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Pulmonary adenocarcinoma of fetal type: alternating differentiation argues in favour of a common endodermal stem cell*

H.K. Müller-Hermelink [1] **and E. Kaiserling** [2]

[1] Institut für Pathologie der Universität Würzburg, Josef-Schneider-Strasse 2, D-8700 Würzburg, Federal Republic of Germany
[2] Institut für Pathologie der Universität Kiel, D-2300 Kiel, Federal Republic of Germany

Summary. Pulmonary adenocarcinoma exhibit different cell types that ultrastructurally, appear to be related to the different epithelial cell types occuring in the terminal lung lobule. The present paper describes the light-microscopical, ultrastructural and immunhistochemical features of a newly defined type of pulmonary adenocarcinoma that resembles an early stage of lung differentiation. The alternating epithelial differentiation within this tumour speaks in favour of a common endodermal stem cell for the different types of epithelial cells within the lung, including endocrine cells. The relationship of this tumour to pulmonary blastoma is discussed.

Key words: Adenocarcinoma of the lung – Neuroendocrine carcinoma – Lung development – Pulmonary blastoma – Endocrine lung

Introduction

The different growth patterns of pulmonary carcinoma are not apparently connected with different histogenetic origins of tumour stem cells. The range of endoderm derived epithelial cells in the lung suggests that a consideration of histogenesis is important in the terminology of pulmonary adenocarcinoma. Several reasons, however, speak against an attempt to use histogenetic terminology in its strict form: Firstly, the cellular differentiation of pulmonary epithelial cells is not readily visible in ordinary light microscopical specimens but requires including electron microscopy and immune cytochemistry etc. (e.g. Mollo et al. 1973; Gould et al. 1983a, b; Fasske 1984; Albain et al. 1985). Secondly, changing differentiation patterns in different parts of individual lung carcinoma are frequently observed (WHO 1981). Thirdly, the prognostic significance of such an attempt is unclear. However those investigators using a refined methodology in the diagnosis of pulmo-

* Dedicated to Professor K. Lennert, Kiel, on the occasion of his 65th birthday

Offprint requests to: H.K. Müller-Hermelink at the above address

nary adenocarcinoma agree that normal differentiation pathways may be recognized, and be related to different growth patterns or functional activites (e.g. Fasske 1984).

Endocrine differentiation in pulmonary carcinoma has traditionally been separated from the "exocrine" tubular or papillary type of adenocarcinoma. The histogenesis of endocrine pulmonary carcinoma, however, has been strongly disputed in recent years (Gould et al.1983a, b; Becker and Gazdar 1982). The traditional view that these tumours represent the neoplastic transformation of separate neurocrest induced cell lines as originally proposed by the APUD concept (Pearse 1968) has been challenged. New findings argue in favour of an endocrine differentiation of commmon endoderm driven stem cells. In view of the selective action of different carcinogens on specific lung cells exemplified experimentally this question is not merely of semantic value (Ward et al.1985).

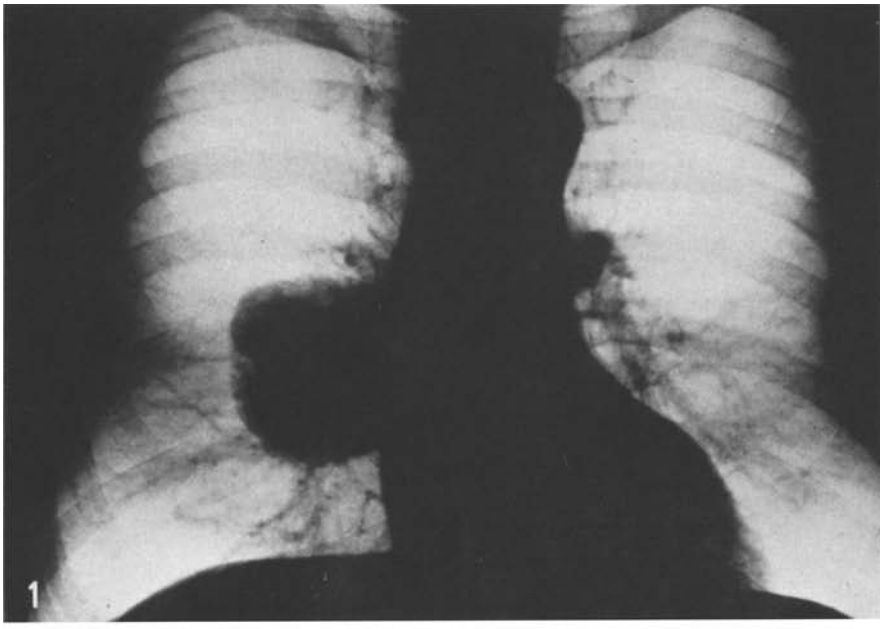
Embryonal tumours of the human lung are very rare. Barnard (1952) described a tumour that he called "embryoma" because of its similarity to the embryonal lung. Spencer (1961) described new cases and inroduced the term "pulmonary blastoma" for tumours composed of a glycogen-rich non-ciliated tubular epithelial component and a primitive mesenchymal component resembling the early fetal stage of lung formation. Endocrine cells, present in the fetal lung from the 10th week of gestation on (Stahlman et al. 1985), were also found in primary pulmonary blastoma (Kodame et al. 1984) and in a purely epithelial neoplasm generated by xenotransplantation of pulmonary blastoma to nude mice (Tameia et al. 1982). Recently primitive appearing pure epithelial tumours similar to the epithelial component of pulmonary blastoma have been recognized (Kradin et al. 1982; Kodame et al. 1984); Manning et al. 1985). A very prominent feature of these tumours is the presence of endocrine cells as seen by electron microscopy, argyrophilia or peptide hormone immune histochemistry. The present study gives a detailed description of this newly described entity, with electron microscopical and immune histochemical findings showing a complex organoid differentiation. This argues in favour of common endoderm driven stem cells for all pulmonary carcinomas.

