Effects of Maternal Nicotine Exposure on Expression of Laminin Alpha 5 in Lung Tissue of Newborn

Mahdi Shariati Kohbanani, Mohammad Reza Nikravesh, Mehdi Jalali, Alireza Fazel, Mojtaba Sankian and Alireza Ebrahimpazheh Bidokhan

1Department of Anatomy and cell biology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
2Instructor of Department of Anatomy, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
3Bu-ali Research Institute, Immunology Research Center, Mashhad, Iran

Abstract: Maternal smoking has been clearly demonstrated to be associated with increased health problems in infants and children. Nicotine is the chemical substance with high level of toxicity. It crosses through the placenta and accumulates in the developing organs of fetus. Previous investigation indicated that maternal nicotine exposures induce decreased fibronectin expression in lung parenchyma. In this study, the effect of maternal nicotine exposure on laminin expression of the newborn mice lungs has been evaluated. Female pregnant Balb/C mice were divided randomly into four groups as follow: Experimental group 1 (Exp D1); was received 3 mg kg\(^{-1}\) nicotine intra peritoneal injection (IP) from gestational day 7 (GD7) to the last day of pregnancy, Experimental group 2 (Exp D14); was received 3 mg kg\(^{-1}\) nicotine from GD7 to postnatal day 14, Groups 3 and 4; as sham control groups (Sha-Con) were received the same volume (3 mg kg\(^{-1}\)) of normal saline parallel to experimental groups. At the end of exposure times, all of newborns were anesthetized; their lungs were removed and prepared for immunohistochemical method and real-time polymerase chain reaction. The finding indicated that laminin alpha 5 (Lama5) mRNA expressions in the lungs of newborn in the nicotine treated Exp D1 decreased by 0.63 fold but increased in Exp D14 by 1.57 fold comparing to Sha-Con groups. Lama5 immunoreactivity was not similar in different parts of the lungs including alveoli and bronchiole, having a significant increase in the experimental groups in contrast to the Sha-Con groups. However, increase in immunoreactivity observed more in Exp D14. Immunoreactivity intensity in small vessels of all experimental groups was not significantly different. These data also indicate that maternal nicotine exposure may induce abnormal laminin expression which may cause defects in lung function during life time.

Key words: Laminin, lung, nicotine, newborn, immunohistochemistry

INTRODUCTION

Maternal smoking has been associated with pregnancy complications, including intra uterine retardation (IUGR), fetal and neonatal death, spontaneous abortion and premature delivery (Haafström et al., 2005; Wickstrom, 2007). Nicotine is the causative agent for these effects, because it is a major pharmacological constituent of tobacco that easily crosses the placenta and is concentrated in the fetus to a higher level than the mother (Chen et al., 2005). The lung has important role as a gas exchanger in survival of the breathing organism. The lungs development occurs in uterus and is prepared to function at birth time but similar to other mammals, final stages of its development do not complete until birth. Disturbance in developmental stages of lung may affect its maturation and resistance to diseases in future life (Sekhon et al., 2004; Wasowicz et al., 1998; Wasowicz et al., 1996). Investigation in animal models showed that maternal nicotine exposures cause a variety of effects on neonatal lungs including, significant suppression of alveolarization (Maritz, 1988; Maritz and Dennis, 1998), abnormal collagen IV expression and defect in bronchopulmonary development (Jalali et al., 2010), decreased expression of fibronectin (Kohbanani et al., 2012) and decreased elastin staining of lung parenchyma (Pierce and Nguyen, 2002). Extracellular matrix (ECM) composition is essential for morphogenesis and
differentiation of virtually all tissues (Gullberg and Ekblom, 1995). Basement membranes are distinctive extracellular matrices having essential roles in tissue organization and development. Components common to all basement membranes include laminin, type IV collagen, entactin/nidogen and sulfated proteoglycans (Kruegel and Mioenge, 2010). Among the matrix molecules found, laminins are glycoprotein’s that mediate adhesion and signaling through integrin binding; additionally, they adhere to other ECM molecules (Aumailley and Smyth, 1998; Suzuki et al., 2005). Each laminin molecule is composed of three non-identical subunits, called the α, β and γ chains (Boceto-Bellemin et al., 2003). At least 15 isoform types of laminin have been identified which substantially were synthesized and expressed during fetal and adult periods (Lefebvre et al., 1999; Miner et al., 1997; Nguyen et al., 2002). Several studies indicated that Alpha 5 chain of laminin is essential for the lung development, both in embryonic and adult lung (Nguyen et al., 2005; Nguyen et al., 2002), natural development of smooth muscle cell types, basal membrane of blood vessels (Vainionpaa et al., 2006) and digestive tract (Boceto-Bellemin et al., 2003). Lama5 is also essential for propagation and polarization of epithelial cells (Fukumoto et al., 2006). Deletion of the gene encoding alpha 5 chain of laminin during fetal development in mouse lead to death (Miner et al., 1998) and imposed abnormality in kidney and digestive tract (Lefebvre et al., 1999). In the lungs it would bring about a delay in its evolution along with abnormality in the growth of the surface alveolar cells and disorganization in growth and development of vessels and alveoli (Rahuel et al., 2008; Rebusini et al., 2007). Because laminins are important BM component essential for morphogenesis of all tissues, this study has evaluated the effect of nicotine on the expression of lama 5 in lung tissue development of the offspring during gestational time and lactation period.

