Thyroid transcription factor-1, hepatocyte nuclear factor-3β and surfactant protein A and B in the developing chick lung

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ABSTRACT

Expression of surfactant proteins SP-A, SP-B and the transcription factors TTF-1 and HNF-3β was identified by immunohistochemistry in the developing chicken. SP-B, a small hydrophobic peptide critical for lung function and surfactant homeostasis in mammals, was detected in the epithelial cells of parabronchi in embryonic chicken lung from the 15th day of incubation, prior to the onset of the breathing movements and was expressed at high levels in the posthatching chicken lung. SP-A, an abundant surfactant protein involved in innate defence of the mammalian lung, was detected in the chick embryo in subsets of epithelial cells in the mesobronchus, starting from d 15 and was detected in the posthatching chicken lung. The transcription factors hepatocyte nuclear factor 3β (HNF-3β) and thyroid transcription factor-1 (TTF-1), both regulators epithelial cell differentiation and gene expression in mammalian species, were detected at the onset of lung bud formation (d 4 of incubation) and throughout lung development. Abundant nuclear expression was detected in nuclei of respiratory epithelial cells of developing bronchial tubules for both transcription factors. In contrast to the surfactant proteins, expression of both TTF-1 and HNF-3β decreased markedly in posthatching chicken lung. The expression of SP-A and SP-B in chick lung demonstrates the conservation of surfactant proteins in vertebrates. The temporospatial pattern of TTF-1 and HNF-3β overlaps with that of SP-A and SP-B, supporting their potential roles in chick lung development and demonstrating the conservation of regulatory mechanisms contributing to gene expression in respiratory epithelial cells in vertebrates.

Key words: Lung development; TTF-1; HNF-3β.

INTRODUCTION

The mammalian lung is formed when epithelial cells derived from foregut endoderm invade the splanchnic mesoderm. The primitive respiratory tubules undergo dichotomous branching to form the conducting airways. In late gestation, the distal regions of the bronchial tubules dilate, forming the alveolar sacs (Adamson, 1991). The respiratory epithelial cells lining the bronchial tubules and alveolar surfaces undergo cytodifferentiation characterised by the increasing expression of a number of secretory products, including surfactant proteins A, B, C, D and Clara cell secretory protein (CCSP) (Weaver & Whitsett, 1991; Ten Have-Opbroek & De Vries, 1993; Whitsett et al. 1995). SP-B and SP-A are expressed in the alveolar type II epithelial cells and/or bronchial epithelial cells and contribute to the surface tension reducing activities of surfactant and host defence functions of mammalian lung, respectively (Weaver & Whitsett, 1991; Pison et al. 1994; Clark et al. 1995; Gaynor et al. 1995; Whitsett et al. 1995; Le Vine et al. 1996). Transcription of the SP-A, B, C and CCSP genes is controlled by a number of nuclear proteins including thyroid transcription factor-1 (TTF-1) and hepatocyte nuclear factor-3β (HNF-3β), which are expressed in the respiratory epithelium throughout lung development in mouse and human (Stahlman et

The anatomy and physiology of the avian lung is highly distinct from that of the mammalian lung. The chick lung is organised around a primary bronchus (mesobronchus). The secondary bronchi branch off the mesobronchus and elongate to give rise to the air sacs and a network of tertiary bronchi (parabronchi), each parabronchus forming the centre of a lobule. Along the walls of each parabronchus, minute branches termed air capillaries project into the lung parenchyma. In contrast to the alveolar respiratory system in mammalian lung that terminates in the alveoli, the bronchial branches and the air capillaries in the avian lung anastomose, allowing respiratory gases to move in an unimpeded manner through the respiratory tubules in only one direction in a manner distinct from the bellows-like action of the mammalian lung (Romanoff, 1960).

