

ULTRASTRUCTURAL AND PROTEIN ANALYSIS OF SURFACTANT IN THE AUSTRALIAN LUNGFISH *NEOCERATODUS FORSTERI*: EVIDENCE FOR CONSERVATION OF COMPOSITION FOR 300 MILLION YEARS

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Summary

The Australian lungfish *Neoceratodus forsteri* is the most primitive member of the lungfish family, with a surfactant lipid composition similar to the actinopterygian fishes, which evolved 400 million years ago. We have analysed the proteins associated with surfactant isolated from lung lavage of this species, and used electron microscopy and immunohistochemistry to examine the surfactant structures and the subcellular localisation of these proteins. The epithelial lining of the gas-exchange region of the lungfish lung consists of one basic cell type, which has characteristics of both mammalian alveolar type I and type II cells and may be the common ancestor of both. It has long cytoplasmic plates containing microvilli, large osmiophilic bodies resembling mammalian lamellar bodies and a cytoplasm rich in metabolic organelles. Extracellular structures

reminiscent of mammalian surfactant forms, but not including tubular myelin, were observed in the airspaces. Immunochemical analysis of the lungfish surfactant and lung tissue, using antibodies to human SP-A and SP-B, showed a similar staining pattern to human surfactant, indicating that SP-A- and SP-B-like proteins are present. Immunohistochemistry revealed that both SP-A and SP-B reactivity was present in the secretory cell osmiophilic bodies. In conclusion, our results suggest that, despite the great diversity in present day lung structures, a common cellular mechanism may have evolved to overcome fundamental problems associated with air-breathing.

Key words: *Neoceratodus forsteri*, surfactant, lung, air-breathing, evolution, immunohistochemistry, electron microscopy

Introduction

Air-breathing is generally regarded as pivotal in the evolution of the tetrapods. Each major group of vertebrates has members which breathe through 'lungs' and air-breathing has probably evolved on two separate occasions, once in the actinopterygian fishes at least 420 million years ago and then approximately 320 million years ago in the sarcopterygian fishes. Each major group of vertebrates has members which breathe through 'lungs' and despite the huge diversity of these lungs, they share a common problem. All maintain gas-containing, aqueous-lined structures which change volume, and in order to keep the work of breathing to a minimum, surface tension at the interface must be reduced. In all species so far investigated, the gas-liquid interface is lined with a surface-active lipid material resembling mammalian surfactant. However, the trends in lipid composition might reflect a convergence of evolution in response to common selective pressures faced with air breathing, rather than a common ancestry (Orgeig and Daniels, 1995). In order to test our hypothesis that all species have solved the problem in a similar way, we used electrophoresis, immunochemical analysis and immunohistochemistry to characterise the

proteins associated with the surfactant in the Australian lungfish, *Neoceratodus forsteri*, the most primitive of air-breathing fish. In addition, we have examined the structure of the gas-exchange region, concentrating on alveolar type II or analogous cells containing lamellar body-type structures and tubular myelin, the characteristic form of surfactant found in most species.

Materials and methods

A female lungfish *Neoceratodus forsteri* (Kreffit), 1040 mm in length and weighing approximately 12 kg, was placed in a small tank of water and anesthetized by adding dissolved ethyl *m*-amino benzoate (final concentration, $1 \text{ g } 101^{-1}$) (Sigma Chemical Company, St Louis, Missouri) to the tank. The fish stopped breathing within 15 min, was removed from the tank, and a midline incision was made exposing the viscera. A tracheal catheter was inserted and a tie placed around the lung and dorsal column of the posterior one third of the lung. The anterior two thirds of the lung were lavaged with a total of 400 ml of 0.15 mol l^{-1} saline. The posterior one third was used

for histology and was not lavaged in order to avoid disrupting any alveolar structures.

Protein sample preparation

Lungfish lavage surfactant

The lungfish lavage fluid was centrifuged at 150 g for 5 min to remove cellular debris and then at 138 000 g (max) for 1 h at 4 °C to obtain a surfactant pellet. The pellet was delipidated in *n*-butanol (50:1 dilution), dissolved in 50 µl of Laemmli (1970) sample buffer and heated at 100 °C for 5 min.

Mammalian surfactant

A 10 ml volume of human (Doyle et al., 1994a) and rat (Doyle et al., 1994b) lavage was centrifuged at 4 °C for 2 h at 100 000 g. The pellet, which contained 50 µg of protein, was dissolved in 50 µl of Laemmli sample buffer and heated at 100 °C for 5 min.

Lungfish and human tissue preparation

A 200 µg portion of *N. forsteri* and of human lung tissue was macerated in 600 µl of Laemmli sample buffer and left at 20 °C overnight. The material was heated at 100 °C for 10 min, cooled, centrifuged at 10 000 g for 10 min and the soluble proteins separated.

