FETAL RAT PULMONARY EPITHELIAL-MESENCHYMAL CELL IMPAIRMENT UPON SMOKE COMPONENT EXPOSURE: α7 NICOTINIC ACETYLCHOLINE RECEPTORS IN THE DEVELOPING PULMONARY SURFACTANT SYSTEM

BY

CRAIG HILLIER

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MASTER OF SCIENCE

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ABSTRACT

Increased prevalence of pediatric smoking-associated lung disorders occur in infants of mothers who smoke. Epithelial-mesenchymal (E/M) interactions are well-established mediators of normal lung development and injury/repair signaling pathways. Cooperative interactions such as these are required for prenatal lung growth, cellular replication, and differentiation. Disruption of one or both of these cell types during lung development may lead to lung damage, decreasing lung function and increasing incidences of respiratory illnesses. Current studies have identified a negative relationship between maternal cigarette smoking and fetal pulmonary function, suggesting an effect of smoke constituents or metabolites on E/M interactions in the fetal lung. Nicotine, the major component in tobacco smoke freely crosses the placental barrier and directly interacts with nicotinic acetylcholine receptors (nAChRs) found within the fetal lung. The purpose of this study was to analyze the effects of the major bioactive component of cigarette smoke, nicotine and its major long-acting metabolite cotinine on fetal rat pulmonary E/M cells. Upon nicotine stimulation nAChRs may lead to alterations in lung development affecting lung function. The most abundant nAChR found within the fetal lung is the α7-nicotinic receptor (α7nAChR). Therefore, we also tested the hypothesis that functional non-neuronal α7-nicotinic receptors are present in fetal lung interstitial fibroblasts and alveolar epithelial type II pneumocytes. α7nAChRs may function, upon nicotine exposure to alter proliferation & DNA damage in fibroblasts and alveolar pneumocytes altering the complex staging of lung development. Our system of study uses primary cultured fetal rat alveolar type II cells and adjacent interstitial fibroblasts. Western blotting and immunocytochemical fluorescent confocal microscopy were used to

examine protein expression and spatial distribution of nicotinic receptors. We have shown that α7nAChR expression does exist in fetal lung fibroblasts and alveolar pneumocytes. These receptors are apparent and functional as determined using Fura-2 fluorescent intracellular calcium imagery. DNA damage was determined using the COMET assay. Our results indicate that exposure of E/M cells to these compounds result in structural impairments and increasing DNA damage in the fetal type II alveolar epithelial component of the alveolus. The mesenchymal or interstitial fibroblast components appear to be unaffected at the cellular DNA level. These and other data suggest that functional α7nAChRs do exist and may be important in nicotinic signaling in pulmonary tissues of the fetus leading to functional changes in cells of developing lung. Additionally, our results suggest that cigarette smoke may disrupt the balance of E/M cells which leads to improper lung development. This fetal disruption of E/M cells during lung development may be important in the pathogenesis of impaired pulmonary function in children who were exposed to cigarette smoke *in utero*.

ABBREVIATIONS

IF – interstitial fibroblast

CT - connective tissue

ER – endoplasmic reticulum

ECM – extracellular matrix

BM – basement membrane

FN – fibronectin

NLIC – non-lipid interstitial cell

LIC – lipid interstitial cell

CIC – contractile interstitial cell

LB – lamellar bodies

PS – pulmonary surfactant

PC – phosphatidylcholine

DPPC – dipalmitoylphosphatidylcholine

PG - phosphatidylglycerol

PE - phosphatidylethanolamine

PI - phosphatidylinositol

SMC – smooth muscle cells

TG – triglycerides

SP – surfactant protein

FGF – fibroblast growth factor

PDGF – platelet derived growth factor

VEGF – vascular endothelial growth factor

FPF – fibroblast-pneumocyte factor

KGF – keratinocyte growth factor

PTH – parathyroid hormone

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INTRODUCTION

RESPIRATORY SYSTEM

The lung tissue surface area in the human has been estimated at 120 m^2, representing the largest body surface area exposed to the environment (Gehr et al., 1978). This respiratory tissue system consists of the trachea, conducting airways and a gas exchange portion of the lung including: bronchi, bronchioles, terminal and respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli (Burri et al., 1997). Since the primary focus of our studies involve the respiratory unit of the lung at the cellular level, only epithelial-mesenchymal cells associated with the alveolar region will be considered in further detail.

2.1 GAS-EXCHANGE UNIT OF THE LUNG – THE ALVEOLUS

The lung is a complex organ composed of more than 40 specialized cell types, all of which contribute to its function of delivering oxygen and removing CO_2 from the bloodstream (Sorokin et al., 1970). The transport of oxygen into the blood occurs at the level of the alveolus with its surface area suggested to cover an area of 70 – 80 m^2 (Crapo et al., 1982). Alveoli are specialized small air-filled sacs considered to be structural gas-exchange units of the lung. These respiratory units are polyhedral, thin walled sacs which open on one side into an alveolar sac or alveolar duct. Alveoli are approximately 200 um in diameter which exchange oxygen across their walls into dense capillary networks forming a blood-air barrier. Alveolar walls are lined by two distinct cell types characterized by morphology and function known as Type I alveolar epithelial cells and

Type II alveolar epithelial cells or pneumocytes. Fibroblasts are located within the connective tissue compartment of the pulmonary interstitium adjacent to alveoli, being intimately linked to the development of these Type II pneumocytes. Alveolar development and structural integrity of the lung is required for proper lung function. These characteristics can be considered a fine balance of cooperative efforts between epithelial-mesenchymal cell types. In our studies these complex interactions involve lung alveolar Type II cells in a close relationship with interstitial fibroblasts (IFs).

A) PULMONARY ALVEOLAR INTERSTITIUM

According to Weibel & Crystal (1991) the pulmonary interstitium is a connective tissue (CT) 'continuum' that encompasses the entire lung. CT fibers serve as a mechanical scaffold on which alveoli along with capillaries are mounted to ensure favorable gas-exchange conditions within the parenchyma. In the alveolar walls, the interstitial space contains a rich cell population, chiefly consisting of mesenchymal cells including myofibroblasts, undifferentiated fibroblast-like cells and central to our studies interstitial fibroblasts or IFs.

a) Pulmonary Interstitial Fibroblasts

Estimated at ~600 um^3 fibroblasts are one of the largest and most prevalent of all cell types found within the lungs connective tissue compartment (Weibel et al., 1981; 1982; 1984; 1991). This accounts for 52-62% of the interstitial population in the pulmonary parenchyma (Pinkerton et al., 1982). The fibroblast is a spindle-shaped cell containing long extensions and a large cigar shaped nucleus with one or more nucleoli (Ross et al.,

1968; Kuhn et al., 1978). A fibroblast engaged in fibrogenesis and/or simple fiber maintenance contains well-developed endoplasmic reticulum (ER) and a large Golgi complex which is consistent with its role in synthesizing and secreting important connective tissue matrix constituents including collagens, proteoglycans and fibronectin (Bradley et al., 1980). These mesenchyme derived cells are thought to maintain the integrity of lung tissue in the alveolar interstitium through deposition, maintenance, degradation and remodeling of the constituents of the CT extracellular matrix (ECM) (Weibel et al., 1991; McGowan et al, 1997). Proliferation and migration of IFs can be induced via interaction with phagocytic and immune cells during lung injury. Pulmonary IF activation plays an important component in the formation of fibrotic tissue in response to injurious agents and during inflammatory interstitial lung diseases (McGowan et al., 1997).

b) Pulmonary Fibroblast Heterogeneity (Maksvytis et al., 1984)

Three subpopulations of IFs have been proposed based on ultra-structural characteristics (Maksvytis et al., 1981; Brody et al., 1983; Kaplan et al., 1985). The first two populations of IFs have been characterized based on the presence or absence of lipid droplets within their cytoplasm referred to as lipid interstitial cells (LICs) and non-lipid interstitial cells (NLICs) respectively (Vaccaro et al., 1978; Brody et al., 1983).

i) Non-lipid Interstitial Cells (NLIC)

NLICs contain no lipid granules, but are rich in organelles for protein synthesis and secretion. They have been implicated in the formation of both the lung interstitial fiber system and the ECM.

ii) Lipid Interstitial Cells (LIC)

LICs accumulate lipid inclusions during late prenatal and early postnatal life. They are most frequently observed on the base of ridges within the rat lung. As the lung matures, LICs decrease in number and becoming absent from the adult lung (Brody et al., 1983). LICs contain microfilaments therefore it has been suggested that a third IF subtype the contractile interstitial cell (CIC) could be derived from the LIC population (Kapanci et al., 1974; Brody et al., 1983). Recently, it has been suggested that pulmonary fibroblast transdifferentiation does occur from lipofibroblasts (LIC) to myofibroblasts (CIC) via epithelial-mesenchymal signaling pathways. This differentiation may result in altered pulmonary development and function (Rehan et al., 2005).

iii) Contractile Interstitial Cells (CIC)

The last fibroblast population referred to as CICs or myofibroblasts exhibit features of both fibroblasts and smooth muscle cells (SMC) (Kapanci et al., 1974, 1976, 1979; Gabbiani et al., 1976, 1981; Alder et al., 1986). Kapanci proves that myofibroblasts contain bundles of thin microfilaments including actin and myosin which enables this contractile interstitial cell (CIC) to contract upon appropriate stimulation.

c) Alveolar Interstitial Fibroblast Matrix

Pulmonary ECM and associated basement membranes (BM) are 3-dimensional architectural frameworks which provide support and structure to the alveoli. IF extracellular matrix (ECM) proteins in the alveolar wall include a number of connective

tissue components including: collagens, elastic fibers or elastin, fibronectin, and proteoglycans (Hance & Crystal et al., 1975; Baum et al., 1977; Bradley et al., 1980; Ruoslahti et al., 1981; McGowan et al, 1997). Importantly, excess depositions of CT matrix molecules are linked to interstitial fibrotic lung disorders (Crystal et al., 1978; 1981; 1984) and tobacco-related lung diseases such as emphysema and chronic bronchitis (Roman et al., 2004). Moreover, tissue remodeling characterized by increased matrix deposition has been linked to decreased pulmonary functions in infants (Taylor et al., 1987; Hanrahan et al., 1992; Tager et al., 1993; Cunningham et al., 1994; Tager et al., 1995, Lodrup et al., 1997; Hoo et al., 1998; Upton et al., 1998; Dezateux et al., 1999; Milner et al., 1999; Sekhon et al., 2001). Lung function will be addressed and discussed in further detail later.

i) Collagen

Collagen corresponds to ~20% of the dry weight of the lung. Structural collagen Types I & III represent 90% of the above total collagen in the lung (Chambers et al., 1997). These two major secretory products of lung fibroblasts polymerize into fibrils providing great tensile strength in the alveolar wall. Chambers has suggested that collagen I predominantly plays a key role in providing tensile strength while collagen III mainly provides compliance. Type I cells are attached to type IV collagen matrix whereas type II cells are located in alveolar corners containing a mixture of type IV and I collagens. Pulmonary fibrosis is associated with a progressive thickening of the alveolar interstitium and a dense fibrotic matrix composed primarily of collagen. Phipps et al. (1989) suggest

that in pulmonary fibrosis deposition of abnormal quantities of collagen may reflect hyperactivity and/or proliferation of a fibroblast subpopulation (Brody et al., 1983).

ii) Elastin

Elastin, an insoluble, hydrophobic cross-linked structural protein produced by fibroblasts forms a random 3-dimensional network creating elastic recoil in the matrix of the lung. Alveolar type II cells are thought to preferentially localize over cables of elastin (Honda et al., 2000). Elastic fiber matrix damage results in deterioration and destruction of the alveolar wall.

iii) Fibronectin (FN)

FN is a cell adhesive glycoprotein implicated in tissue injury and repair (Bitterman et al., 1983; Kuhn et al., 1989; Roman et al., 1997; 1998). Formation of its fibrils is associated with nearby matrix components and interstitial mesenchymal cells. Specific binding sites on the FN molecule promote cell adhesion, migration, differentiation and cell growth. Moreover, FN modulates branching morphogenesis, early in fetal lung development (Chen et al., 1986; Snyder et al., 1987). High levels of FN expression exist in tissue remodeling states of tobacco-related and interstitial lung diseases (Roman et al., 2004).

iv) Proteoglycans

Proteoglycans are complex macromolecules comprised of a protein core and large sugar groups called glycosaminoglycans (GAGs). Heparin sulphate, chondroitin sulfate and dermatan sulfate are the major proteoglycans in the alveolar wall. These bulky complexes

take up water molecules influencing fluid balance and lung compliance. Aside from modulation of lung development, proteoglycans have been implicated in cell-matrix adhesion, cell proliferation and migration (Scott et al., 1988).

v) Basement membranes (BM)

BMs are continuous barriers composed of Type VI collagen, laminin, nidogen or entactin and heparin sulfate proteoglycans defining the outer limits of the interstitium. They play important roles in protection from particulates, pathogens and metastatic cells and are thought to modulate the differentiation of the epithelium.

B) PULMONARY ALVEOLAR EPITHELIAL CELLS

a) Type I Pneumocytes

Type I pneumocytes are squamous cells accounting for 8% of the total cells of the lung while covering a large ~92% of the alveolar surface area (5000 um^2) (Crapo et al., 1982). Type I cells attach to endothelial cells via thin basement membranes (Bertalanffy et al., 1964) providing intact gas permeable surfaces as a part of the blood-air barrier. This structural arrangement creates a 0.3 um thin filter through which both O_2 and CO_2 can be exchanged through capillary walls (Burri et al., 1985). These cells are considered to be terminally differentiated, not possessing the ability to divide (Kauffman et al., 1974). Replacement occurs by the de-differentiation of the type II alveolar cell during lung injury as well as during lung development (Evans et al., 1973; 1975; Kauffman et al., 1980).

b) Type II Pneumocytes

Type II cells are the most prevalent cell type of pneumocyte found in the alveoli comprising 15% of the lung cell population (Crapo et al., 1982)(covering a surface area of 7% (183 um^2)). Though Type II pneumocytes are more abundant than Type I alveolar epithelial cells they cover only ~ 8% of the alveolar surface area. Type II pneumocytes are cuboidal in shape, located in the corners or niches of the alveolus where higher amounts of mechanical strain exist. The type II apical surface contains microvilli which protrude into the alveolar luminal space whereas the basal surface is securely bound to a fixed basal lamina or basement membrane (BM).

Precursors

It is widely accepted that Type II pneumocytes are stem cells or precursors of type I cells which terminally differentiate during normal fetal lung growth and upon alveolar epithelial injury (Adamson and Bowdon, 1974; 1975; Kauffman et al., 1974; 1980; Evans et al., 1975; Crapo et al., 1980; Burri et al., 1985; Bishop et al., 2004). A number of investigators believe that transition of a Type II cell into a Type I cell is reversible (Borok et al., 1998), occurring via an intermediate cell type which combines the characteristics of both cells (Flecknoe et al., 2000).

Distinctive Feature

The most distinguishing feature of alveolar Type II cells are their large intracellular inclusions or concentric membrane bound storage units known as lamellar bodies (LB)

(Wright et al., 1989; Rooney et al., 1994). Appearance of these structures is linked to the appearance of pulmonary surfactant (PS) (Wang et al., 1971; Engle et al., 1982; Adamson et al., 1984). LBs first appear in cytoplasm at the basal region of the type II cell and are correlated with a decrease in glycogen content within the cell (Buckingham et al., 1962; Maniscalco et al., 1978; Engle et al., 1982).

Function

Type II cell stimulation leads the intracellular storage organelles of surface active material or pulmonary surfactant (PS) to become exocytosed from the apical surface of Type II pneumocytes to the lungs air-liquid interface (Gil et al., 1973). It is at this interface or hypophase layer that LBs unravel to form tubular myelin, an intermediate reserve pool of PS (Sanders et al., 1980). Sufficient amounts of properly functioning PS within alveoli are vital for normal lung function (Wright et al., 1987).

2.2 PULMONARY SURFACTANT

Function

Type II pneumocytes prevent lung collapse or atelectasis through production and secretion of a complex 'lung liquid' known as pulmonary surfactant (PS). PS stabilizes alveoli and becomes spread upon lung expansion to coat the alveolus, reducing the surface tension imposed upon the walls of the alveolus. This prevents lung collapse leading to a decrease in the work of breathing.

Composition

PS is an amphipathic material comprised of ~80% phospholipids including diphosphatidylcholines (DPPC), ~12% neutral lipids such as cholesterol and ~8% surfactant proteins (SP-A, B, C, D) which collectively line the air-liquid interface to decrease surface tension within the lung (Creuwels et al., 1997; Possmayer et al., 2001; Orgeig et al., 2004).

a) Surfactant Phospholipids

Mammalian surfactant is 80-90% lipid in composition, the majority being phospholipids (King et al., 1984). Surfactant phospholipids consist of 80% phosphatidylcholine (PC), 40-50% of PC occurs in a saturated form known as dipalmitoylphosphatidylcholine (DPPC). It is DPPC which gives surfactant its surface tension lowering abilities. The remaining phospholipids consist of 8-15% phosphatidylglycerol (PG) and phosphatidylinositol (PI), 3-5% phosphatidylethanolamine (PE) (Veldhuizen et al., 1998) and 10% neutral lipids, the majority of which is cholesterol (90%) (Rooney et al., 1991; Orgeig et al., 2001). Recycling of the above phospholipids occurs within Type II alveolar epithelial cells (Wright et al., 1987). Conversely, evidence suggests that macrophages play an important role in clearing spent surfactant from alveoli (Sueishi et al., 1977; Desai et al., 1978; Coalson et al., 1987).

b) Surfactant Proteins (SP-A,-B,-C,-D)

The four main surfactant proteins include: SP-A; being of most abundance, SP-B; most essential, SP-C; lung specific and most recently found SP-D (Haagsman et al., 2001).

Refer to table for surfactant protein structures along with relative molecular weights. (Table 1.)

Table 1. Surfactant protein structures along with relative molecular weights.

Surfactant Protein (SP)	Structure	Hydrophobic/Hydrophilic	Relative Molecular Weight
A	hexadecamer	hydrophilic	26 - 38 kDa glycoprotein
B	dimer	hydrophobic	6.5 - 9 kDa
C	monomer	hydrophobic	4 - 5 kDa
D	dodecamer	hydrophilic	43 kDa glycoprotein

i) Hydrophobic Surfactant Proteins B & C

Surfactant proteins (SP) -B & -C are hydrophobic and of low molecular weight. SP-B is involved in spreading of the surfactant proteins upon the alveolar surface (Tokieda et al., 1999) and has involvements in lung antioxidant function (Weaver et al., 2001). Deficiencies in SP-B protein lead to lethal respiratory failure upon birth suggesting that SP-B protein is important being the only surfactant protein essential for lung function (Nogee et al., 1993). SP-C expression is exclusive to the Type II alveolar epithelial cell in the lung (Weaver et al., 2001). Individuals with reduced SP-C protein levels were found to suffer from interstitial lung fibrosis (Nogee et al., 2001).

ii) Hydrophilic Surfactant Proteins A & D

Surfactant proteins A & D are hydrophilic members of the collectin family, thought to play an immunologic role against pathogens in the body's defense system (Korfhagen et al., Lawson et al., 2000; Crouch et al., 2001). These SP proteins are not specific to Type II cells of the lung, they can also be found at mucosal surfaces throughout the body in

places such as the sub-mucosal glands of conducting airways and in the gastrointestinal tract (Rubio et al., 1995; Goss et al., 1998; Khubchandani et al., 2001; van Rozendaal et al., 2001). Furthermore, SPA & D are thought to play important roles in surfactant lipid recycling (Johansson et al., 1997; Crouch et al., 1998).