The distinctive anatomical features of avian as compared with mammalian species raise interesting questions regarding the presence and functions of the surfactant proteins and whether the molecular mechanisms regulating the surfactant specific gene expression in mammals might also be conserved in avian lung. The presence of pulmonary surfactant material was previously demonstrated lining the cell surface of the parabronchial, atrial and air capillary spaces in the avian lung (Pattle, 1978). As in mammals, the major class of phospholipids in avian surfactant is phosphatidylcholine (PC) (Tyler & Pangborn, 1964; Marin et al. 1978). Cells resembling mammalian type I and type II pneumocytes were described in the posthatching and embryonic chick lung (Tyler & Pangborn, 1964; Lambson & Cohn, 1968). Lamellar bodies, the storage organelles for surfactant were also detected in type II cells in avian lung (Hylka, 1989). Since SP-B is required for the formation of lamellar bodies in mammals (Clark et al. 1995), the presence of lamellar bodies in chick lung also suggests that SP-B may be expressed in chick lung.

In the present study, the presence of SP-B and SP-A was demonstrated in developing chicken lung. Temporal and spatial patterns of expression of SP-B, SP-A, TTF-1 and HNF-3β were compared at various developmental stages in the developing chicken. The overlapping pattern of expression of HNF-3β, TTF-1, SP-B and SP-A in subsets of epithelial cells in parabronchi of chick lung supports the roles of both transcription factors in epithelial cell differentiation and surfactant expression in the chicken lung.

**MATERIALS AND METHODS**

**Eggs and tissue preparation**

Fertilised chicken eggs were obtained from SPAFAS, Inc. All eggs were incubated in 38 °C in a high humidity incubator. The developmental stages of chicken were determined by days of incubation. Whole chick embryos from d 4 to d 9 of incubation or lungs dissected from the d 15 to posthatching d 3 were washed briefly in phosphate buffered saline (PBS) and fixed in neutral buffered paraformaldehyde (4%) for 16 h. Tissues were washed 3 times in PBS and dehydrated through a graded series of ethanol and embedded in paraffin. Sagittal sections (5 µm) of whole embryos or lung tissue alone were mounted on the poly-lysine-coated slides.

**Antibodies and immunohistochemical staining**

Rabbit polyclonal antiserum, generated against a synthetic peptide spanning amino acid residues 110–122 of rat TTF-1 (Guazzi et al. 1990) was kindly provided by Dr Robert Dilauro (Zoological Station, Naples, Italy) and was used at a dilution of 1:8000. Rabbit polyclonal antiserum against amino acids 7-86 of rat HNF-3β was generated by Dr Robert H. Costa (University of Chicago, Chicago, IL) and was used at a dilution of 1:100. Rabbit antiserum against SP-B (R28031) was generated to the mature active SP-B peptide isolated from bovine lung (Lin et al. 1996) and was used at a dilution of 1:1000. The SP-A antiserum was generated against the rat SP-A and was absorbed against human blood of blood group type A antigen. Paraffin sections (5 µm) were rehydrated and immunohistochemical staining for SP-B was performed as previously described (Khoor et al. 1994). Immunostaining for SP-A was performed as described for SP-B except the sections were incubated with 6N guanidine-HCl for 30 min and with 0.02 % trypsin, pH 7.6 for 30 min prior to the incubation with the primary antibody to retrieve the antigen. Immunostaining for TTF-1 and HNF-3β was performed as described for SP-B except that microwave antigen retrieval was performed before the incubation with the primary antibody (Zhou et al. 1996). The incubation and blocking conditions were as described for specific reactivity of each antiserum previously demonstrated in human and mouse tissue (Stahlman...