Surfactant proteins

Human SP-A (Doyle et al., 1994a) and rat SP-A (Doyle et al., 1994b), mature human SP-B (Yogalingam et al., 1996) and full-length human recombinant SP-B (a gift from Dr T. Weaver) (Weaver, 1996) were similarly dissolved in Laemmli sample buffer to a concentration of 1 µg µl⁻¹.

Electrophoresis

Proteins were separated employing a Laemmli buffer system with 0.75 mm thick 12 % polyacrylamide gels using a Bio-Rad Mini-Protean II apparatus (Laemmli, 1970). Gels were either stained with silver or transferred onto nitrocellulose.

Protein blotting and immunochemical staining

After electrophoresis, gels for immunological characterization were equilibrated at 4 °C for 30 min in a solution of 25 mmol l⁻¹ Tris, pH 8.3, 192 mmol l⁻¹ glycine in 20 % (v/v) methanol. Proteins were transferred at 100 V for 1.5 h onto nitrocellulose membrane (Trans-Blot, Bio-Rad Laboratories Pty Ltd) using a Bio-Rad Mini-Protean II apparatus.

Non-specific protein-binding sites on the nitrocellulose were saturated by incubating the membranes for 2 h in a solution of 10 mmol l⁻¹ Tris, pH 7.5, 150 mmol l⁻¹ NaCl and 0.05 % (v/v) Tween 20 (TBST) containing 5 % (w/v) bovine serum albumin (BSA) (fraction V; Sigma Chemicals). The membranes were incubated overnight with polyclonal antibodies Po-A or Po-B diluted 1:1000 in TBST containing 1 % (w/v) BSA. The membranes were washed four times for 5 min in TBST and incubated for 90 min with alkaline phosphatase-conjugated sheep anti-rabbit polyclonal IgG (Silenus, Melbourne,

Australia) diluted 1:30000 in TBST. The wash procedure was repeated and the membranes developed using a Protoblot Immunoscreeing System (Promega; Madison, WI).

Antibodies

Polyclonal antibody Po-A (Doyle et al., 1994a) was raised against human proteinosis SP-A. Polyclonal antibody Po-B was raised against the mature SP-B peptide extracted from human proteinosis lavage fluid (Yogalingam et al., 1996).

Light immunohistochemistry

Lung sections were fixed in 0.1 mol l⁻¹ phosphate buffer (pH 7.4) containing 4 % paraformaldehyde. Blocks of tissue approximately 5 mm³ were snap-frozen in liquid nitrogen and embedded in OCT compound (Sakura Fine Chemicals, Tokyo, Japan). Sections approximately 12 µm thick were cut at -20 °C using a cryostat and placed on gelatine-coated slides. The sections were blocked with 20 % normal horse serum in Tris-buffered saline (TBS), pH 7.4, for 1 h and then incubated overnight with either anti-human SP-A antibodies (Po-A; diluted 1:2000) or anti-human SP-B antibodies (Po-B; diluted 1:1000) in TBS containing 1 % normal horse serum. The sections were washed and then incubated for 2 h with a biotinylated goat anti-rabbit second antibody (1:500; Tago Inc, Burlingame, USA) and then with streptavidin peroxidase (1:1000; Zymed Laboratories Inc, San Francisco, USA) for 1 h. The resulting antibody complex was developed with 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB; BioRad) and the sections examined and photographed with an Olympus microscope (BH2) linked to a Kodak EOS-DCS digital camera.

Electron microscopy immunohistochemistry

Sections of lungfish lung were immersion-fixed with 0.1 mol l⁻¹ phosphate buffer (pH 7.4) containing 2 % glutaraldehyde (w/v) and 1 % paraformaldehyde (w/v), with post-fixation in 1 % osmium tetroxide for 24 h at 4 °C, and stained with 1 % uranyl acetate (w/v) for a further 24 h at 4 °C. The samples were dehydrated and embedded in Taab resin (Taab Laboratories, Reading, UK). Semi-thin sections (1 µm) were cut for light microscopy and ultrathin sections (0.1 µm) for electron microscopy. For immunohistochemistry, tissue was fixed in 2.0 % paraformaldehyde, 0.25 % glutaraldehyde (w/v) in 0.1 mol l⁻¹ phosphate buffer at pH 7.4, post-fixed in 0.5 % osmium tetroxide (w/v) for 60 min and embedded in L. R. White resin (London Resin, Basingstoke, UK). The sections were incubated in primary antibody for 16 h at 4 °C and for 2 h in goat anti-rabbit colloidal gold-conjugated secondary antibody (Janssen Life Sciences; Piscataway, NJ; Auroprobe EM GAR G15). The sections were stained and examined using a JEM 1200 EX electron microscope (Jeol, Tokyo, Japan).

