Four-Week Continuous Nicotine Treatment of Immortalized Bronchial Epithelial Cells Does Not Contribute to Tumorigenesis

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Abstract

Cigarette smoking is one of the major risk factors for the development of lung cancer [1]. However, little is known about the effects of nicotine, a major constituent of cigarette smoke, on lung epithelial cells in the context of lung tumorigenesis.

In order to investigate whether nicotine elicits differential effects on mechanisms promoting or leading to carcinogenesis, human immortalized non-tumorigenic BEAS-2B and tumorigenic BZR bronchial epithelial cells were continuously exposed to low, medium, and high concentrations of nicotine (10, 100, and 1000 nM) for four weeks. Proliferation, apoptosis, and expression of metalloproteases were assessed weekly by real-time impedance measurements using the xCELLigence® platform, high-content imaging, and Luminex technology. Gene expression analysis was performed on nicotine-treated and untreated cells and collected weekly using microarrays together with a computational network approach. In addition, soft agar assays were performed at the end of the treatment period to examine anchorage independence.

Nicotine did not increase the proliferation of immortalized BEAS-2B or BZR cells as determined by cell counting. Real-time impedance data indicate a small but transient pro-proliferative nicotine effect on BEAS-2B cells at week 3. Nicotine treatment had no impact on staurosporine-induced apoptosis of BEAS-2B and BZR cells. Furthermore, nicotine treatment did not increase the levels and activity of metalloproteases. Systems toxicological analysis indicated a small but non-consistent impact when treating BEAS-2B cells with nicotine, while BZR cells seemed to be more responsive to nicotine than BEAS-2B cells. Finally, anchorage-independent growth was not observed in BEAS-2B or BZR cells when treated for four weeks with nicotine.

In conclusion, chronic nicotine treatment of immortalized non-tumorigenic BEAS-2B and tumorigenic BZR cells did not promote cell proliferation, suppress apoptosis, or initiate any mechanisms that favor tumorigenesis. Longer exposures might be necessary to elucidate the contribution of nicotine to cancer promotion and progression.

Material and Methods

Non-tumorigenic BEAS-2B cell stocks and tumorigenic BZR cell stocks were cultured in cell culture plates over four weeks and continuously treated with nicotine at three selected doses. Cells containing only medium or cells exposed to tumor necrosis factor alpha (TNF-α) were cultured separately and included as controls.

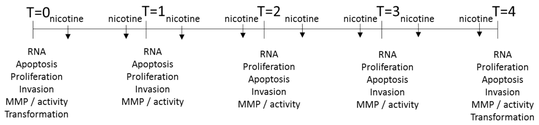

Figure 1. Experimental design of chronic nicotine exposure of tumorigenic and non-tumorigenic BEAS-2B cells. At the designated time points, cells and supernatants were collected for further analysis.

Cell Culture - Non-tumorigenic human bronchial epithelial BEAS-2B (CRL-9609) and tumorigenic BZR (CRL-9483) cells were obtained from the American Type Culture Collection (LGC Standards, Wesel, Germany). Cells were cultured in Bronchial Epithelial Growth Medium (BEGM™) (consisting of Bronchial Epithelial Basal Medium (BEBM™) supplemented with a SingleQuots™ kit) following the recommendations of the manufacturer (Lonza, Basel, Switzerland). Cells were grown in collagen I-coated 6-well plates or cell culture flasks (VWR, Dietikon, Switzerland) at 37°C in an atmosphere of 5% CO_2 until 75%-80% confluence was reached.

Chronic Nicotine Treatment - Three independent BEAS-2B and BZR stocks were cultured in cell culture flasks over four weeks and treated continuously with three different concentrations of nicotine (10, 100, and 1000 nM). Cells containing only medium or TNF-α at a final concentration of 10 ng/mL were included as negative and controls, respectively. At the designated time points (Figure 1), after reaching 75%-80% confluence (twice a week), media were removed, and cells were detached with trypsin/EDTA solution, counted with a CASY cell counter (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, and passaged while continuing each treatment.