RESULTS

The embryonic to posthatching development of chick lung

The lung primordium first appeared on d 3 of incubation in chick embryo. The endodermally derived lung buds diverged from the ventral region of the oesophagus and elongated, and grew into the splanchnic mesoderm on d 4–5 of incubation of the chick embryo (Fig. 1 A, B). Lung development at this early stage in the chick embryo was similar to that seen in the mouse embryo at d 10 to d 11. After d 5 of incubation, the primary bronchus gave off secondary bronchi of various sizes along the length of its wall (Fig. 1 C, D), a pattern that was highly distinct from the successive dichotomous branching pattern in mammalian lung. The bronchial buds were lined by columnar or squamous epithelial cells. The airsacs were formed at the distal end of the secondary

Fig. 1. Development of chick lung from the 4th day of incubation to posthatching d 3. Sagittal sections (5 µm) of chick embryos at incubation d 4 (A), 5 (B), 7 (C) and 9 (D) and lung tissue from the incubation d 15 (E) and posthatching d 3 (F) were stained with haematoxylin and eosin and photographed under bright field microscopy. On the d 4 of incubation, the tracheal bud diverges from the ventral aspect of the oral pharyngeal region (A) and elongates into the splanchnic mesoderm on d 5 (B). On d 7 of incubation, the secondary bronchi branch off the primary bronchus (C), forming bronchial buds of various sizes around the primary bronchus on d 9 (D). On the d 15 of incubation, the tertiary bronchi (parabronchi) and the air capillaries comprise the major portion of lung mass (E). On posthatching d 3, the air capillaries grow extensively and form a network surrounding each parabronchus (F). a, atrium; ac, air capillary; as, air sac; es, oesophagus; mb, mesobronchus; op, oral pharyngeal region; pb, parabronchus; sb, secondary bronchus; tr, trachea. Bar for A, B, C, D = 50 µm; bar for E, F = 100 µm.
Fig. 2. Immunohistochemical staining for TTF-1 in the developing chick lung. Immunostaining for antibody against rat N-terminus of TTF-1 (1:8000 dilution) was performed on paraffin sections of whole chick embryos at incubation d 4 (A), 5 (B), 7 (C) and 9 (D) and lungs from chick embryos of incubation d 15 (E, F) and posthatching d 3 (G, H). The staining of embryos or lung sections reflects results from 4 individual animals and representative results are shown in the photomicrograph. TTF-1 was detected in the tracheal epithelial cells, thyroid primordium and forebrain but not the liver or gut at incubation d 4 (A) and 5 (B). At d 7–15 (C, D, E, F), TTF-1 was expressed at high levels in the nuclei of the epithelial cells lining the developing bronchial tubules, including mesobronchus (arrow), parabronchi and atria. No TTF-1 was detected in the surrounding splanchnic mesodermal cells. On posthatching d 3 (G, H), TTF-1 expression was weaker but detectable in the respiratory epithelial cells of both mesobronchus (arrow) and atria of parabronchi. No TTF-1 staining was seen in the air.
bronchi. At d 9 of incubation, the airsacs were enlarged and projected beyond the lung surface but did not branch (Fig. 1D). The secondary bronchi gave off tertiary bronchi (parabronchi) of nearly uniform calibre at d 15 of incubation (Fig. 1E). The wall of each parabronchus had numerous openings (atria) leading to minute branches (air capillaries) extending into the surrounding mesenchyme (Fig. 1E). At the posthatching d 3, the atria further dilated and the air capillaries grew extensively, forming a network surrounding each parabronchus. Structurally, the air capillaries in chicken lung corresponded to the alveoli in mammalian lung. However, the air capillaries in chicken lung did not terminate as blind-ended sacs in mammalian lung. The air capillaries in chick lung grew towards and fused with each other and formed an intricate network with blood capillaries, facilitating air exchange in both inspiration and expiration.

