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Biochemical and Biophysical Research Communications 353 (2007) 121–126
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STAT3 activation inhibits human bronchial epithelial cell apoptosis in response to cigarette smoke exposure ☆

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Received 20 November 2006
Available online 8 December 2006

Abstract

We have previously reported that cigarette smoke can induce DNA damage in human lung cells without leading to apoptosis or necrosis. In this study, we report that STAT3 is required for the survival of human bronchial epithelial cells (HBECs) following cigarette smoke-induced DNA damage. Cigarette smoke extract (CSE) exposure increases STAT3 phosphorylation (Tyr 705) and DNA binding activity in HBECs. CSE also stimulates IL-6 release and mRNA expression. Anti-IL-6 neutralizing antibody partially blocks STAT3 activation and renders the cells sensitive to CSE-induced DNA damage. Suppression of STAT3 by siRNA results in severe DNA damage and cell death in response to CSE exposure. These findings suggest that STAT3 mediates HBEC survival in response to CSE-induced DNA damage, at least in part, through the IL-6/STAT3 signaling pathway.

Keywords: Cigarette smoke; STAT3; Apoptosis

Cigarette smoking is associated with many diseases including lung cancer and emphysema. The mechanisms by which cigarette smoking causes these alterations in the lung remain to be elucidated. *In vitro* cigarette smoke exposure results in DNA damage, which may be followed by apoptosis or necrosis depending on the concentration of cigarette smoke exposure [1–4]. We have previously reported that human lung fibroblasts or human bronchial epithelial cells (HBECs) do not undergo either apoptosis or necrosis following DNA damage induced by low concentrations of cigarette smoke exposure. In contrast, these DNA-damaged cells survive, undergo DNA repair, and can proliferate clonogenically [3,4].

Many factors may modulate the cell survival or apoptosis in response to DNA damage. Among these, signal transducer and activator of transcription 3 (STAT3) is a likely candidate factor regulating cell survival or death in response to cigarette smoke exposure. STAT3 activation is triggered by either binding of IL-6 family of cytokines to their receptors or intra-cellular activation of Src kinases [5,6]. Accumulating evidence indicates that STAT3 activation plays an important role in cell proliferation, differentiation, apoptosis, inflammation, and wound repair [5,7,8]. Over-expression of STAT3 protects the lung epithelium from hyperoxic injury while down-regulation of STAT3 or *Stat3*-deficiency renders lung cells sensitive to external insults and accelerates apoptosis [9,10].

The current study was designed to investigate the role of the STAT3 in modulating cell survival in response to CSE exposure. CSE stimulates IL-6 release as well as STAT3 tyrosine phosphorylation (Tyr 705) and DNA binding activity. Anti-IL-6 neutralizing antibody partially blocks STAT3 phosphorylation induced by CSE. Suppression of STAT3 by siRNA renders the cells sensitive to CSE-induced cell death. These results demonstrate that STAT3 activation, which results in part from the autocrine/paracrine activity of IL-6, plays an important role in mediating the blockade of apoptosis or necrosis in response to CSE-induced DNA damage.

☆ Funded by Larson Endowment, University of Nebraska Medical Center, Omaha, Nebraska, and by the State of Nebraska LB506.
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doi:10.1016/j.bbrc.2006.11.147

Materials and methods

Cell culture. Normal human bronchial epithelial cells (HBECs) were acquired from bronchial biopsies using a previously published method with modifications [11]. HBECs were cultured using the medium of a 1:1 mixture of LHC-9/RPMI 1640 [12].

Cigarette smoke extract (CSE) preparation. CSE was prepared with a modification of the method of Carp and Janoff [13]. Briefly, one 100 mm cigarette without filter (Research Grade Cigarette, University of Kentucky) was combusted through 25 ml distilled water. The resulting suspension was filtered through a 0.22 μm pore filter (Lida Manufacturing Corp., Kenosha, WI). This solution was considered to be 100% CSE and diluted with LHC-D/RPMI 1640 medium [12].

Comet assay. Comet assay was performed using the CometAssay™ Kit (Trevigen, Inc., Gaithersburg, MD). Briefly, CSE or camptothecin-treated cells were suspended in cold PBS at 10^5 cells/ml. The cell suspension (50 μL) was then mixed with 500 μL LMAgarose and 75 μL of the agarose/cells was pipetted over the sample area of the CometSlides. The samples were lysed, electrophoresed, and stained following the manufacturer's instructions.