Apoptosis - For the assessment of apoptosis, BEAS-2B and BZR cells were seeded in black collagen I-coated, clear-bottom 96-well tissue culture plates (BD, Allschwil, Switzerland) at a density of 12,000 cells/well. The next day, BEAS-2B cells were stimulated with 0.5 µM staurosporine, and BZR cells were stimulated with 5.0 µM staurosporine for four hours. After the stimulation, cells were stained with fluorescent caspase 3/7 substrate (Thermo Fisher Scientific Inc.). Following staining of the cells, fluorescence data were acquired with a Cellomics® ArrayScan™ VTI High Content Screening Reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) and vHCS view software (Thermo Fisher Scientific Inc.).

Proliferation - The effects of nicotine on BEAS-2B and BZR cell proliferation were evaluated using a CASY cell counter or a RTCA xCELLigence® instrument and electrical impedance plates (E-plates, Bucher Biotec, Basel, Switzerland). The E-plate is a cell culture plate covered with a gold microelectrode network that generates an impedance signal upon cell adhesion and proliferation. The bottom of the well was coated with collagen A (0.5 mg/mL) for one hour; 25,000 BEAS-2B or BZR cells were seeded on each insert of collagen-coated E-plates containing BEGM™. During cell growth and proliferation, changes in the cell index (impedance) were recorded in real time using RTCA software v2.0 (Bucher Biotec). The slope of the cell growth curve during a chosen time period was calculated by fitting the points to a straight line.

Cell Transformation - After four weeks, nicotine-treated and control BEAS-2B and BZR cells were processed for the CytoSelect™ 96-well Cell Transformation Assay (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Fluorescence was monitored with an excitation wavelength of 485 nm through a 520 nm bandpass filter in a 96-well fluorometer (Fluostar Omega Microplate reader, BMG Labtech, Ortenberg, Germany).

Total Protein Levels and Activity of Matrix Metalloproteases (MMP) in the Supernatant - Total MMP-1 and MMP-9 protein levels were determined in the cell culture supernatants using the Luminex® Performance Human MMP magnetic bead panel according to the manufacturer's instructions (Merck Millipore, Schaffhausen, Switzerland), using Luminex® technology (Luminex Corporation, Austin, TX, USA). Activity of MMPs was determined in the cell culture supernatants using the MMP Activity Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions .

RNA Extraction and Microarray Hybridization - Transcriptomics analysis was carried out on cellular material collected every week during the four-week treatment. A full randomization was applied across all collected samples briefly prior to sample processing to minimize batch effects. Cells (0.5×106) were lysed in 240 µL RLT lysis buffer containing 1% β-mercaptoethanol followed by RNA extraction using a Qiagen miRNeasy Mini Kit (Qiagen, Hilden, Germany). The quantity of purified RNA was determined using the NanoDrop™ ND1000 spectrophotometer (Thermo Fisher Scientific Inc.), while the quality of the RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Only RNA samples with a RIN>9.5 were processed further. Total RNA (50 ng) was reverse-transcribed to cDNA using the Nugen Ovation RNA Amplification System V2 (Nugen, Leek, The Netherland). The cDNA was then fragmented and labeled. Successful amplification was checked on the Fragment Analyzer (Advanced Analytical, Heidelberg, Germany), and the fragmentation step was confirmed using the Agilent 2100 Bioanalyzer. Hybridization cocktails were prepared for each sample according to the manufacturer's instructions. The final cocktails were hybridized to GeneChip® Human Genome U133 Plus 2.0 Arrays (Affymetrix) at 45°C for 16 hours while rotating at 60 rpm. Arrays were then washed and stained on a GeneChip® Fluidics Station FS450 DX (Affymetrix) using the protocol FS450_0004 and scanned using a GeneChip® Scanner 3000 7G (Affymetrix) to generate the CEL files containing the gene expression raw data. Differential gene expression comparing nicotine- or TNF-α-treated cells vs. DMSO controls was computed with the limma R package employing a false-discovery rate (FDR) cut-off value of 0.05. Network perturbation amplitude analysis was conducted using two-layer causal network models representative of different biological processes, as described previously [2-5].