**Distribution of TTF-1 in developing chick lung**

TTF-1 was readily detectable in the early lung bud on d 4 and d 5 of incubation in the chick embryo (Fig. 2A, B). TTF-1 staining was localised in the nuclei of the epithelial cells of the tracheal and main bronchi. No staining was seen in the surrounding splanchnic mesodermal cells. Consistent with the expression pattern of TTF-1 in mouse and rat embryos (Lazzaro et al. 1991; Zhou et al. 1996), TTF-1 staining was also detectable in the ventral region of diencephalon and the thyroid primordium. No staining for TTF-1 was detected in other organs. From d 7 to d 15, TTF-1 was abundantly expressed in the epithelial cells in developing bronchial tubes, including the developing mesobronchus (Fig. 2F), parabronchi and the atria of chick lung (Fig. 2E). TTF-1 staining was weaker in the developing airsacs (Fig. 2D). At posthatching d 3, the TTF-1 staining was weaker than in the previous developmental stages and was restricted to epithelial cells in the mesobronchus and the atria of parabronchi (Fig. 2G, H). TTF-1 was not detected in the epithelial cells of air capillaries of the posthatching chick lung (Fig. 2G).

**Distribution of HNF-3β in developing chick lung**

Similar to TTF-1, HNF-3β was detectable on d 4 of incubation in the chick embryo and was localised in the epithelial cells of main bronchi, but was absent from the surrounding mesenchymal cells (Fig. 3A, B). HNF-3β was also detectable in the liver primordium and in the gut. No staining for HNF-3β was observed in the thyroid primordium. During d 7 to d 15, HNF-3β was detected in the epithelial cells of developing parabronchi and atria extending into the lung parenchyma (Fig. 3C, D). In contrast to TTF-1, staining for HNF-3β was not detectable in the mesobronchus at d 15 of incubation in the chick lung (Fig. 3F). At posthatching d 3, the expression of HNF-3β in parabronchi was greatly decreased (Fig. 3E). In contrast to TTF-1, HNF-3β was not detected in epithelial cells of mesobronchi and air capillaries in the posthatching chick lung (Fig. 3G, H).

**Distribution of SP-B and SP-A in developing chick lung**

Mature SP-B was first detected in the epithelial cells of parabronchi and atria in the developing lung at d 15 of incubation (Fig. 4A). Staining for mature SP-B increased in posthatching d 3 chick lung (Fig. 4B) and colocalised in the same subsets of the epithelial cells in parabronchi that stained for TTF-1 and HNF-3β. SP-B was not detectable in the epithelial cells of mesobronchi or the epithelial cells of air capillaries in the posthatching chicken lung (Fig. 4B, C). SP-A was readily detectable at high levels in subsets of epithelial cells in the mesobronchi in the posthatching chick lung (Fig. 4D), but was not detected in the respiratory epithelial cells of peripheral airways, including the parabronchi and air capillaries.

**Discussion**

The temporal and spatial patterns of expression of TTF-1, HNF-3β, SP-A and SP-B were determined in the developing chicken lung by immunohistochemistry. HNF-3β and TTF-1 were detected in the respiratory epithelial cells from the initiation of lung bud formation and throughout embryonic lung development. Posthatching, TTF-1 staining decreased markedly and HNF-3β was undetectable. SP-B was first detected late in lung development, starting from d 15 of incubation, before the onset of respiratory movement. SP-B was expressed at high levels in subsets of respiratory epithelial cells in the posthatching chick lung. SP-A was detected at high levels
Immunohistochemical staining for HNF-3β in the developing chick lung. Tissue sections were prepared as described in Fig. 2 except that the staining was performed with antibody against rat HNF-3β (1:100 dilution). HNF-3β was detected in the epithelial cells of the trachea, liver and gut on incubation d 4 (A) and 5 (B). On incubation d 7 and 9 (C, D), HNF-3β continued to be expressed in the epithelial cells lining the developing airways. On d 15 (E, F), staining for HNF-3β was restricted to the epithelial cells of parabronchi and atria but not those of the mesobronchus. The HNF-3β expression was decreased in chicken lung at posthatching d 3 and was undetectable in the air capillaries or mesobronchus (G, H). a, atrium; ac, air capillary; g, gut; li, liver; mb, mesobronchus; pb, parabronchus; tr, trachea. Bar for A, B, C, D = 50 µm; bar for E, F, G, H = 100 µm.
in subsets of respiratory epithelial cells in the mesobronchus in the posthatching chicken lung. Crossreactivity of bovine SP-B and rat SP-A antisera with the chicken lung demonstrates the conservation of these surfactant proteins in mammals and avian vertebrates. The overlapping pattern of expression of TTF-1 and HNF-3β with SP-A and SP-B in late embryonic lung development supports their roles in the regulation of SP-A and SP-B gene expression in chicken lung, and may reflect conservation of the regulatory mechanisms that contribute to respiratory epithelial specific gene expression in the vertebrate lung.