2.3 PULMONARY ALVEOLAR EPITHELIAL-MESENCHYMAL (E/M) INTERACTIONS

Epithelial-mesenchymal interactions are well-established mediators of normal lung development and injury/repair signaling pathways (Shannon et al., 1997; Wright et al., 1999; Demayo et al., 2002). Cooperative interactions such as these are required for prenatal lung growth, replication and differentiation (Shannon et al., 2001) including the development of Type II cells (Scott and Das, 1994). Interactions between lung type II cells and IFs occur via physical contacts, ECM, metabolic substrates, growth factors and hormones.

a) Physical Contact Points

Developing rat lung IFs express membrane contact points, thereby providing a physical mechanism of epithelial-fibroblast interaction via molecular transfer (Bluemink et al., 1976; Leung et al., 1980). Foot processes from type II cells are within 30 Å of fibroblast cell membranes and are concentrated adjacent to lamellar bodies. Cell contacts increase during epithelial cell differentiation and initiation of surfactant synthesis (Grant et al., 1983; Adamson et al., 1984) corresponding to days 20 & 21 when rat lung structure consists of tubules of type II cells overlying IFs. This suggests that direct cell-cell

communication may be involved in the induction of surfactant synthesis in type II cells (Adamson et al., 1984).

b) Extracellular matrix (ECM)

Fibroblast secreted ECM establishment beneath alveolar epithelium in the alveolar wall has dramatic effects on the differentiation state of Type II cells (Roman et al., 1997). It is known that cellular behaviors differ on varying substrates (Kleinman et al., 1981) and type II cell function and morphology can be affected by underlying collagen types (Rannels et al., 1987; Messmer et al., 1982; Adamson et al., 1990). Density and substrata are additionally important in lung Type II to Type I cell transdifferentiation in vitro (Reynolds et al., 1999). Laminin substrata in particular promote the retention of alveolar type II cell differentiation in vitro (Schuger et al., 1997).

c) Metabolic Substrates

IFs provide lipid substrate for Type II cell surfactant phospholipid synthesis (Torday et al., 1995). IFs serve as a reservoir for pulmonary surfactant (PS) substrates such as triglycerides (TG) (Nunez et al., 1995). During late fetal lung development differentiation of IFs is marked by neutral lipid vacuoles containing triacyclglycerols that reach peak concentrations prior to the onset of adjacent Type II maturation and in association with the appearance of LBs (Torday et al., 1995). This suggests that other pulmonary surfactant components may be transferred from fibroblasts to alveolar Type II cells, thereby facilitating pulmonary surfactant synthesis and secretion into the air-liquid interface within the alveolus.

d) Growth Factors & Mediators

During fetal development, fibroblasts appear to play an essential role in regulating differentiation of type II cells via lung maturation or growth factors and production of secreted matrix. This matrix assembles the groundwork on which type II pneumocytes are appended. A mediating protein factor called fibroblast-pneumocyte factor (FPF) (Smith et al., 1978; 1979; Post et al., 1984); is shown to increase the rate of lung maturation (Smith et al., 1979) and phospholipid synthesis in fetal type II cells (Post et al., 1984). Lung fibroblasts have a large impact on Type II cell surfactant protein (SP) and phospholipid synthesis. Syntheses of these components are additionally mediated by keratinocyte growth factor (KGF) otherwise known as fibroblast growth factor (FGF-7) (Shannon et al., 2001).

e) Hormones

Glucocorticoids are one of the major regulators of normal lung development (Bolt et al., 2001). These steroid hormones act on fetal fibroblasts promoting epithelial maturation factor secretion (Smith et al., 1979), which facilitates lung maturation and increased frequency of epithelial-interstitial cell contacts. This maturation factor slows down epithelial growth and enhances differentiation (Adamson et al., 1986). Thus Glucocorticoids promote alveolar wall thinning, expression of enzymes associated with phospholipid metabolism (Ballard et al., 1982), and lamellar bodies formation in type II alveolar epithelial cells (Adamson et al., 1986). This correlates with glucocorticoid ability to increase PS phospholipid and SP levels (Bolt et al., 2001). Additional hormones and growth factors play important roles in normal lung development including parathyroid

hormone (PTH), platelet derived growth factor (PDGF), vascular endothelial growth (VEGF) and retinoic acid (Adamson et al., 1986). Details of these hormones are beyond the scope of this study along with developmental involvements with transcription factors.

2.4 LUNG DEVELOPMENT

Lung development can be subdivided into three chronological periods – an early embryonic period, a fetal period and a postnatal period. The fetal lung development period consists of three phases first being a pseudoglandular phase, the second a canalicular phase and finally a saccular phase. The subsequent and final postnatal period consists of alveolarization to which the timing of the end of pulmonary development has yet to be elucidated (Burri et al., 1984; McGowan, 2001). Distinction of the phases and timing of lung development are characterized within the human, but times of gestation vary depending on the species of mammalian lung (Table 2.).

Table 2. Fetal lung development phases and timing from gestation to full term

Morphologic Stage	Human (weeks)	Rat (days)	Rabbit (days)
Embryonic (lobar division)	0 - 5	0 - 13	0 - 18
Glandular (airway passages)	5 - 16	13 - 18	18 - 24
Canalicular (bronchiole & epithelial branching)	16 - 26	18 - 20	24 - 27
Saccular (alveolar sacs & ducts)	26 - Term	20 - Term	27 - Term

Modified from THE LUNG: Scientific Foundations, 1991

Development and maturation of the lung occurs from the proximal to the peripheral segments of the airway tree and there may be overlap between the stages, making determinations of the precise stage in a given lung extremely difficult (Burri et al., 1991). The canalicular/saccular period of fetal lung development is of most importance in the following study which allows us to study cellular functions in the surfactant system at a time when development of the lung and its surfactant system is most critical.

FIVE STAGES OF LUNG DEVELOPMENT

a) Embryonic Period

The embryonic period corresponds to a period of organ development or organogenesis and early conducting airway formation. Next, the fetal period of development uses three phases to reach functional maturity required at birth including the pseudoglandular, the canalicular and finally the saccular.

Fetal Period – Stages 2, 3 & 4

b) Pseudoglandular Stage

The first fetal stage or pseudoglandular gland-like stage forms all prospective conducting airways, including bronchial tree formation and the beginning of round clusters of cells known as acini (acinus). Tissues originating from the ventral diverticulum of the forgut grow into the surrounding mesenchyme. It is at this stage that epithelial-mesenchymal interactions play a determining role in regulating growth and branching patterns (Bluemink et al., 1976). Furthermore, collagen is required for this branching (Spooner et

al., 1980) and the amount of mesenchyme influences the differentiation of the adjacent epithelium at this time (Master et al., 1976).

c) Canalicular Stage

Future gas-exchange functions of the lung depend on the formation of a thin blood-air barrier and sufficient quantities of surfactant material during this late fetal development stage. First, future airways containing approx. three orders of branching known as canaliculi continue to divide and enlarge at the expense of the mesenchyme. Second, at the periphery of the airway tree, the epithelium differentiates into flat Type I pneumocytes and/or cuboidal Type II pneumocytes containing lamellar bodies. Concurrently, capillaries of the mesenchyme come into close contact with the Type I pneumocytes in the epithelium forming a thin air-blood barrier. Lung airspace surface area is increasing rapidly and Type II pneumocytes containing lamellar bodies are secreting surfactant material into the air spaces. In most mammals, surfactant production starts relatively late in pregnancy at approx. 80-85% of gestation time.

d) Saccular or Terminal Stage

Rapid development and maturation of the pulmonary periphery, including the surfactant system, at this time increases the chances of a prematurely born baby to survive via enhanced ability of the lung to exchange gases. This stage name stems from the fact that its widening airways end in clusters of thin-walled terminal saccules, separated by thick inter-saccular septa forming the primitive lung parenchyma. This last fetal development stage gives the lung its spongy appearance and can be characterized by the enormous

enlargement of the peripheral air spaces. The tremendous expansion of airspaces leads to a marked decrease in mesenchymal mass otherwise known as the thinning of the interstitial tissue during this stage. The lung parenchyma or interstitial cell population is very cellular, responsible for the extracellular fibrous material and matrix, appears to play a role in growth, repair and differentiation of epithelial cells along with the control of surfactant production (Adamson et al., 1984; 1985; Rannels et al., 1989). Elastin formation plays an essential role in the further morphological development of the lung as the initiating step for alveolar formation (Dubreuil et al., 1936). Cell proliferation slows down during this late fetal period, therefore, it may be that the increased surface area is achieved by the rearrangement of the available tissue. At day 21 in the rat lung there is a sharp drop in division of the epithelial cell population and high proliferation rates in IFs. Furthermore, some data suggests that the lung is born with a given number of interstitial cells which are subsequently distributed over the increasing lung volume. By the end of gestation the lung contains functional alveoli or gas exchange units along with a functional pulmonary surfactant system (McGowan et al., 1997). Finally, the last developmental step is postnatal alveolarization.

e) Postnatal Period - Alveolarization

At birth the lung houses approx. 50 million alveoli (Langston et al., 1984) although > 80% of alveoli must be produced postnatally to reach the adult number of 300 million (Weibel et al., 1963). The beginning of alveolarization is unclear, although some studies show that it may begin as early as the 32-34th week of gestation in humans (Langston et al., 1984). During this period alveoli are formed through a septation process that greatly

increases the gas exchange surface area. Alveoli increase in diameter with age. For example rat alveoli double in size between the 3rd and 4th postnatal weeks. It is currently unclear how far into childhood the period of alveolar formation extends, but it has been postulated that new alveoli continue to be added until the ages of 8-10 years (Dunhill et al., 1962; McGowan et al., 1997). Changes in lung mechanics resulting from in utero developmental alterations in the lung are thought to persist into adulthood (Upton et al., 1998).

2.5 MATERNAL SMOKING DURING PREGNANCY: FETAL OUTCOMES

Detrimental fetal outcomes including increased risk of spontaneous abortion, stillbirth, preterm delivery, low birth weight, early neonatal morbidity and mortality, sudden infant death syndrome and abnormal pulmonary alteration is directly related to maternal smoking during pregnancy (Wen *et al.*, 1990; Hanrahan et al., 1992; Cunningham et al., 1994; Cliver *et al.*, 1995; Mathews *et al.*, 1998; Higgins et al., 2002; Gilliland et al., 2003; Hofhuis et al., 2003). Despite these facts twelve percent of women still smoke during pregnancy (Spindel et al., 2002). Strong evidence now suggests that smoking during pregnancy has direct and adverse long-term consequences on fetal lung growth and is predicted to affect future lung development. Consequences include increased respiratory illness and altered pulmonary function in children born of smoking mothers (Collins et al., 1985; Chen et al., 1987; Taylor et al., 1987; Maritz et al., 1988; Tager et al., 1992; Hanrahan et al., 1992; Cunningham et al., 1994; Walsh et al., 1994; Hoo *et al.*, 1998; Wisborg *et al.*, 2000; Sekhon et al., 2001; Spindel, 2002; Scott, 2004). It has been estimated that annual costs of increased respiratory illness and consequences associated with maternal smoking is well above 660 million dollars per year (Stoddard & Gray, 1997). As mentioned above, infants born to mothers who smoke during pregnancy are more prone to respiratory ailments and compromised lung function (Taylor *et al.*, 1987; Tager *et al.*, 1993). Correspondingly, in monkeys, prenatal nicotine exposure similarly alters pulmonary function at birth (Sekhon *et al.*, 2001). The tobacco constituent nicotine freely passes the placenta and is detected in the amniotic fluid of smoking mothers (Luck *et al.*, 1985). This sensitivity is most likely due to the expression of nicotinic acetylcholine receptors in the developing lung. In accordance with this hypothesis,

airway epithelium in fetal monkey lung has been shown to have abundant expression of nAChR (Maus *et al.*, 1998; Sekhon *et al.*, 1999, 2002).

2.6 NICOTINIC ACETYLCHOLINE RECEPTORS (nAChRs)

Nicotinic acetylcholine receptors (nAChRs) are members of the superfamily of ligand-gated ion channels (LGIC). The nAChR is the best understood of the superfamily of ligand-gated ion channels including similar glycine, γ-aminobutyric acid-a ($GABA_A$), and 5-hydroxytryptamine 3 (5HT3) receptors (Lindstrom *et al.*, 1996). Five identical subunits are arranged symmetrically to form a central transmembrane cation channel. LGICs open transiently in response to the binding of a neurotransmitter or ligand. Ligands such as nicotine and/or the physiological agonist acetylcholine bind to the nAChR changing its conformation to allow cations to move through its channel. Prior studies have focused on nicotinic acetylcholine receptor mediation of fast synaptic transmission in the brain and the effects of nicotine on neural activity (Dani *et al.*, 2001). More recently, members of this receptor family have been found to be expressed in a wider variety of cell types and to mediate an array of downstream effects (Conti-Fine *et al.*, 2000). For our study it is important to note that non-neuronal cells, particularly those derived from lung, are reported to express nicotinic acetylcholine receptors (nAChRs) (Conti-Fine et al., 2000). Nicotine, the most addictive component of tobacco smoke signals through this family of receptors (Lindstrom et al., 1987). Sekhon and colleagues (1999) have demonstrated the ability of nicotine to cross the placental barrier and interact with nAChRs altering structure of the developing fetal lung. Fetal lung during development may be the most susceptible to damaging influences as it is exposed to smoke constituents through

circulation and from accumulation in the amniotic fluid (Scott & Thliveris, 2005). The amniotic fluid acts as a reservoir for prolonged fetal exposure bathing the fetus in high concentrations of smoke constituents (Scott and Thliveris, 2005). Exposure to concentrated levels of constituents such as nicotine may alter complex processes including epithelial-mesenchymal interactions occurring within the developing fetal lung via nAchRs. Nicotinic receptor downstream effects have been implicated in human small cell lung carcinomas and smoking-associated pediatric lung disorders including bronchopulmonary dysplasia (BPD), cystic fibrosis (CF), sudden infant death syndrome (SIDS) (Elliot et al., 1998) and in chronic obstructive pulmonary diseases (COPD), for example asthma (Schuller et al, 2000; Roman et al., 2005).

a) nAChR Structure

The shape of the acetylcholine receptor and the arrangement of its subunits were determined by electron microscopy. Receptor molecules have a diameter of 9 nm with a central 2 nm pore (Unwin, 1993, 1995, 2000). Half of the receptor protein mass protrudes into the extracellular space approximately 7 nm from the membrane lipid matrix and 3 nm into the cytoplasm (Mol. Cell Bio., 1986). The classical nAChR is a 290 kDa membranal heteropentameric glycoprotein (glycosylated) consisting of five subunits in the stoichiometry of $\alpha 2\beta\gamma\delta$ (Karlin, 1993; Corringer *et al.*, 2000). The molecular weights of the 4 different polypeptide subunits in Daltons include: α (40,000), β (49,000), γ (57,000) and δ (65,000) (Darnell *et al.*, 1986). Subunits are arranged in the clockwise order $\alpha\gamma\alpha\beta\delta$ to create a cylindrical complex around the central ion channel. Four respective transmembrane domains are found within each subunit including: M1, M2, M3

& M4 (Samson *et al.*, 2004). These domains play a key role in determination of ion selectivity (Galzi et al., 1992; Corringer et al., 1999) and ion transport rates (Imoto et al., 1986). Outer M4 domains are exposed to the bilipid layer while the innermost alpha helical M2 domains form the ion conducting channel or pore (Unwin, 2000). Negatively charged side chains at both ends of the pore allow only positively charged ions such as calcium (Ca^{2+}) to pass through the channel. Nicotinic receptors contain large extracellular agonist-binding domains referred to ligand binding domains (LBD). Agonists such as acetylcholine bind cooperatively into two separate pockets found on the receptors alpha subunits. Nicotinic receptors are appropriately named seeing that nicotine binds twice as strongly the physiological agonist, acetylcholine. Agonist sensitivity is dependent on which α subunit(s) are present, although β subunits can modify that sensitivity (Sekhon et al., 1999). Binding initiates rotational movements in both alpha subunits opening the receptors channel by causing a change in pore size via outward movement of hydrophobic leucine and valine side chains (Unwin, 1998). The pore considered to be occluded or in closed conformation when the α helix side chains form a gate near the middle of the lipid bilayer. Nicotinic receptors exist as homomeric (same subunits) or heteormeric (combination of subunits) isotypes. Nicotinic receptor isotypes become desensitized upon prolonged exposure to agonists (Conti-Fine et al., 1994; Galzi and Changeux, 1995). Functional properties of these receptors such as ligand affinities, channel conductances, opening and closing rates, toxin sensitivities and degree and severity of receptor inactivation are distinct to the composition or the specific nicotinic receptor isotype (Galzi et al., 1995).

b) nAChR Isotypes (Subtypes)

Subtype diversity arises from nAChR assembly as homomeric or heteromeric pentamers containing different subunits (α1-α10, β1-β4, ε, γ, δ) encoded by a presently identified family of seventeen genes (Sekhon et al., 1999; Peng *et al.*, 2004). Nicotinic receptor isotypes exist in muscle, neurons and non-neuronal cell types. Muscle nAChRs are comprised of four different types of subunit (α, β, γ or ε and δ) whereas neuronal nAChRs include two types of subunits (α and β) or five copies of the same α subunit (Conti-Tronconi et al., 1994; Galzi and Changeux, 1995; Lindstrom et al., 1995, 1996; Maelicke et al., 1996; Albuquerque et al., 1997). Neurons express all ten α subunits and four β subunits providing an array of α , β receptor combination possibilities (Conti-Tronconi et al., 1994; Lindstrom, 1996; Maelicke et al., 1996; Albuquerque et al., 1997). Examples of nAChR subtypes exist in brain neurons (McGehee *et al.*, 1995) and most importantly in our studies non-neuronal cell types of the lung. The most abundant isotypes within the brain include the heteromeric form $(\alpha4)_3(\beta2)_2$, and the homomeric form is $(\alpha7)_5$ (Lindstrom *et al.*, 1996).

c) nAChR Function

Nicotinic receptors play important roles in classical excitatory neurotransmission at the neuromuscular junction (NMJ) facilitating skeletal muscle contraction, via autonomic ganglia and central nervous system cholinergic pathways thought to contribute to processes such as cognition, perception and emotion (Zhao et al., 2003). Non-neuronal intracellular mechanisms by which smoking produces its adverse effects on developing lung is unclear. The recent identification of abundant nicotinic cholinergic receptors in

developing lung by the Sekhon group suggests that nicotine may trigger signaling mechanisms that can alter developing lung (Sekhon *et al.*, 1999). Direct chronic stimulation of α7nAChR receptor by nicotine and/or NNK (byproduct) is thought to initiate an autocrine loop in mammals which may cause pediatric smoking associated pulmonary diseases and incidences of small cell lung carcinoma (Maritz, 2004).

d) Non-neuronal nAChR Distribution

Non-neuronal cells lining the internal and external surfaces in humans express nAchRs similar to those expressed by neurons (Conti-Fine et al., 2000). Using radiolabeled agonist, studies have shown that nicotine binding sites definitely exist in the lung (Shriver *et al.*, 2000). Nicotinic receptor expressing cells include skin keratinocytes, airway fibroblasts and endothelial cells (EC). Lung specific receptor cell distributions include such cell types as: airway epithelial cells, human and mouse bronchial epithelial cells (BECs), alveolar epithelial cells (AECs) or type II cells, pulmonary neuroendocrine cells (PNECs), submucosal glands, airway and vascular smooth muscle cells (ASMCs & VSMCs), interstitial fibroblasts, pulmonary vessels and pulmonary macrophages (Grando et al., 1995; Zia et al, 1997; Maus *et al.*, 1998; Macklin et al., 1998; Sekhon *et al.*, 1999, 2004; Heeschen et al., 2002; Carlisle et al., 2004). Fu and colleagues (2003) show presence of nAChR α_3,α_4,α_7 & β_2-subunits in neuroepithelial bodies (NEB). NEBs are small groups of pulmonary neuroendocrine cells located in airway epithelium typically at bifurcations of small airways. NEBs function as chemo- and mechanoreceptors and are thought to play a trophic role during lung development. Active nAChRs within NEB proposes mechanisms by which smoking during pregnancy impairs normal lung

development and suggests links between smoking during pregnancy and SIDS and links smoking with lung cancer (Spindel, 2003). Expression of nicotinic receptor expression in the lung is as summarized in the table below.

Table 3. Nicotinic expression found in lung.

Lung Cell Types	Subunits	References
Bronchial epithelial cells	alpha - 3, 5, 7 beta - 2, 4	Sekhon et al., 1999; Zia et al., 1997; Maus et al., 1998; Reynolds et al., 2005
Alveolar epithelial cells	alpha - 3, 4, 5, 6, 7	Zia et al., 1997; Reynolds et al., 2005
Pulmonary Neuroendocrine Cells (PNEC)	alpha 3, 4, 7 beta - 2	Sekhon et al., 1999; Fu et al., 2003
Neuroepithelial Bodies (NEB)	alpha 3, 4, 7 beta - 2	Sekhon et al., 1999; Fu et al., 2003
Submucosal glands	alpha 7	Zia et al., 1997
Airway smooth muscle cells	alpha 7	Sekhon et al., 1999
Vascular smooth muscle cells	alpha 7	Sekhon et al., 1999
Fibroblasts (mesenchyme)	alpha 7	Sekhon et al., 1999; Reynolds et al., 2005
Pulmonary macrophages	alpha 7	Sekhon et al., 1999
Small Cell Lung Carcinomas (SCLC)	alpha 3, 5, 7 beta 2	Quik et al., 1994; Song et al., 2003

2.7 ANIMAL MODELS OF SMOKE EXPOSURE

Smoke exposure in the rat during gestation causes hypoplasia leading to reductions in lung volume, decreased numbers of saccules, septal crests and elastin fibers in fetal lungs (Collins et al., 1985). Although pulmonary damage caused by maternal smoking is thought to be multifactorial, Sekhon and colleagues (1999) use a fetal monkey model to prove that a large part of the effect of maternal smoking on the lung is mediated by nicotine and its interaction with pulmonary nicotinic receptors in the lung of the developing fetus. This proves that nicotine is a major player in the effects of tobacco on the developing fetal lung. Furthermore, nicotine exposure during pregnancy and lactation caused emphysema-like alterations in rat pup lung structures such as elastic tissue reduction and increased numbers of lamellar bodies in the alveolar epithelial type II cells of the lungs (Maritz et al., 1993, 1995). Congruent to the above, our lab has shown

decreased numbers of lamellar bodies in fetal rat lung occur upon exposure to nicotine. Mechanisms responsible for inducing the above cellular structural and functional changes are yet to be elucidated. Further studying nAChR expression in lung development will provide receptor-mediated mechanisms enabling us to more fully understand effects of nicotine at a molecular level including structural and cellular changes associated with the developing lung.