Results

Effect of Chronic Nicotine Treatment on Cell Proliferation

- Cell counting on a weekly basis did not show a time or dose effect of nicotine on BEAS-2B or BZR total cell number. TNF-α treatment decreased BEAS-2B cell number but not BZR cell number in the first two weeks. Thereafter, cell numbers recovered (Figure 2A-C).
- Cell proliferation was assessed on a weekly basis using xCELLigence® impedance technology. During the first two weeks of nicotine stimulation, no effects were observed in BEAS-2B cells. After three weeks of treatment, a significant dose-dependent effect was detected, but at week four this was no longer seen, although large sample-to-sample variability may have masked a potential difference (Figure 2D). Pro-proliferative effects were not observed when BZR cells were stimulated with nicotine (Figure 2E). Treatment with TNF-α decreased proliferation of BEAS-2B and BZR cells in the first week, but this effect was not sustained over the following three weeks.

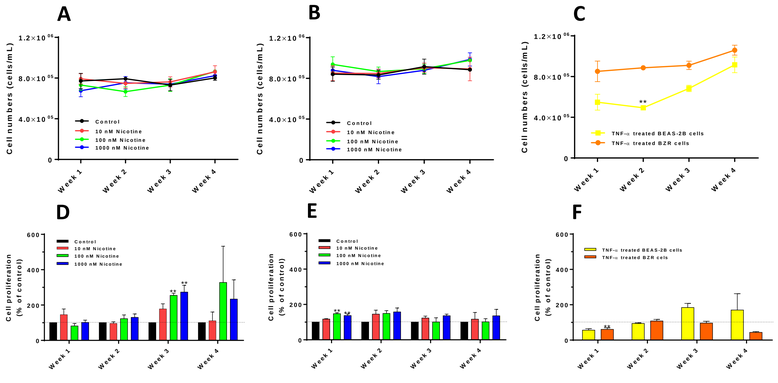

*Figure 2. Effect of chronic nicotine treatment on proliferation using standardized cell counting and impedance technology. Standardized cell counting using CASY technology was performed on a weekly basis in **A)** BEAS-2B and **B)** BZR cells. Cell proliferation was assessed on a weekly basis during the exponential growth phase using xCELLigence® impedance technology in **D)** BEAS-2B and **E)** BZR cells. Chronic TNF-α treatment, which promotes proliferation, was used as a positive control in the **C)** standardized cell counting and **F)** cell proliferation assay. Data shown are mean values ± SEM from three independent experiments. **P<0.01 vs control by Dunnett's multiple comparison test. Nic: nicotine; TNF-α: tumor necrosis factor alpha.*

Effect of Chronic Nicotine Treatment on Staurosporin-Induced Apoptosis

- Staurosporine, a potent PKC inhibitor, was used to induce caspase 3/7 activity. The EC50 values for a four-hour period treatment of BEAS-2B and BZR cells with staurosporine were 0.5 µM and 3.7 µM, respectively (data not shown).
- Chronic nicotine stimulation did not suppress staurosporine-induced caspase 3/7 activity in BEAS-2B or BZR cells. Although low and high doses of nicotine significantly decreased apoptosis in BEAS-2B cells in week two, this suppressive effect disappeared in the following weeks (Figure 3).

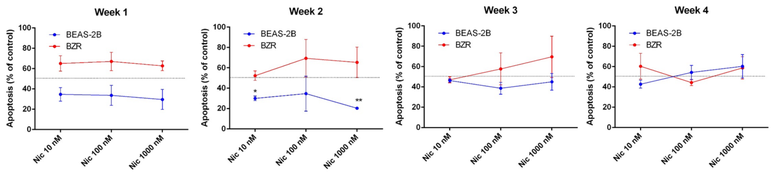

*Figure 3. Effect of chronic nicotine treatment on staurosporine-induced apoptosis. Caspase 3/7 activity was measured weekly as an indicator of apoptosis. Data shown are mean values ± SEM from three independent experiments. *P<0.05 vs control, **P<0.01 vs control by t-test. Nic: nicotine. The dotted line represents the EC50 values of staurosporine-induced caspase 3/7-mediated apoptosis.*

Effect of Chronic Nicotine Treatment on MMP levels

- Nicotine and nAChRs have been found to promote metastasis by upregulating the expression of various MMPs. Nicotine did not induce the release of MMPs in BEAS-2B or BZR cells.
- TNF-α, a known inducer of MMPs in lung epithelial cells, stimulated MMP-1 and MMP-9 secretion.

