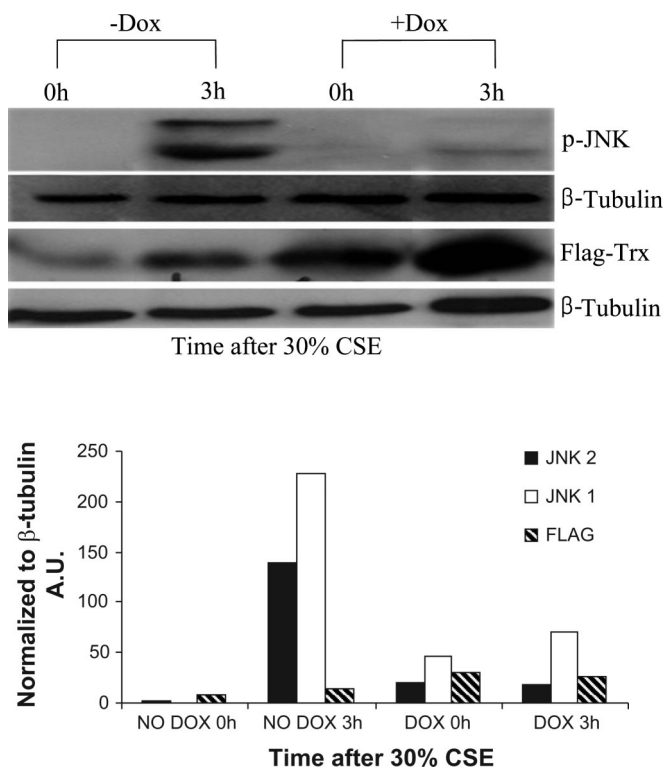


Fig. 8. Western blot analysis of tetracycline (Tet)-on inducible thioredoxin (TRX) stable HBE1 cell line after CSE exposure. Western blots were performed to measure the phosphorylation of JNK on HBE1-Tet-TRX cells after 3 h of 30% CSE exposure either with or without doxycycline (DOX) treatment. Tet-on inducible TRX cells overexpress Flag-tagged TRX after the addition of DOX as shown in lanes 3 and 4 with mild constitutive production by the cells without DOX (lanes 1 and 2). β -Tubulin was measured to assure equal loading in each lane. Expression of p-JNK and Flag-TRX were detected with multi-gauge software and normalized to β -tubulin in each lane.

our results is that there are different cellular responses between these two culture conditions. These alterations included metabolic changes in ATP and GSH levels and the activation of various MAPK signaling proteins, which include the phosphorylation of ASK1, JNK, p38, and ERK.

Only the reduction of GSH and the phosphorylation of ASK1, JNK, and p38 could account for the cell density-dependent cell death in our model as these responses were not seen in cells of the CSE-exposed confluent cultures. Interestingly, we demonstrate that inhibition of ASK1/JNK signaling by induced overexpression of TRX, pretreatment of cells with a JNK inhibitor, and siRNA of JNK1 and JNK2 ameliorated cell survival. These results are consistent with the known roles of ASK1/JNK and p38 in stress-induced signaling pathways. Similar to ASK1/JNK, p38 phosphorylation was also differentially expressed in CSE-exposed subconfluent and confluent cultures in our study. However, an inhibitor of p38 phosphorylation failed to ameliorate cell survival.

We found JNK upregulation in subconfluent cultures within 3 h of exposure to CSE. These findings are consistent with data in the literature showing later (1–6 h) activation of JNK mediates proapoptotic signaling as opposed to more immediate (1 h) activation of JNK, which has been shown to be associated with cell survival (37). Our finding that the JNK pathway is involved in cell death is also consistent with other ROS-induced cell injury models (4, 15, 23) including a recent

publication on CSE-induced endothelial injury (9). Studies have shown that JNK is activated by various types of stresses such as UV irradiation, heat shock, proinflammatory cytokines, osmotic stress, and ROS (2, 34). Although the detailed signaling mechanisms leading to JNK activation are not defined, Tobiume et al. (33) suggested that ROS activate JNK through the ASK1 pathway. Furthermore, Saitoh et al. (26) showed that expression of TRX inhibited ASK1 kinase activity whereas inhibition of TRX resulted in the activation of endogenous ASK1 activity, suggesting that TRX is a physiological inhibitor of ASK1 function.

Based on these data, we hypothesize that CSE will oxidize TRX leading to activation of ASK1/JNK and subsequent cell death. This is consistent with our findings of rapid ASK1 phosphorylation (within 1 h) before JNK activation and cell death and that overexpression of TRX, but not the mutant TRX, can ameliorate CSE-induced cell death and JNK phosphorylation in subconfluent cultures. Taken together, these results suggest that ASK1 and TRX are regulators of JNK-mediated CSE cytotoxicity in subconfluent cultures.