Nicotine Upregulation of nAChRs

Prolonged nicotine exposure upregulates nicotinic acetylcholine receptor expression in adult humans, monkey, rodents and in vitro cell lines (Marks et al., 1992; Lindstrom et al., 1996; Breese et al., 1997; Peng et al., 1997; Slotkin et al., 2002). Pregnant rats exposed to nicotine show increases in nicotinic receptor expression in the brains of their offspring (Slotkin et al., 1987, 2002; van de Kamp et al., 1994). Recently, upregulation of the alpha-7 nicotinic receptors was similarly shown by Sekhon (1999) using a fetal monkey model of lung development. A significant upregulation of α7 nAChR in fetal monkey lung following maternal nicotine exposure was found by Sekhon (1999) suggesting a role of α7 nAChR in modulating functional changes at the cellular level in airway and alveolar cells in the developing lung. In the non-neuronal developing cells of the lung, α7 nAChRs are by far the most abundant form of nAChR and are found in airway vessel and alveolar wall cells (Sekhon *et al.*, 1999).

2.8 ALPHA-7 NICOTINIC RECEPTORS

Several functional subtypes of heteromeric α7-containing nAChRs exist in chick sympathetic neurons, rat superior ganglion and intracardiac ganglion neurons (Yu et al., 1998; Cueves et al., 2000). Neuronal nAChRs particularly the α7 subtype, exhibit high relative permeability to Ca^{++} (Seguela *et al.*, 1993; McGehee *et al.*, 1995). Therefore, opening of the nAChR channel can lead to a direct increase in cytoplasmic Ca^{++} concentration. Recently, intracellular Ca^{++} stores have been shown to contribute to the sustained Ca^{++} signals activated by nAChR stimulation in chick cilliary ganglion neurons (Shoop *et al.*, 2001) and substantia nigra brain slices (Tsuneki *et al.*, 2000). In parallel, functional nicotinic receptors of airway fibroblasts and human bronchial epithelial cells (HBE) regulate Ca^{++} upon ligand binding and lead to downstream activation of signaling pathways MAPK or PKC when bound by nicotine. These downstream effects can be blocked by use of nicotine antagonists (Carlisle *et al.*, 2004), confirming that the above signal pathways function via an α7 nAChR specific mechanism. Other studies have shown that the activation of the cell survival Akt signaling pathway occurs in cultured lung epithelial cells via nicotine and NNK stimulation (West *et al.*, 2003). It has also been shown that nicotine stimulates the phosphorylation of key residues in the anti-apoptotic protein B cell lymphoma gene 2 (BCL2) through activation of the protein kinase C α and phospholipase C pathways to inhibit apoptosis (Mai *et al.*, 2002). Cholinergic signaling has been reported in lung cancers, and nicotine activation appears to stimulate lung cancer cell growth (West *et al.*, 2003; Song *et al.*, 2003). As well as effecting lung growth, autocrine cholinergic-signaling likely plays a role in lung development.

a) Role of α7 nAChR in Lung Cancer

Lung cancer demonstrates a strong etiologic association with smoking. Small cell lung carcinoma (SCLC) is found almost exclusively in smokers, rendering this histologic type of lung cancer tobacco-specific (Weiss *et al.*, 1983). SCLC are tumors derived from pulmonary neuroendocrine cells (PNEC) or related precursor cells. SCLCs express α_7 nAChR and their proliferation or cell growth can be stimulated by nicotine. This cell proliferation can be modulated by a serotonergic autocrine loop, inhibitable by α-bungarotoxin (α-BTXN) (Schuller *et al.*, 1999), confirming the α7 nAChR specificity. Others have shown that α7nAChR regulates the release of 5-hydroxytryptamine (5-HT, serotonin) in PNECs and SCLC causing bronchoconstriction. Nicotine and its nitrosated tobacco smoke carcinogenic derivative 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) binds to the α7nAChR in SCLC and PNECs, resulting in activation or the influx of Ca^{++}, release of 5-HT, and activation of a mitogenic pathway mediated by protein kinase C (PKC), Raf-1, mitogen activated protein kinase (MAPK) and c-myc. NNK exposure up-regulated the α_7nAChR and its associated serotonergic mitogenic pathway in PNECs. Upregulation of α_7nAChR and its serotonergic mitogenic signal transduction pathway via chronic exposure to nicotine and NNK of pregnant mothers, may contribute to the development of SCLC in smokers and pediatric lung disorders such as bronchitis and asthma in children of mothers who smoke. Also, this α7 nicotinic receptor upregulation may increase the vulnerability of infants to the development of paediatric lung disorders (Schuller *et al.*, 2003; Maritz, 2004).

b) Role of α7 nAChR in Fetal Development Lung Alterations

Impaired lung development and function in children whose mothers smoked cigarettes during pregnancy is well documented (Zucker, 2004) and the nicotine component of tobacco is now thought to be the major player. Nicotine exposure alters fetal monkey lung development via *1) upregulation of nAChRs within the fetal lung (previously discussed), 2) increased deposition of matrix from interstitial lung fibroblasts and by 3) decreasing or compromising pulmonary lung function mechanics.*

2) Increased Fibroblast Matrix Deposition

Most tobacco-related lung diseases are associated with alterations in tissue remodeling and are characterized by increased matrix deposition. Fibroblasts are the primary cells that synthesize lung connective tissues or matrix. Nicotine has been shown to increase the expression of α7nAChRs on fibroblasts (Roman *et al.*, 2004). Changes in the synthesis & secretion of essential extracellular matrix (ECM) components such as fibronectin (FN) & collagen (COL I & III) have recently been implicated within lung fibroblasts upon nicotine stimulation.

High levels of Fibronectin (FN) expression exist in states of tobacco-related lung disease. FN is a cell adhesive glycoprotein implicated in tissue injury and repair. Nicotine has recently been shown to stimulate the expression of FN in lung fibroblasts via the activation of intracellular signals that lead to increased FN gene transcription. Nicotine's stimulatory effect was associated with activation of both PKC and MAPK cell signal pathways. This stimulatory effect of nicotine on FN expression was abolished by α-

bungarotoxin, the specific inhibitor of α_7nAChRs. This suggests that nicotine induces lung fibroblasts to produce FN by stimulating α_7nAChR-dependent signals that regulate the transcription of the FN gene (Roman *et al.*, 2004).

Nicotine exposure increases connective tissue expression in fetal monkey pulmonary vessels (Sekhon *et al.*, 2004) suggesting that collagen expression may become altered under similar conditions in the developing lung. Nicotine exposure during pregnancy not only increases airway wall thickness, but it also stimulates the synthesis and accumulation of collagen type I and III in airway walls and alveolar compartments (Sekhon, 2001). In lung, collagen types I & III represent 90% of collagen (Chambers *et al.*, 1997). Chambers has suggested that collagen I predominantly plays a key role in providing tensile strength while collagen III mainly provides compliance. Nicotine-induced alterations in collagen would thus be consistent with the changes in lung function test in the premature infants who show a decrease in maximal force expiratory flow in infants who were exposed to smoke *in utero* (Hoo *et al.*, 1998). In accordance to this, Sekhon et al. (1999) has shown that prenatal nicotine exposure in monkeys alters pulmonary function at birth as manifested by decreased expiratory flows and increased specific airway resistance. These ECM alterations may in part contribute to the observed lung function abnormalities in *utero* smoke-exposed infants (Sekhon, 2001). These changes also persist through late childhood (Cunningham *et al.*, 1994) and may even exist into adulthood (Upton *et al.*, 1998).

SUMMARY

Nicotinic acetylcholine receptors of the superfamily of ligand-gated ion channels are well understood in terms of structure and basic functions. The focus of studies on these receptors in the past has been done within brain tissue and assessed their role in neural activities. Recently receptor expression has been shown in a wider variety of cell types, such as non-neuronal lung cells and play an integral role in lung development. Nicotine exposure to lung cells expressing nAChRs lead to signals that regulate an array of downstream effects. Specifically, α7 nAChRs found in prenatal tissues are upregulated causing an increase in matrix deposition and alterations in cell proliferation. These α_7nAChR-dependent signals may cause the compromised lung function apparent within monkeys and human infants. In conclusion, nicotine may act to promote alterations in lung development and/or increase lung disease by acting to change cell growth via nAChRs. In airway fibroblasts this may lead to thickening of the airway wall as seen in the pathogenesis of COPD (Carlisle *et al.*, 2004) and has been linked with SIDS (Spindel, 2002). Nicotinic acetylcholine receptor regulation and their downstream effectors are major players in the developing lung. This has been shown by the increases in growth within small cell lung carcinomas (lung cancer) and compromised lung functions leading to increased matrix deposition found within tobacco-related pediatric lung disorders. For example disorders include bronchopulmonary dysplasia (BPD), cystic fibrosis (CF), sudden infant death syndrome (SIDS) and asthma (Schuller et al, 2000).

RATIONALE

Approximately 1.3 billion people smoke worldwide. The World Health Organization estimates that based on current smoking trends, tobacco use will soon become the leading cause of morbidity and mortality in the world. Babies born to smoking mothers have detrimental fetal outcomes including: increased risk of severe respiratory diseases including sudden infant death syndrome and reduced lung function (Health Canada, 2005). Increased prevalence of lower respiratory illnesses and reduced lung function in the fetus is correlated with **prenatal** smoke exposure. Further studies are clearly required in order to elucidate the complex mechanisms of the prenatal or fetal developing lung. Our system of study uses primary cultured fetal rat TII AECs and adjacent interstitial fibroblasts (IF) considered epithelial-mesenchymal (E/M) cells. These epithelial-mesenchymal (E/M) interactions are well-established mediators of lung functions such as prenatal lung growth, cellular replication, differentiation, injury and repair signal pathway. Fibroblasts have been shown to impact the ability of Type II alveolar epithelial cells (AECs) to synthesize surfactant proteins and phospholipids. Extracellular matrix (ECM) composition has effects on differentiation state of AECs ex. Laminin making the mechanisms of these two particular cell types very important with respect to the developing lung.

HYPOTHESIS

Smoke constituent exposure has a negative effect on fetal pulmonary epithelial-mesenchymal cells.

OBJECTIVE

Analyze the effects of cigarette smoke constituents on the fetal rat pulmonary epithelial-mesenchymal cell system.

SPECIFIC AIMS

Specific Aim # 1 – Identify the two cell types in our culture system and differentiate them using morphology and cell type specific proteins.

Specific Aim # 2 – Quantify the effects of smoke constituents on both cell types using assessments of DNA damage and cell proliferation.

Specific Aim # 3 – Identify the presence or absence of nicotinic acetylcholine receptors in both cell types and determine whether or not they are functional.

MATERIALS & METHODS

3.1 MATERIALS

Random-bred pregnant Sprague-Dawley rats gestational day 22 were used in our studies. All animals were obtained from Central Animal Care Services, University of Manitoba and treated according to protocols approved through the Canadian Council of Animal Care and their local representative agencies. Cell culture reagents (media, antibiotics, fungizone & newborn calf serum) were obtained from Life Technologies – Gibco/BRL (Burlington, ON), chemicals used from Sigma Chemicals (St. Louis, Mo.), plastic tissue culture flasks from Fisher Scientific (Nepean, ON) and Western Blotting reagents purchased from Biorad Laboratories (Mississauga, ON) unless otherwise stated. Enhanced Chemiluminescence reagents were used for chemiluminescent detection of proteins on immunoblots and developed on HyperFilm ECL film from Amersham Life Science (Oakville, ON). Use of confocal microscope and calcium imaging systems and the Molecular Probes Inc. (Eugene, OR) fluorescent calcium indicator fura-2 was a generous donation from Dr. Andrew Halayko, University of Manitoba Asthma and COPD Center and Manitoba Institute of Child Health (MICH). Primary horseradish peroxidase and fluorescently labeled secondary antibodies used in Western Blotting and Immunocytochemistry were obtained from Santa Cruz (California, U.S.A.). Prolong Antifade Gold and propidium iodide were purchased from Molecular Probes (Eugene, OR). Nylon filters used in preparation of cells for flow cytometry were purchased from BioDesign Inc. (Carmel, New York). Fluorescein (FITC)-conjugated anti-BrdU antibodies used for flow cytometric studies were purchased from Becton Dickenson (San

Jose, CA). Bromodeoxyuridine was obtained from Amersham Life Science (Burlington, ON).

3.2 METHODS

a) Dissection and Primary Cell Culture

Fetal Interstitial Fibroblasts (IFs) & Fetal Type II Alveolar Epithelial Cells (AECs)

Fetal rat lung Type II AECs were isolated and grown as described by Batenburg et al. 1988 and Scott et al. 1994. Fetuses were removed from pregnant gestational day 22 (day 0 was the day of breeding) rats obtained from Central Animal Services, University of Manitoba. The thorax was opened, the trachea and lungs removed, and stored in sterile, ice cold Hanks Balanced Salt Solution (HBSS). Mincing of the lung was performed using a Sorval tissue chopper (Sorval Instruments, Newton, CT) then thoroughly minced with fine scissors. The tissue slurry was placed into 100 mL of digestion buffer (90% - HBSS, 10% trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05%/0.02%)). Cells were incubated at 37°C for 1 hour while being stirred. The cell suspension was immediately diluted 3:1 with medium consisting of Minimal Essential Medium (MEM), 10% carbon stripped Newborn Calf Serum (sNCS) along with antibiotics and fungizone. Cells were filtered through three layers of 150 um Nitex gauze. The cell suspension was pelleted by centrifugation (250 x g for 10 minutes). Supernatant was discarded and pellet resuspended in a medium consisting of MEM and 10% sNCS. Cells were plated on 2 x 75 cm^2 plastic cell culture flasks. Cells were incubated for 1 hour at 37°C in a humidified atmosphere consisting of 95% air/5% CO_2 to allow fibroblasts to adhere to the plastic.

Media containing the nonadherent AECs was removed from 2 x 75 cm^2 flasks and replaced with NCS medium promoting mesenchymal interstitial fibroblast growth. Cell numbers of AECs was estimated by counting using a Coulter Counter and cells were plated onto 75cm^2 cell culture tissue flasks at a density of 1 x 10^5 cells/flask. AECs were grown in MEM/10% sNCS and IFs were grown in an incubator with MEM/10% NCS culture medium. Replacement of media occurred 24 hours later and every 48 hours thereafter. For all studies unpassaged cultures of AECs were used. At confluence IFs were passaged using trypsin/EDTA to remove the cells and replated in new culture flasks containing MEM/10%NCS medium along with antibiotics and fungizone. A ratio of 1:5 flasks was used for passaging. For every one flask of cells lifted, five new flasks were plated. IFs were used at the second passage (P2). Cultured fetal AECs and IFs were treated with nicotine and cotinine at concentrations ranging from 10^{-4} - 10^{-7} M over a 24 hour period.

b) Whole Cell Lysate Preparation

AECs and IFs were grown in 75cm^2 tissue culture flasks. Whole cell lysates were obtained in the following manner. Cells were washed three times in 5 mL of ice cold HBSS. RIPA lysis buffer (200 uL/flask) was used. RIPA lysis buffer consists of 40 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Igepal (octylphenyl-polyethylene glycol), 1% Deoxycholic acid, 1 mM PMSF (phenylmethanesulfonyl fluoride) and 5ug/mL each of pepstatin A, leupeptin and aprotinin. Cells were removed from plates by scraping with a cell scraper on ice. Buffer containing cells and cell debris were transferred to 1.5 mL microcentrifuge tube for sonication using a Heat Systems Microscon Ultrasonic Cell

Disrupter at setting # 7 with 3 x 10 second pulses using a sonicator. Samples were stored at -70° C up to 2 months.

c) Protein Assay

Sample protein concentrations of crude extracts were estimated spectrophotemetrically using the BioRad DC Protein Assay kit based on the coomassie dye-binding protocol method first described by Bradford (1976). Absorbances were estimated at 595 nm on a Beckman DU Series 640 spectrophotometer. Bovine serum albumin (BSA), Fraction V was used as a relative protein standard for all assays. Assay results were used to calculate dilutions required to bring all samples to equivalent protein concentrations for use in subsequent Western Blot analyses.

d) Protein Electrophoresis: SDS-PAGE

Sample proteins in crude extracts were size fractionated by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the buffer system first described by Laemmli (1970). The BioRad mini-gel vertical electrophoresis apparatus was used for all applications. Depending on the protein of interest lower separating gels ranged between 10% - 12% acrylamide and 4% acrylamide upper stacking gels were used. Both upper and lower gels were prepared from a 30% acrylamide/bis-acrylamide monomer stock solution, the concentration of the crosslinker (bis-acrylamide) was 3.7% w/w. Separating gels contained 375 mM Tris, pH 8.8 and 0.1% (w/v) sodium dodecyl sulphate (SDS). Stacking gel buffer included 125 mM Tris, pH 6.8, 0.1% (w/v) SDS. Samples were prepared by diluting crude extracts 1:4 in sample buffer (1.0 M Tris-HCl, pH 6.8,

8% (w/v) SDS, 45% (v/v) glycerol, 2.86 M β-mercaptoethanol, 0.02% (w/v) bromophenol blue) and boiled at 95°C for 5-10 minutes. Protein samples of 30-50 ug were fractionated by running gels at constant voltage (200V) for 1 hour at room temperature. The electrode running buffer used was of the following composition: 25 mM Tris, 192mM Glycine and 0.1% (w/v) SDS.

e) Western Blotting of Whole Cell Protein Lysates

Sample proteins obtained were size fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 8 cm x 10 cm mini-gels as described above. Quantitative profiles of protein markers were obtained by electroblotting SDS-PAGE fractionated proteins to nitrocellulose as described by Towbin and Gordon (1984). The transfer buffer was comprised of 25mM Tris, 192mM Glycine, 0.05% SDS and 20% methanol. The transfer of proteins from the gel onto nitrocellulose paper was done at a constant voltage of 100 V at 4°C for 1 hour. After transfer, blots were immediately washed for 5 minutes, stored in Tris-buffered saline (20mM Tris, 500 mM NaCl, pH 7.5) containing 0.1% Tween-20 (TBST) and immunostained at a later time.

f) Blot Immunostaining

Blots were blocked in TBST including 5% skim milk powder overnight at 4°C. Next blots were placed into TBST/3% skim milk powder with appropriate diluted primary antibody for 1 hour at room temperature with constant shaking of the antibody-containing solution. Blots were rinsed 3 x 15 minutes with TBST and incubated for one hour with horseradish peroxidase conjugated secondary antibody diluted in TBST/1% of skim milk

powder. Primary and secondary antibodies used are summarized in Table 3 along with dilutions used for each. Blots were rinsed 3 x 15 minutes. To detect the protein of interest, chemiluminescence techniques were used with ECL reagents as specified by the manufacturer. Chemiluminograms were captured on Hyperfilm ECL High Performance Chemiluminescence Film. The film was exposed from 5 seconds to 10 minutes.

g) Fluorescent Immunocytochemistry

Cultured cells were grown on coverslips in multiwell culture dishes. The cells were washed three times with warm 37°C phosphate-buffered saline (PBS) (2.7 mM KCl, 1.2 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2PO_4, pH 7.4) and fixed with cold (4°C) 3% paraformaldehyde in PBS (pH 7.6) for 15 minutes. Cells were permeabilized with cold 3% paraformaldehyde in PBS + 0.3% Triton X-100 for 5 minutes at room temperature. Coverslips were rinsed twice with cold CB buffer (MES 10 mM, NaCl 150mM, EGTA 5 mM, $MgCl_2$ 5 mM) and stored in 1X cyto-TBS (10X stock cyto-TBS; Tris base 200mM, NaCl 1.54 M, EGTA 20 mM, $MgCl_2$), in parafilm sealed chambers at 4°C for up to 1 month. Prior to labeling the fixed cells, samples were blocked by incubating in cold cyto-TBS containing 3% normal donkey serum (Cedarlane) for 30-60 minutes. The species of serum was matched to that of the secondary antibody. Prior to primary antibody application coverslips were rinsed once with cyto-TBS. Coverslips were turned face down onto ~25uL of primary antibody diluted appropriately in cyto-TBS containing 0.1% tween-20 placed on a piece of hydrophobic parafilm. Cells were incubated with primary antibody for 1 hour at room temperature. Next cells were washed three times with cyto-TBST and incubated with appropriate secondary antibodies for 2 hours at room

temperature in the dark in a humidified chamber. Thereafter, coverslips were rinsed three times with cyto-TBS. Some cells were labeled with 5 ug/mL propidium iodide to stain nuclei. After rinsing in ddH_2O coverslips are mounted cell-side down on microscope slides using ProLong Antifade Gold mounting medium. Slides were sealed with clear nail polish and stored for up to a month in 4°C before imaging with an Olympus IX70 inverted confocal microscope with Nomarski DIC optics to evaluate staining. Images were analyzed using FluoView Software.