IL-6 quantification by ELISA. IL-6 was quantified by ELISA with capturing anti-IL-6 antibody (R&D Systems, Cat# AB-206-NA, Minneapolis, MN), bridging anti-IL-6 antibody (CalBiochem, Cat# 407670, EMD Biosciences, San Diego, CA), and HRP-conjugated (goat) anti-rabbit IgG (Rockland, Cat# 611-1302, Gilbertsville, PA).

Real-time RT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and 1 μg of total RNA was treated with DNAse I (Invitrogen, Carlsbad, CA). For cDNA synthesis, 600 ng of total RNA was reverse transcripted using reverse transcription reagents (Applied Biosystem, Foster City, CA). Real-time PCR was conducted in a total volume of 50 μL using ABI Prism 7500 (Applied Biosystem, Foster City) as described previously [14] . Primer and probe sequences were as follows: IL-6/47forward: CTCCAGGAGCCCAGCTATGA IL-6/112 reverse: CCCAGGGAGAAGGCAACTG IL-6/68probe: 5′-FAM-C TCCTTCTCCACAAGCGCCTTCGGT-BHQ1-3′. For internal control, rRNA control kit (Applied Biosystem, Foster City, CA) was used.

Immunoblots. Whole cell lysate was used. After heating for 5 min at 95 °C, 10 μg of total protein was subjected to electrophoresis. After transferring, the PVDF membrane was blocked and allowed to react with primary antibodies (Cell signaling) at 4 °C overnight. Target proteins were subsequently detected using horseradish peroxidase-conjugated IgG with an enhanced chemiluminescence plus detection system (ECL plus) and Typhoon Scanner (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England).

Electrophoretic mobility shift assay (EMSA). Electrophoretic mobility shift assay (EMSA) was performed with a kit (Panomics, Inc., Redwood city, CA) following the manufacturer's instructions. Briefly, nuclear extract (5 μg) was incubated with a biotin-labeled STAT3 probe. Protein–DNA complexes were then resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE). After transferring to Pall Biodyne B® membrane (Pall Corporation, East Hills, NY), proteins were immobilized in UV cross-linker. After blocking, avidin-HRP was applied and detected by enhanced chemiluminescence (ECL, Amersham).

RNA interference. STAT3-targeting siRNA and non-targeting control siRNA (Dharmcon, Inc., Lafayette, CO) were introduced into the cells as described previously [15,16]. Briefly, HBECs were cultured in V30-coated 60 mm dishes to 60–70% confluence. After washing with PBS, cells were treated with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) containing STAT3-siRNA or control-siRNA (final concentration 100 nM in Opti-MEM) for 6 h followed by re-feeding with LHC-9/RPMI medium. On the next day, cells were used to examine the silencing effect by immunoblot or exposed to CSE as designed.

LIVE/DEAD cytotoxicity/viability assay. Cell viability was evaluated by ethidium homodimer-1 dye exclusion using the LIVE/DEAD Kit, following the manufacturer's instruction (Invitrogen, Carlsbad, CA). Briefly, cells were detached by trypsinization and incubated in PBS containing Calcein AM (green) and ethidium homodimer-1 (EthD-1, red) at 37 °C for 15 min. Cells were then spun onto slides by cytospin followed by observation under fluorescence microscopy within 24 h. Nuclei stained by EthD-1, which appeared red, were counted as dead cells.

Clonogenic assay. Assay of clonogenic growth was performed with a modification of the previously reported methods [17]. Briefly, CSE or camptothecin-treated cells were harvested and plated in a 6-well tissue culture dishes at 2000 cells/well in LHC-9/RPMI. Cells were maintained in culture for 7–10 days with medium changes every 3–4 days. Cells were then fixed with PROTOCOL (Fisher Diagnostics. Middletown, VA) and photographed. All colonies presented on the dishes, defined as a cluster of 20 or more cells, were counted visually.

Statistical analysis. Statistical comparisons of multi-group data were analyzed by analysis of variance (ANOVA) followed by Tukey's (one-way) comparison using PRISM4 software. A value of $p < 0.05$ was considered significant.

Results

Effect of cigarette smoke on STAT3 activation

CSE exposure increased STAT3 phosphorylation (tyrosine 705) in a concentration- and time-dependent manner (Fig. 1A and B). CSE also stimulated DNA binding activity of STAT3 as evidenced by the electrophoresis mobility shift assay (Fig. 1C).