Results

Histology

The lung of *N. forsteri* consists of a single elongated chamber,

compartmentalized by a thick cartilaginous structural framework (Grigg, 1965a; Gannon et al., 1983). The epithelial lining of these supporting structures comprises abundant capillaries interspersed with cells resembling alveolar type II cells (Fig. 1). However, at the electron microscopy level, these cells (TI/TII) exhibited features of both alveolar type I cells and alveolar type II cells and appear to be the only cell type lining the gas-diffusing surface (Fig. 2). They have long cytoplasmic plates bearing microvilli, which form part of the gas-exchange membrane. In addition, these cells contained large numbers of osmiophilic bodies resembling mammalian lamellar bodies, although most of these had a granular core, no limiting membrane and appeared to be in the process of either forming spontaneously or degrading in the cytoplasm (Fig. 3). Some osmiophilic bodies did appear to have a limiting membrane (Fig. 2). Although a few osmiophilic bodies were observed unravelling in the folds of the airspaces, no material resembling tubular myelin was detected (Fig. 4). A montage showing two such TI/TII cells containing lamellar bodies and three capillaries clearly shows these unique cells forming the gas-diffusing barrier (Fig. 5). The cell-cell junction is clearly visible in the high power insert. This cell was the only cell type observed in the epithelial lining and appeared between each capillary.

Immunochemical staining

SP-A

Po-A reacted strongly with whole human and rat surfactant, purified human and rat SP-A, and human lung tissue (Fig. 6). Po-A also reacted with lungfish lung tissue with an almost identical pattern of immunostaining to human lung tissue, but with less intensity (Fig. 6). The major reaction was with a protein of approximately 35 kDa, and a lesser reaction with a protein of approximately 70 kDa. Similarly, the immunostaining of lungfish lavage proteins was almost

identical to human surfactant separated under reducing conditions, but with a much lower intensity (data not shown).

SP-B

Po-B reacted strongly with mature SP-B (approximately 8 kDa), the processing intermediate (25 kDa) and the pro-protein in human and rat whole surfactant, and with purified SP-B separated from human surfactant under reducing conditions (Fig. 6). Po-B also reacted with the human full-length recombinant SP-B. In human lung tissue, Po-B reacted strongly with the mature protein, the processing intermediate and a range of translation products. In lungfish lung tissue there was a strong reaction with two proteins; one had an apparent molecular mass of 30 kDa and the other of approximately 15 kDa (Fig. 6). There was no reaction with the 25 kDa processing intermediate or with the range of translation products present in human lung tissue. In lungfish lavage surfactant there was a faint reaction with the mature protein and proprotein, but like the lung tissue there was no reaction with the processing intermediate.

Light microscopy immunohistochemistry

SP-A reactivity

SP-A reactivity was localised in the epithelial lining cells, which resembled the TI/TII cells seen with electron microscopy (Fig. 7A,B). Not all TI/TII cells were labeled; in some cases it appeared that the cells had a granular appearance and may in fact have been monocytes in the capillaries. No other cells were labeled and there was no labeling in the negative controls, which also had a lighter background (Fig. 7E,F).

SP-B reactivity

The SP-B reactivity was specifically localised to the TI/TII cells in the lung epithelial lining (Fig. 7C,D). In some cells the labeling appeared in the cytoplasm, while in others both the

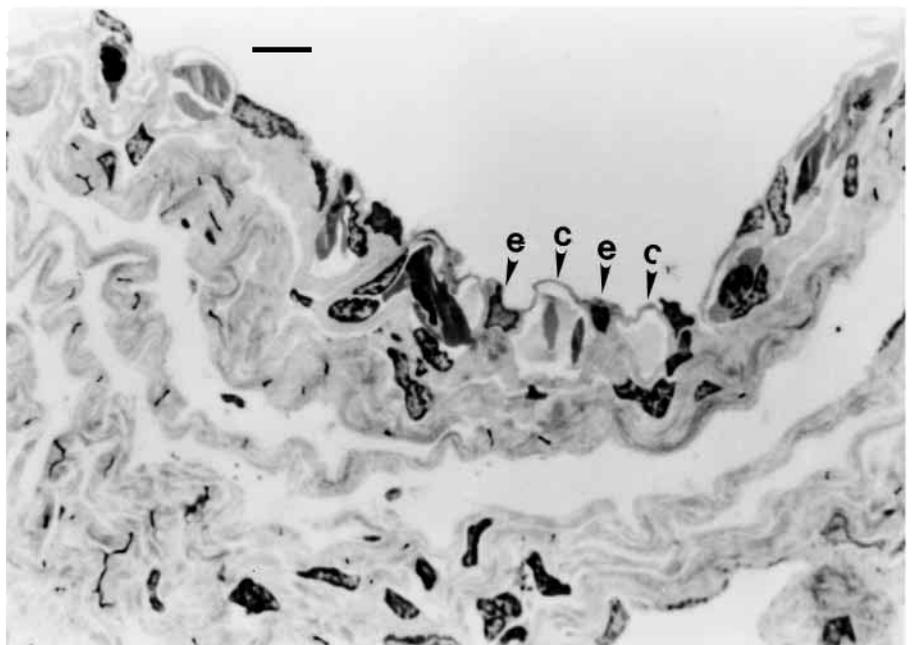


Fig. 1. Light micrograph showing the gas exchange region of the lungfish lung. The secretory epithelial cells (e) are interspersed between the capillaries (c). Scale bar, 20 μ m.

