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Evidence for in Vivo Nicotine-Induced Alveolar Interstitial Fibroblast-to-Myofibroblast Transdifferentiation

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Introduction

There is strong epidemiologic and experimental evidence that fetal exposure to maternal smoking during gestation results in detrimental long-term effects on lung growth and function (1). For example, significant suppression of alveolarization, functional residual capacity, tidal flow volume, and increased predisposition to asthma have been demonstrated in the offspring of nicotine-exposed pregnancies (2–12). However, the lung cell-types affected, the underlying molecular mechanisms involved, and the effector molecules leading to these effects are not fully understood (1,13).

We have previously hypothesized that in utero nicotine exposure disrupts specific molecular paracrine communications between the alveolar epithelium and interstitium that are driven by epithelial Parathyroid Hormone-related Protein (PTHrP) and mesenchymal Peroxisome Proliferator-Activated Receptor (PPAR) γ , resulting in transdifferentiation of lung alveolar interstitial fibroblasts (AIFs)-to-myofibroblasts (MYFs), *i.e.*, the conversion of the AIFs to a phenotype that is not conducive to alveolar homeostasis, and is the cellular hallmark of chronic lung disease (14–17). Though we have previously provided evidence for this phenomenon in vitro, at present it is not known if the same phenomenon also takes place in vivo (14). Here we provide the first evidence of nicotine-induced in vivo AIF-to-MYF transdifferentiation, which may possibly explain the offspring pulmonary phenotype following in utero exposure to smoke during pregnancy.

Methods and Materials

Animals

Time-mated first-time pregnant Sprague Dawley rat dams weighing 200 – 250 g received either placebo (diluent, n = 12) or nicotine (1 mg/kg, n = 12) intraperitoneally in 100 μ l volumes once daily from embryonic day (e) 6 of gestation until they were killed either following cesarean delivery at term (e22) or following spontaneous delivery at postnatal day 1, 8, or 16. Control and nicotine-treated dams were pair-fed with free access to water, and were maintained in a 12H: 12H::light: dark cycle. Lungs from e22 fetuses and postnatal day 1, 8, and 16 pups were removed and processed for either fibroblast culture and later RT-PCR and Western analysis, on extracted mRNA and protein, respectively, or paraformaldehyde-fixed for histology and immunochemistry. All animal procedures were performed following National Institutes of Health guidelines for the care and use of laboratory animals, and

approved by the Los Angeles Biomedical Research Institute Animal Care and Use Committee.

Lung morphometry

An investigator unaware of the treatment groups performed lung morphometry. Fifty randomly selected non-overlapping fields from sections obtained from twelve blocks from each treatment group were included for the measurements. Each field was viewed at 200-fold magnification, scanned with a digital camera and projected onto a monitor. For each field, the numbers of air saccules were counted visually. An air saccule was defined as a lung structure bounded by septa, and having an opening through which it communicated with a common air space (the most distal airway that is discrete, i.e., has three walls) (18). Small structures occasionally seen opening into a saccule were considered part of the saccule, and not a separate independent structure. To distinguish between saccules and saccule ducts, gas-exchange structures were followed visually through a complete set of prints through a serial set of lung tissues. Secondary crests were identified as described previously (19).