Role of IL-6 in cigarette smoke activation of STAT3 and effects on cell viability

Since IL-6 family of cytokines are the major stimulator of STAT3 signaling, the effect of CSE on the IL-6/STAT3 signaling pathway was investigated in the current study. CSE, at concentrations of 2.5%, 5%, and 10%, significantly

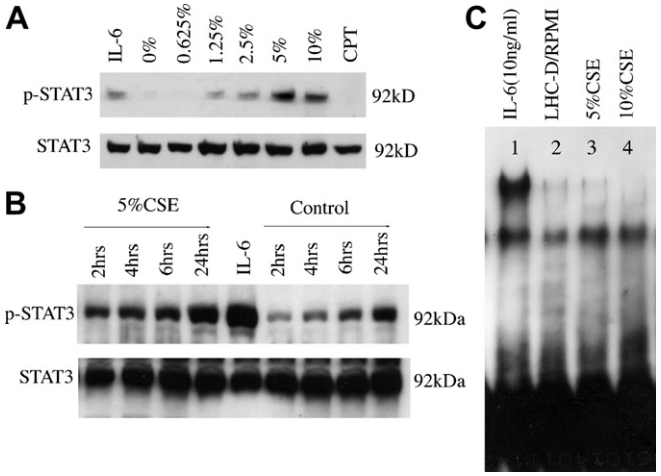

Fig. 1. STAT3 phosphorylation and DNA binding activity in response to CSE. (A) CSE concentration dependent effect. Cells were treated with varying concentrations of CSE, IL-6 (10 ng/ml) or camptothecin (CPT, 0.5 μM) for 6 h. Data presented is one representative from four separate experiments. (B) Time-dependent effect. Cells were treated with or without 5% CSE for 2, 4, 6 and 24 h, or with IL-6 (10 ng/ml) for 24 h. Data presented is one representative from three separate experiments. (C) CSE stimulates STAT3-DNA binding activity. Cells were treated with IL-6 (10 ng/ml) or 0%, 5%, and 10% CSE in LHC-D/RPMI for 30 min, and electrophoresis mobility shift assay (EMSA) was performed.