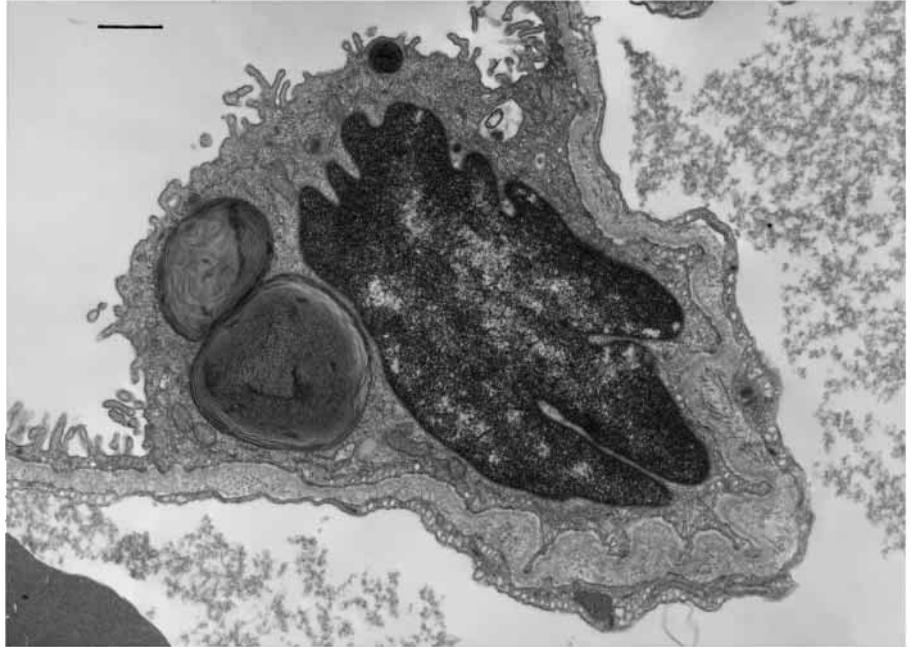


Fig. 2. An electron micrograph of the TI/TII secretory cell with long cytoplasmic extensions forming the gas-diffusing barrier. These cells also contain osmiophilic bodies and display microvilli. Scale bar, 1 μ m.

cytoplasm, particularly the apical regions, and the lamellar bodies were labeled. Whereas not all cells were labeled, many more were labeled than was found with SP-A. There was no labeling in the negative controls (Fig. 7E,F).

Electron microscopy immunohistochemistry

Both the SP-A and SP-B antibodies reacted with lungfish lamellar bodies and the surrounding cytoplasm, with the SP-B antibody showing a stronger reaction than the SP-A antibody (Figs 8, 9). Very little SP-A antibody-associated gold was seen in the alveolus, whereas SP-B antibody-associated gold appeared in the region around the surfactant-secreting cell, but was not associated with any structures. Apart from the occasional grain, gold was not seen associated with the TI/TII cell nucleus or other organelles and was not seen associated with other cells.

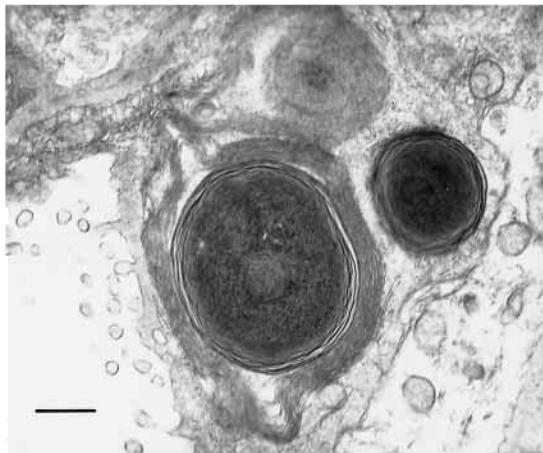


Fig. 3. An electron micrograph showing different forms of the osmiophilic bodies within the cytoplasm of the TI/TII secretory cell. Scale bar, 500 nm.

Discussion

The Australian lungfish, *N. forsteri*, differs from other lungfish in that it can air-breathe for only short periods. Whereas the gross anatomy and aeration of the lung of this species has been well documented by Grigg (1965a) and the general architecture and microcirculation extensively studied by Gannon et al. (1983), the microscopic details of the gas-exchange surface has received meagre attention.