Material and methods

A 64 year old man was admitted with a well circumscribed rounded mass in the lower lobe of the right lung, known to be present by X-ray investigations for 4 years (Fig. 1). Right lower lobe resection was performed with lymph node dissection. The postoperative course was without complications. 18 months after resection the patient is recurrence-free and working, complaining only of exertion dyspnea a resulting from moderate pre-existing emphysema.

The specimen consisted of the right lower lobe with a peripherically localized sharply delineated tumour of maximal 6 cm in diameter, covered by lung tissue. No vascular invasion was noticed and there were no lymph node metastasis (pT_3 N_0 MX).

The tumour tissue was fixed in 10% neutral formalin and embedded in paraffin as usual. Tumour specimens were also fixed in 5% glutaraldehyde (0,1 M Phosphate buffer pH 7,3) for electron microscopy after post-osmification with 2% OsO_4, dehydration in acetone and embedding in Araldite. Fresh tumour tissue was also snap frozen in liquid nitrogen and stored at $-80°$ C until cryostat section-immune histochemistry could be performed.



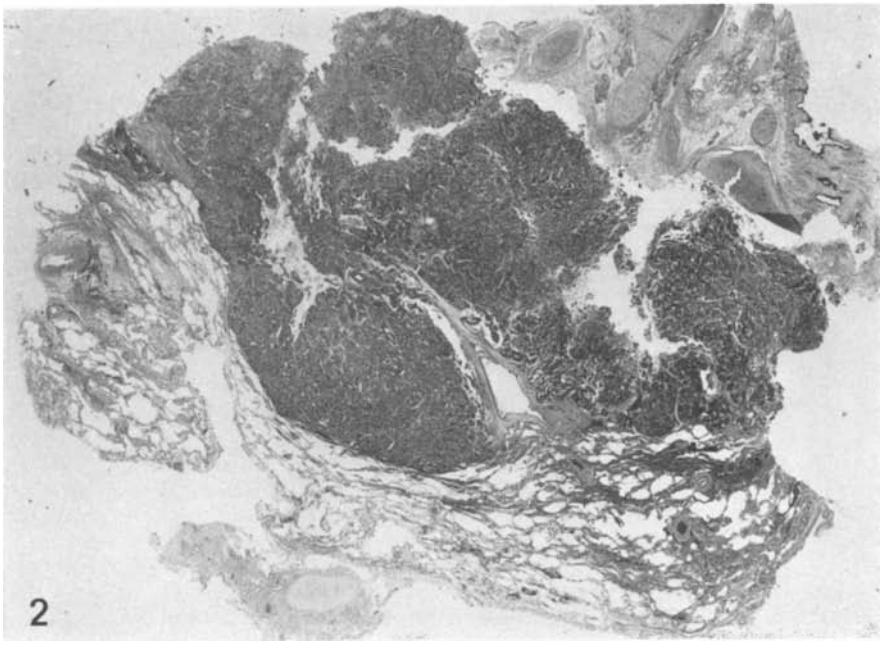


Fig. 1. Chest roentgenogram with an almost circular tumour shadow near to the hilus within the right lower lobe of the lung

Fig. 2. The tumour shows a multinodular structure histologically. Note the sharp demarcation from the surrounding lung tissue. HE 4×

Table 1. List of monoclonal antibodies used in this study

Antibody	Specifity	Source
A Intermediate filaments		
34β E 12	57/66 Kd keratins in squamous epithelium ductal epithelium, parabasal glandular cells	Gown and Vogel J Cell Biol 95:414 (1982)
35β H 11	54 Kd keratin(s) in most non-squamous epithelia	dto.
anti 160 Kd NF	Neurofilaments	Amersham, Braunschweig, (FRG)
B Melanoma-associated antigens		
M–2–7–6	Melanoma, naevi, peripheral nerves	Dr. E.B. Bröcker, Dr. C. Sorg, unpublished
M–2–10–15	Melanoma and naevi, peripheral nerves	dto.
A–1–43	130 Kd protein on melanoma and other tumours, basal layer of epidermis	Brüggen and Sorg (1983) Cancer Immunol. Immunther. 15:200–205
C Others		
Anti-Leu 7	HNK1: natural killer cells, nerve and CNS-antigen, some epithelial cells	Becton-Dickinson, Oxnard, Calif., USA
Anti-epithelial membrane antigens	Diverse epithelial cells	Dakopatts, Hamburg (FRG)
Anti-HLA-A,B,C	Class I – MHC-antigens	Bethesda Research Laboratories, Neu-Isenburg (FRG)
Anti-HLA-DR	Class II – MHC-antigens	New England Nuclear, Dreieich (FRG)

Light microscopic study was done on paraffin section using H. & E., Giemsa, PAS, Gomori silver stain and argyrophilic (Grimelius technique) and argentaffin reactions following postfixation with Bouin's solution. Immunhistochemical studies were worked out on formaldehyde fixed paraffin embedded tissue using the unconjugated peroxidase-antiperoxidase technique described by Sternberger et al.(1970). Primary rabbit antisera (Immuno Nuclear Corporation) were utilized against the following amines and peptides: neuron specific enolase, protein S 100, neurotensin, serotonin, leu-enkephalin, met-enkephalin, somatostatin, bombesin calcitonin, ACTH, beta-endorphin, substance P, gastrin, glucagon, vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), and human chorionic gonadotropin, alpha$_1$-fetoprotein (Dakopatts, Hamburg). Peroxidase-reaction was performed according to Graham and Karnovsky (1966). Specificity was demonstrated by positive staining of control tissue and by negative reaction when primary antisera were replaced by rabbit normal serum.