Isolation of pulmonary fibroblasts

Neonatal rat lung fibroblasts were cultured with slight modifications of our previously described method (20). Briefly, the lungs were trimmed to remove major airways, and rinsed with calcium- and magnesium-free Hanks' balanced salt solution (HBSS). Pooled lung tissue from 3 to 5 pups was minced into 1 to 2-mm³ pieces and was suspended in pre-warmed (37°C) digestion buffer containing 2.5 ml of heat-inactivated chicken serum (2.5 ml), Hepes (1.25 ml of 500 mM, pH 7.4), collagenase I (12.5 mg, Sigma), Collagenase 1A (12.5 mg, Sigma) in Waymouth's medium (final volume 25 ml). The tissue was triturated 100 times with a 10 ml pipette, 100 times with a 5 ml pipette, and 100 times with a 9" Pasteur pipette. The tissue was further dissociated in a 37°C water bath using a Teflon™ stirring bar to disrupt the tissue mechanically. Once the tissue was dispersed into a unicellular suspension, the cells were pelleted at 500 × g for 10 min at room temperature in a 50 ml polystyrene centrifuge tube. The supernatant was decanted, and the pellet was resuspended in Minimal Essential Medium (MEM) containing 20% fetal bovine serum (FBS) to yield a mixed cell suspension of ca. 3 × 10⁸ cells, as determined by Coulter particle counter (Beckman-Coulter, Hayaleah, FL). The cell suspension was then added to culture flasks (75 cm²) for 30–60 min to allow for differential adherence of lung fibroblasts. These cells are greater than 95% pure fibroblasts based upon their morphologic appearance when viewed at the light microscopic level, and by immunohistochemical staining for vimentin.

RNA extraction and semi-quantitative and real time reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted using a standard protocol (21). RNA integrity was assessed from the visual appearance of the ethidium bromide-stained ribosomal bands following fractionation on a 1.2% (wt/vol) agarose-formaldehyde gel, and quantitated by absorbance at 260 nm. Semi-quantitative RT-PCR probes used included PTHrP receptor: 5'-ATGTGGATGTAGTTGCGCGTGCAGT-3' and 3'-GGGAAGCCCAGGAAAGATAAGGCAT-5' (445 bp); PPAR γ : 5'-CCCTCATGGCAATTGAATGTCGTG and 3'-TCGCAGGCTCTTTAGAACTCCCT-5' (757 bp); ADRP: 5'-GTTGCAGTTGATCCACAACCG-3' and 3'-TGGTAGACAGGGATCCCAGTC-5' (666 bp); and α -smooth muscle actin (α -SMA): 5'-CGCAAATATTCTGTCTGGATCG-3' and 3'-TCACAGTTGTGTGCTAGAGACA-5' (167 bp). cDNA was synthesized from 1 μ g of total RNA by RT using 100 units of SuperScript reverse transcriptase II (Invitrogen, Carlsbad, CA) and random primers (Invitrogen) in a 20- μ l reaction mixture containing 1× SuperScript buffer (Invitrogen), 1 mM dNTP mix, 10 mM

dithiothreitol, and 40 units of RNase inhibitor. Total RNA and random primers were incubated at 65°C for 5 min, followed by 42°C for 50 min. A denaturing enzyme at 70°C for 15 min terminated the reaction. For PCR amplification, 1 µl of cDNA was added to 25 µl of a reaction mix containing 0.2 µM of each primer, 0.2 mM dNTP mix, 0.5 units of AccuPrime *Taq* DNA polymerase (Invitrogen), and 1× reaction buffer. PCR was performed in a RoboCycler (Stratagene, La Jolla, CA). The PCR products were visualized on 2% agarose gels by ethidium bromide staining, and gels were photographed under UV lights. Band densities were quantified using the Eagle Eye II System (Stratagene). The expression of different mRNAs was normalized to 18s mRNA levels.