Table 3. Antibodies and dilutions used in Western Blotting & Immunocytochemistry

Primary Antibodies (Type)	Type	Source	Cytochem. Dilut.	Western Dilut.	Blocking
anti - alpha7nAChR	Rabbit polyclonal	Santa Cruz	1:100	1:100	3% Donkey Serum
anti - SPA	Goat polyclonal	"	1:100	1:200	"
anti - SPB	"	"	1:100	1:200	"
anti - SPC	"	"	1:100	1:200	"
anti - SPD	"	"	1:100	1:200	"
Secondary Antibodies					
FITC (Green Fluorescent Dye)	Donkey-anti-Goat	"	1:100	x	"
FITC (Green Fluorescent Dye)	Donkey-anti-Rabbit	"	1:100	x	"
TR (Texas Red Fluorescent Dye)	Donkey-anti-Goat	"	1:100	x	"
HRP (Horse Radish Peroxidase)	Donkey-anti-Goat	"	x	1:1000	"
HRP (Horse Radish Peroxidase)	Goat-anti-Rabbit	"	x	1:1000	3% Goat Serum

h) [^{3}H]thymidine incorporation into DNA

The [^{3}H]thymidine incorporation protocol has been previously established by Smith and colleagues (1980). IFs were grown on multiwell culture dishes. Cells were treated with nicotine or cotinine concentrations ranging from 10^{-4} - 10^{-7} M solubilized in NCS medium for 24 hours. Incubation of cells with 1uCi of [^{3}H]thymidine was performed overnight. Medium was discarded and enough trypsin/EDTA to cover the cells was added. Cells

were placed in the incubator for 2-5 minutes and agitated until they detached from the culture surface. Thereafter NCS/MEM medium was added in a 3:1 ratio. Samples were placed in test tubes and 5 mL of absolute methanol was added before vortexing and refrigerating overnight at 4°C. Samples were centrifuged at 1000 x g for 30 minutes. Supernatants were discarded without disturbing the pellet. Pellets were resuspended in 1 mL of 2% Triton X-100 and 2% SDS and left overnight. Samples were transferred to counting vials and Beckman Ready Protein Liquid Scintillation Cocktail for protein, peptides and nucleic acids was added. Scintillation cocktail levels were subtracted to control for background. Measurements of [^{3}H]thymidine were performed on a Beckman LS 5801 Scintillation Counter using H# for quench compensation (Beckman Instruments, Palo Alto, CA).

i) MTS Formazan Cell Proliferation Assay

IFs were grown on 96-well assay plates. Cells were treated with nicotine or cotinine concentrations ranging from 10^{-4} - 10^{-7} M solubilized within MEM/10% NCS medium for 24 hours. MTS formazan reagent was thawed and 20 uL was added to each well. Incubation of the plate occurred for 1-4 hours at 37°C, 5% CO_2. Absorbances at 490 nm were recorded every hour for 4 hours using a Biorad Model 550 Microplate Reader with Biorad Microplate Manager 5.1 plate reader software.

j) Flow Cytometry

Flow Cytometric Analysis & Cell Cycle Distribution – Bromodeoxyuridine (BrdU) / Propidium Iodide (PI) Staining

Cells were grown at 37°C in MEM media until confluence was reached. Cells were rinsed using Hanks Balanced Salt Solution (HBSS) and lifted using trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05%/0.02%)). Prior to analysis the solutions were vortexed and filtered through a 70 um nylon mesh. Samples were analyzed using an EPICS Model 753 fluorescence activated cell sorter (Coulter Electronics, Hialeah, FL) equipped with a 488 nm (50 mW) line from an argon laser. Forward versus 90° light scatter histograms were used to gate on intact cells and eliminate debris. For basic cell sorting experiments, sorting parameters were set manually based on the distribution of cells observed on the bivariate light scatter histograms obtained for each sample.

To enable the detection of FITC fluorescence (emission peak ~520 nm) for the cell cycle distribution analysis, a 525 ± 10 nm band pass filter was used. PI fluorescence (emission peak ~620 nm) was determined through a 610 long pass filter. Analysis was carried out at rates between 500 and 1200 cells/sec. Peak versus integrated PI fluorescence emission signals were used for doublet discrimination gating criteria. The mean fluorescence intensities of each parameter, i.e. channel number on the abscissa (PI fluorescence) and ordinate (FITC fluorescence), were determined for cell clusters seen in bivariate histograms.

k) Measurement of Intercellular Free Calcium ($[Ca^{++}]_i$)

Mobilization of free $[Ca^{++}]_i$ was measured using fura-2 loaded cells grown in 8-well chamber dishes (Labtek, Nalge Nunc International, Naperville, IL). Cells were loaded

with fura-2 by incubation in HBSS/0.1% BSA/10uM fura-2 AM for 1 hour at 37°C. Cells were washed with 37°C HBSS/0.1% BSA and left in the dark at RT for an additional 30 minutes in order to de-esterify and activate the fluorescent molecules. Cells were then mounted on an Olympus IX70 inverted microscope equipped with an Olympus-LSR Calcium ion digital system, including an Olympix Interline 12-bit CCD camera and UltraView computer software. An Olympus x20/0.75 objective was used for all studies. Cells were alternately exposed to excitation light for 100-400 msec using 340 and 380 nm, with Sutter Filter system; emitted fluorescence (510 nm) was acquired for ~400 msec at each excitation wavelength and used to calculate calcium concentrations (nM) from an *in vitro* calibration curve of known free Ca^{++} (0 to 1.35 uM) that had been determined for the system. During experiments an equal volume of buffer containing twice the desired concentration was pipetted into the chambers to ensure rapid mixing in the chamber. In experiments, fetal cells were exposed to nicotine 10^{-5} M, an acetylcholine receptor agonist. Alpha-7 subunit antagonist α-bungarotoxin 10^{-9} M was used to block stimulation of the homomeric α7 nicotinic receptors. Calcium responses were analyzed using Ultraview software and compared between ~50 individual cells per well.

Calcium Calibration Procedure

To make quantitative assessment of data from Ca^{++} studies, a calibration procedure was used for the calcium system. This allowed for the conversion of light detected by the camera as gray scale ratios into $[Ca^{++}]_i$ in mM. For calibration the Molecular Probes (F-6774) Fura-2 Calcium Imaging Calibration Kit was used which allowed the measurement

of known concentrations of calcium mixed with fura-2. These data were manually imported into the Ultraview software thus enabling the calculation of calcium concentration for any given ratio obtained within the maximum and minimum calibration sample concentrations.

l) Single Cell Gel Electrophoresis or Comet Assay – Alkaline Method

AECs and IFs were grown in 75 cm^2 plastic culture flasks with appropriate media and treatments as described previously. Cells were removed, counted and resuspended in 1X PBS (Ca^{++} and Mg^{++} free) at concentrations of 1 x 10^5 cells/mL. Protocols were followed according to Trevigen CometAssay™ Reagent Kit for Single Cell Gel Electrophoresis Assay Instruction Manual (Trevigen, Inc., MD). Pre-warmed microcentrifuge tubes were filled with molten agarose at 37°C to keep it from solidifying during processing. Cells were added at 1 x 10^5/mL to the molten LMAgarose (37°C) at a ratio of 1:10 (v/v) and immediately 75 uL was pipetted onto a CometSlide™. The pipette tip was used in a circular motion to spread the agarose/cells over the sample area to ensure complete coverage. After the agarose solidified for approximately 30 minutes in the dark at 4°C, the slides were immersed in prechilled Lysis solution (2.5 M sodium chloride, 100 mM EDTA, pH 10, 10 mM Tris base, 1% sodium lauryl sarcosinate, and 1% Triton X-100) at 4°C for 45 min in the dark. Slides were removed from the buffer and submerged in freshly prepared alkaline solution (NaOH, 200 mM EDTA, dH_20, pH >14) at RT for 30 min in the dark to allow DNA unwinding. Slides were placed in a horizontal gel apparatus equidistant from the electrodes and equilibrated with alkaline running buffer (300 mM NaOH, 1 mM EDTA, pH 13) before electrophoresing at 27V/300 mA for 25

min at 4°C in darkness. Slides were removed, dipped several times in ddH_20 and dehydrated in 70% ethanol for 5 minutes and air-dried overnight. SYBR Green with an excitation and emission spectrum of 494nm/521nm respectively allowed visualization of fixed nuclear material as comets. Slides were stored for up to 2 weeks in 4°C before imaging with an Olympus IX70 inverted confocal microscope with Nomarski DIC optics to evaluate staining. Coverslips were mounted on comet slides using ProLong Antifade Gold mounting medium to decrease confocal laser photo-bleaching. Images were visualized using FluoView and analyzed using CASP data analysis (Comet Assay Software Project) software. CASP Software is free of charge and can be found at website: http://free.of.pl/c/casp/index.php?contents=doc.inc&title=Documentation.

m) Transmission Electron Microscopy (TEM)

Cells exposed to nicotine or cotinine 10^{-4} – 10^{-7} M for 24 hours were fixed in 2% buffered gluteraldehyde followed by post-fixation in 1% osmium tetraoxide for examination by electron microscopy. Samples were dehydrated in a graded ethanol series, transferred to propylene oxide and embedded in Epon 812 resin. Blocks were cured at 60°C for 24 hours. Thin sections were cut, placed on nickel grids and viewed in a Phillips CM10 electron microscope.

RESULTS

FETAL RAT PULMONARY PRIMARY CELL CULTURES

Established cell cultures including a fetal pulmonary epithelial culture and an interstitial fibroblast culture were used in these studies. A stripped medium (10% newborn calf serum spun down with carbon) and differential adherence technique was used to isolate pulmonary epithelial cells in culture. The second fibroblast cell type was grown in 10% newborn calf serum. It is advantageous to study specific lung cell types to enable us to tease out the cell type specific mechanisms that are occurring in the lung. In this study both cell types were used to assess DNA damage but only characteristics regarding the fibroblast component will be assessed in further detail. Primary cells isolated from gestational day 22 fetal rat lungs are shown in Figure 1A. Cultured fetal cell types included alveolar epithelial type II cells (AECs) and occasional mesenchymal interstitial fibroblasts (IFs). Cultured cells were grown to confluence over a period of five days. AECs are cuboidal in appearance and generally occur in circular clusters (Figure 1B). Mesenchymal interstitial fibroblasts (IFs) are elongated cells growing parallel to one another and forming circular bundles surrounding the AEC clusters. An enlarged image of a gestational day 22 fetal rat pulmonary AEC culture cluster suggests direct contact with mesenchymal IFs (Figure 1B). To verify that the cells within our culture system were in fact AECs, cell specific expression of surfactant protein A (SPA) and its overall distribution within individual cells *in vitro* is shown in Figure 2A. Antibody localization occurred as circular fluorescent arrangements evenly distributed throughout the cytoplasm and suggested association with specific intercellular organelles. Western

analysis of AEC exposed to nicotine or cotinine (10^{-4} and 10^{-5} M) suggests that expression levels of SPA appeared to be slightly increased (Figure 2B). Most prominent increases occurred at nicotine or cotinine levels of 10^{-5} M. Fluorescent cross-reactivity using propidium iodide and a red (CY5) conjugated secondary antibody occurs in gestational day 22 fetal rat lung AECs and IFs (Figure 1C).

EFFECTS OF NICOTINE OR COTININE ON FETAL RAT PULMONARY EPITHELIAL-MESENCHYMAL CELLS

COMET ASSAY or SINGLE-CELL GEL ELECTROPHORESIS (SCGE)

Single-cell gel electrophoresis (SCGE) or comet assay was the method utilized to evaluate DNA damage in cultured epithelial alveolar type II and mesenchymal interstitial fibroblast cells. An example of the images generated by SCGE from cells exposed to nicotine (Figure 3A) or cotinine (Figure 3B) are shown. Quantitative analysis of DNA damage was determined by measuring the tail length (TL) and tail moment (TM) using the Comet Assay Software (CASP) Analysis program. CASP allowed us to assess and interpret comet tail shape and migration pattern data using defined parameters and are shown in Figure 4G. Tail length (TL) is defined as the distance of DNA migration from the middle of the nuclear core to the end of the DNA tail and it is used to evaluate the extent of DNA damage. Tail moment (TM) is the product of tail length and the percentage of DNA in the tail of the total DNA (Olive et al., 1990; Ashby et al., 1995; De Boeck et al., 2000). In other words the Comet Tail Moment measurement involves

(Comet Tail Distance x Intensity) / Tail Length reflects a combined and overall measurement of DNA damage.

The denatured and cleaved DNA fragments migrated from the right of the nucleoid under the influence of an electric field as represented in Figures 3A and 3B. Undamaged DNA migrates slower and largely remains in the confines of the nucleoid when a current is applied as shown in Figure 4A and 4D. Twenty four hour exposure to nicotine or cotinine was done at concentrations of 10^{-4} M – 10^{-7} M. Untreated fetal AECs displayed DNA confined to the nucleoid as represented in Figure 4A. Fetal AECs exposed to nicotine (Figure 4B) or cotinine (Figure 4C) at concentrations of 10^{-4} M displayed increased levels of DNA damage as visualized by the extent of the comet tails compared to the control samples (Figure 4A). Conversely exposure of mesenchymal interstitial fetal fibroblasts to nicotine or cotinine at concentrations of 10^{-4} M – 10^{-7} M did not result in any alterations in the migration patterns of DNA from the nucleoid (Figures 4E and 4F) compared to control samples (Figure 4D).

Comet Analyses

Quantitative analyses of the SCGE results were done using CASP software (Figure 4G). Random pictures were generated from confocal TIFF images and saved as individual files. Each file was imported into CASP and all comets in the field were analyzed. For each treatment image 3 replicates were used to generate 3 random files from which 30-50 comets were analyzed.

Nicotine or cotinine at concentrations of 10^{-4} or 10^{-5} M did not alter TL and TM in IFs (Figure 5A and 5B). Conversely, TII AECs induced significant changes in fetal AEC TL and TM compared to control ($p < 0.05$) (Figure 5A and 5B). Tail Length reflects the migration of small DNA fragments from the nucleoid (De Boeck et al., 2000). Cotinine had a significantly greater effect on TL and TM compared to nicotine treated fetal AECs.

FLOW CYTOMETRY

Cell Culture Light Scatter Distributions

Flow Cytometric cell distribution analyses was used to display that populations of alveolar epithelial type II cells and fibroblasts co-exist in our cell culture system. Cultured pulmonary alveolar epithelial type II cell and mesenchymal interstitial fibroblast cells were analyzed by flow cytometry and the bivariate histograms are displayed in Figures 7A & B respectively. The separated epithelial and mesenchymal cell cultures showed different distributions as measured by flow cytometric forward (cell size) and side light scatters (granularity). Within each culure, two clusters of cells were designated as either Type A (relatively low forward and 90° light scatter) or Type B (relatively high forward and 90° light scatter). Three populations of cultured fetal pulmonary cells identified include the interstitial fibroblasts represented by low light scatter, alveolar epithelial type II cells represented as having high light scatter and an intermediate population of some form of differentiated cell type displaying medium amounts of light scatter. The red areas represented as Type B cells (~14%) in Figure 7A are considered to be lamellar body containing fetal type II alveolar epithelial cells as they display larger

cell sizes and higher levels of granularity. The green areas delineated by rectangles represented as Type A cells (~90%) are thought to contain the majority of the fibroblast population showing small cell size and low granularity (Figure 7B). Upon 24 hours exposure to nicotine or cotinine at concentrations of 10^{-4} M fetal rat interstitial fibroblast distributions within the gating parameters were altered (Figures 8B & C) compared to the untreated samples (Figures 8A). The distribution of cells with high light scatters increased from ~7% to ~30% upon nicotine treatment and increased from ~7% to ~31% with exposure to cotinine (Figures 8B & C).

NICOTINIC ACETYLCHOLINE RECEPTORS

Expression

Using antibody to the α7-nicotinic receptor (α7-nAchR) nicotinic protein expression was identified within cultured AECs and mesenchymal IFs as shown in Figure 6. Punctuate staining was observed evenly distributed throughout the cytoplasm of the AECs and elongated IFs isolated from the fetal lung Figure 6B. The fetal rat AEC receptor protein appears to be constitutively expressed and expression levels do not change after 24 hours of exposure to nicotine or cotinine at concentrations of 10^{-4} M and 10^{-5} M (Figure 6C). Rat brain was used as a positive control to the α7-nAChR. Additionally, a total adult lung cell lysate was used to verify expression within lung. Fetal rat mesenchymal IF receptor expression levels were similar to those of the AECs (Figure 6D).

Nicotinic Receptor Functionality using Fura-2 Calcium Indicator Dye

Fluorescent ratio-metric calcium indicators are routinely used to identify a role of calcium in cell metabolism and regulation. Fura-2 is the dye of choice for ratio-imaging microscopy, where the excitation wavelengths can be changed (Silver, 1998). Upon binding calcium, fura-2 exhibits an absorption shift that is observed by scanning the excitation spectrum between 300 and 400 nm, while monitoring the emission at ~510 nm. Fura-2 is preferred to other indicator dyes due to its resistance to photo-bleaching (Becker et al., 1987), its reactivity at physiological calcium concentrations (~100 nm), and its high selectivity for calcium binding relative to Mg^{2+} (Grynkiewicz et al., 1985). In our study fluorescently labeled Fura-2 AM $[Ca^{2+}]_i$ mobility dye was used to determine functionality of α7 nAChRs. Fetal pulmonary epithelial-mesenchymal (E/M) cells were loaded with FURA-2 fluorescent dye for 1 hour and visualized by the Perkin Elmer Ultraview Software shown in Figure 9A. Fetal pulmonary E/M cells displayed increased intracellular calcium levels upon nicotine stimulation (10^{-7} M) (Figure 9B). Cells were loaded with FURA-2 for 1 hour and pre-treated with the α7-specific antagonist α-bungarotoxin for 10 minutes at a concentration of 10^{-9} M. Cells were again exposed to nicotine. Intracellular calcium Fura-2 mobility was not observed (Figure 9C). Cells used in the experiments were stimulated with acetylcholine (Ach) or Bradykinin (BK) (10^{-7} M) and the calcium responses indicated that the cells were viable.

[^{3}H]THYMIDINE INCORPORATION

The [^{3}H]thymidine incorporation is used as a measure of DNA synthesis levels by determining the amount of radioactively labeled thymidine incorporated into the cells during the S phase of the cycle. The effects of nicotine or cotinine on [^{3}H]-thymidine

incorporation into DNA and MTS formazan formation by isolated fetal rat pulmonary mesenchymal interstitial fibroblasts are shown in Figures 10 and 11 respectively. These cells were grown in medium containing 10% newborn calf serum (NCS). Nicotine 10^{-4} treatment DNA radiolabelling in Figure 10 was comparable to that of the control samples. At concentrations 10^{-5} - 10^{-7} M nicotine decreased levels of the radiolabelling of DNA (Figure 10). Cotinine inhibited thymidine incorporation at 10^{-7} M but increased levels at 10^{-4} M (Figure 11). At concentrations central to this study (10^{-4} and 10^{-5} M) no significant changes in [^{3}H]-thymidine incorporation after exposure of fetal rat lung interstitial fibroblasts to nicotine or cotinine were found ($p < 0.05$). This suggests that the rate of DNA synthesis was not affected in the interstitial population of cells entering the S phase.

FORMAZAN ASSAY

We used the formazan (MTS) assay to examine the ability of cells to convert a tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] into formazan as a measure of mitochondrial activity (Promega, Ontario, Canada). The formazan assay measures cellular mitochondrial dehydrogenase activity within a cell as a measure of cellular viability. Formazen conversion was not significantly altered upon exposure to nicotine suggesting no changes in mitochondrial activity (Figure 12) ($p < 0.05$). At all cotinine concentrations 10^{-4} - 10^{-7} M generally decreased mitochondrial activity over time (Figure 13).

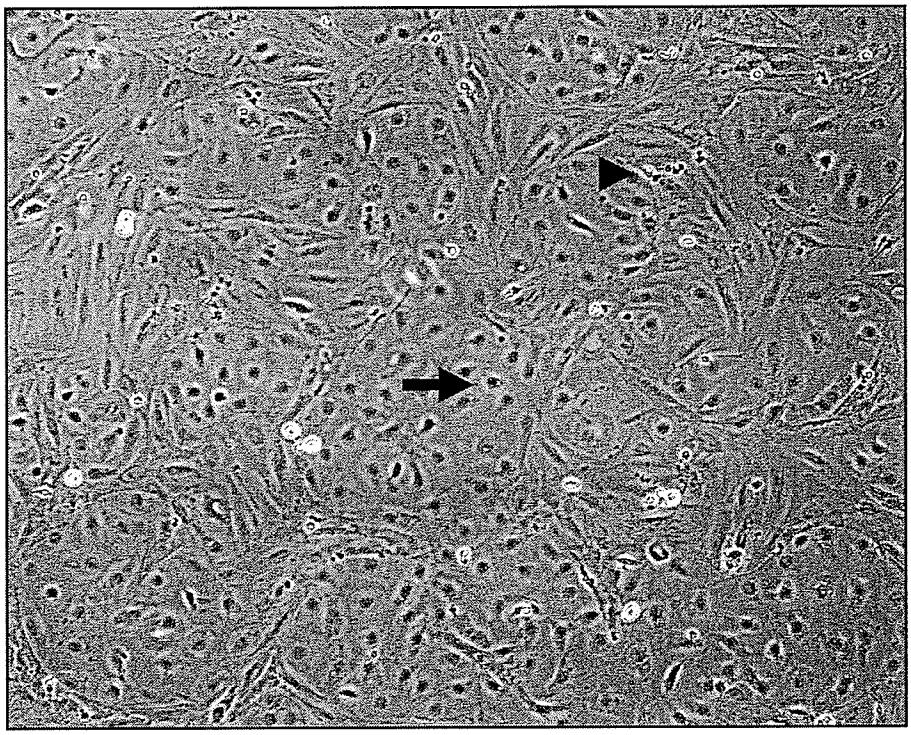


Figure 1A. Fetal rat alveolar epithelial type II cells and mesenchymal interstitial fibroblasts in primary culture. Phase contrast image of P1 type II alveolar epithelial cells or pneumocytes along with adjacent interstitial fibroblasts upon confluence (Day 3-5). Pockets of fetal type II alveolar epithelial cells are incubated in serum containing MEM media with 10% stripped carbon newborn calf serum (sNCS) inducing pneumocyte growth while restricting fibroblast overgrowth. (X20) Black arrow type II alveolar epithelial cell. Arrow head mesenchyme interstitial fibroblast.