MATERIALS AND METHODS

Nicotine administration and tissue preparation: From January 2011 for about three months female Balb/c mice were randomly divided into 2 experimental and 2 control groups (n = 6). Sperm positivity in vaginal plaque was designated as day zero of pregnancy. The animals were maintained at the animal house under controlled conditions (12 h light and dark cycle, 21°C and 50% relative humidity) with laboratory chow and water provided ad libitum. The experimental group1 (Exp D1) received 3 mg kg⁻¹ of nicotine (N 3876, sigma.com) daily intra peritoneally (IP) from day 7 of gestation to the last day of pregnancy and experimental group 2 (Exp D14) was received nicotine from day 7 of gestation to two weeks postnatal (lactation period) (Jalali et al., 2010). The sham control groups (Sh-Con) were received nicotine solvent (Normal saline) at the same period. Finally, the animals were rapidly sacrificed by cervical dislocation and their lungs were removed in postnatal days one (PD1) and fourteen (PD14), then fixed for 24 h at room temperature in formalin 10% to use for immunohistochemistry (IHC) study.

Immunohistochemistry method: The 5 μm thickness sections were deparaffinized, rehydrated and then washed in PBS (pH 7.4) for 10 min, Antigen retrieval were carried out with Heat-induction by Tryps/EDTA buffer, pH 9.0 for 20 min. The slides were washed in Phosphate buffered saline (PBS) plus 0.025% Triton X100 for 5 min and blocked in 1% normal serum (goat, Sigma, USA) with 1% BSA (Sigma, USA) in PBS for 2 h at room temperature. All the sections were incubated with monoclonal anti laminin antibody (Abcam, 75344, USA) diluted 1: 150 in PBS with BSA 0.1% for overnight at 4°C and then washed three times with PBS. For blocking endogenous peroxidases activity the slides were incubated in 0.03% H₂O₂ (Merk, Germany) dissolved in methanol (Bidesten, Iran) for 30 min. Next, tissues were incubated for 2 h with secondary antibody (Abcam, 97051, USA) diluted 1:800 in PBS with BSA 0.1% for 2 h. After incubation, the sections were washed extensively with PBS for 3 min and treated with DAB (Sigma, USA) solution (0.03 grDAB in 100 mL PBS and 200 μL H₂O₂/100 mL PBS) for 15 min at room temperature in dark. After being washed in running water, all the sections were counterstained with hematoxylin for 1 min. Finally the sections were dehydrated in increasing graded ethanol, cleared in xylene and mounted in glass slide. Laminin reaction in alveoli and lung parenchyma were graded blind by three separate observers (Table 1), (Jalali et al., 2010; Kranenburg et al., 2006). Percentile median intensity reactivity was calculated and presented in the form of 50% (25, 75%).