In the present studies the lung was lavaged *in situ*. The lung has a rigid structural framework and hence did not inflate as a mammalian lung would. Since inflation is a major stimulus for surfactant release in the mammal, the question remains whether this is the case in the lungfish. The total lung capacity in this fish was approximately 150 ml (12.5 ml kg⁻¹). When the harvested lavage fluid, which contained no trace of blood, was first centrifuged to remove any cells or cellular debris and the

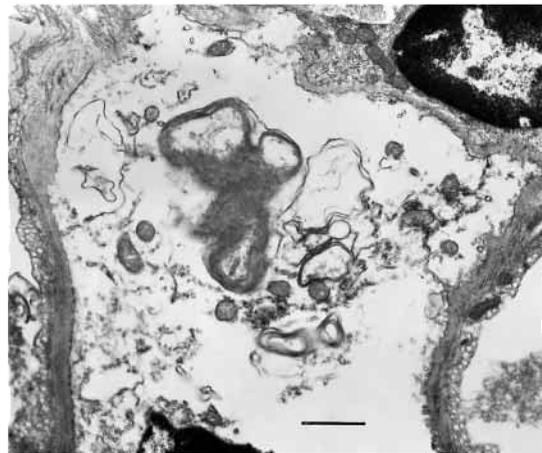


Fig. 4. An electron micrograph showing the remnants of an unraveling osmiophilic body in the fold of an airspace. Scale bar, 1 μ m.

resulting supernatant centrifuged to pellet any tubular myelin, as for mammalian lavage fluid ($1000g_{av}$ for 25 min), no pellet resulted. A further centrifugation at $138\,000g_{max}$ for 2 h was then employed to pellet the surfactant lipids. The total amount of phospholipid recovered was markedly less than that found in mammalian lungs, when expressed per lung mass (Orgeig and Daniels, 1995).

We have found that the epithelial lining of the gas-exchange region of the lung of *N. forsteri* resembles that of the reptilian lung, in that secretory cells are interspersed between capillaries. However, whereas in the reptile these cells are distinctly cuboidal and resemble mammalian alveolar type II cells, in the lungfish there is a single cell type with characteristics of both alveolar type I and type II cells. These cells contain microvilli and a cytoplasm rich in secretory

vesicles and metabolic organelles but with thin cytoplasmic plates. Some cells were positioned between the capillaries with cytoplasmic extensions anchoring the cell onto the basal lamina (Fig. 2), while other cells protruded directly from the gas-exchange surface with little anchoring support. In the mammalian lung, the type II cell is the progenitor cell for the type I cell, but the two cell types are distinctly different in both morphology and function. Possibly the epithelial cell in the lungfish is the common ancestral cell for the alveolar type I and type II cells found in the mammalian lung.

Whereas some of the secretory-type vesicles in the lungfish are osmiophilic and resemble mammalian lamellar bodies, others exhibited a more granular core with the appearance of glycogen granules. Many of these lamellar bodies were very large (up to $3\mu m$ in diameter), lacked a limiting membrane, and