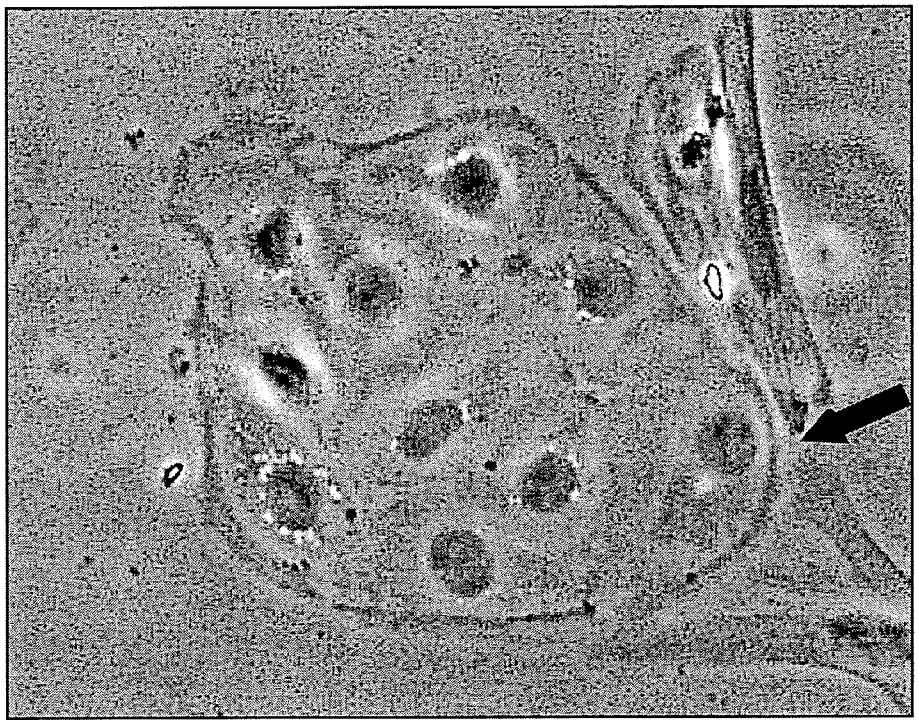


Figure 1B. Cultured fetal epithelial-mesenchymal cell clusters and cell-to-cell contacts. Phase contrast image of cultured type II alveolar epithelial cells (AECs) prior to confluence (Day 3). Cells were plated in serum containing MEM media with 10% sNCS **Arrow:** indicates a close association between alveolar epithelial type II cells and adjacent interstitial fibroblasts. (X100)

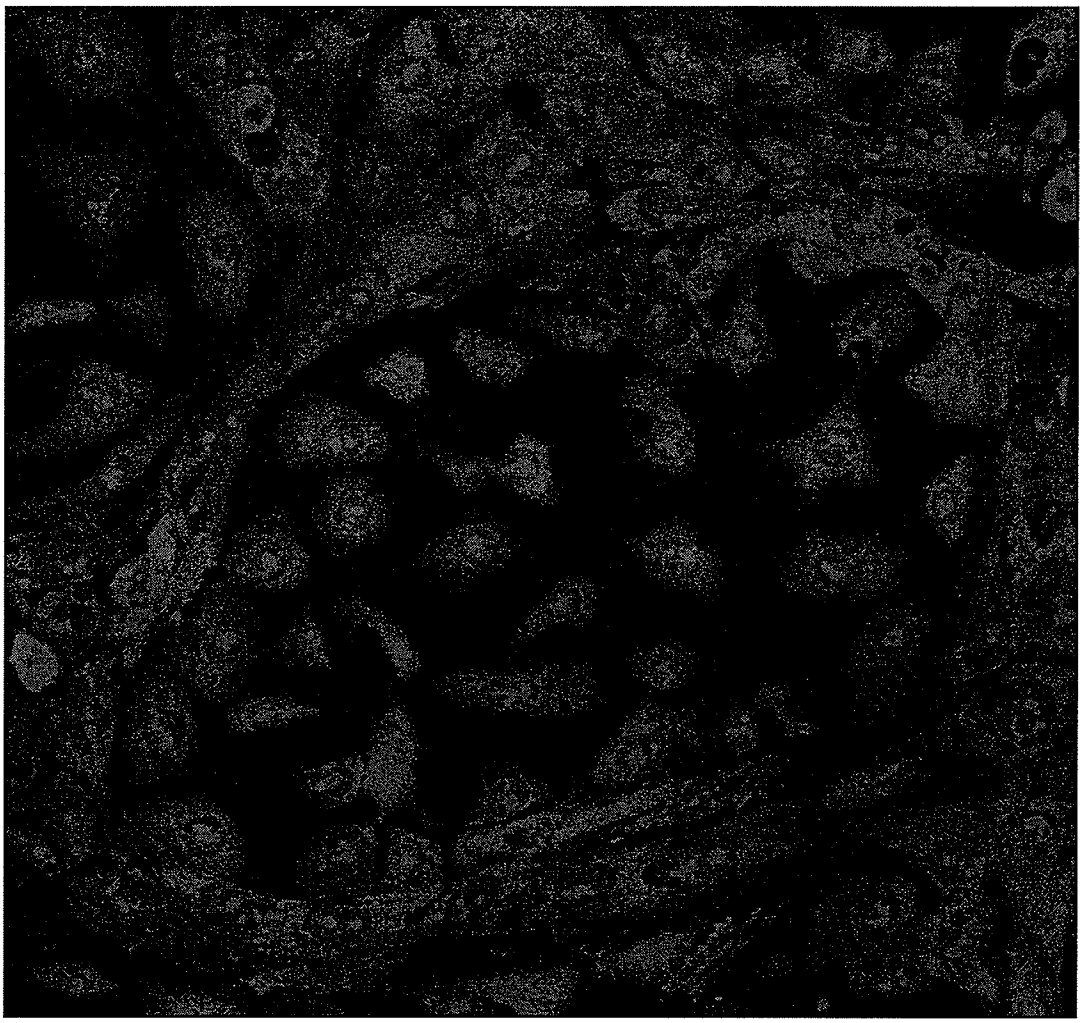


Figure 1C. Fluorescent fetal rat epithelial-mesenchymal organization in culture. Immunofluorescent image taken with a confocal microscope showing red (Cy5) immuno-staining for surfactant proteins A and B. Primary antibodies to SP proteins, secondary antibodies and their concentrations are outlined in Materials and Methods. (X20)

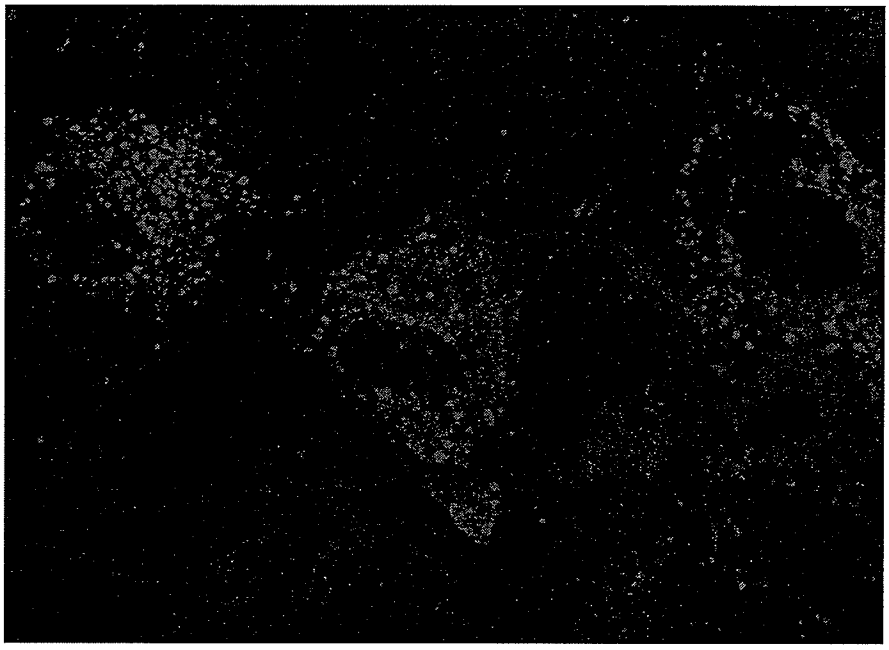


Figure 2A. Surfactant protein A (SPA) expression in cultured fetal rat alveolar type II cell or pneumocytes using Immunocytochemistry. Confocal image displaying the pattern of distribution of SPA in fetal type II alveolar epithelial cells stained using red fluorescence. SPA is localized to specific intracellular organelles. (X100 oil)

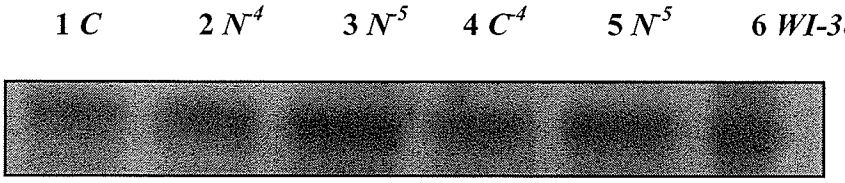
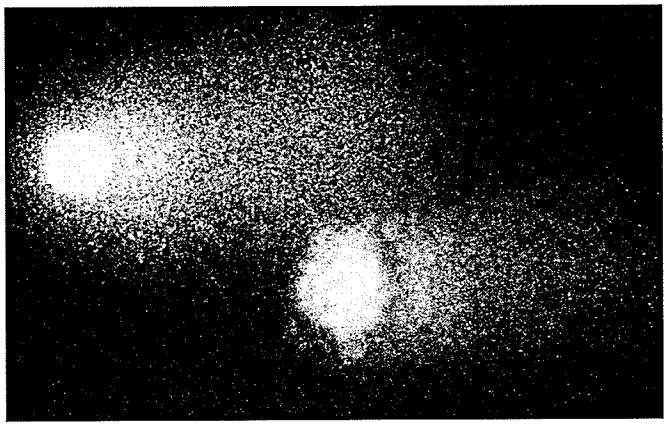


Figure 2B. SPA expression upon exposure to nicotine or cotinine using western blot analysis. Western blot analysis of cultured fetal rat AECs following treatment with nicotine or cotinine (10^{-4} and 10^{-5} M) for 24 hours. Upon reaching confluence crude cell lysates were harvested using RIPA lysis buffer. Primary antibodies to SPA, secondary antibodies and their concentrations are outlined in Materials and Methods. Chemilumigrams were developed on Hyperfilm-ECL. **Lane 1** - control levels **Lane 2 and 3** – nicotine 10^{-4} and 10^{-5} M treatments **Lane 4 and 5** – cotinine 10^{-4} and 10^{-5} M treatments **Lane 6** – WI-38 total adult lung cell lysate (positive control)

3A



3B

Figure 3. DNA damage in cultured fetal rat pulmonary alveolar epithelial type II cells using the Single-Cell Gel Electrophoresis (SCGE) or Comet assay. Immunofluorescent images using confocal microscope generated SPEC1 and SPEC2 coloring to clearly represent comet DNA stained using SYBR green fluorescence. The cultured fetal rat lung AECs shown in Figure 3A were treated for 24 hours with nicotine while Figure 3B shows cells exposed to cotinine both at concentrations of 10^{-4} M. Fluoview software and an Olympus IX70 inverted confocal microscope were used. (X100 oil)

Figure 4. Immunofluorescent confocal images of "comets" and "tails" contain SYBR green fluorescent stain to visualize and quantify all cellular DNA within nucleoid bodies and fragments. (X10)

(A) Control – Cultured fetal rat pulmonary alveolar type II pneumocytes in sNCS medium for 24 hours shows minimal DNA migration.

(B) Cultured fetal rat pulmonary alveolar type II pneumocytes in sNCS medium containing nicotine 10^{-4} M for 24 hours induced the formation of "comets" and shows DNA migration from the nuclear core.

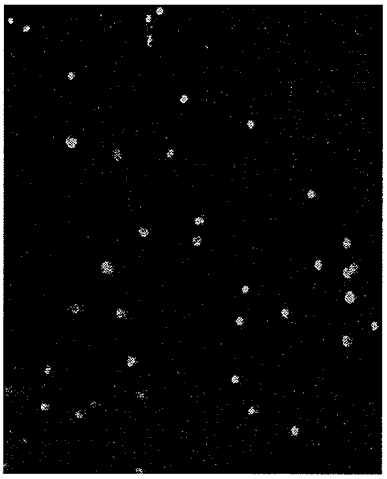
(C) Cultured fetal rat pulmonary alveolar type II pneumocytes in sNCS medium containing nicotine metabolite cotinine 10^{-4} M for 24 hours also induced "comet" formation.

(D) Control – Cultured fetal rat pulmonary interstitial fibroblasts in NCS medium for 24 hours shows minimal DNA migration.

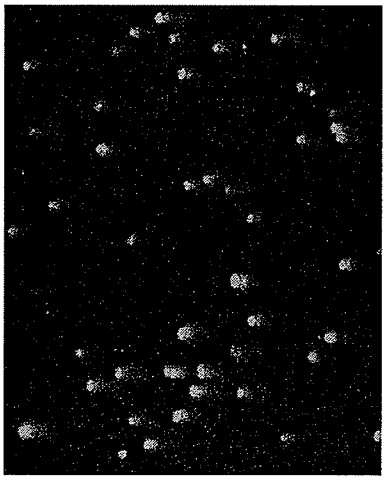
(E) Cultured fetal rat pulmonary interstitial fibroblasts in NCS medium containing nicotine 10^{-4} M for 24 hours did not induce "comet" formation

(F) Cultured fetal rat pulmonary interstitial fibroblasts in NCS medium containing cotinine 10^{-4} M for 24 hours did not induce "comet" formation.

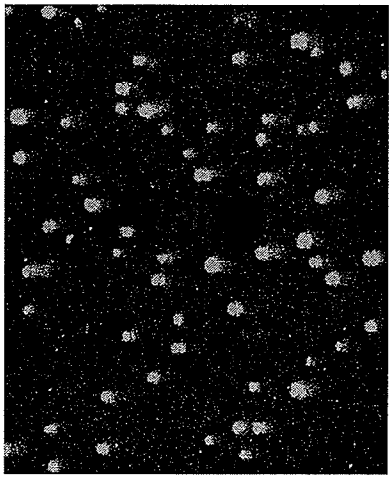
4A



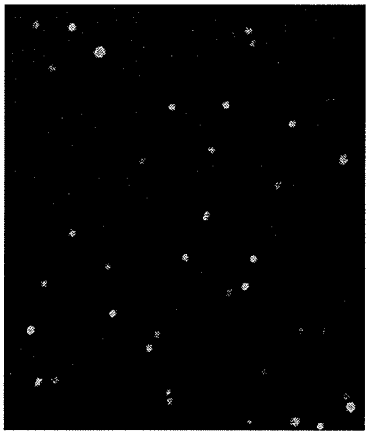
4B



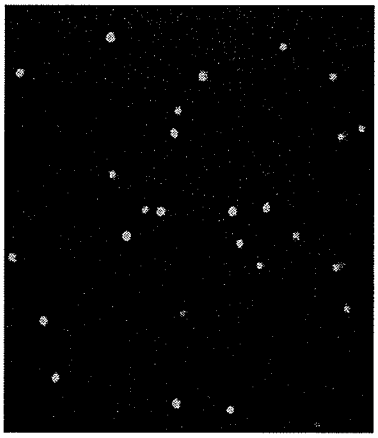
4C



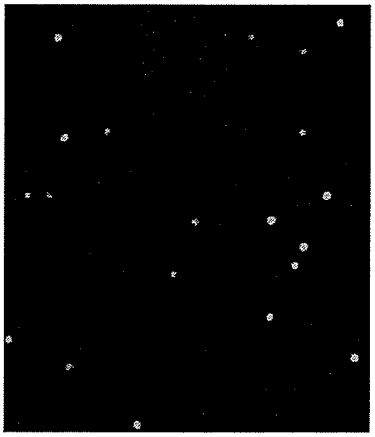
4D



4E



4F



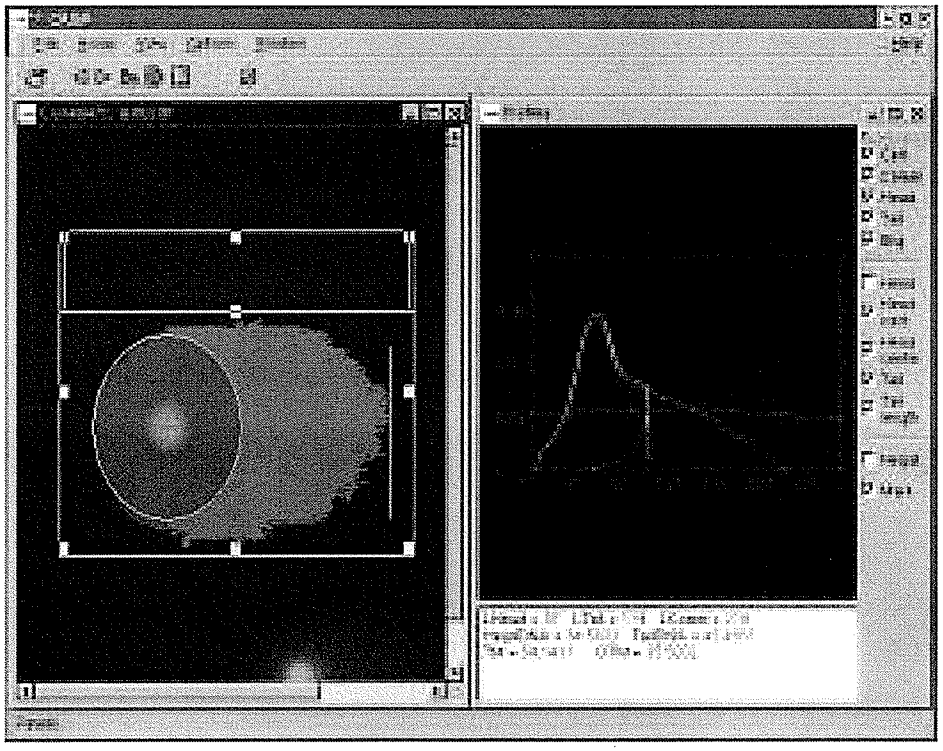


Figure 4G. CASP Software Analysis Program CASP Software Analysis Program Display showing a selected comet (left) and data analysis of the comets selected parameters (right).

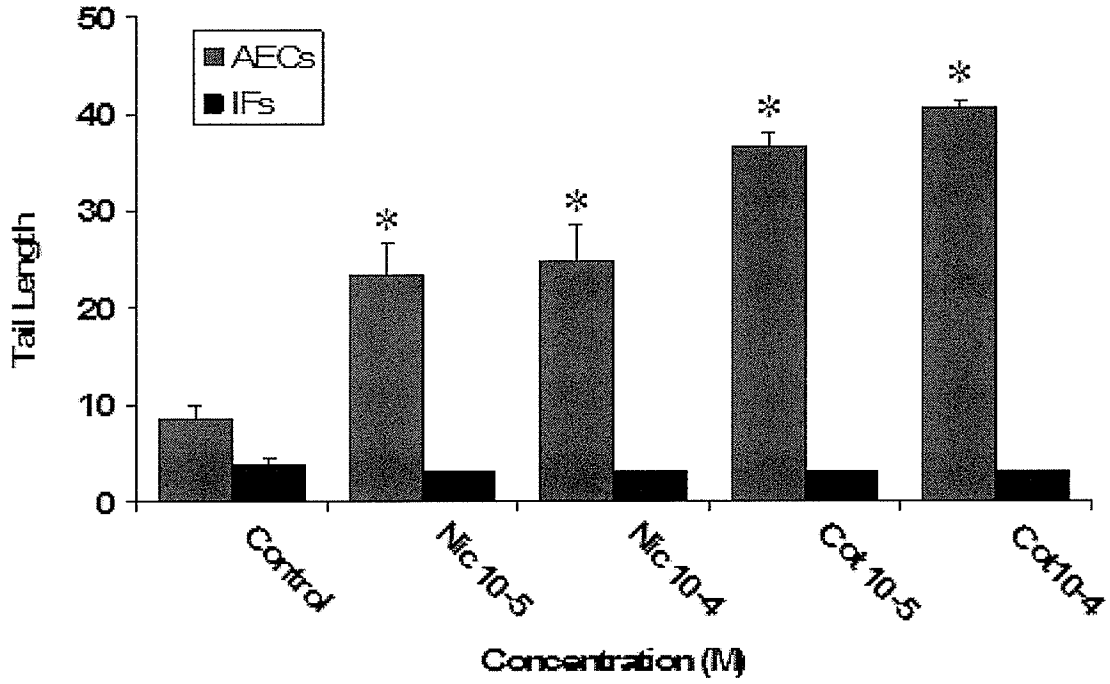


Figure 5A. Effect of nicotine or cotinine exposure on fetal rat epithelial and interstitial mesenchymal fibroblast Comet Tail Length. Comet tail length (number of pixels) of fetal rat alveolar type II cells and interstitial fibroblasts following exposure to nicotine or cotinine at concentrations of 10^{-4} and 10^{-5} M for 24 hours. CASP software was used to determine mean tail length. Each bar represents the mean ± standard error of the mean of a minimum of 40 comets. Experiments were repeated in triplicate and significant differences between epithelial control and treatments ($p<0.05$) are indicated (*).