Monoclonal antibodies to melanoma associated antigens (Bröcker et al.1985) which react in normal tissue with melanocytes, nerves and some cells of the diffuse endocrine system and with different epithelial cells, were used to demonstrate endocrine differentiation on cryo-

stat sections. In addition anti-Leu 7 (HNK1) (Beckton and Dickinson) and monoclonal antibodies to different intermediate filaments (different cytokeratins, vimentin, neurofilaments, were demonstrated (Table 1). Double indirect immuno-peroxidase technique using Peroxidase labeled rabbit anti-mouse and goat anti-rabbit immunoglobulin (DAKO, Hamburg) was used to demonstrate the immunreactivity.

Results

The surgical specimen showed a well circumscribed tumour mass surrounded on all sides by pulmonary tissue or covered by pleura. The maximal diameters were $6 \times 3 \times 3$ cm. On the cut surface a multi-nodular structure became evident (Fig. 2). Microscopically the tumour consisted of interconnected tubular and glandular formations (Fig. 3) that often demonstrated a double row of nuclei. The cytoplasm of these epithelial cells was clear and contained moderate amounts of PAS+substances. The tubular and acinar growth pattern showed regular solid nests of another cell type, namely densely packed, sometimes spindle-shaped or prismatic cells of higher density. These cells were localized either in the continuity of the rows of tubular cells or in the intertubular connective tissue often in direct connection to tubular or acinar areas. Mitosis were very rare. Atypia was not marked. The border of the tumour was sharp. Although some tumour-invasion in adjacent alveolar spaces was seen, the tumour grossly compressed adjacent alveolar and lung tissue thus forming a pseudocapsule. Using the Grimelius technique for the demonstration of argyrophilia densely packed morula-like nests of cells or small aggregates of cells in between the clear tubular epithelial cells showed argyrophilic granules (Fig. 4). Staining intensity for argyrophilic granules varied from very strong reactions to weak ones hardly visible at oil immersion. The cell shape of positive cells varied from single cells with basal nuclei and narrow cytoplasm reaching the tubular lumen to aggregates of cells in the intertubular space without obvious relationship to the tubular lumen. In addition spindle-shaped positive cells with bipolar processes were seen running along axis of the tubules.

The ultrastructure of the immature looking tubular epithelial cells showed loose nuclei mainly localized at the cellular base (Fig. 5). The nucleoli were rather small and sharply delineated. The cytoplasm contained large amounts of glycogen. At the luminal surface these cells were connected to each other by a junctional complex consisting of desmosomes and tight junctions. In the cytoplasm short strands of rough endoplasmatic reticulum were seen. The luminal surface was smooth. In other areas tubular epithelial cells contained pleomorphic large granular inclusions comparable to the granules of Clara-cells in the normal lung. Sometimes concentric lamellated bodies were seen in the cytoplasm reminiscent of the inclusion in pneumonocytes type II. No cilia were found.

At the ultrastructural investigation the large number of cells containing dense-core granules was also remarkable (Fig. 6). Typical dense-core granules of different diameters ranging from very small granules of 50 mμ to those of 150–200 mμ in diameter were seen. The size distribution of these