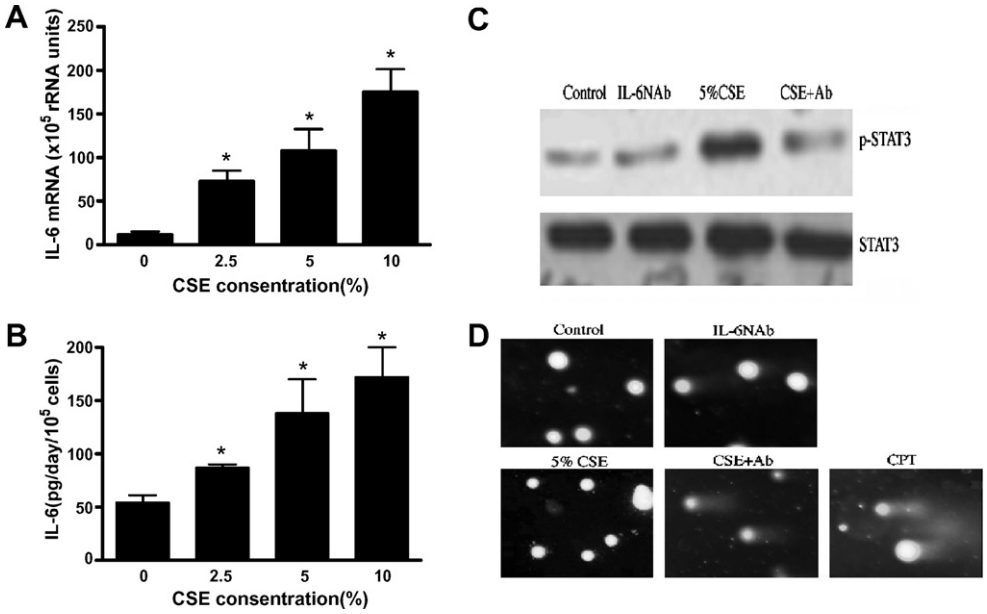

Fig. 2. IL-6 mediated STAT3 activation and cell survival in response to CSE exposure. Confluent HBECs were treated with varying concentrations of CSE for 6 h (mRNA) or 24 h (protein). (A) IL-6 protein quantification by ELISA. Horizontal axis: CSE concentration (%); vertical axis: IL-6 production expressed as pg/day/10^5 cells. Data presented are means ± SEM of three separate experiments. $^*p < 0.05$ compared to 0% CSE (LHC-D/RPMI). (B) IL-6 mRNA quantification by real-time RT-PCR. Horizontal axis: CSE concentration (%); vertical axis: IL-6 mRNA normalized versus rRNA. Data presented are means ± SEM of three separate experiments. $^*p < 0.05$ compared to 0% CSE. (C) Effect of anti-IL-6 neutralizing antibody on STAT3 activation by CSE. Data presented is one representative from two separate experiments. (D) Effect of anti-IL-6 neutralizing antibody on cell viability assessed by Comet assay. Data presented is one representative from two separate experiments.

increased IL-6 release (Fig. 2A, $p < 0.05$) and IL-6 mRNA expression (Fig. 2B, $p < 0.05$). Anti-IL-6 neutralizing antibody (IL-6NAb) partially blocked cigarette smoke-induced STAT3 phosphorylation (Fig. 2C). Furthermore, when HBECs were exposed to CSE in the presence of IL-6NAb, apoptotic cells characterized by a small DNA head with fan-like tail were observed by COMET assay, identical to the cells treated with camptothecin (Fig. 2D).

Role of STAT3 in modulating cell survival in response to CSE exposure

To confirm the role of STAT3 in modulating cell survival, STAT3 was depleted by siRNA. Twenty-four hours after transfection with STAT3-siRNA, STAT3 protein was significantly decreased while non-targeted p65 and β-actin levels were unchanged (Fig. 3A). In contrast, STAT3 levels were unchanged when the cells were transfected with control-siRNA (Fig. 3A). Suppression of the STAT3 by STAT3-siRNA remained effective for at least 72 h (Fig. 3A).

In response to CSE exposure, cells with STAT3 depletion started to detach at 4 h after exposure and most of the cells were detached from the culture dish after 16 h exposure. Severe DNA damage and apoptosis were observed by Comet assay (Fig. 3B) in the cells lack of STAT3 and exposed to CSE. LIVE/DEAD staining further demonstrated that over 50% of the cells were dead (red nuclei) in the cells lack of STAT3 and exposed to CSE (Fig. 3C, $p < 0.01$ compared to control-siRNA transfected cells). Similarly, a very high percentage (32.5 ± 1.8%) of the camptothecin-treated cells were stained red (Fig. 3C, $p < 0.01$ compared to control-siRNA transfected cells).

To further evaluate cell survival and proliferation, clonogenic assay was performed. Neither CSE exposure of the control-siRNA transfected cells nor the STAT3-siRNA transfection alone altered cellular ability to form colonies (97.8 ± 20.3 and 100.6 ± 30.1 colonies/dish, respectively). However, CSE exposure of STAT3-depleted cells resulted in a significant decrease in colony formation (1.4 ± 1.1 colonies/dish, $p < 0.001$ compared to control-siRNA transfection and CSE exposure). Similarly, colony formation was significantly decreased in camptothecin-treated cells (5.6 ± 3.1 colonies/dish, $p < 0.001$ compared to control-siRNA transfection and CSE exposure) (Fig. 4).

Discussion

We have previously reported that low concentrations of CSE-induced DNA damage without leading to apoptosis [3,4]. Here, we report that IL-6 and its downstream key signal transduction mediator, STAT3, are required for the survival of HBECs in response to cigarette smoke exposure. CSE stimulated IL-6 mRNA expression and protein production by HBECs in a concentration-dependent manner. CSE also induced STAT3 (tyrosine 705) phosphorylation in a time- and concentration-dependent manner, and increased DNA binding activity of STAT3. Anti-IL-6 neutralizing antibody (IL-6NAb) partially blocked CSE-in-