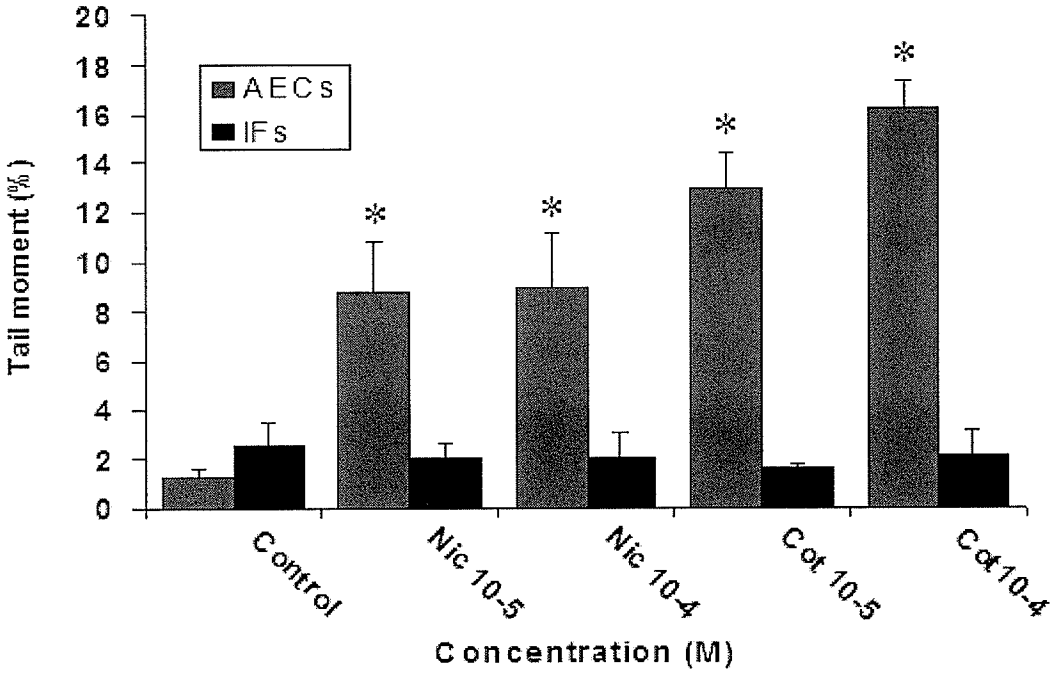
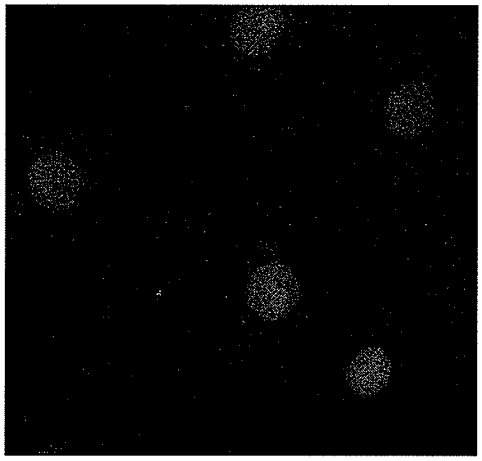


Figure 5B. Effect of nicotine or cotinine exposure on fetal rat epithelial and interstitial mesenchymal fibroblast Comet Tail Moment. Comet tail moments of isolated fetal rat alveolar type II cells and interstitial fibroblasts after exposure to nicotine or cotinine at concentrations of 10^{-4} and 10^{-5} M for 24 hours. Each bar represents the mean ± standard error of the mean for a minimum of 40 comets. Experiment was repeated in triplicate and significant differences ($p<0.05$) are indicated (*) compared to control cell samples not exposed to the smoke constituent or metabolite.

6A



6B

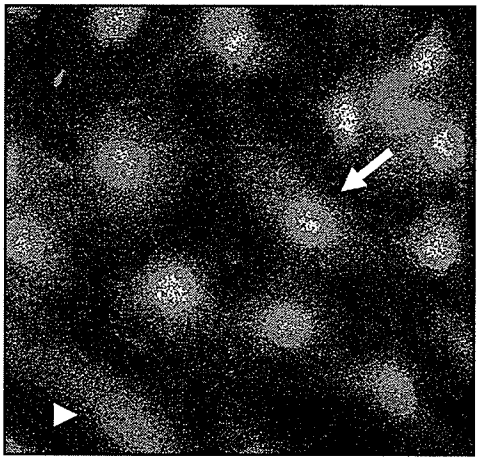


Figure 6. Alpha-7 nicotinic acetylcholine receptor (nAChR) expression in fetal pulmonary epithelial-mesenchymal cells. Figure 6A. Immunocytochemical confocal image of negative control sample containing secondary antibody only. Red fluorescence of propidium iodide (PI) was used as the nuclear stain. (X40 oil immersion)
Figure 6B. Confocal image of alpha-7 nAChR expression in cultured fetal rat pulmonary epithelial-mesenchymal cells. FITC-labelled antibody to the α7-nAchR was used to visualize receptor subunit expression. Punctate staining appears to be evenly distributed throughout the cytoplasm and on the cell surface of the fetal alveolar type II cells (arrow) and interstitial fibroblasts (arrow head). (X40 oil immersion)

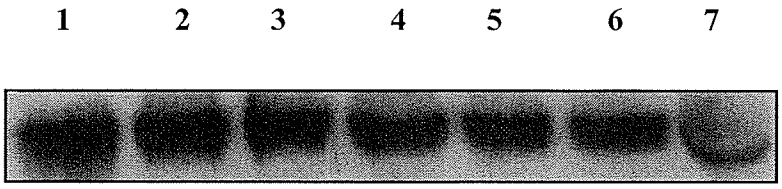


Figure 6C. Alpha-7 nicotinic acetylcholine receptor expression in fetal rat alveolar type II cells (AECs) after exposure to nicotine or cotinine using western blot analysis. Western blot analysis of α-7 nAChR expression in cultured fetal rat type II alveolar epithelial cells after 24 hours exposure to nicotine or cotinine. Expression levels were unchanged after 3, 6 & 12 hours of exposure at concentrations of 10^{-4} - 10^{-7} M (data not shown). Primary and secondary antibodies and concentrations used are outlined under Materials and Methods. Chemilumigrams were developed on Hyperfilm-ECL. **Lane 1** – rat brain (positive control), **Lane 2** – untreated AEC's, **Lane 3 & 4** - nicotine 10^{-4} and 10^{-5} M, **Lane 5 & 6** - cotinine 10^{-4} & 10^{-5} M, **Lane 7** – total lung cell lysate

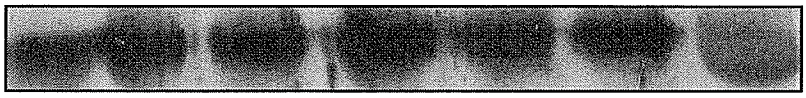
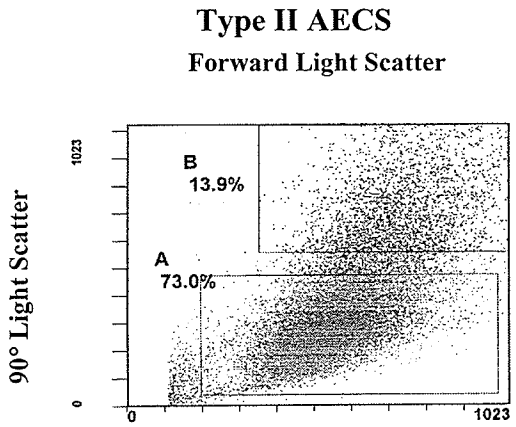


Figure 6D. Alpha-7 nicotinic acetylcholine receptor expression in fetal rat pulmonary mesenchymal interstitial fibroblasts (IFs) after exposure to nicotine or cotinine using western blot analysis Western blot analysis of α-7 nAChR expression in cultured fetal rat mesenchymal interstitial fibroblasts cells after 24 hours exposure to nicotine or cotinine. Expression levels are similarly unchanged upon 3, 6 and 12 hour exposures at concentrations of 10^{-4} - 10^{-7} M (data not shown). Primary and secondary antibodies and concentrations used are mentioned under Materials and Methods. Chemilumigrams were developed on Hyperfilm-ECL. **Lane 1** – rat brain (positive control), **Lane 2** – untreated IFs, **Lane 3 & 4** - nicotine 10^{-4} & 10^{-5} M, **Lane 5 & 6** - cotinine 10^{-4} & 10^{-5} M, **Lane 7** – total lung cell lysate

7A



7B

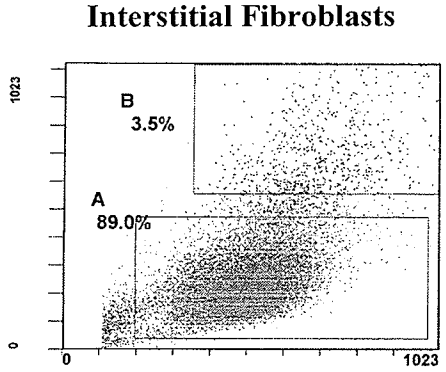
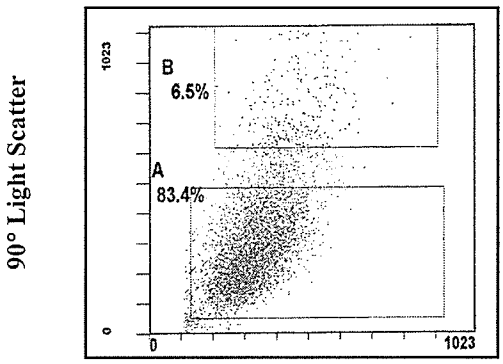


Figure 7. Flow Cytometric distribution of cultured fetal rat pulmonary alveolar epithelial type II cells and mesenchymal interstitial fibroblast cells using flow cytometric forward and side light scatter. Figure 7A. Cultured fetal rat pulmonary type II alveolar epithelial cell distribution using forward light scatter (cell size) on the x-axis and side light scatter (cell granularity) on the y-axis. Rectangle B includes the alveolar type II cells and rectangle A includes alveolar type II cells along with mesenchymal interstitial fibroblasts.

Figure 7B. Cultured fetal rat pulmonary interstitial fibroblast distribution using forward light scatter (cell size) on the x-axis and side light scatter (cell granularity) on the y-axis. Rectangle A represents interstitial fibroblasts and rectangle B represents alveolar type II cells

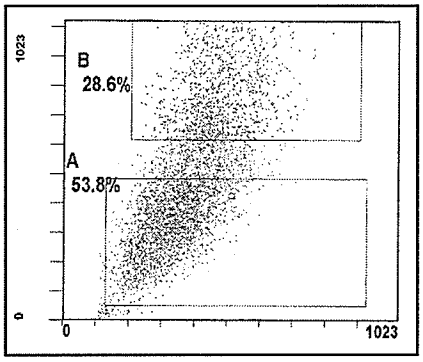
8A

Control IFs



8B

Nicotine 10^{-4} M



8C

Cotinine 10^{-4} M

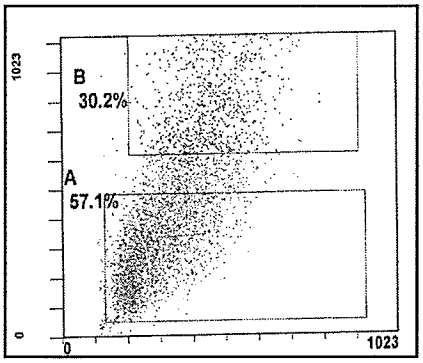


Figure 8. Flow cytometric analysis of cultured fetal rat interstitial fibroblasts upon exposure to nicotine or cotinine.

Figure 8A. Untreated fetal rat pulmonary interstitial fibroblast distribution using NCS medium for 24 hours.

Figure 8B. Fetal rat pulmonary interstitial fibroblast distribution upon 24 hour exposure to nicotine 10^{-4} M.

Figure 8C. Fetal rat pulmonary interstitial fibroblast distribution upon 24 hour exposure to cotinine 10^{-4} M.

9A

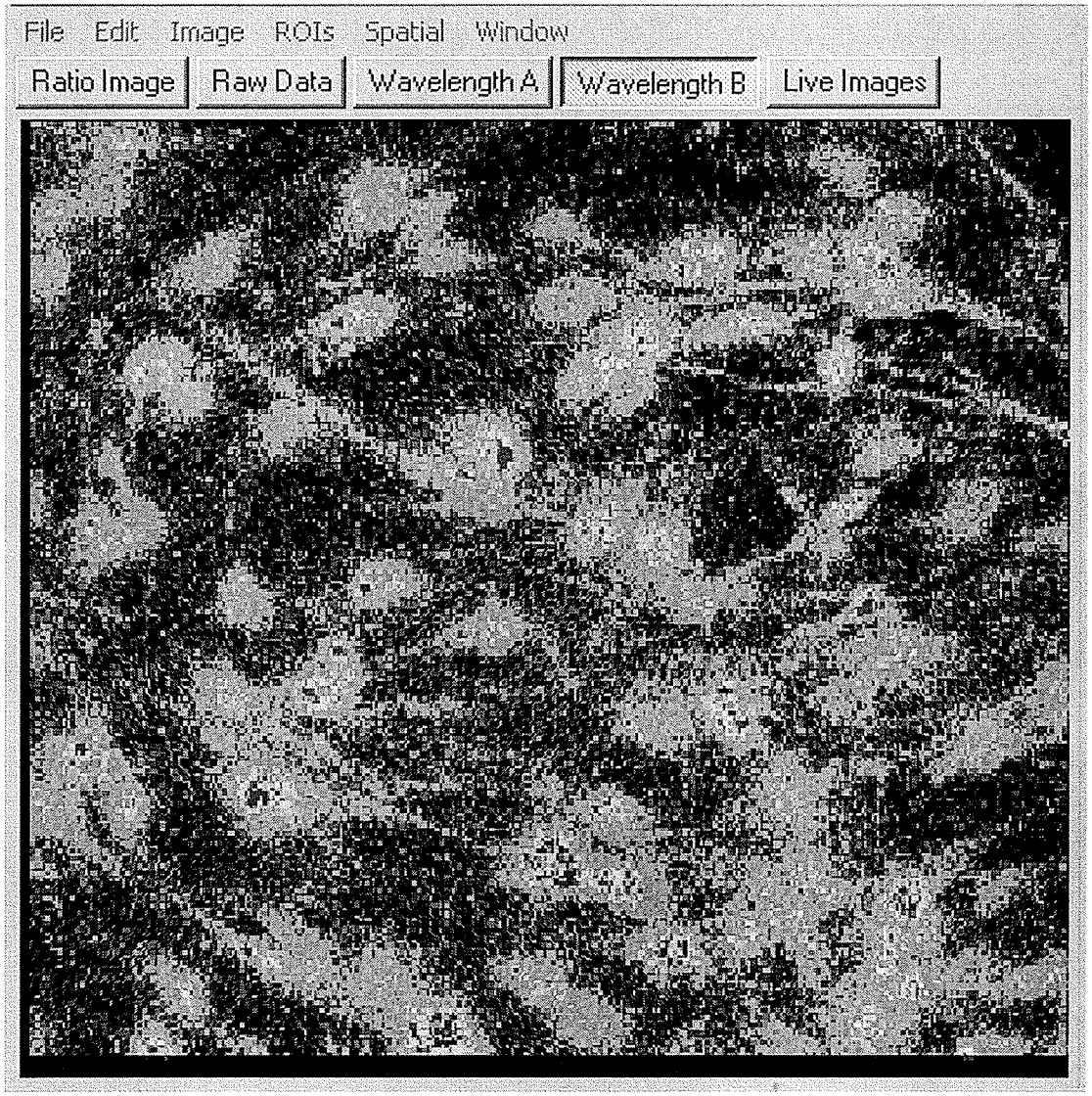


Figure 9. Fura-2 fluorescent calcium mobility assay in cultured fetal pulmonary epithelial-mesenchymal cells.
Figure 9A. Fluorescently loaded cultured fetal pulmonary epithelial-mesenchymal cells using 10 uM of Fura-2 AM visualized using Perkin Elmer Ultraview Software.

9B

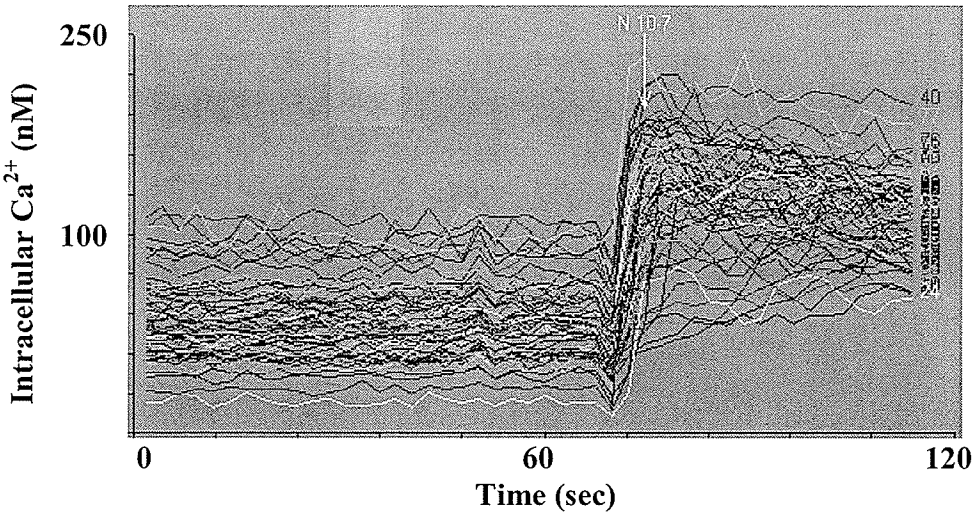


Figure 9B. Effect of nicotine on the levels of intracellular calcium in cultured fetal epithelial-mesenchymal cells. Cells were loaded with 10 uM Fura-2 AM 1 hour before nicotine. Nicotine 10^{-7} M produced an increase in $[Ca^{2+}]_i$. Each colored line represents $[Ca^{2+}]_i$ within a single cell. All cells in each field were stimulated.

9C

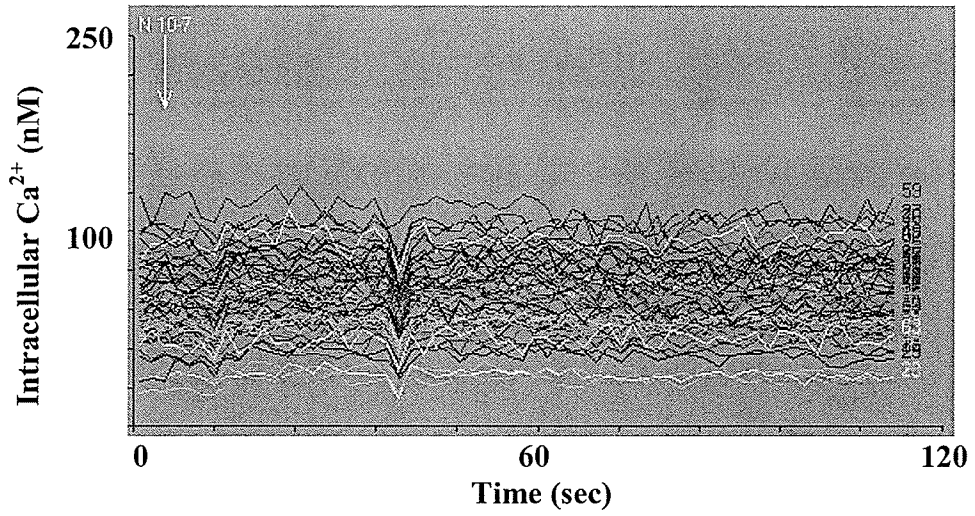


Figure 9C. Effect of a nicotinic antagonist on the levels of intracellular calcium in cultured fetal epithelial-mesenchymal cells. Cells were loaded with 10 uM fura-2 AM for 1 hour before nicotine. Pretreatment with α-bungarotoxin at a concentration of 10^{-9} M for 10 minutes inhibited the nicotine-induced increase in $[Ca^{2+}]_i$. Each colored line represents $[Ca^{2+}]_i$ within a single cell. Cells were stimulated post-experimentally with acetylcholine (ACh). All cells were shown to respond in order to determine if cells were viable.