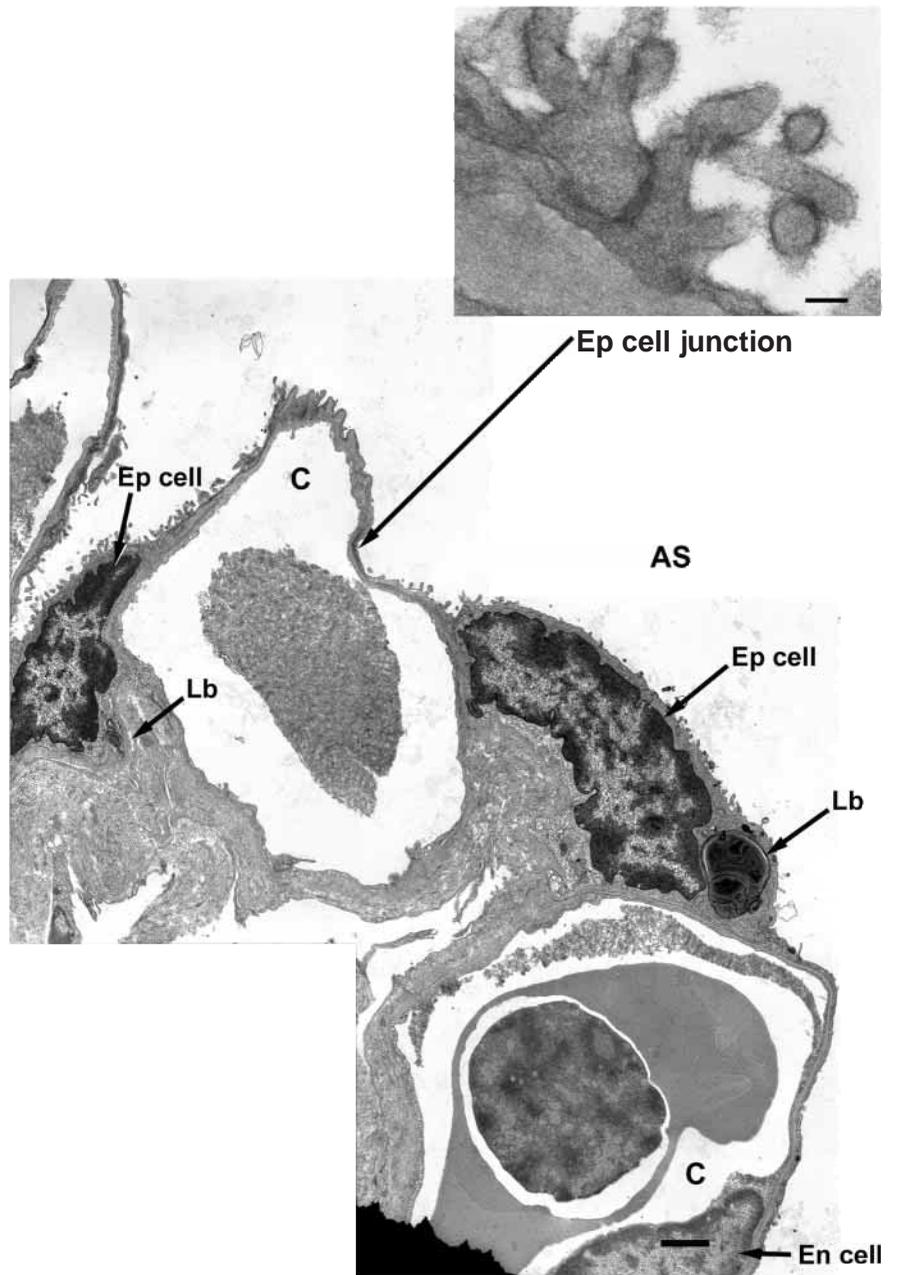


Fig. 5. A montage showing the epithelial cells of the gas exchange region of the lungfish lung. AS, airspace; C, capillary; Ep cell, epithelial cell; En cell, endothelial cell; Lb, lamellar body. Scale bar, $2\mu m$. The high magnification insert shows the junction between the two epithelial cells; scale bar, 100 nm.

Fig. 6. SDS-PAGE silver stain (left panel) and immunochemical staining (centre and right panels) of whole human surfactant (1), whole rat surfactant (2), human SP-A (3), rat SP-A (4), mature human SP-B (5), human full-length recombinant SP-B (6), *N. forsteri* lung tissue (7,8), and human lung tissue (9,10). The volume loaded was approximately 5 μ l except for lanes 8 and 10, where 10 μ l was used. The immunochemical staining was obtained using Po-A (antibody raised against human alveolar proteinosis SP-A), and Po-B (antibody raised against human alveolar proteinosis SP-B). Molecular mass standards (std) shown are (from top to bottom) phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α -lactalbumin (14.4 kDa).



their rings of osmiophilic material appeared to merge directly with the cytoplasm in a manner not seen in the mammalian type II cell. The lung of *N. forsteri* is very much an accessory organ, which is only used during periods of high activity in its natural habitat (Grigg, 1965b). When *N. forsteri* are kept in aquaria they rarely use their lungs, relying entirely on their gills. As lung inflation is the major stimulus for surfactant release in the mammal (Nicholas and Barr, 1981; Nicholas et al., 1982; Power et al., 1986), and the lungs of *N. forsteri* normally remain inactive for long periods, the question arises as to the nature of the turnover of surfactant. We suggest that in the absence of

air-breathing the lamellar body-type vesicles are degraded within the cytoplasm of the secretory cell. The lungfish we examined had been kept in an aquarium for an extended period and had only been air-breathing in a small tank for less than 2 h before it was anaesthetised. We found only a limited amount of surfactant structures in the airspaces, possibly reflecting this brief period of air-breathing. A longer period of air-breathing might have both increased surfactant in the airspaces and reduced the number of lamellar bodies apparently undergoing degradation. Uhlig et al. (1995) reported that in their isolated perfused rat lung model, lipopolysaccharides induce changes in