It has been suggested that phosphorylated JNK can activate the downstream transcription factor c-Jun, which, in turn, regulates the expression of Fas ligand, which binds to Fas receptors and ultimately leads to cell death (6, 7). This hypothesis is consistent with our results in which c-Jun was phosphorylated in subconfluent cultures after CSE treatment but not in confluent cultures. These data also support the JNK pathway as a key player regulating CSE cell density-dependent cytotoxicity.

Another study using mouse fibroblasts exposed to hydrogen peroxide implicates EGF receptor signaling in cell density-associated cytotoxicity (18, 23). Cells in a logarithmic growth phase responding to EGF will have intrinsic tyrosine kinase activity, whereas static growth phase cells will not (30). Furthermore, the EGF receptor can be dimerized by a peroxynitrate in cigarette smoke (22, 35). Cell density-dependent cytotoxicity is presumably due to EGF receptor activation, and cigarette smoke may dimerize and activate the EGF receptor leading to cell death.

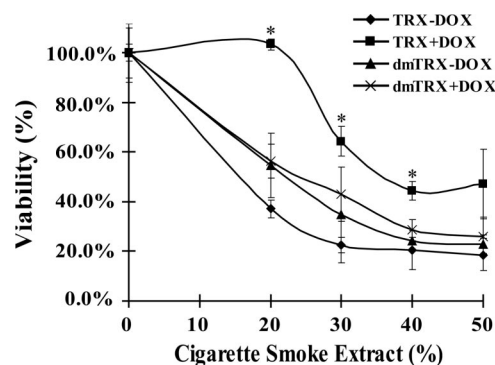


Fig. 9. Cell viability assay of Tet-on inducible TRX and mutant TRX stable HBE1 cell line after CSE exposure. Dose-dependent cell viability assay was performed on HBE1-Tet-TRX and HBE1-Tet-mutant TRX cells after 24-h CSE exposure. DOX-treated HBE1-Tet-TRX cells (closed square) show significant increase in viability compared with the no-DOX-treated HBE1-Tet-TRX cells (closed diamond). In contrast, DOX-treated HBE1-Tet-mutant TRX ("x") did not show any significant difference compared with the no-DOX-treated HBE1-Tet-mutant TRX (closed triangle). The values are expressed as percent viable cells and represent means \pm SD of triplicate samples. * $P < 0.05$ relative to no DOX TRX HBE1 culture.