For Real Time PCR, 2 µg of total RNA was reverse-transcribed into single-stranded cDNA using the TaqMan Gold RT-PCR Kit at 50°C for 30 min in a total volume of 20 µl. The PCR reaction mix consisted of 4 µl of 10-fold diluted cDNA for all genes except 18s, for which the dilution was 1:5000, PCR Gold DNA polymerase reagent mix, and optimized for forward and reverse gene specific primers (900 nMs each) with a gene-specific probe (250 nM, FAM dye label). All real time PCR primer and probe sets were purchased as pre-designed or, if needed, custom designed TaqMan Gene Expression Assays (Applied Biosystems) were performed. Real-Time PCR reactions were run in triplicate on 96 well plates using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Reactions proceeded by activation of DNA polymerase at 95°C for 10 min, followed by 38 PCR cycles of denaturing at 95°C for 15 sec, and annealing/extension at 60°C for 1min. Normalization control was the 18s ribosomal RNA TaqMan Gene Expression Assay. Data were analyzed to select a threshold level of fluorescence that was in the linear phase of the PCR product accumulation [the threshold cycle (C_T) for that reaction]. The C_T value for 18s was subtracted from the C_T value of the gene to obtain a delta C_T (ΔC_T) value. The relative fold-change for each gene was calculated using the $\Delta\Delta C_T$ method. Results were expressed as the mean $\pm$ SE, and considered statistically significant at $p < 0.05$. RT-PCR probes used included-Rat β -Catenin: F 5'-CCGTTTCGCCTTCATTATGGA-3' and R 5'-GGGCAAGGTTTCGGATCAAT-3'; Rat LEF-1: F 5'-GAGCACGAACAGAGAAAGGAACA-3' and R 5'-TTGATAGCTGCGCTCTCCTTTA-3'; Rat Fibronectin: F 5'-AGCACACCCGTTTTTCATCCA-3' and R 5'-TTTCACGTCGGTCACTTCCA-3'; Rat α -SMA: F 5'-TATCCGATAGAACACGGCATCA-3' and R 5'-CACGCGAAGCTCGTTATAGAAG-3'; and Rat 18s: F 5'-GGACAGGATTGACAGATTGATAGC-3' and R 5'-TGGTTATCGGAATTAACCAGACAA-3'.

Protein Determination and Western Blot Analysis

Protein determination was made using the Bradford dye-binding method (22). Western blotting was performed with modifications of methods described previously (14–16). Briefly, cells were lysed using an extraction buffer [10 mM Tris (hydroxymethyl) aminomethane (Tris, pH 7.5), 0.25 M sucrose, 1 mM EDTA, 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 10 µg/ml each of pepstatin A, aprotinin, and leupeptin] and centrifuged at $140 \times g$ for 10 min (4°C). Equal amounts of the protein (25 µg) from the supernatant were dissolved in electrophoresis sample buffer, and were subjected to SDS-PAGE (4–12% gradient), followed by electrophoretic transfer to a nitrocellulose membrane. Nonspecific binding of antibody was blocked by washing with Tris-buffered saline (TBS) containing 5% milk for 1 h. The blot was then subjected to two brief washes with TBS plus 0.5% Tween 20, incubated in TBS plus 0.1% Tween 20, and the specific primary antibodies (PTHrP-Receptor 1:100, MILLIPORE, Temecula, CA; PPAR γ 1:2,000, Alexis Biochemicals, San Diego, CA; β -catenin 1:1000, Cell Signaling, Danvers, MA; LEF-1 1:500, Santa Cruz, CA; Fibronectin 1:300, Santa Cruz, CA; α SMA 1:10,000, Sigma, St. Louis, MO; ADRP 1:3,000, a kind gift from Dr. Constantine Londos, National Institute of

Diabetes and Digestive and Kidney Diseases) overnight at 4°C. Blots were then washed in TBS plus 0.1% Tween 20, and then incubated for 1 h in secondary antibody, washed, and developed with a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL) following the manufacturer's protocol. The densities of the specific protein bands were quantified using a scanning densitometer (Eagle Eye II still video system, Stratagene). The blots were subsequently stripped and reprobed with anti-GAPDH (1:5,000; Chemicon, Temecula, CA) antibody to confirm equal loading of samples.

Immunohistology

The lungs were inflated by intratracheal instillation of 4% paraformaldehyde at a pressure of 20 cm of water. After overnight fixation, the tissue was processed through standard paraffin embedding. Five-micron tissue sections were stained with hematoxylin-eosin. Dual staining was performed for lipid droplets (oil red O) and α SMA was performed as follows: The slides mounted with lung tissue sections were fixed in 10% formalin, and then washed in phosphate buffered saline (PBS), blocked with 3% normal goat serum (Jackson ImmunoResearch Lab, West Grove, PA,) in PBS for 30 min at room temperature to block non-specific binding, and then incubated in primary antibody (α SMA 1:100, Sigma, St. Louis, MO) overnight at 4°C. Secondary goat-antimouse IgG_{2a} conjugated Fluorescein (FITC) was used at 1:100 dilution for 30 min. The slides were then washed $\times$ 3 with PBS, with double distilled water $\times$ 2, and were then incubated with oil red O (Sigma, St. Louis, MO) for 15 to 30 minutes. Slides were rinsed $\times$ 3 for 5 minutes and then mounted and coverslipped with Vestashield mounting medium, with DAPI (Vector Laboratories, Inc, CA) visualization under fluorescence microscope.