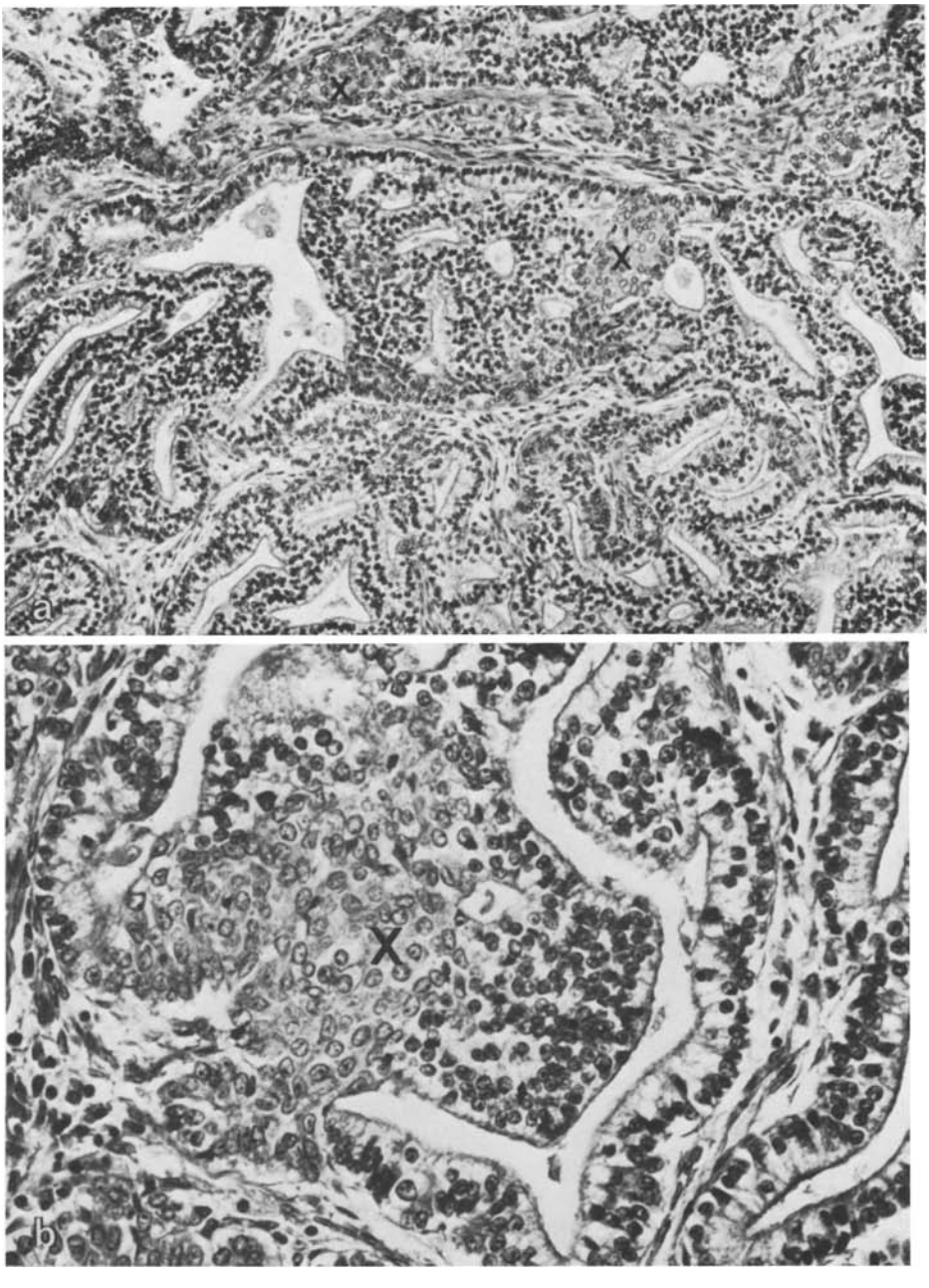


Fig. 3a, b. The tumour shows a predominantly glandular and tubular pattern with cylindroid-shaped epithelial cells with clear cytoplasm. Intermingled are solid morula-like nest of cells with irregularly arranged epithelial cells (X). **a** 140 × ; **b** 350 ×

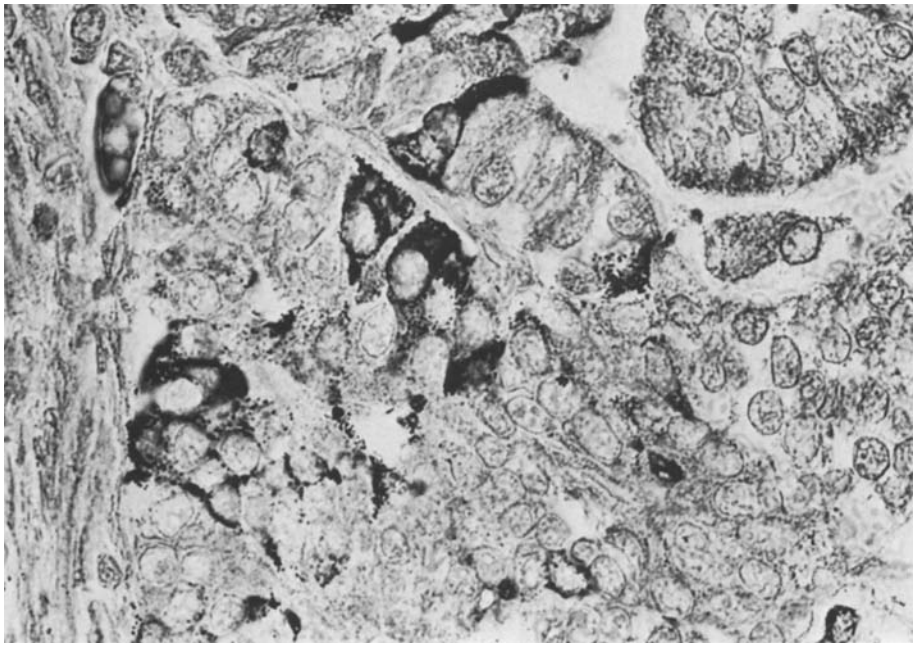


Fig. 4. Single cells and morula-like nests of epithelial cells show argyrophil granules in the cytoplasm. Grimelius-reaction. 880 ×

granules in individual cells suggested the occurence of different endocrine activities. In the areas of very densely packed aggregates of endocrine cells the number of granules was very low whereas single cells intermingled in between the tubular and ductal cells and single endocrine cells in intertubular localization showed large numbers of granules. The endocrine cells were connected to adjacent cells of similar or of differing differentiation by small desmosomes. Sometimes small aggregates of intermediate filaments were seen in the cytoplasm. The nuclei of endocrine differentiated cells showed a coarser chromatin structure than the ductal epithelium. Within the masses of endocrine cells, sometimes densely packed, were very immature looking small cells containing only very few dense-core granules.

Table 2 shows the endocrine activity as demonstrated by the immunreactivities on immunhistochemical investigation on paraffin sections. High or moderate activity was seen to neuron-specific enolase (Fig. 7a), leu-encephalin (Fig. 7b) and met-enkephalin, neurotensin and somatostatin. The few positive cells were seen with anti-alpha-fetoprotein, bombesin and serotonin. Negative results were observed with protein S 100, calcitonin, corticotropin, beta-endorphin, substance P, glucagon, VIP, CCK and βHCG.

The reactivity of tubular and endocrine epithelial cells to a variety of monoclonal antibodies is shown in Table 3. Monoclonal antibody to melanoma associated antigens reacting with nerves and the cells of the diffuse endocrine system also showed weak activities in the distribution of endocrine