Real time study

RNA extraction: Total RNA was isolated by RNA plus (Cinnagen, Iran) according to the manufacturer's

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>(+)</td>
</tr>
<tr>
<td>Weak</td>
<td>(++)</td>
</tr>
<tr>
<td>Moderate</td>
<td>(+++)</td>
</tr>
</tbody>
</table>
| Strong   | (+++++)

(−), no reaction; (+), weak reaction: light brown; (++), moderate reaction: brown; (+++), strong reaction: dark brown and (++++), very strong reaction: very dark brown
instructions briefly, 50-70 mg of lung tissue was homogenized in RNA plus using homogenizer (polytron PT 1200E, Switzerland). The homogenate was centrifuged at 12000 xg for 10 min at 4°C to remove insoluble debris and the supernatant was transferred to a fresh micro centrifuge tube (Eppendorf, Germany). Samples were allowed to sit at room temperature for 5 min and 0.2 mL of chloroform was added per 1 mL of RNA plus™. The Samples were vortexed (Velp, Italy) for 15 s and allowed to stand for 5 min at room temperature. The mixture was centrifuged at 12000 xg for 15 min at 4°C. The aqueous phase was transferred to a fresh micro centrifuge tube and an equal amount of isopropanol (Merk, Germany) was added. After 30 min incubation at -20°C, the mixture was centrifuged at 12000 xg for 15 min at 4°C. The pellet was washed with 75% ethanol, air-dried and resuspended in 50 μL of diethylpyrocarbonate-treated water. The total RNA was examined by measuring the optical density at 260/280 nm.

**cdDNA synthesis:** First strand cdDNA was made using a cdDNA synthesis kit (Fermentas, Lithuania) according to the manufacturer's instructions. RNA (3 μL) was mixed with 1 μL of Dnase and incubated for 30 min at 37°C. (Incubator, Memmert, Germany) and then 1 μL of 100 pmol μL⁻¹ Oligo (dT) and 8 μL of H₂O were added to each incubated 10 min at 70°C (Thermal cycler, Bioer, China). After the above step, 2 μL of 10 mM dNTP Mix 4 μL (Fermentas, Lithuania) of 5x reaction buffer, 1 μL of Ribolock (Fermentas, Lithuania) and 1 μL of reverse transcriptase (Fermentas, Lithuania) were added to each sample tube. The tubes were sequentially incubated at 42°C for 60 min and 70°C for 5 min. (Thermal cycler, Bioer, China) and stored at -20°C (Freezer, Sikat, Iran).

**Primers and real-time PCR:** The designed primers were as follows:

- Lama α-5-F, CGTCCCACAGGAATGCT, Lama α 5-R, TACCAACGAGGGCTGCG, GAPDH-F, AACTCCCATATTCCACCTTG, GAPDH-R, CTGTAGCCATAATTTCATTGTCA

Real-time PCR was performed using 10 μL SYBR®. Real-time PCR Master Mix (Paris Bioscience, Iran), 1 μL of each primer (10 pmol μL⁻¹), 1 μL cdDNA and 7 μL DEPC-water. Reactions were run using Stratagene Mx-3000P (Stratagene, La Jolla, CA, USA). After a RT-PCR for each target gene, cdDNAs from high quality samples for target genes were selected and they serially diluted to obtain six standard solutions to generate the standard curve in the real-time PCR assay. All data were normalized to the house-keeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the relative mRNA expression ratios were determined using the method that introduced by Pflaff (2001) and Wong and Medrano (2005).

**Statistical analysis:** On the basis of staining intensity, sections were graded and Mann-Whitney non-parametric statistical test was used to compare differences between samples. Student t test used for real time PCR. The p-values <0.05 were considered statistically significant.