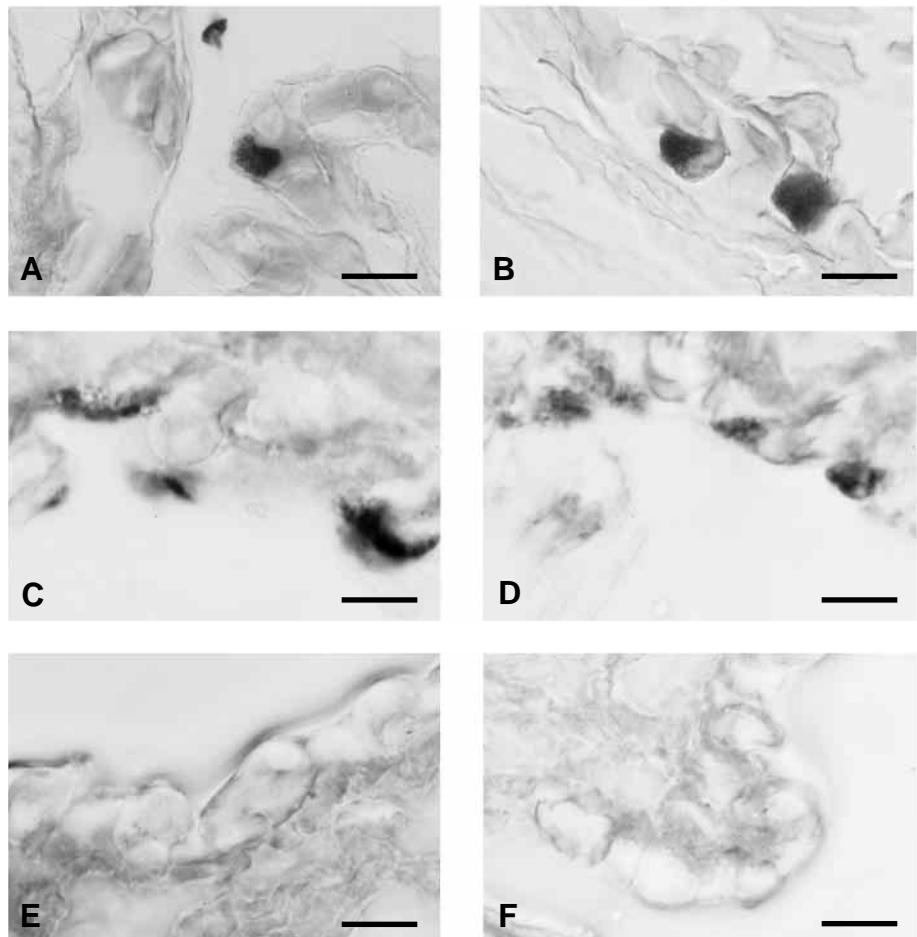


Fig. 7. (A,B) Light immunohistochemistry showing specific SP-A antibody reactivity in surfactant-secreting cells in the lung epithelium. (C,D) Light immunohistochemistry showing specific SP-B antibody reactivity in surfactant-secreting cells in the lung epithelium. (E,F) Negative controls (normal horse serum) showing no labeling of any cells in the lungfish lung. Scale bars, 20 μ m.

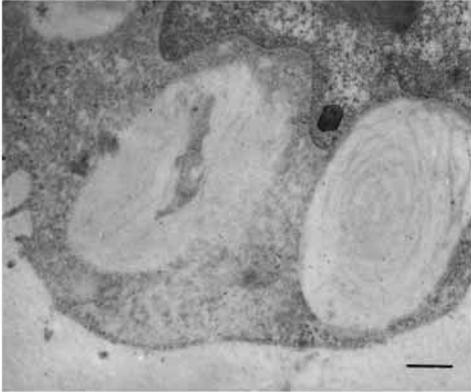


Fig. 8. Electron micrograph of a section through a lungfish secretory cell showing specific human SP-A antibody reactivity with lungfish osmiophilic bodies. SP-A reactivity was detected with 15 nm goat anti-rabbit colloidal gold-conjugated antibody. Scale bar, 500 nm.

surfactant metabolism that result in very large lamellar bodies similar to those seen in the lungfish. In future studies it would be informative to compare the lung and the surfactant levels of a lungfish which had been air-breathing more frequently.

It is difficult to see how the lamellar bodies lacking limiting membranes could be exocytosed. Possibly, unlike the mammalian lamellar bodies, the limiting membrane only appears in the final stages of maturation of the osmiophilic bodies, and only mature osmiophilic bodies are released. We suggest that this is unlikely, as structures resembling dense multivesicular bodies and composite bodies were present in some cells. Numerous small vesicles were apparent in the cell membrane, which may represent surfactant in the process of being released or recycled. Possibly this species normally relies on a constitutive release of unstructured surfactant.

The surfactant lipid profile of the Australian lungfish has been compared with other members of the Dipnoi family (Orgeig and Daniels, 1995). Two obligatory air breathers, *Prototerus annectens* and *Lepidosiren paradoxa*, have a profile similar to amphibians, whereas *N. forsteri* is similar to actinopterygian fish which evolved 400 million years ago. In this sense, the detection of the surfactant-associated proteins in the lung of *N. forsteri* has particular evolutionary significance, as these proteins must then be conserved for at least 300 million and possibly 400 million years.

The protein components of lungfish lung tissue and the lavage surfactant pellet were separated electrophoretically and analysed using western blotting with antibodies directed against human SP-A and SP-B. The SP-A antibodies reacted in an identical manner with both lungfish and human proteins in the lung tissue and lavage surfactant, suggesting that this protein is highly conserved. Preliminary N-terminal sequencing revealed a gly-X-Y repeating unit for at least 18 amino acid residues, which is consistent with the collagenous domain of SP-A. These data suggest that, even in this primitive lung, SP-A is probably processed in the same manner as in mammals. Indeed, Sullivan et al. (1998) have shown using northern blot analysis that a mouse cDNA probe

to SP-A hybridises with RNA from a range of species, including the Australian lungfish.