Statistics

Analysis of variance for multiple comparisons with Newman-Keuls post hoc test and Student's *t*-test, as indicated, were used to analyze the experimental data. $P < 0.05$ was considered to indicate statistically significant differences in the expression of lipogenic and myogenic markers among the control, nicotine, and nicotine plus treatment groups.

Results

The body weights of rat dams at the beginning and at delivery were similar in control and nicotine-treated groups (235 ± 16 vs. 227 ± 20 g; $p > 0.05$; $n = 12$). Similarly, there were no group differences in body weight between the control and nicotine treated pups on postnatal day (PND) 1 (6.5 ± 0.9 vs. 6.2 ± 0.9 g; $p > 0.05$; $n = 12$), 8 (16.8 ± 1.5 vs. 16.4 ± 1.4 g; $p > 0.05$; $n = 6$), and 16 (26.3 ± 2.1 vs. 27.5 ± 2.3 g. $p > 0.05$; $n = 6$). Initially, using H & E staining on lung sections from pups sacrificed on PND1, we confirmed the previously described lung morphometric changes following in utero nicotine exposure (23,24). In agreement with the observations of others, we found that saccules in the lungs of in utero nicotine-exposed animals were larger, but fewer in number, per unit area [29.4 ± 2.1 vs. 22.5 ± 1.7 saccules/measured grid (grid size = 0.099 mm squared) in control vs. nicotine-exposed lungs; $p < 0.01$; $n = 6$] and contained fewer secondary septal crests (Fig. 1). Then, utilizing semi-quantitative RT-PCR and Western hybridization, we determined the markers of AIF-to-MYF transdifferentiation on the lung tissue from control and nicotine exposed pups. Similar to our in vitro data on cultured WI38 cells (14), there was clear evidence of nicotine-induced in vivo AIF-to-MYF transdifferentiation, based on significant decreases in PTHrP receptor, PPAR γ , and ADRP expression, and significant increases in α SMA, at both the mRNA (Fig. 2A) and protein levels (Fig. 2B). This was further supported by the Western blot analysis for PTHrP receptor, PPAR γ , and fibronectin on protein lysates from cultured AIFs, clearly suggesting AIF-to-MYF transdifferentiation by in vivo nicotine exposed fibroblasts (Fig. 3).

Nicotine-induced in vivo AIF-to-MYF transdifferentiation was further corroborated by immunofluorescence for the markers of AIF-to-MYF transdifferentiation on lung sections from the control and nicotine-exposed pups (Fig. 4). To follow AIF-to-MYF transdifferentiation temporally, lung sections from control and in utero nicotine-exposed pups were examined at e18, e22, and at postnatal days 8 and 16 by immunofluorescence staining for the key markers of AIFs [lipid staining by oil red O (ORO)] and MYFs (α SMA). With perinatal nicotine exposure, compared to controls, there was a progressive decrease in AIFs that stained solely for ORO. This was accompanied by a progressive increase in AIFs that stained positively for both as a lipid-containing, fibroblast (~lipofibroblast) and as a MYF, *i.e.*, transdifferentiating AIFs (ORO- and α SMA-positive cell, pink arrows) up to PND8 (6% at e18, 12% at e22, and 23% at PND8), which was followed by a decrease in the percentage of cells staining for both ORO and α SMA (7% at PND16). There was also an accompanying increase in the percentage of fibroblasts staining solely as MYFs (α SMA positive, white arrows), and a decrease in the percentage of cells staining solely as lipofibroblasts (ORO positive, yellow arrows), suggesting that nicotine drives lipid-containing fibroblasts to MYFs rather than affecting MYF proliferation preferentially.