The temporospatial pattern of expression of TTF-1 and HNF-3β in the early chick embryo is similar to that seen in the mouse (Zhou et al. 1996). TTF-1 was expressed in the lung bud, thyroid primordium, ventral region of the diencephalon. HNF-3β was detected in endodermal derivatives, including the lung bud, liver primordium and gut. The distinct pattern of TTF-1 and HNF-3β expression in early embryos suggests that TTF-1 and HNF-3β play distinct roles in lung organogenesis. TTF-1 is expressed at the onset of lung bud formation from the foregut endoderm. In the mouse, disruption of the TTF-1 gene caused severe lung hypoplasia. These lungs consisted of cystic tubules lacking the lung parenchyma (Kimura et al. 1996), indicating the essential role of TTF-1 in lung organogenesis and the subsequent respiratory epithelial cell differentiation. HNF-3β is expressed early in embryogenesis in the node, notochord, floor plate of the neural tube and gut (Sasaki & Hogan, 1993). Homozygous HNF-3β gene targeted mice fail to form the anterior–posterior body axis and lack the gut endoderm (Weinstein et al. 1994), supporting a critical role of HNF-3β in the foregut endoderm formation. HNF-3β was found to bind to TTF-1 gene regulatory elements and activate the transcription of TTF-1 gene in vitro (Ikeda et al. 1996). Therefore, the coexpression of TTF-1 and HNF-3β in early lung organogenesis may provide signals involved in the specification of the respiratory epithelium along the foregut axis as suggested previously (Bohinski et al. 1994).

In embryonic chick lung, TTF-1 and HNF-3β are coexpressed in the respiratory epithelial cells of developing bronchial tubules. TTF-1 and HNF-3β
Fig. 5. Schematic representation of the developing lung structure in mammals and birds. (A) The bronchial tree in mammalian lung is formed by successive dichotomous branching from the ends of the bronchial tubules. After the last division, the alveolar sacs are formed as the distal end of the bronchial tubules dilate. (B) The mature alveoli of mammalian lung are lined by differentiated type II and type I epithelial cells. Surfactant proteins are expressed by alveolar type II cells. The air flow (arrow) in mammalian lung is bidirectional. (C) In contrast to mammalian lung, the bronchial tree in chicken lung is formed by nondichotomous branching of the secondary bronchi along the primary bronchi (mesobronchi). The parabronchi further branch off the secondary bronchi and give off numerous fine and elongated air capillaries. (D) The air capillaries from parabronchi grow towards each other and anastomose. The epithelial cells of air capillaries proximal to parabronchi (atria) are type II cells, which express SP-B and TTF-1. The cells lining the rest of air capillaries are type I cells. In chicken lung, the air flow (arrow) is unidirectional and continuous.

expression precedes the expression of SP-A and SP-B by several days, supporting the potential regulatory roles of both transcription factors in respiratory epithelial cell specific gene expression and lung morphogenesis. From d 15 of incubation, TTF-1 and HNF-3β expression overlaps with that of SP-B in the epithelial cells of parabronchi, consistent with the previous in vitro studies demonstrating that both TTF-1 and HNF-3β bind to and activate the SP-B gene (Bohinski et al. 1983; Clevidence et al. 1994). TTF-1 is also expressed in the mesobronchus of chick lung, where SP-A is also expressed. This finding is consistent with the previous finding that TTF-1 binds to the promoter region of the mouse SP-A gene and activates SP-A gene transcription (Bruno et al. 1995; Zhang et al. 1997). However, SP-A expression in
chicken lung is restricted to subsets of epithelial cells in the mesobronchus, in contrast to the more dispersed pattern of expression of TTF-1 in the mesobronchus, suggesting that combinations of TTF-1 with other cell specific and/or ubiquitous regulatory factors may contribute to the cell selective pattern of SP-A expression. Since HNF-3β is not expressed in the mesobronchus, it is possible that HNF-3β is not required for the expression of SP-A in chick lung.