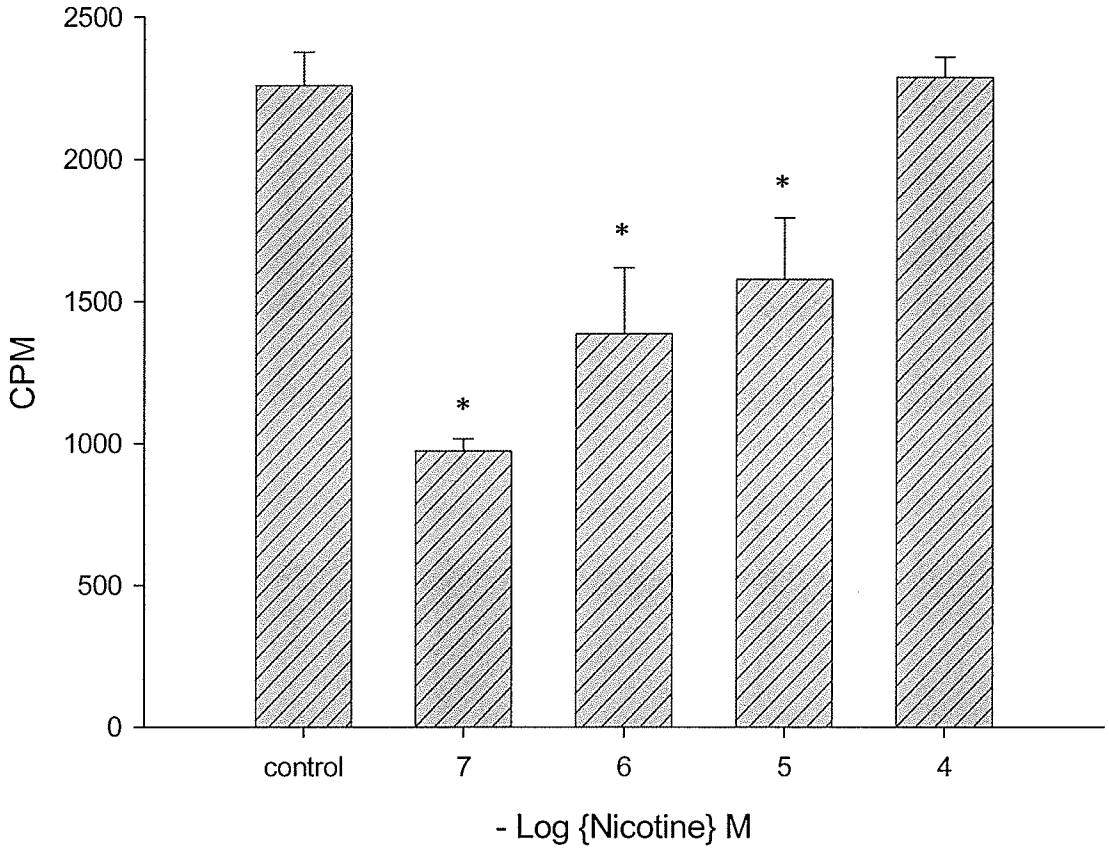


Figure 10. Cultured fetal pulmonary mesenchymal interstitial fibroblast [^{3}H] thymidine incorporation upon exposure to nicotine. [^{3}H]-thymidine incorporation by fetal pulmonary mesenchymal interstitial fibroblast cells measuring levels of DNA synthesis in control samples and those exposed to nicotine concentrations $10^{-4} \rightarrow 10^{-7}$ M for 24 hours. All cells were grown in medium containing 10% NCS. Scintillation counts were obtained using the method of H number (Beckman LS501, Palo Alto, CA) for quench compensation. Results are expressed as mean disintegrations per minute +/- SD for 3 replicates. Significant differences ($p<0.05$) are indicated (*).

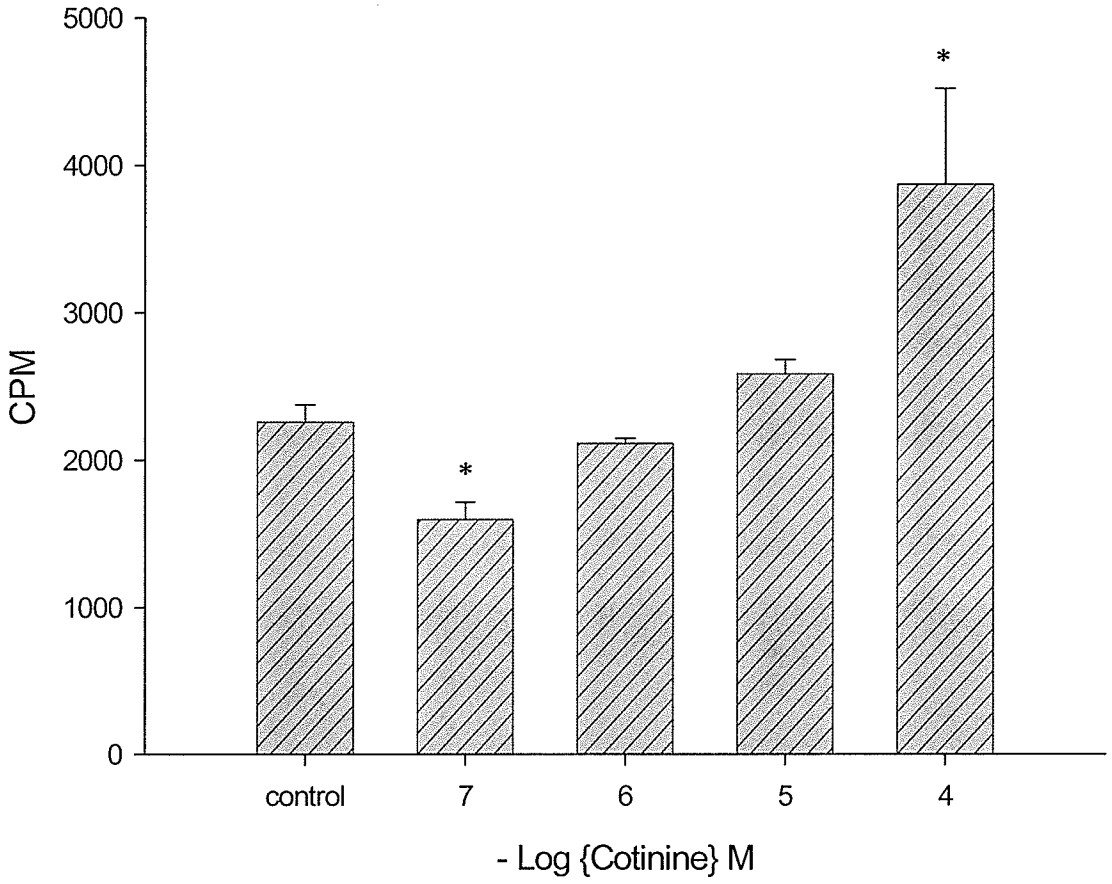


Figure 11. Cultured fetal pulmonary mesenchymal interstitial fibroblast [^{3}H] thymidine incorporation upon exposure to cotinine. [^{3}H]-thymidine incorporation by fetal pulmonary mesenchymal interstitial fibroblast cells measuring levels of DNA synthesis in control samples and those exposed to cotinine concentrations 10^{-4} - 10^{-7} M for 24 hours. Scintillation counts were obtained using the method of H number (Beckman LS501, Palo Alto, CA) for quench compensation. Results are expressed as mean counts per minute +/- SD for 3 replicates. Significant differences ($p<0.05$) are indicated (*).

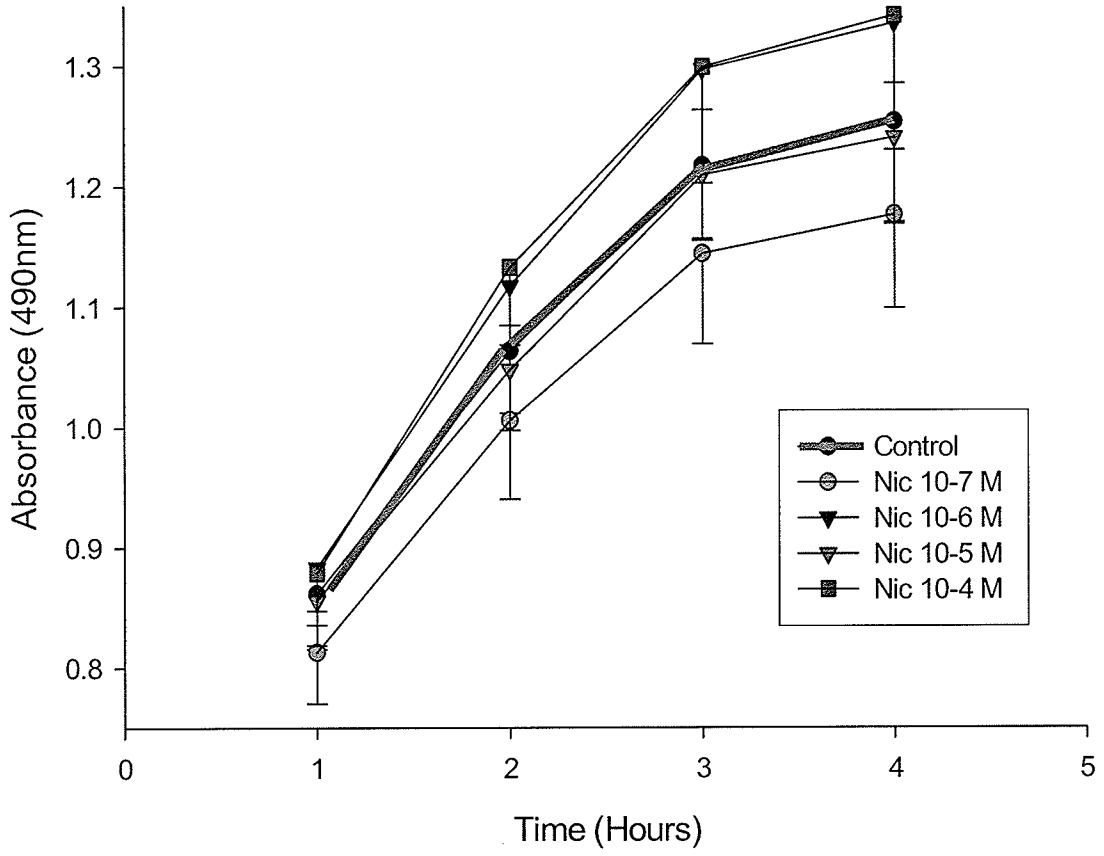


Figure 12. Nicotine treated fetal interstitial fibroblast mitochondrial activity. Absorbance values at 490 nm reflecting mitochondrial dehydrogenase activity obtained using the MTS formazan assay in isolated fetal rat lung interstitial fibroblast cells following exposure to nicotine at 10^{-4} - 10^{-7} M for 24 hours. Absorbance values were obtained after 1, 2, 3 and 4 hours using the Bio Rad Model 550 microplate reader. Results are expressed as mean absorbance values +/- SD for 24 replicates at each time point. No significant differences were found ($p>0.05$).

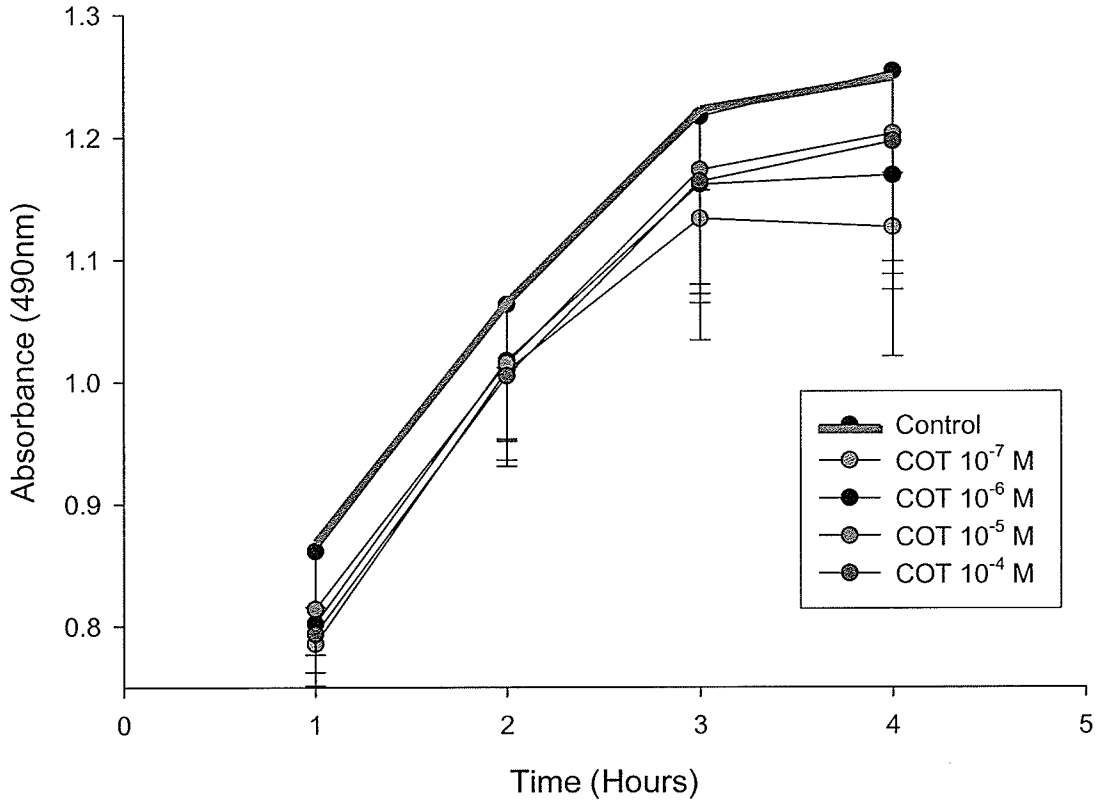
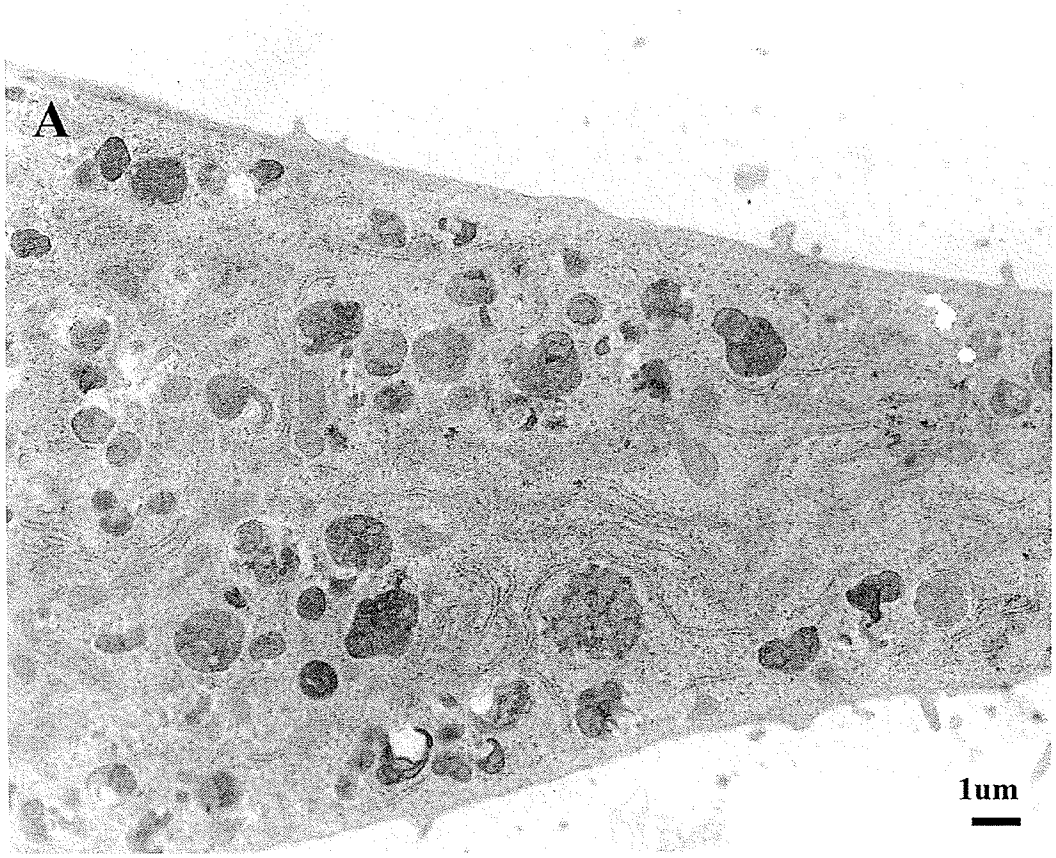
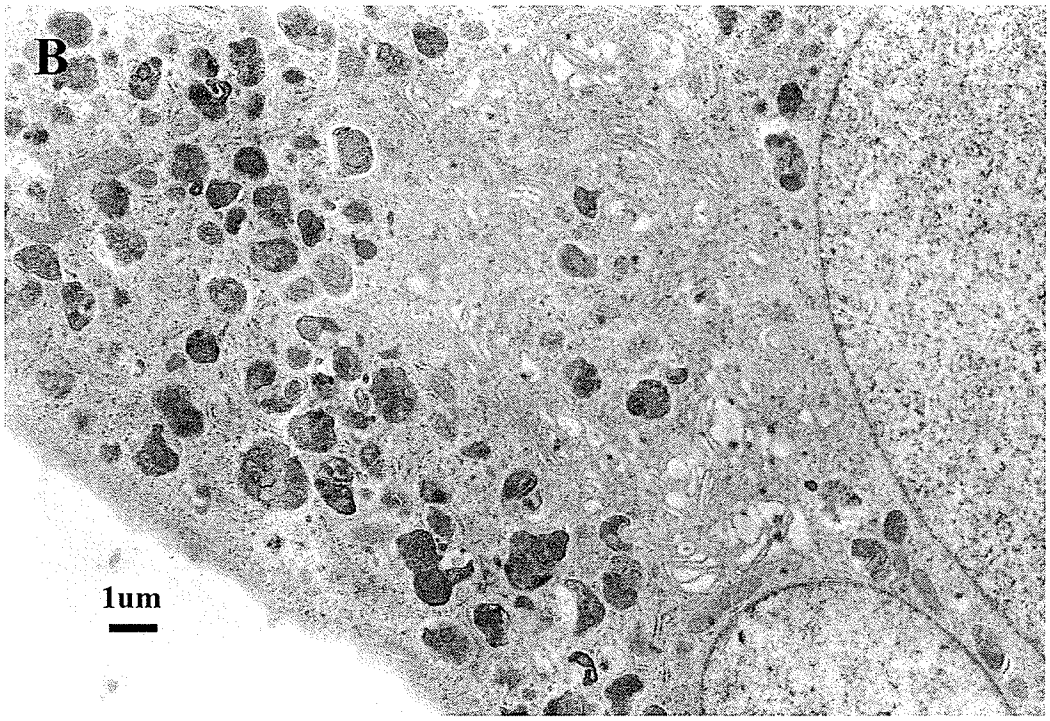


Figure 13. Cotinine treated fetal interstitial fibroblast mitochondrial activity. Absorbance values at 490 nm reflecting mitochondrial dehydrogenase activity obtained using the formazan assay in isolated fetal rat lung interstitial fibroblast cells following exposure to cotinine at 10^{-4} - 10^{-7} M for 24 hours. Absorbance values were obtained after 1, 2, 3 and 4 hours using the Bio Rad Model 550 microplate reader. Results are expressed as mean absorbance values +/- SD for 24 replicates at each time point. No significant differences were found ($p>0.05$).

Figure 14A - C. Electron Microscopic images of cultured fetal interstitial fibroblasts. Cells contained numerous membrane-bounded secretory granules similar in appearance to developing lamellar bodies as seen in adult type II cells. All cells had well developed organelles, such as rough endoplasmic reticulum, Golgi membranes, and mitochondria as shown in A, B and C. Administration of nicotine or cotinine at concentrations of 10^{-4} – 10^{-7} M (data not shown) seemed to have very little effect on morphological appearance or on numbers of organelles and secretory bodies in the fibroblasts after 24 hour exposures.