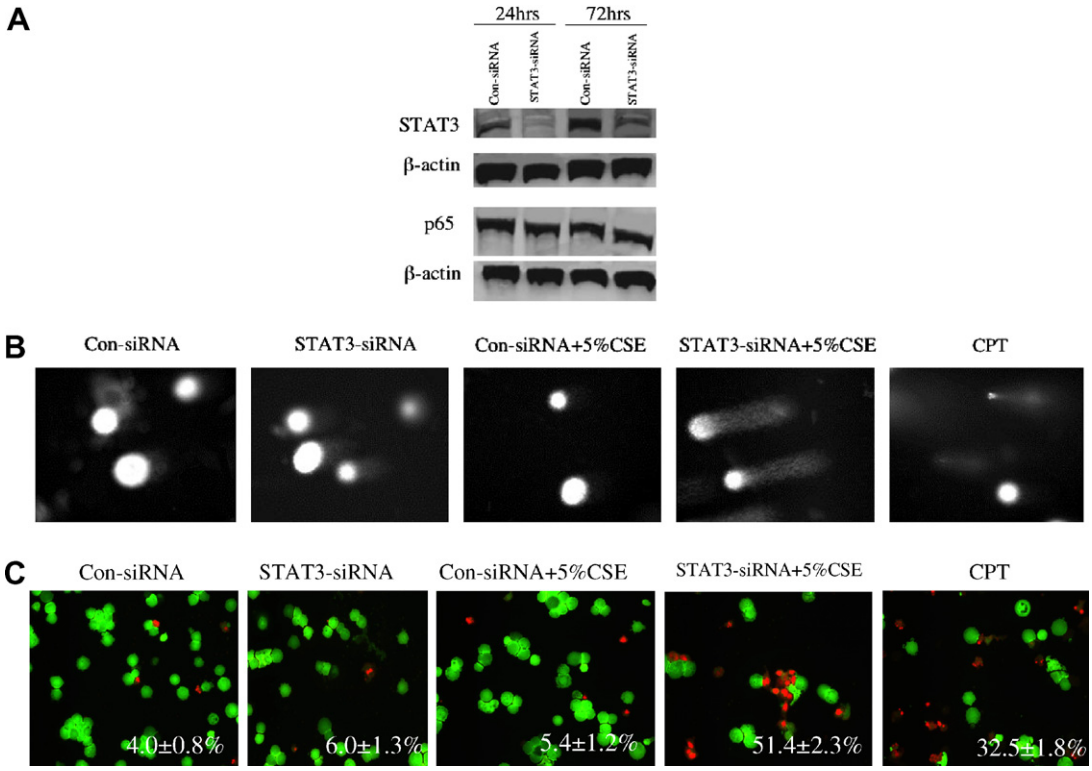

Fig. 3. STAT3 suppression and cell survival in response to CSE. (A) STAT3-siRNA effect on STAT3 synthesis. Cells were transfected with STAT3-siRNA or non-targeting control-siRNA. Cell lysates were obtained 24 and 72 h after transfection. Immunoblots to total STAT3, NF-kB (p65) and β-actin were performed. Data presented is one representative from five separate experiments. (B,C) Suppression of STAT3 by siRNA resulted in severe DNA damage (B) and cell death (C) in response to CSE exposure. Twenty-four hours after transfection with STAT3-siRNA or control-siRNA, cells were exposed to 5% CSE or CPT (0.25 μM) for 16 h. Comet assay (B) or LIVE/DEAD viability/cytotoxicity assay (C) were then performed. Green, live cells; red, dead cells. Data presented is one representative from three separate experiments.

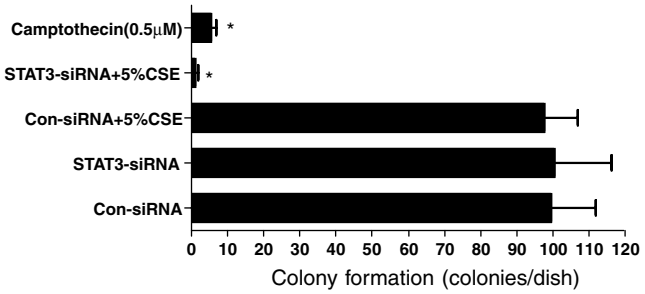

Fig. 4. Clonogenic assay. Cells were transfected with STAT3- or control-siRNA followed by exposure to 5% CSE for 16 h. Cells were then harvested and clonogenic assay was performed. $^{*}p < 0.001$ compared to Con-siRNA transfection. Data presented are means ± SEM of three separate experiments.

duced STAT3 phosphorylation, and importantly, in the presence of IL-6NAb, CSE-induced severe DNA fragmentation and apoptosis as evidenced by Comet assay. Furthermore, suppression of STAT3 by siRNA also resulted in HBEC death following exposure to CSE. These results indicate that HBEC survival following CSE exposure depends on STAT3 signaling, which is activated by CSE, at least partially, through IL-6 release.