**RESULTS**

Our results showed that decrease in lung and body weight of mice offspring born from mothers exposed to nicotine was significant compared to control groups. Therefore nicotine had highest impact on body weight (5.28±0.39) and lung (0.60 ± 0.007) in PD14 (p<0.05) (Table 2). Analysis of laminin α5 mRNA expression in lung tissue showed that mRNA expression decreased by 0.63 fold in Exp D1 and increased by 1.57 fold in Exp D14 comparing with the Sh Con groups. Statistical analysis indicated that the laminin α5 expression increased significantly from PD1 to PD14 in experimental group (p<0.05) (Fig. 1). Thus laminin expression in PD14 was effected in most (1.5 fold) when exposed to nicotine. Immunohistochemical reactivity of lung tissue using rabbit monoclonal antibody against mice was specific for

![Fig. 1: Relative transcription level of laminin mRNA expression in lung tissue of the experimental groups under treatment with nicotine (3 mg kg⁻¹) on different days (days 1 and 14 of newborn) using Real-time PCR. Sh-Con. (Sham control), Exp D1 (postnatal day one) and Exp D14 (postnatal day fourteen). Values represent the Means±SE (n = 6), * p<0.05](image)

**Table 2: Effect of nicotine treatment (3 mg kg⁻¹) from the 7th day of gestation to the 14th day postnatal in the new born mice. The body weight and Lung weight index in new born mice at different days are compared to control groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control group PD1</th>
<th>Experimental group PD1</th>
<th>Control group PD14</th>
<th>Experimental group PD14</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. wt (g)</td>
<td>1.5±0.05</td>
<td>1.43±0.04 *</td>
<td>5.84±0.33</td>
<td>5.28±0.39 *</td>
</tr>
<tr>
<td>L. wt (g)</td>
<td>0.025±0.001</td>
<td>0.025±0.004 *</td>
<td>0.11±0.006</td>
<td>0.09±0.007 *</td>
</tr>
</tbody>
</table>

B. wt: body weight, L. wt: Lung weight, Values is Means±SD, * p<0.05
laminin α5 in alveolar septum and bronchioles, showing positive reactivity in immunohistochemical method. The locations of laminin expression in lung tissue were determined according to the intensity of color darkness. Immunohistochemistry data showed that intensity of immunoreactivity of laminin α5 in infant lung alveoli of Sh-Con D1 group reacted moderate with median of 2(2,3) while at the same position in infants lung born from mothers affected under nicotine Exp D1 was in average reactivity with median of 3(3,4). Statistical analysis showed that this increase was significant (p = 0.01). In addition median of reaction intensity of alveoli in Exp D14 was very intense 4(3-4) compared with Sh Con D14 (p = 0.001). Also expression of laminin α5 between Exp D1 and Exp D14 increased significantly (p = 0.01) (Fig. 2-5). Immunoreactivity of laminin α5 in bronchioles of Exp D14 and Exp D1 showed a decrease reactivity in Exp D1 with median 2(2,3) comparing with Sh-con 3(2, 4) (p = 0.002). However, intensity reactivity in bronchioles Exp D14 with median 4(3, 4) increased significantly comparing with Sh-con 3(2, 3) (p = 0.001). Statistical analysis showed that the expression of laminin α5 in bronchioles increased significantly between Exp D1 and Exp D14 (p = 0.001) (Fig. 3-5). Increase in immunoreactivity of laminin α5 in small vessels of Exp D14 and Exp D1 observed with median 3(2,3) and 2(2, 3), respectively comparing with Sh-con groups (Fig. 4-5).