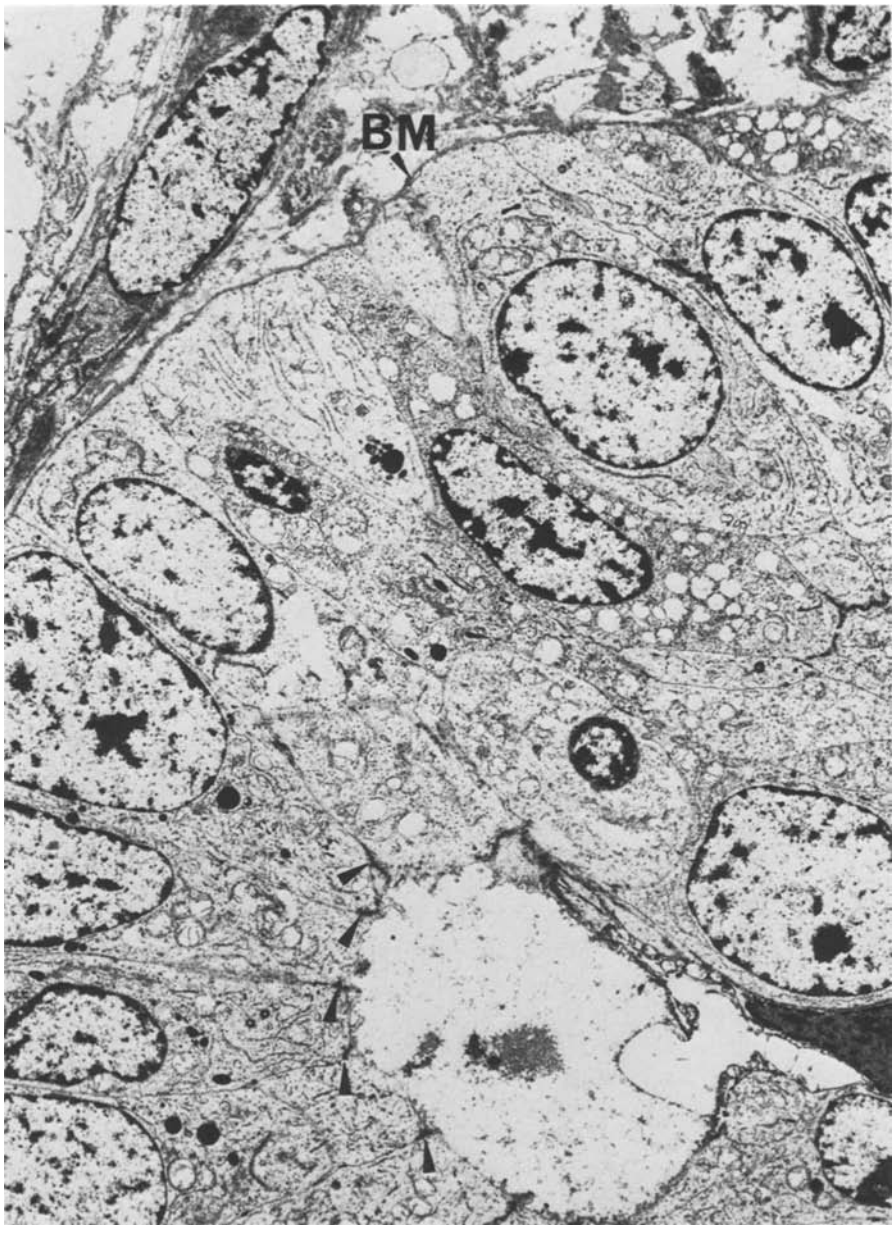


Fig. 5. Electron microscopic demonstration of a tubular area with a central lumen. The epithelial cells are connected by desmosomes and a junctional complex (*arrows*). Nuclei of epithelial cells are at the cell basis. The tubule is surrounded by a basement membrane (*BM*). 7000 ×

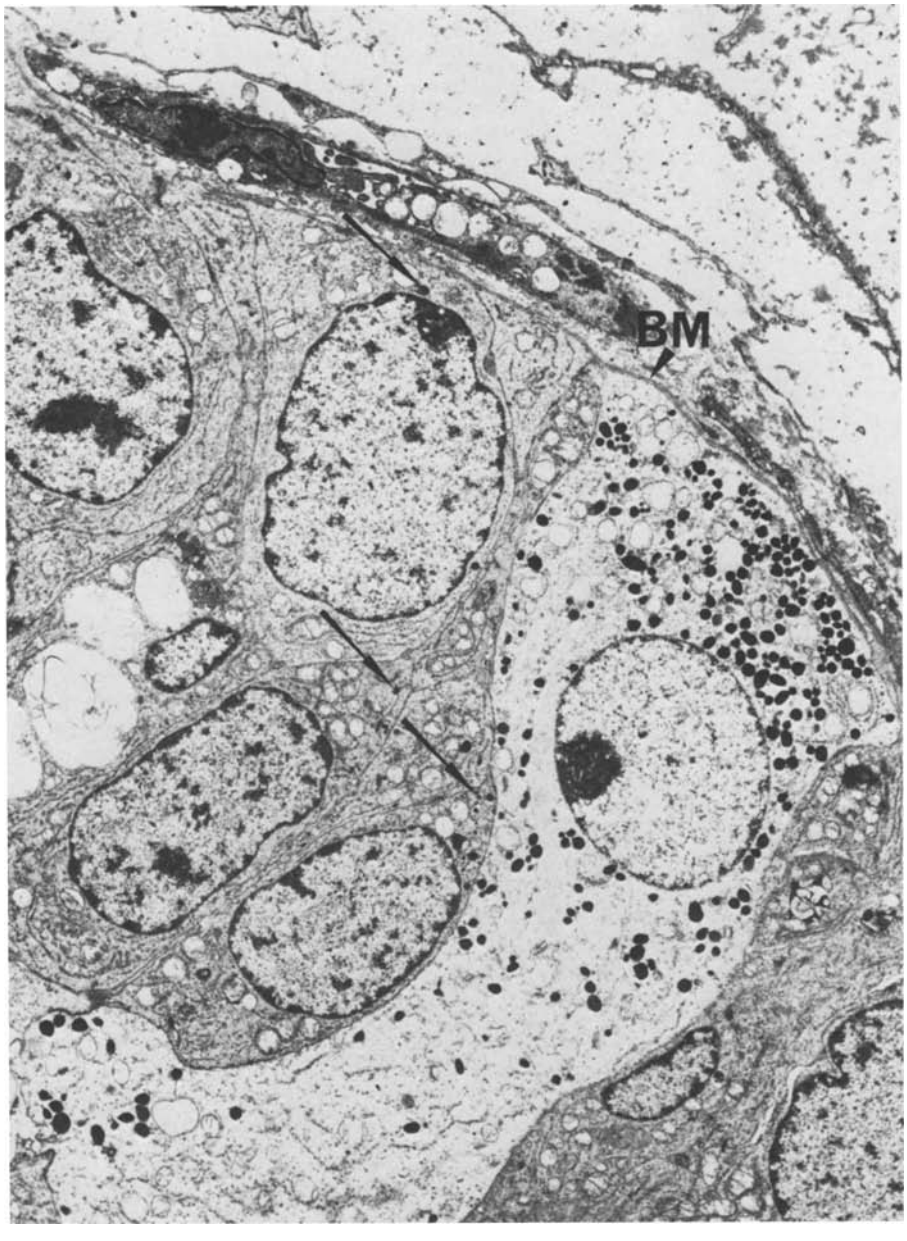


Fig. 6. In between the tubular epithelial cells numerous cells are intermingled containing electron dense granules of endocrine typ. Epithelial cells in nest-like areas contain also albeit much fewer endocrine granules (*arrows*). The basement membrane surround the tubule (*BM*). 7500 ×