Mature SP-B is a hydrophobic peptide that is relatively insoluble in aqueous solutions and is first synthesized as a water-soluble precursor. Signal peptide cleavage, probably in the endoplasmic reticulum after translocation (Barritt, 1992), and glycosylation yields a hydrophilic proprotein (42 kDa). It is generally accepted that processing of the proprotein involves at least two distinct proteolytic events: (1) cleavage of 200 amino acid residues from the N terminus to generate an intermediate of approximately 25 kDa and (2) cleavage of 102 residues from the C terminus to yield the mature protein. Lamellar bodies in the mammalian lung are associated with mature SP-B, but with little, if any, of the proprotein or the approx. 25 kDa intermediate (Weaver and Whitsett, 1989; Voorhout et al., 1992, 1993). Although the majority of mature SP-B is currently thought to be secreted *via* lamellar bodies *in vitro*, pulse-chase labelling studies indicate that some hydrophilic precursor is also secreted (Weaver and Whitsett, 1989). We have recently confirmed that the proprotein and the processing intermediate are present in the human alveolus (Doyle et al., 1997).

The human SP-B antibodies cross-reacted with two peptides of approximately 15 kDa and 42 kDa in lungfish lung tissue and surfactant but not with the 24 kDa fragment, nor with any of the translation products as found in humans. This suggests that the mature protein is slightly larger in the lungfish lung and that SP-B is processed differently. Since SP-B is also present in Clara cells in the mammalian lung (Horowitz et al., 1991), perhaps the 24 kDa fragment is secreted by this cell type. As the trachea is very short in the lung fish and does not have a complex airway system, this cell type would neither have contributed to the lavage fluid nor been present in the tissue analysed.

Light immunohistochemistry showed that both SP-A and SP-B reactivity was present in a lung epithelial cell that shared features with both mammalian type I and type II cells in shape and location. The SP-B antibody labeled many, but not all, the lungfish TI/TII cells, but we suspect that this was due to

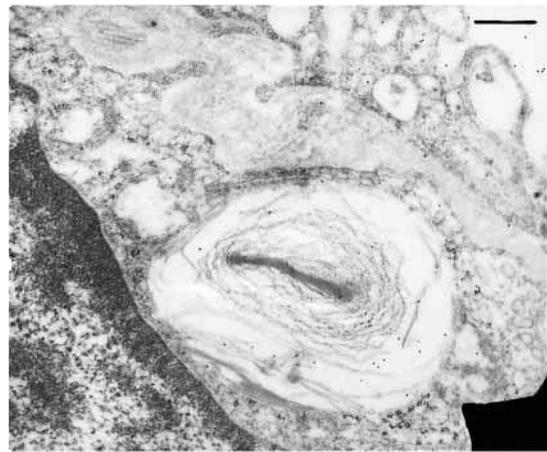


Fig. 9. Electron micrograph of a section through a lungfish secretory cell showing specific human SP-B reactivity with lungfish osmiophilic bodies. SP-B was detected with 15 nm goat anti-rabbit colloidal gold-conjugated antibody. Scale bar, 500 nm.

antibody penetration, as the staining was carried out on frozen sections 12–15 µm thick. Some cells probably remained intact in the middle of the section while others were sectioned and therefore more easily accessible to the antibody. In some cells the lamellar bodies were clearly stained while in others the cytoplasm was stained and the lamellar bodies appeared as vacuoles; again, we think this was due to differences in antibody penetration. While the SP-B antibody clearly stained the cells observed at the EM level, the SP-A antibody staining was more variable. The SP-A antibody stained some of the same cells as did the SP-B antibody but on occasions the stained cells appeared to be in the capillaries and may have been monocytes.

Immunohistochemistry at the electron microscope level with SP-A and SP-B antibodies revealed slight reactions in both lamellar bodies and the cytoplasm, confirming the light immunohistochemical observations. The staining with the SP-B antibodies in lamellar bodies was greater than with the SP-A antibody, which is consistent with what we see in rat. The light staining with the SP-A antibody was similar to that reported in rat lung (Walker et al., 1986; Haller et al., 1992). Certainly in the case of SP-B, the majority of the gold particles were localised to the lamellar bodies and the adjacent cytoplasm with only the occasional grain associated with other structures. These observations strongly supported the observations at the light immunohistochemical level. The tubular myelin fraction is greatly enriched with SP-A in mammalian lungs (Haller et al., 1992), but we did not observe any tubular myelin-like structures, and the few alveolar structures present were not labeled with gold to any extent. This again may reflect the short period of air-breathing.

In summary, the lungfish contains a surfactant-like material containing both SP-A and SP-B like proteins, suggesting that even in this primitive lung these proteins are still involved in surfactant homeostasis. The surfactant-secreting cell in the lungfish has structural features of mammalian type I and type II cells and may be the ancestor of both. Despite the great diversity in lung structures, our results suggest that a common cellular mechanism may have evolved to overcome fundamental problems associated with airbreathing.

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