**DISCUSSION**

The results of this study show that nicotine administration during gestation and lactation could change expression of laminin α5 as one of the most important proteins of basement membrane. According to immunohistochemistry (IHC) analysis, maternal nicotine exposure causes laminin reaction intensity increase in the alveolar septum and decrease in bronchiole during gestation period. Therefore, the pattern of laminin expression could be different in various parts of the newborn lungs. Also decrease in expression of laminin would only occur in bronchioles of experimental group 1 while in experimental group 2 increased in both alveoli and bronchioles. Although a little difference in lamin expression was observed in experimental groups using Real time PCR but it was not significant. Changes found
for protein levels followed the same time course as those described for mRNA expression. Data obtained from real time PCR and IHC analyses indicated that lama5 expression increased in experimental groups 2 follow the same developmental pattern. Thus, we concluded that nicotine could have an inhibitory impact on laminin transcription during the gestation period and an stimulatory effect on expression of laminin in lactation period. Schuger et al. (1990) indicated that laminin expression increased during late development. In our investigation also laminin expression in alveolus and bronchiole had an increase from day 1 to day 14 which more or less is in conjunction with above studied.

Luck and Nau (1984) showed that nicotine in maternal smoking results in milk concentrations between 1.5 and 3 times the simultaneous maternal plasma concentration. The nicotine in breast milk is rapidly absorbed through the infant’s gut and accumulates in some tissues.
Accordingly a reason for increase in lama5 expression in this study might be because of the presence of high nicotine concentration in mother breast milk.

Several studies indicated that nicotine acts on nAChRs, which are ligand-gated ion channels controlling influx of calcium and sodium into cells (Akaike et al., 2010). Neuronal nicotinic receptors are present in bronchial epithelium and vascular endothelial cells as well as in the cholinergic nerves innervating the bronchial smooth muscle (Sekhon et al., 1999; 2002). Sekhon et al. (2002) showed that nicotine administration to pregnant rhesus monkeys caused increase in expression of α7 nicotinic cholinergic receptors within the lungs, which was accompanied by increases in collagen deposition in the airway wall in the lung. The study of Maritz (2009) showed that nicotine increase the production of free radicals parallel with a decrease in the lung antioxidant capacity. Furthermore, suppression of glycolysis and an increase in cAMP results in changes in lung growth (Maritz, 2008). Therefore, we propose that the activation or suppression of intracellular signals by nicotine and change in expression of α7 nicotinic cholinergic receptors could lead to increased or decreased laminin gene transcription. In addition, nicotine exposure during gestation and lactation may result in remarkable low pups birth weight. This finding was consistent with the results of other researchers (Ozokutan et al., 2005; Sekhon et al., 2004).

Based on results of other researchers, lung laminin alpha5 can be expressed by endothelial cells, smooth muscles of vessels (Bolato-Bellemín et al., 2003) and airways epithelial (Nguyen et al., 2005), our results also support these findings. Decrease in lama5 expression observed in this study may also result from malfunctioning of another lung component such as smooth muscles and endothelial vessels.

Several studies showed that laminins affect lung development at multiple stages and in different cellular compartments (Schuger et al., 1990; Willem et al., 2002). Laminin α2 has been shown to be important for bronchial smooth muscle cell differentiation (Relan et al., 1999). Mice lacking laminin γ2 or α3 die 1-3 days after birth from malnutrition (Meng et al., 2003). A reduction in laminin α5 expression was reported in breast cancers (Martin et al., 1998), Prostate (Calaluce et al., 2001), lung (Akashi et al., 2001; Manda et al., 2000) and colon (Sordat et al., 1998). Increases in expression of laminin β2 and alphal chains have been reported in airway of asthma patients, allergic airway remodeling and chronic obstructive pulmonary disease (COPD). Therefore, change in laminin expression in embryonic period may cause functional defects especially asthma during either childhood or puberty period. Although above studies highlight the important of laminin in cancer studies but there have not found any record about the effect of nicotine in laminin gene expression.

CONCLUSION

This study has found that maternal nicotine exposure during pregnancy and postnatal produce variable changes in Laminin α5 gene expression at different stages of lung development. This implies that maternal nicotine exposure might change the future development of lung dysfunction.

ACKNOWLEDGMENT

The provided data in this article are from PhD student thesis supported by the grant (89566) from vice chancellor for research, Mashhad University of Medical Sciences, Iran. In addition, the authors would like to thank Mrs. F. Motejaded for her technical assistance.

REFERENCES