Table 2. Endocrine activities of pulmonary adenocarcinoma of fetal type

Specificity	Results
Neurone specific enolase (NSE)	++
Protein S 100	−
Neurotensin (NT)	+
Serotonin (SER)	(+)
Leu-enkephalin (Leu-E)	+
Met-enkephalin (Met-E)	+
Somatostatin (Som)	+
Substance P	−
Gastrin	−
Bombesin (Bomb.)	(+)
Calcitonin	(+)
ACTH	−
β-endorphin	−
Glucagon	−
Cholecystokinin (CCK)	−
Vasoactive intestinal peptide (VIP)	−
βHCG	−
AFP	(+)

++: many, +: some, (+): a few,
−: no positve cells

Table 3. Reactivities of tubular and morula-like areas of pulmonary adenocarcinoma of fetal type

Specificity	Tubular	Single cells and morula-like nests
Intermediate filaments		
34β E 12	(+)	+
35β H 11	+	(−)
Neurofilaments	−	+
Melanoma associated antigens		
M-2-7-6	−	+
M-2-10-15	−	+
A-1-43	+	−
Others:		
Anti-Leu 7	−	−
Anti-EMA	−/(+)	+
Anti-HLA − A,B,C	−	−
Anti-HLA-DR	−	−

cells in this tumour. The reactivity to anti-Leu 7 was negative. Antibodies reacting with membrane antigens of squamous and glandular epithelium (A 1–43) showed the distribution of epithelial cells of tubular and acinar differentiation while leaving the endocrine morula-like nests of cells unstained. A similar contrasting staining pattern was found with different keratins: The 34-beta H11 antibody staining the pre-keratin of squamous and some glandular epithelial cells reacted positively in the tubular epithelium with weak activities in morula-like areas whereas 35-beta H12 antibody

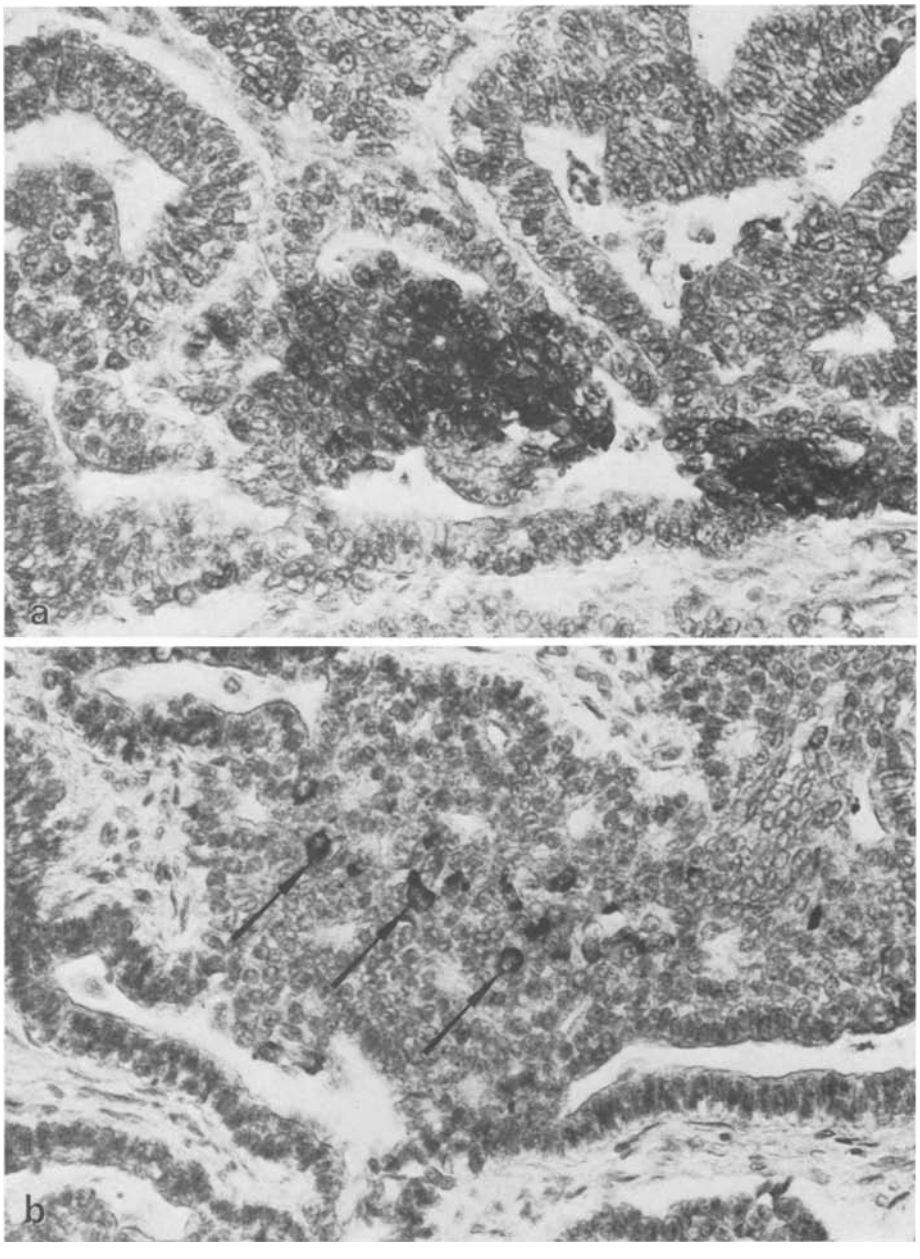


Fig. 7a, b. Immunhistochemical demonstration of endocrine activities. **a** Demonstration of neuron-specific enolase reacting predominantly with morula-like epithelial complexes. The glandular areas are completely negative (800 ×). **b** Demonstration of leu-enkephalin immune reactivity (*arrows*) within tumour cells lying within morula-like nest (350 ×)