B
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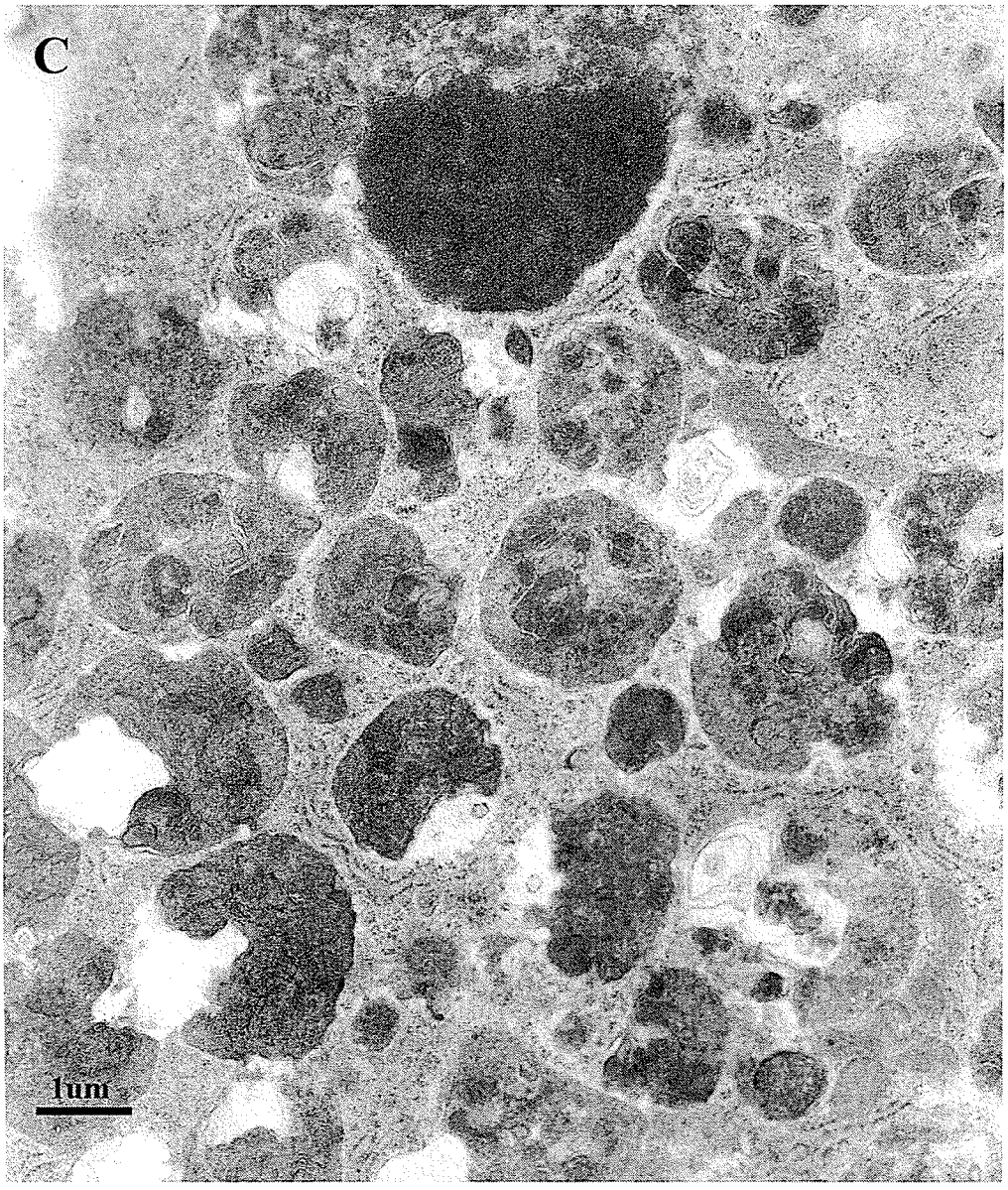
C
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DISCUSSION

It has been estimated that approximately 1.1 billion people smoke worldwide (900 million men and 200 million women). This number is expected to rise to more than 1.6 billion by the year 2020. The World Health Organization (WHO) estimates 10 million tobacco-related deaths yearly by the early 2030s, and based on current smoking trends, tobacco use will soon become the leading cause of morbidity and mortality in the world (Health Canada, 2005). Tobacco smoke contains approximately 2000 – 4000 chemical agents (Robin et al., 2002), and the dangers of tobacco are magnified when smoke from tobacco is inhaled. First-hand smokers are approximately three times more likely to develop cancer than non-smokers. Cancers are widespread throughout the body and do not develop solely in areas directly exposed to tobacco smoke. Cancers are found in locations such as the oral cavity, esophagus, breast, stomach, pancreas, larynx, bladder, kidney and lung. These have been identified as directly associated with tobacco use, furthermore it is clearly established that cigarette smoking is a direct cause of lung disease. In North America tobacco is the cause of one-third of all deaths among middle-aged women. Lung cancer due to tobacco kills more women in North America each year than breast cancer. Babies born to smoking mothers have lower birth weights, face greater risks of respiratory diseases and are more likely to die of sudden infant death syndrome than babies born to non-smokers (Health Canada, 2005). Evidence suggests that smoking during pregnancy has direct and adverse effects on lung development. Research has now established that the detrimental fetal outcomes include increased risk of spontaneous abortion, stillbirth, preterm delivery, early neonatal morbidity and

mortality and abnormal pulmonary alterations all directly related to maternal smoking during pregnancy (Wen *et al.*, 1990; Hanrahan et al., 1992; Cunningham et al., 1994; Cliver *et al.*, 1995; Mathews *et al.*, 1998; Higgins et al., 2002; Gilliland et al., 2003; Hofhuis et al., 2003). Infants under the influence of smoke during pregnancy show decreased body mass (weight), reduced lung function and show an increased risk of the development of childhood asthma and other severe respiratory illnesses. Exposure to tobacco smoke components during pregnancy may play a much greater role in altered lung function than postnatal or childhood exposure (Tager et al., 1995). Further studies are clearly required in order to elucidate the complex mechanisms of the prenatal or fetal developing lung.

PRENATAL OR FETAL RAT PULMONARY CELL CULTURES

Epithelial-mesenchymal (E/M) interactions are well-established inducers and mediators during embryonic development and function as important mechanisms of normal lung development and injury/repair signaling pathways (Demayo et al., 2002). Cooperative interactions such as these are required for prenatal lung growth, cellular replication, and differentiation (Shannon et al., 2001). Our system of study shown in Figure 1 (A, B, C) contains fetal rat AECs and adjacent IFs isolated during the prenatal saccular/canalicular stage of lung development, a time when the development of the pulmonary surfactant system and its surfactant is most critical. Presence of fibroblasts impacts the surface tension properties of the lung via type II alveolar epithelial cells in synthesizing both surfactant proteins and phospholipids. The surfactant system and efficient surfactant

secretions are crucial in order to decrease the large surface tensions imposed on the lung at birth. Presence of fibroblasts impacts the ability of TII AECs to differentiate and synthesize both surfactant proteins and phospholipids (Rannels et al, 1989; Torday et al., 1995; Shannon et al., 2001). Additionally, extracellular matrix (ECM) composition beneath the alveolar epithelium has dramatic effects on the differentiation state of AECs in conjunction with a combination of fibroblast growth factors (FGFs) (Mason et al., 2001). Disruption in cell numbers or intimate associations of these cell types during lung development may lead to lung damage, decreasing lung function and increasing incidences of respiratory illnesses. Smoking during pregnancy impairs development of fetal lungs resulting in altered lung function within children of smoking mothers. The increased frequency of lower respiratory illnesses and reduced lung function correlate well with prenatal, but not postnatal maternal smoking (Tager et al., 1993). This suggests an *in utero* effect of smoke constituents or metabolites on epithelial-mesenchymal interactions in the fetal lung. To our knowledge we are the first to analyze the effects of the major bioactive components of cigarette smoke, nicotine and its major long-acting metabolite, cotinine, on fetal pulmonary epithelial-mesenchymal cells. Our cultures contain both type II alveolar epithelial cells with their expression of surfactant proteins localized to specific intracellular organelles, the lamellar bodies (Figure 2A) and interstitial fibroblasts, which display an elongated morphology, thus enabling us to replicate *in vivo* interactions and study developmental E/M cellular mechanisms *in vitro*.

NICOTINE OR COTININE EFFECTS ON FETAL PULMONARY TISSUES

In vivo, fetal lung may be most affected by the damaging influences of tobacco smoking as it is dually exposed to smoke derived compounds through the circulation and from accumulated compounds in the amniotic fluid. Nicotine, the major bioactive component of tobacco smoke, is implicated in adverse effects of tobacco smoke on lung structural development (Maritz et al., 2002; Spindel et al., 2002) although the specific effects have not been established. Nicotine is highly soluble in water, and an aqueous layer lines the lung and the bronchial surfaces (Lindell et al., 1993). Nicotine in plasma is quickly metabolized with a half-life of 2 to 3 hours (Benowitz et al., 1996). Nicotine readily crosses the placental barrier, accumulating in the amniotic fluid where it is detectable at concentrations ranging from nM to uM (Luck et al., 1985). In addition, slower amniotic fluid removal results in nicotine and other metabolites to become concentrated thus resulting in prolonged fetal exposure beyond the direct maternal smoking period. In fact, concentrations are higher in amniotic fluid than those found in maternal blood. The ability of maternal nicotine to cross the placenta allows it to bind and modulate nicotinic acetylcholine receptors found within fetal developing lung cells (Maus et al., 1998; Sekhon et al., 1999, 2001, 2002, 2004; Fu et al., 2003; Spindel, 2003). Another transferable smoke component is cotinine, a long-acting nicotine metabolite found in smoke-exposed individuals. Cotinine levels are about 10 times higher than those of nicotine although its biological activity is about tenfold less (Benowitz et al., 1983). In addition to pregnant women that smoke, exposure to Environmental Tobacco Smoke

(ETS) also contributes to amounts of cotinine and nicotine exposure of the developing fetus.

Nicotine administration to pregnant monkeys appears to alter fetal lung development causing reduced structural complexity and surface area of the alveoli (Sekhon et al., 1999). Chen and colleagues (2005) showed that nicotine exposure during pregnancy may reduce lung surfactant lipids and produce variable changes of surfactant protein gene expression at different stages of lung development. In the present study surfactant protein A (SPA) levels appeared to be increased slightly upon nicotine or cotinine treatment when compared to control samples suggesting that type II alveolar cell surfactant-related functions may be altered (Figure 2B). Nicotine has both inhibitory and stimulatory effects on surfactant gene expression that appear to be related to the stage of lung development (Chen et al., 2005). Thus, changes within pulmonary surfactant composition will lead to altered surface tension within the lung producing improper lung expansion and deflation at the onset of respiration. Chen's data suggest that nicotine exposure *in utero* has the potential to alter the programming of future lung development resulting in susceptibility to lung dysfunction and diseases in later life.

NON-NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR EXPRESSION IN FETAL PULMONARY TISSUES

Non-neuronal nicotine acetylcholine receptors (nAChRs) exist in fetal animal lung models such as monkey used by Sekhon et al. (1999), hamster used by Fu et al., (2003) and others have used lamb. Similar to these models of development we have established

that nAChRs exist within our pulmonary rat E/M culture system (Figure 6). Activation of nicotinic receptors in prenatal tissues seems to be cell system dependent. Chronic nicotine exposure leads to receptor inactivation in some systems (Olale et al., 1997; Quick et al., 2002; Fu et al., 2003) causing a lack of functional cell surface nicotinic receptors. Maus and colleagues (1998) suggest that this desensitization is related to bronchial dissociation facilitating further entry of carcinogens present in tobacco smoke into the lung. Similar effects may be occurring in the lung of the fetus where alveolar type II cells become impaired presenting additional carcinogens to the adjacent interstitial fibroblasts in the lung. Maus and colleagues suggest an alternate explanation where hyper-stimulation of nicotinic receptors causes excessive intracellular Ca^{2+} leading to cell degeneration and calcium-mediated cell toxicity. Our DNA damage analysis data corresponds with the idea that cell-mediated toxicity may be occurring in the epithelial portion of the fetal lung while the mesenchymal or fibroblast portion is not damaged (Figure 4 and 5). Nicotinic receptors nAChRs in pulmonary fibroblasts exposed to nicotine *in utero* induces an alteration of collagen and elastin expression in the developing lung (Sekhon et al., 2001). Our nicotine or cotinine exposed cultured E/M cells showed no significant changes in DNA synthesis (Figure 10 and 11) or cell proliferation (Figure 12 and 13) in addition to the lack of DNA damage suggests that no impairment or cell-mediated toxicity occurred. Roman and colleagues (2004) results support the contention that connective tissue is altered as nicotine appears to stimulate fibronectin expression in lung fibroblasts. The increased fibronectin gene transcription is thought to occur via activation of alpha-7 nAChR which begins a cascade of intracellular signals. This suggests downstream alterations in lung architecture including such

tobacco-induced disease alterations as abnormal lung extracellular matrix composition. Schuller and colleagues have suggested that upregulation of components of the nicotinic receptor pathway in smokers may be an important factor in the progression of small cell lung carcinoma (SCLC). More recently, expression of the alpha-7 nicotinic acetylcholine receptor has been shown not only in an animal model but more importantly in human lung cells (Schuller et al., 2003; Anat Rec/Schuller et al., 2005).

ALPHA-7 NICOTINIC ACETYLCHOLINE RECEPTOR

In the developing lung the α7 is the most abundant form of nAChR found in airway, vessel, alveolar epithelial and associated alveolar wall cells (Sekhon et al., 1999) which includes interstitial fibroblasts. Nicotine administration to the mother increases expression of alpha-7 subunit binding in fetal lung. This suggests that maternal nicotine interacts with nicotinic receptors in the lung of the developing fetus affecting proper lung development (Sekhon et al., 1999). Immunohistochemical localization of the α7-nicotinic subtype was detected in fetal monkey lung and similarly we found these receptors in our AECs and adjacent IFs using Immunocytochemistry (Figure 6B). Recent identification of the expression of α7-nicotinic acetylcholine receptors in human lung cells by Plummer and colleagues (2005) suggest important roles in both normal lung cells and lung cancer cell lines. Nicotinic receptor expression in fetal monkey lungs after nicotine administration to the mother increases expression of α7-nAChR binding in fetal lung (Sekhon et al., 1999). Our results indicate constitutive expression of α7-nAChR over a 24 hour period with no changes in protein expression levels (Figure 6C and 6D). This result

may reflect the requirement and importance of a sustained period of nicotine exposure in the fetal lung cells to increase α7 nAChR receptor levels. Animal models use prolonged exposure to nicotine which could not be replicated in our system due to the four to five day window of growth of our epithelial or AEC component. Alpha-7 nicotinic receptors in particular demonstrate a high permeability for Ca^{2+} (Gopalakrishnan et al., 1995).

NICOTINIC RECEPTOR ACTIVATION

Calcium influx is a hallmark of the activation and opening of the nAChR ion channel (Carlisle et al., 2004). We determined nicotinic receptor functionality in our culture system by loading cells with fura-2 fluorescent calcium molecules to measure intracellular calcium mobility (Figure 9A). An increase in intracellular calcium from the extracellular fluid occurs by a direct influx of calcium through ligand-gated nAChR channels (Seguela et al., 1993; Michelmore et al., 2002). Nicotinic receptors in our system were considered functional as determined by the fura-2 mobility study increases in intracellular Ca^{2+} (Figure 9B). Nicotinic receptor antagonist alpha-bungarotoxin (α-BGT) blocked all nicotine-induced fetal cultured epithelial-mesenchymal intracellular calcium responses (Figure 9C) suggesting that responses were nicotinic receptor specific. This functional expression of fetal nAChRs in the lung is in agreement with work done by Fu and colleagues showing the expression of functional nicotinic receptors in neuroepithelial bodies of neonatal hamster lung (Fu et al., 2003). Further studies are required to delineate patterns of nAChR activation during various stages of fetal lung development.

NICOTINE OR COTININE EFFECTS ON FETAL PULMONARY E/M DNA: COMET ASSAY OR SINGLE-CELL GEL ELECTROPHORESIS (SCGE)

Smoking effects DNA within certain cells. Cigarette smoke components have various injurious effects on AECs including: suppression of proliferation, augmentation of detachment of cells, and most applicable for our study DNA single-strand breaks in cells and suppression of surfactant secretion (Leanderson et al., 1992; Lannan et al., 1994; Wollmer et al., 1994). This suggests that the injury of AECs may contribute to the lung diseases induced by cigarette smoking. Assays to identify DNA damage in cells include DNA laddering using gel electrophoresis (Bagchi et al., 2002) although changes are not quantifiable, DNA unwinding assay utilizing fluorescence to detect double stranded DNA (Weitberg and Corvese, 1993). The traditional terminal transferase-mediated DNA nick end labeling (TUNEL) assay also detects cells containing DNA strand breaks. It could have been used however; it does not discriminate between apoptosis, necrosis and autolytic cell death and is not considered to be a specific marker of apoptosis (Grasl-Kraupp et al., 1995). We utilized another assay known as the comet assay or single-cell gel electrophoresis (SCGE), a sensitive genotoxic method used for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. The COMET assay or SCGE was chosen for its simplicity, sensitivity, speed and it quantifiability (Collins, 2004). DNA strand breaks provide a "comet tail"-like appearance to each cell upon low voltage electrophoresis (Figures 3A, 3B). Our COMET data suggests that nicotine or cotinine both induced levels of DNA damage in cultured pulmonary AECs but not in the

IF component of fetal lungs. Resistance found in the IFs play a greater role in the abnormal matrix deposition characteristic of lung disease (Roman et al., 1998; 2004) when compared to the strand breaks found in the AEC component of the lung. Parallel to Lams study (2005) on immortalized hamster check pouch (POII) cells we observed variable susceptibilities of our fetal pulmonary cells to the above mentioned tobacco-related compounds. The most inclusive measurement of DNA single strand breaks, the tail moment is defined as the length of the Comet tail multiplied by the percentage DNA in the tail. Congruent with smoke treated AECs cells (Leanderson et al., 1992; Lannan et al., 1994) our Tail Length (TL) and Tail Moment (TM) measurements indicate increased DNA damage by nicotine or cotinine in cultured AECs (Figures 5A and 5B). These smoke-derived agents have a destructive impact on the epithelial but not the mesenchymal portion of the developing fetal lung cellular genome.

FLOW CYTOMETRIC ANALYSIS

The lung is a highly complex organ consisting of many types of cells including fibroblasts, lymphocytes, macrophages, Clara cells, endothelial cells, and type II alveolar epithelial cells (Phipps et al., 1989). Our fetal pulmonary AECs and IFs were isolated and grown using a differential adherence technique as described by Batenberg et al., 1988. Isolated AEC cultures display increased in cell size and cell granularity when compared to pure cultures of IFs (Figures 7A, 7B). The AEC larger cell sizes and higher levels of granularity may be indicative of the higher levels of intracellular organelles or lamellar bodies distinctive of this particular cell type. Small cell sizes and relatively

lower levels of granularity include the majority of IFs. We consider light scatter found between these two groups an intermediate population of some form of differentiated fetal fibroblast pulmonary cell subtype. Previous works by Brody (1983), Kaplan (1985) and Maksvytis (1981) have ultrastructurally characterized three subpopulations of interstitial fibroblasts including: the lipid interstitial cell or lipofibroblast, the non-lipid interstitial cell or contractile interstitial cell and the myofibroblast. Others suggest that only one type of fibroblast exists (Spit et al., 1983). We believe that our fibroblasts are of the LIC subtype due to their high abundance during the late fetal period (Maksvytis et al., 1981) and electron microscopic images indicate pre-lamellar-like lipid pools or granules within these cells (Figures 14A, B, C). These droplets contain primarily neutral lipids, triglycerides, cholesterol and retinal esters (McGowan et al., 1995) and their abundance at this fetal period is not understood. Torday and colleagues (1997) believe these cells to be supportive cells providing nutrients to AECs for synthesis and release of phospholipid and protein rich surfactant necessary for normal lung function (Vaccaro et al., 1978). Upon exposure to nicotine or cotinine the fibroblast cellular distributions change increasing the number of cells by increasing their cell size and granularity (Figures 8A, 8B, 8C). Cells may have trans-differentiated towards another subtype of fibroblast or even towards AECs. This may occur in the same way transdifferentiation occurs upon pulmonary lipofibroblast exposure to hyperoxia (Rehan et al., 2001). This suggests that smoke constituents may allow the cells to differentiate from interstitial fibroblasts into other fibroblasts or type II alveolar epithelial cells. This may be demonstrated using flow cytometric cell sorting to sample various cell populations and visualize them under the

light microscope. The individual cell populations could be further characterized using cell specific fluorescent markers for each individual cell type.

SUMMARY

Nicotine activates functional alpha-7 nicotinic receptors in fetal pulmonary E/M cells. Nicotinic receptors may lead to the nicotine or cotinine induced cellular disruptions at the epithelial level of the alveolus. The mesenchymal interstitial fibroblast component of the lung seems to be unaffected. It is now suggested that prolonged exposure to nicotine may change function of the mesenchymal component of the fetal lung including overproduction of extracellular matrix production. Our flow cytometric and electron microscopic results suggest that fibroblasts may trans-differentiate to AECs upon exposure to nicotine and cotinine. This nicotine-induced fetal disruption of E/M cells during lung development affects the development of the surfactant system and may in part contribute to the pathogenesis of impaired pulmonary function and incidences of respiratory illnesses in children exposed to cigarette smoke *in utero*.

CONCLUSIONS

Through no choice of their own, infants of mothers who smoke during pregnancy show reduced lung function and become more prone to development of severe respiratory illnesses and disease during childhood. Despite these findings some women continue to smoke during pregnancy. Currently the complex mechanism by which smoking affects fetal development is unclear. Research studies now demonstrate that many of the effects

of smoking during pregnancy are mediated by carcinogenic compounds such as nicotine crossing the placental barrier and accumulating in the amniotic fluid. The bathing of the fetus in smoke-derived toxins specifically nicotine appears to localize to the pulmonary tissues. It has now been established that nicotine crosses the placental barrier and interacts with nicotinic receptors in the developing lung. Elucidation of mechanisms of maternal smoking alterations in prenatal lung development may provide us with a molecular approach to its prevention.

Our study is the first to show the expression of the specific α7 nicotinic acetylcholine receptor protein in fetal rat pulmonary AECs and IFs. The alpha-7 nicotinic receptor has been found in high abundance in Spindel's nicotine treated fetal monkey model. Parallel to this and other animal smoking models our results support the contention that nicotinic receptors are functional and become activated mobilizoing $[Ca^{2+}]_i$ upon nicotine stimulation. Secondly, our study shows the coexistence and intimate association of two populations of cells fetal AEC and IFs in culture at the crucial time when development of the surfactant system is initiated. This E/M culture model is an important tool in helping us to tease out specific cellular mechanisms behind abnormal fetal lung development. Further studies are required to characterize this model. Thirdly, our results indicate that exposure of fetal E/M cells to the tobacco smoke constituent nicotine and its metabolite result in structural impairments and increased DNA damage in the fetal AEC cell component of the alveolus. Nicotine or cotinine exposed mesenchymal or IF cells appear to be unaffected structurally or at the cellular DNA level. Our thymidine and formazan results indicate that DNA synthesis of IFs is not affected by these smoke components.

Taken together our results provide evidence for cell-type specific impairment within the fetal lung strongly suggesting that the function of one cell type may be inhibited (AECs) while the other enhanced (IFs). Nicotine-induced changes to fetal lung AECs will affect the synthesis and secretion of surfactant compromising the main function pulmonary surfactant system. The disruption in cellular function may in part contribute to the altered pulmonary mechanics and increased incidences of respiratory related illnesses and disease found in infants.

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