The PPAR γ and the Wnt signaling pathways are central in determining the lipofibroblastic phenotype vs. the myofibroblastic phenotype of AIFs (25,26), respectively, and since we have recently demonstrated activation of Wnt signaling in nicotine-induced AIF-to-MYF transdifferentiation (25), we next examined if there was evidence of Wnt activation in nicotine-exposed cultured lung fibroblasts. Indeed, an increase in α -SMA and fibronectin expression (by Real Time-PCR) was accompanied by increases in total β -catenin and LEF-1 expression, demonstrated by both Real-Time PCR and Western blot analysis, suggesting activation of canonical Wnt signaling by the in vivo nicotine-exposed AIFs (Fig. 5).

Discussion

Until now, there has been no specific intervention to prevent nicotine-induced morbidity in the developing fetus. This is mainly because of the failure to eradicate maternal smoking during pregnancy, compounded by a lack of understanding of the molecular mechanisms involved in nicotine-induced lung morbidity (1,13). We have previously demonstrated that in vitro nicotine exposure causes AIF-to-MYF transdifferentiation (14). Alveolar interstitial fibroblast-to-MYF transdifferentiation results in failed alveolarization in the developing lung, which leads to an arrest of pulmonary growth and development, the hallmarks of in utero nicotine-induced lung damage (26). More importantly, we have previously shown that by targeting specific AIF molecular intermediates, we can not only block, but also reverse nicotine-induced AIF-to-MYF transdifferentiation, at least under in vitro conditions (14–16). Based on the data presented here, we now extend the documentation of nicotine-induced AIF-to-MYF transdifferentiation to in vivo conditions, providing a specific mechanism that can be targeted to prevent detrimental pulmonary effects of in utero nicotine exposure.

The effects of in utero nicotine exposure on the developing lung are extremely complex. On the one hand, there is evidence of enhanced functional pulmonary maturity at birth, possibly contributing to a decrease in the incidence of Respiratory Distress Syndrome (27–30). In contrast, clearly, reduction in both prenatal and postnatal lung growth occurs in children of women who smoke (2–12). Mechanistically, our data complement the previous observations in a rhesus monkey model of in utero nicotine exposure from day 26 to day 134 of gestation (term = 165 days), which results in altered fetal lung development, characterized by smaller lung volume, decreased alveolar surface area, increase in the size of gas exchange units, altered lung mechanics, and increased collagen deposition and airway wall dimensions in the fetal lung. Now we provide data that following in utero nicotine exposure, there is a shift in the lung mesenchyme phenotype from a lipogenic to a myogenic phenotype. Based on our

previous observations that MYFs are unable to sustain alveolarization (17), this would not only explain the increased parenchymal collagen expression reported by Sekhon et al, but would also provide a molecular mechanism for the altered postnatal pulmonary mechanics observed by them (31,32).

The hypothesis that nicotine disrupts the specific homeostatic epithelial-mesenchymal pulmonary communications, inhibiting PTHrP/PPAR γ signaling and stimulating Wnt signaling, culminating in AIF-to-MYF transdifferentiation, could potentially explain virtually all of the known long-term pulmonary effects following in utero nicotine exposure, including the advanced pulmonary maturity at birth and later poor long-term outcome (1).

Our data suggest that the likely cellular/molecular mechanism involved in in utero nicotine exposure-induced fetal lung damage includes up-regulation of the Wnt signaling pathway. This is accompanied by decreased PTHrP/PPAR γ signaling, which would otherwise normally induce a lipogenic phenotype in the AIF, characterized by the expression of such lipogenic features as triglyceride accumulation, and expression of PPAR γ and ADRP. Importantly, we have also shown that lipofibroblasts sustain alveolar type II cell growth and differentiation, whereas MYFs do not, providing a specific cellular mechanism for the failed alveolarization observed in association with neonatal smoke exposure (17).