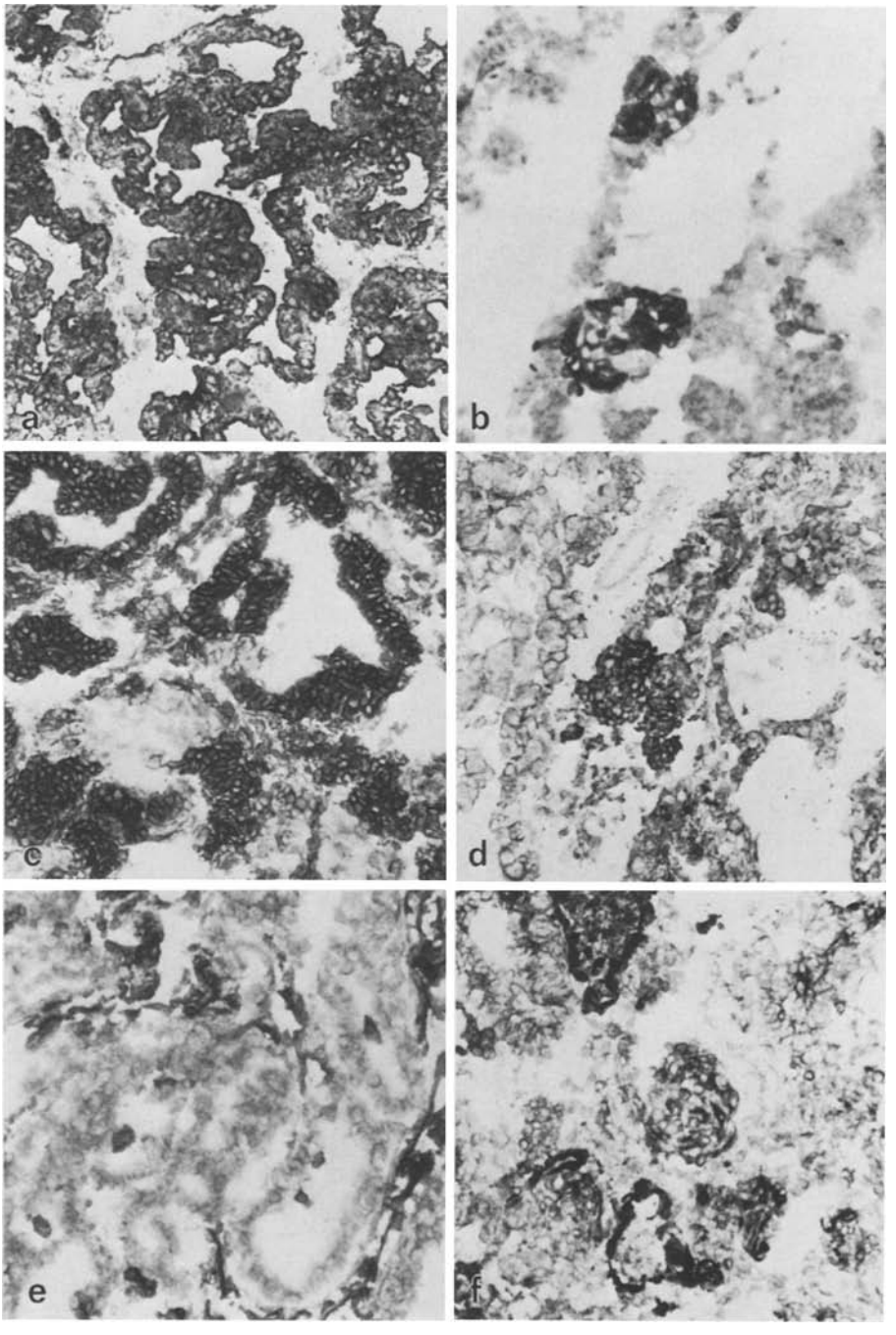


Fig. 8a–f. Immunhistochemical demonstration of different immune-reactivities on cryostat sections by monoclonal antibodies. **a** Antikeratin 35β H11; **b** Antikeratin 34β E 12; **c** Anti A1 43; **d** Anti M2 10 15; **e** Anti HLA ABC; **f** Anti EMA. For the description of the different reactivities within tubular and morula-like areas of this tumour see Table 3 and text. 160 × Immunoperoxidase-reaction

showed only a focal activity in small cell aggregates suggestive of the reactivity with endocrine cells in this tumour. EMA showed a positive reaction in some tubular epithelial cells and nests of cells suggestive of endocrine differentiated cell aggregates (Fig. 8).

Discussion

Malignant epithelial neoplasms of the lung resembling the structural pattern of an early stage of lung development have been reported recently (Kradin et al. 1982; Kodame et al. 1984; Manning et al. 1985). In 7 of 8 published cases evidence of neuro-endocrine differentiation was evident (Table 4). In all cases but that reported by Manning et al. (1985) and our case endocrine cells were present scattered as single cells among the tubular epithelial cells. The case reported by Manning et al. and our case showed morula-like densely packed aggregates of cells differing on light microscopical appearance from the tubular epithelium. As in the reported cases endocrine differentiation is visible by the demonstration of argyrophilia by the Grimelius technique within these cell aggregates, whereas the argentaffin-reaction is negative. The demonstration of peptide hormone immune reactivity in the earlier published cases was heterogeneous. In Kodame's et al. series four of six cases were positive for calcitonin and three of six for gastrin releasing peptide, which probably is also demonstrated by the bombesin-like immune reactivity (Tsutsumi et al. 1983). Manning et al., however, could not find