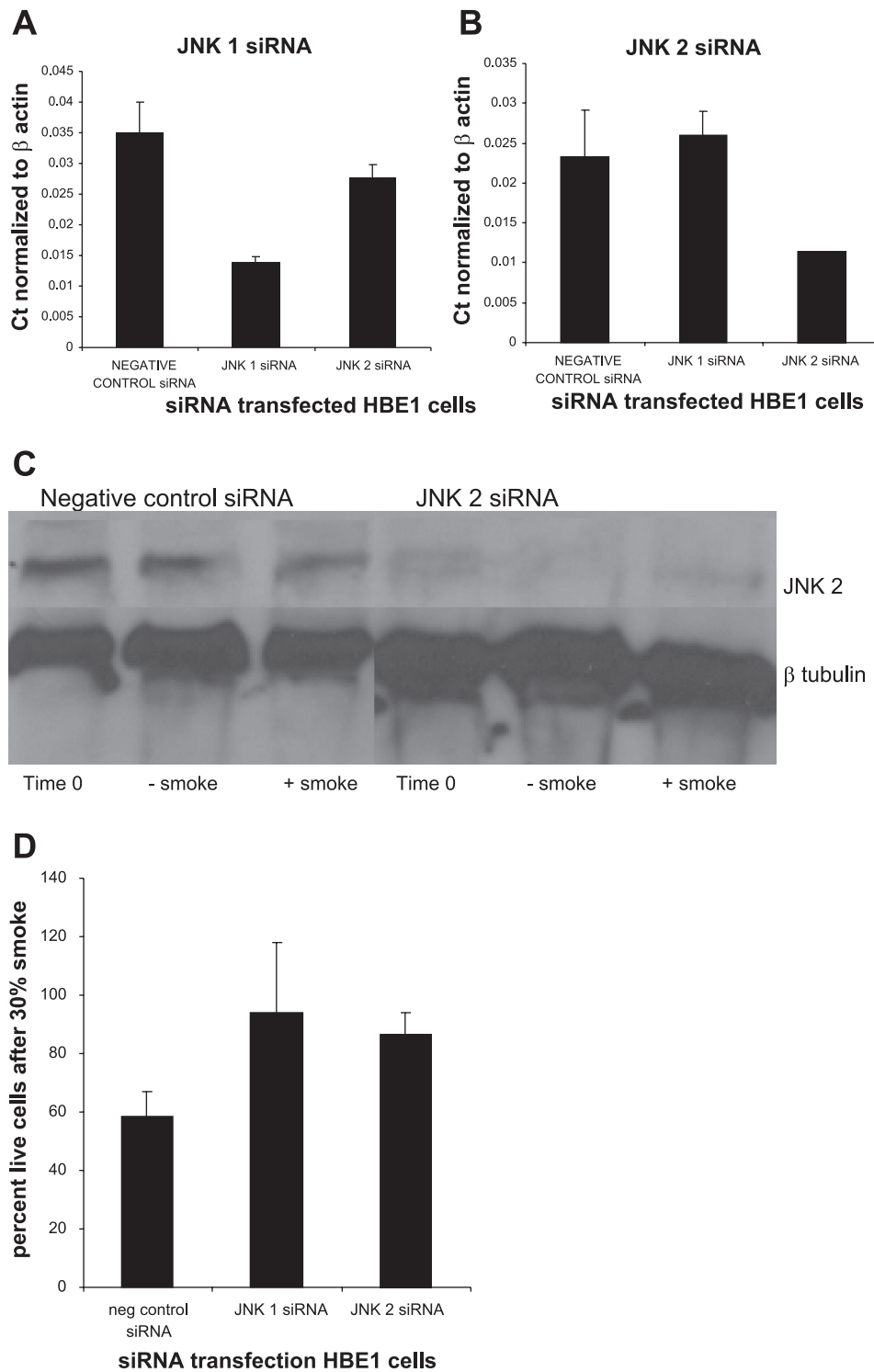


Fig. 10. Short interfering RNA (siRNA) mediated knockdown of JNK1 and JNK2 in HBE1 cells. HBE1 cells were transfected with siRNA to either JNK1, JNK2, or a random oligomer [negative (neg) control] while at a subconfluent state. *A* and *B*: 48 h later, RNA was isolated using TRIzol, and mRNA was reverse-transcribed to cDNA using random primers. Real-time PCR was performed with primers to JNK1, JNK2, and β -actin. The cycle threshold (Ct) values were normalized to β -actin and show knockdown of JNK1 and JNK2 mRNA. Mean values of duplicate wells are presented \pm SE. *C*: protein was isolated from the transfected cells at 48 h (*time 0*) or 24 h following exposure to 0% or 30% CSE. Forty micrograms of protein was run on an SDS-PAGE gel followed by a blot for total JNK. The first 3 lanes are HBE1 cells transfected with random oligomers, and the last 3 lanes are transfected with JNK2 siRNA and show knockdown of JNK2 at all time points. β -Tubulin is shown for normalization. *D*: a LIVE/DEAD assay was performed following a 24-h exposure to 0% or 30% CSE while the cells were subconfluent. The bars show percent of live cells after 30% CSE normalized to 0% CSE for each of the transfected cells and demonstrate increased cell survival in cells transfected with JNK1 and JNK2 compared with negative control siRNA-transfected cells. Mean values of triplicate wells are presented \pm SE.

EGF receptor-mediated signaling includes activation of various MAPK pathways including ERK, JNK, and p38. In this study, we were unable to detect the activation of the EGF receptor by CSE in either the confluent or subconfluent cell culture models (data not shown). This discrepancy could be due to different cell types as breast carcinoma and cytotrophoblast cell lines were used in some studies compared with our study using bronchial epithelial cells (20, 21).

ERK activation was demonstrated in both CSE-exposed subconfluent and confluent cultures. It has been suggested that ERK activation is associated with cell proliferation and is often activated through a receptor tyrosine kinase (12, 31). Although in our model we did not detect EGF receptor activation, it is likely that ERK is activated by another receptor tyrosine kinase in both subconfluent and confluent cultures and that this pathway was independent of the cell death.

In summary, we have demonstrated a cell density-dependent cytotoxicity in both primary human and monkey TBE cells and a human bronchial epithelial cell line, HBE1, after exposure to cigarette smoke. We have observed that subconfluent cultures are more sensitive to CSE than confluent cultures even within the same culture plate. Associated with this finding is differential MAPK pathway activation of the subconfluent cultures in response to CSE. We have demonstrated a close correlation between activation of ASK1, JNK, and p38 and the sensitivity of subconfluent cells to CSE cytotoxicity. In contrast, ERK is activated in both subconfluent and confluent cultures. Furthermore, we demonstrate with MAPK inhibitors and TRX overexpression that the TRX/ASK1/JNK pathway is important in the regulation of cell density-dependent cell death. Further studies of the downstream signal transduction pathway are needed to elucidate the molecular basis of the susceptibility of subconfluent cultures to cell death after cigarette smoke exposure.

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