It is important to point out that although the dose of nicotine used to study its systemic effects in various in vivo studies has ranged from 0.25 to 6 mg/kg, the range of nicotine intake in habitual smokers in one study was 0.16 to 1.8 mg/kg body weight (33,34). Therefore, the dose of nicotine used in this study is approximately equivalent to that used by moderately heavy human smoker ~ 1 mg/kg/body weight. In addition, the pulmonary morphologic and structural changes following in utero nicotine exposure observed by us are similar to the pulmonary changes described by others following in utero smoke exposure, further validating the clinical significance of our findings (35,36).

In summary, our data, for the first-time, provide evidence for in utero nicotine-induced AIF-to-MYF transdifferentiation. Coupled with this, our previous data, demonstrating an up-regulation of Wnt signaling and down-regulation of PTHrP/PPAR γ signaling in this process, provides an integrated mechanism for in utero nicotine-induced lung damage, and how it could permanently alter the “developmental program” of the developing lung by disrupting critically important epithelial-mesenchymal interactions. More importantly, specific interventions that augment the pulmonary mesenchymal lipogenic pathway could, at least partially, ameliorate such very complex nicotine-induced in utero lung injury.

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Figure 1. Hematoxylin and Eosin staining of the lungs of PND1 pups show that the lungs of the in utero nicotine-exposed animals have larger, but fewer saccules per unit area [29.4 ± 2.1 vs. 22.5 ± 1.7 saccules/measured grid (grid size=0.099 mm squared; $p<0.01$ vs. control; $n=6$, Magnification 20 $\times$).

Figure 2.

Time-mated pregnant SD rat dams were treated with either a placebo or nicotine (1 mg/kg) once daily from e6 of gestation until spontaneous delivery at e22. Alveolar interstitial fibroblast and myofibroblast markers were determined in whole lung tissue by semi-quantitative RT-PCR (**Fig. 2A**) and Western analysis (**Fig. 2B**). There were significant decreases in PTHrP receptor, PPAR γ , and ADRP expression and significant increases in α SMA mRNA and protein expression (*= $p < 0.05$ vs. controls; $n=4$).

Figure 3.

Western blot analysis for PTHrP receptor, PPAR γ , and fibronectin on protein lysates from cultured alveolar interstitial fibroblasts isolated from PND1 lungs obtained from placebo and nicotine (1 mg/kg administered to dams once daily from e6 of gestation until spontaneous delivery at term) treated pups showed significant decreases in PTHrP receptor and PPAR γ , and significant increase in fibronectin levels (*=p<0.05 vs. controls; n=3).

Figure 4.

Lung sections from control and in utero nicotine exposed pups were examined at e18, e22 and postnatal day (PND) 8 and 16 by immunofluorescence for the key markers of alveolar interstitial fibroblast (AIF) [lipid staining by oil red O (ORO)] and myofibroblast (MYF) (α SMA). With perinatal nicotine exposure, compared to controls, there is a progressive decrease in AIFs that stained solely for ORO. This was accompanied by a progressive increase in AIFs that stain positively for both a lipofibroblast and a MYF, *i.e.*, a transdifferentiating AIFs (ORO and α SMA positive cell, pink arrows) up to PND8 (6% at e18, 12% at e22, and 23% at PND8), which was followed by a decrease in the percentage of cells staining for both ORO and α SMA (7% at PND16). This was also accompanied by an increase in the percentage of AIF cells staining solely for a MYF (α SMA positive, white arrows) and a decrease in percentage of cells staining solely for a lipofibroblast (ORO positive, yellow arrows), suggesting that nicotine drives lipofibroblasts to MYFs rather than affecting MYF proliferation preferentially (representative lung sections are shown; red fluorescence represents ORO, green fluorescence α SMA, and blue fluorescence DAPI staining; $n = 6$; $*p < 0.05$; magnification $\times 10$).

Figure 5.

RNA and protein isolated from alveolar interstitial fibroblasts cultured at PND1 from placebo or nicotine treated pups demonstrated increases in α -SMA and fibronectin expression (by Real Time-PCR), which was accompanied by increases in total β -catenin and LEF-1 expression, demonstrated by both Real Time PCR and Western blot analysis (*=p<0.05 vs. controls; n=3).