Table 4. Endocrine activity of the report cases of pulmonary adenocarcinoma of fetal type

Case no.	Author	Age/ sex	Evidence of endocrine activity									
			E.M.	Argyro-philia	Argent-affinity	NSE	Bomb.	SER	CT	Leu/E Met	Som	NT
1	Kradin et al. (1982)	35/f	+*	n.t.**	n.t.	n.t.	n.t.	n.t.	n.t.			
2	Kodame et	73/m	?	−	n.t.	n.t.	−	n.t.	+			
3	al. (1984)	67/m	n.t.	−	n.t.	n.t.	+	n.t.	+			
4		49/m	n.t.	−	n.t.	n.t.	+	n.t.	+			
5		59/m	?	−	n.t.	n.t.	−	n.t.	−			
6		54/m	?	−	n.t.	n.t.	+	n.t.	+			
7		23/m	n.t.	+	n.t.	n.t.	−	n.t.	−			
8	Manning et a. (1985)[a]	12/m	n.t.	+	−	−	−	+	−	+	+	+
9	present study[b]	64/m	+	+	−	+	(+)	+	(+)	+	+	+

* + = demonstration of dense core granules
** n.t. = not tested

[a] negative results were found at the demonstration of: corticotropin, insulin, glucagon, gastric I and II, VIP, substance P, CCK, β-endorphin, pancreatic polypeptide, secretin, gastrin
[b] negative results for: S 100, ACTH, β-endorphin, substance P, glucagon, VIP, CCK, gastrin

these hormones in his case but found somatostatin, neurotensin, serotonin, leu- and met-enkephalin instead. A comparable immunoreactivity to peptide hormones was also found in our case. Additionally bombesin-like immunoreactivity was found. Kodame et al. (1984) also investigated the epithelial component in pulmonary blastoma. They showed that in their cases similar immune reactivities (calcitonin and gastrin-releasing peptide) was found in pulmonary blastoma as in the fetal type adenocarcinoma.

Recently the ontogeny of neuro-endocrine cells in human fetal lung has been investigated by immunhistochemistry (Stahlman et al. 1985). It has been shown that after the 10th week of gestation peptide hormones and serotonin were demonstrable in the fetal lung. In the early fetal lung bombesin and serotonin was found whereas calcitonin was only found after the 20th week of gestation. Leu-enkephalin was found only in one infant who survived 7 postnatal months. The diversity of endocrine specificities of the different cases of this tumour is yet unexplained. One may speculate that in Kodame's series a later stage of fetal differentiation already was achieved whereas in Manning et al. (1984) and in our case the reactivities resembled more the lung tissue of the first trimester. No investigation so far has been done on neurotensin and somatostatin immunoreactivities of the early fetal lung. Therefore it cannot be decided whether these reactivities represent normal differentiation of the early fetal fore gut-derived lung tissue or whether this reactivity has to be interpreted as evidence of heterotopic hormone immune reactivity as found in many endocrine neoplasms of medium to high malignancy (Gould et al. 1983a, b). In well differentiated (benign) carcinoid tumors of the lung we did not find somatostatin and neurotensin immune reactivities in 14 cases (unpublished results) whereas others found somatostatin rarely (Gould et al. 1983; Bosman et al. 1984).

Different stages of cellular differentiation were visible not only in the endocrine part of this tumour but also in the tubular epithelium. Similarly to the ultrastructural features reported by Kodame et al. (1984) some granular inclusions in the cytoplasm were visible in addition to very undifferentiated epithelial cells. These granular inclusions were partly comparable to immature Clara-cells and to the concentric lamellar bodies of pneumonocytes type II. The functional relationship of Clara-cell granules to the granules in pneumonocyte II has been recently demonstrate by specific immunoreactivity to lung surfactant apoprotein in both types of granules (Balis et al. 1985).

The coexistence of tubular and acinar epithelium and regularly distributed foci of endodermal cells open the question of the histogenesis of these components. One possibility would be the coexistence of neoplastic (tubular) epithelium and of a nonneoplastic reactive hyperplasia of endocrine cells. The findings of Tameia et al. (1982) are very important evidence against this interpretation which showed endocrine differentiation in xenotransplanted purely epithelial tumours derived from pulmonary blastoma. Other tumours whose stem cell is thought to be derived from primitive endoderm may also contain endocrine cells. This is wellknown for some ovarian adenomas and carcinomas (Robboy 1984; Ueda et al. 1984). The endocrine

and tubular differentiation of epithelial cells in pulmonary adenocarcinoma of fetal type goes along also with changes of membrane bound immunoreactivities to monoclonal antibodies as well as of immunreactivities to intermediate filaments. This is demonstrated by the different pattern of immunoreactivity with melanoma-assoziated antigens M 2–7–6, M 2–10–15 and A 1–43 and cytokeratins 34β E 12 and 35β H 11.

Its interesting that endocrine cells are negative when anti-Leu 7 (HNK1) is used that has been reported to label cells of neuroendocrine differentiation (Lipinski et al.1983). A recent re-evaluation on different tumour cell lines of lung tumours, however, showed a broader reactivity with tumours of different cellular origin (Cole et al. 1985). The complex organoid structure of this tumour shows limited differentiation along the different cell lines that have usually been separated from each other in the original APUD cell concept, namely alveolar epithelium and endocrine cells. Similar to other evidence recently discussed for the endocrine differentiation of lung tumours (Gould et al. 1983a, b) this tumour gives additional evidence in favour of a common endoderm-derived stem cell for endocrine and tubular epithelium of the normal lung. From these arguments it follows that it would be very interesting to look for endocrine differentiation in other less well organized pulmonary adenocarcinomata to see whether abortive forms of endocrine differentiation might be present in these tumours which could account for the fact that lung tumour cell lines of human carcinoma of different histological type not related to small cell carcinoma of the lung were found to produce peptide hormones (Luster et al. 1985).

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