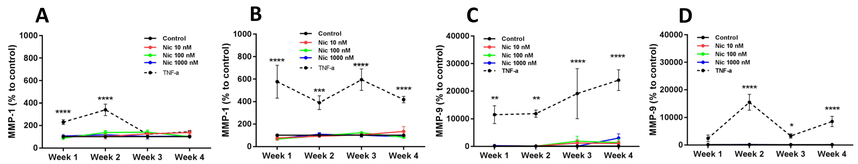

*Figure 4. MMP levels in the supernatant of nicotine- and TNF-α-treated BEAS-2B and BZR cells. Total MMP-1 and 9 levels (measured as % of control) over time in **A), C)** BEAS-2B and **B), D)** BZR cells treated with different concentrations of nicotine. Data are mean values ± SEM from three independent experiments. **P<0.01 vs control, ****P<0.01 vs control by Dunnett's multiple comparison test.*

Effect of Chronic Nicotine Treatment on Anchorage-Independent Growth

- Chronic nicotine treatment of BEAS-2B and BZR cells did not induce any colony forming capabilities. The human lung adenocarcinoma cell line A549 was used as a positive control.

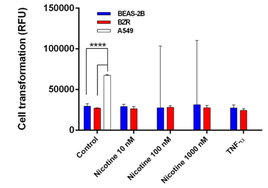

*Figure 5. Anchorage-independent growth of BEAS-2B, BZR, and A549 cells. BEAS-2B and BZR cells, treated with nicotine, TNF-α, or vehicle control for four weeks, were seeded in soft agar and cultured for 10 days. Cellular transformation was determined by measuring formation of cell colonies using a fluorescent dye. The lung adenocarcinoma cell line A549 was used as positive control for anchorage-independent growth. Data are median values ± range from three independent experiments. ****P<0.01 vs control by Dunnett's multiple comparison test.*

Effect of Chronic Nicotine Treatment on Gene Expression

- Nicotine treatment did not change the transcriptome in BEAS-2B or BZR cells significantly over time. Treatment with TNF-α decreased the numbers of differentially expressed genes in BEAS-2B cells significantly but increased them in BZR cells over time.

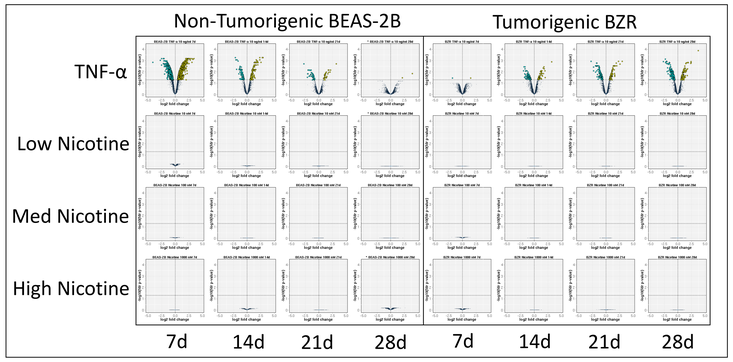

Figure 6. Gene expression changes in non-tumorigenic BEAS-2B and tumorigenic BZR cells exposed to three doses of nicotine (10, 100, and 1000 nM) or TNF-α (used as positive control) over a four-week period are shown. The gene expression change, calculated as the log2 fold change, is plotted on the x-axis, and the statistical significance, proportional to the negative log10-adjusted p-value, is plotted on the y-axis. Negative fold changes are shown in the volcano plots in cyan and positive fold changes in yellow. Changes below an FDR of 0.05 are shown as black dots. Low Nicotine: 10nM; Med Nicotine: 100nM; High Nicotine: 1000nM.

Conclusions

- Chronic nicotine treatment of immortalized non-tumorigenic BEAS-2B and tumorigenic BZR cells did not promote cell proliferation or survival, did not suppress apoptosis, and did not initiate any mechanisms that favor tumorigenesis.

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Competing Financial Interest

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