In human and mouse embryos, SP-B is expressed at low levels in epithelial cells of bronchi and bronchioles at early gestation. Later in gestation (24–36 wk in man), SP-B mRNA and protein concentrations increased dramatically, and are expressed primarily in differentiated type II cells (Whitsett et al. 1995). In chick, SP-B was first detected after d 15 of incubation, before the initiation of respiratory movement. The expression of SP-B increased in posthatching chick lung, consistent with the temporal pattern of SP-B expression in mouse and human lung. In chick lung, SP-B staining was detected in epithelial cells of parabronchi, but not the epithelial cells of the air capillaries, while SP-B was detected primarily in differentiated type II cells in mammals (Khoor et al. 1994; Vorbrocker et al. 1995; Zhou et al. 1996). The identification of SP-B in epithelial cells in the parabronchi of the chicken lung is consistent with previous electron microscopic localisation of lamellar bodies in these epithelial cells (Hylka, 1989). The temporal expression of SP-B in the chicken lung is also coincident with the formation of lamellar bodies, which are first detected in the type II cells in avian lung on the d 16 of incubation, with the numbers increasing with subsequent development.

SP-A mRNA and/or protein have been detected in the lungs of all major vertebrate groups, including the primitive air breathing lung fish, amphibians, reptiles, birds and mammals (Rubio et al. 1996; Sullivan et al. 1998). In the chicken lung, SP-A was highly expressed in subsets of epithelial cells of the mesobronchus but not the epithelial cells of peripheral airways, in sharp contrast to the expression pattern of HNF-3β, TTF-1 and SP-B. In mammals, SP-A is expressed primarily in the nonciliated bronchiolar epithelial cells (i.e. Clara cells) and type II epithelial cells, overlapping with the site of SP-B expression. Cells staining for SP-A in the mesobronchus of chick lung may represent the homologue of Clara cells. However, antiserum against rat CCSP (Singh et al. 1988), which stains the Clara cells in mouse and rat (Zhou et al. 1996), failed to crossreact with the antigen on chicken tissue (unpublished observations). In the mammalian lung, SP-A was also detected in alveolar type II epithelial cells. Since the differentiated epithelial cells of parabronchi contain SP-B and lamellar bodies, the lack of SP-A staining in the epithelial cells of parabronchi may indicate that SP-A is not essential for storage, processing and function of surfactant in chicken lung.

Genetic ablation of SP-A in mice demonstrated that SP-A is not essential for surfactant function or lamellar body formation (Korfhagen et al. 1996). An extensive body of evidence suggests that SP-A, a member of the collectin family of lectins, plays an important role in innate defence of the lung (Tenner et al. 1989; Pison et al. 1994; Gaynor et al. 1995). Of interest, homozygous SP-A knockout mice are highly susceptible to bacterial infection (LeVine et al. 1996). Therefore, SP-A may play an important role in host defence in chicken lung.

In summary, development of the lung structure in the chick is highly distinct from that of mammals (Fig. 5). However, the similar group of lung specific genes, including SP-A and SP-B and their transcriptional regulators TTF-1 and HNF-3β are expressed in the developing chicken lung. The pattern of expression for SP-A, SP-B, TTF-1 and HNF-3β in the chick was consistent with that observed in mammals, supporting the chick as a suitable model system to study lung development and the regulation of lung specific gene expression.

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