Cigarette smoke stimulates the release of many pro-inflammatory cytokines including IL-6 *in vitro* and *in vivo* [18–20]. IL-6 is a member of cytokine family that signals through the JAK/STAT3 pathway to activate target genes [21]. Several studies indicate that IL-6 signaling through the STAT3 pathway plays an important role in cell both survival and inflammation [8,22]. For example, IL-6 and the IL-6 family member IL-11 protect A549 cells and endothelial cells from death in response to hydrogen peroxide exposure [23,24]. IL-6 has an anti-apoptotic effect in cancer cells and fibroblasts from idiopathic pulmonary fibrosis but not in normal lung fibroblasts [5,25,26]. It has also been reported that alveolar epithelial cells and endothelial cells from IL-6 deficient mice are sensitive while cells from the IL-6 over-expressing transgenic mouse are resistant to hydrogen peroxide-induced DNA fragmentation [27,28]. Here, we report that CSE stimulated IL-6 mRNA expression and protein production by HBECs and did not result in cell death. More importantly, in the presence of anti-IL-6 neutralizing antibody, CSE-induced severer DNA fragmentation and apoptosis. These results indicate that IL-6, acting through autocrine or paracrine manner, partially contributes to the survival of HBECs in response to cigarette smoke exposure.

STAT3 is activated by tyrosine/serine phosphorylation through either binding of the IL-6 family of

cytokines to their receptors or alternatively, by intracellular activation of Src kinases [6,29]. Tyrosine phosphorylation is required for STAT3 dimerization and subsequent nuclear translocation where STAT3 binds to DNA and modulates targeted gene expression [5,30]. A variety of toxic insults including hydrogen peroxide and hyperoxia trigger STAT3 activation [9,23]. In the current study, we report that CSE-induced STAT3 tyrosine phosphorylation in a time- and concentration-dependent manner. CSE also stimulated DNA binding activity of STAT3. Furthermore, CSE-induced STAT3 tyrosine phosphorylation was partially blocked by anti-IL-6 neutralizing antibody, indicating CSE activated STAT3 signaling partially through IL-6 release, which may act through autocrine or paracrine mechanisms.

Activated STAT3 is involved in diverse biological processes and the effect of STAT3 suppression or over-expression on cells response to injury resembles those described above for IL-6. For example, STAT3 is activated in acute lung injury induced by hydrogen peroxide [23]. Over-expression of STAT3 protects pulmonary epithelial cells from hyperoxic injury [9]. In contrast, suppression of constitutively activated STAT3 leads to apoptotic cell death in cancer cells [24,31]. In the current study, we report that suppression of STAT3 by short interfering RNA (siRNA) resulted in cell death when these HBECs were exposed to CSE. Furthermore, cells lacking STAT3 and exposed to CSE could not proliferate clonogenically in normal medium containing growth factors. In contrast, cells transfected with non-targeting control-siRNA and exposed to cigarette smoke were able to survive and proliferate clonogenically. These results suggest that STAT3 is required for the cell survival in response to CSE-induced DNA damage. The downstream mechanisms by which STAT3 mediates cell survival, however, remain to be defined.

Suppression of STAT3 by RNAi technology has been reported previously [32,33]. In the current study, we used a pool of siRNA targeting STAT3 mRNA, and demonstrated that STAT3-siRNA pool very efficiently and specifically suppressed STAT3 production in HBECs. Suppression of STAT3 by RNAi *per se* does not alter cell viability or the ability of cells to proliferate. Cell death only occurred when the cells lacking STAT3 were exposed to cigarette smoke, indicating STAT3 may not be a key transcription factor for cell survival under normal conditions, but that it is required for protection of the cells from toxic insult.

Taken together, CSE induces IL-6 release from HBECs, which, in turn, by an autocrine/paracrine mechanism activates STAT3. STAT3 activation, in turn, mediates cell survival in response to CSE-induced DNA damage. By damaging DNA and simultaneously inhibiting apoptosis, cigarette smoke may be able to cause somatic cell mutation. The present study helps to define the pathways involved in this process.

Acknowledgments

The author specifically thanks Dr. Stephen I. Rennard for his support in many aspects of performing experiments, analyzing data and writing the manuscript. The author also thanks Dr. Peter B. Bitterman for his helpful comments and suggestions on the performing experiments and data explanation. The author appreciates Ms. Lillian Richard for her excellent secretarial support for the manuscript.

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