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NORMAL RESPIRATORY MUCOSA, PRECURSOR LESIONS AND LUNG CARCINOMAS: DIFFERENTIAL EXPRESSION OF HUMAN MUCIN GENES

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1. ABSTRACT

Mucins are glycoproteins synthesized by epithelial cells and thought to promote tumor-cell invasion. Eight human mucin genes have been well characterized: *MUC2*, *MUC5AC*, *MUC5B*, *MUC6* map to 11p15.5 and encode secretory gel forming mucins while *MUC1*, *MUC3*, *MUC4*, *MUC7* are scattered on different chromosomes and encode membrane-bound or secreted mucins. The expression pattern of the mucin genes is complex in normal airways involving six genes, mainly *MUC5AC* and *MUC5B* in mucus-producing cells and *MUC4* in a wide array of epithelial cells. *MUC5AC* overexpression in metaplasia, dysplasia and normal epithelium adjacent to squamous cell carcinoma provides additional arguments for a mucous cell origin of preneoplastic squamous lesions. *MUC5AC* and *MUC5B* expression is related to mucus formation in adenocarcinomas. Mucinous bronchioloalveolar carcinoma (BAC) has a particular pattern of mucin gene expression indicating that it has sustained a well-differentiated phenotype similar to the goblet cell, correlated with distinctive features i.e. a noninvasive pattern and a better prognosis than nonBACs. *MUC4* is the earlier mucin gene expressed in the foregut, before epithelial differentiation and is expressed independently of mucus secretion both in normal adult airways and carcinomas. These findings are in favor the histogenetic theory of non-small-cell carcinoma originating from a pluripotent mucous cell.

2. INTRODUCTION

2.1. Secretory Cells in Normal Airways and the Repair Process

The major cell types constituting the pseudostratified, ciliated and columnar surface epithelium which line trachea and bronchi are basal cells, secretory cells, and ciliated cells (Figure 1). The surface epithelium becomes simple cuboidal in bronchioles. Ciliated cells predominate, interspersed by goblet cells which are more numerous in trachea than bronchi and are rarely found in bronchioles less than 1 mm diameter (1).Two cell types secrete mucus in the surface epithelium: the goblet cell and the mucus-small-granule cell which is less differentiated (2). This latter type might correspond to the serous cell described in animals and fœtal humans and infrequently found in adult human small bronchi and bronchioli (1). Transitional cells, serous-mucous cells, have already been described, in rats made bronchitic by inhalation of cigarette smoke or in areas of human normal epithelium in lungs resected for carcinoma (3). Ultrastructural examination has

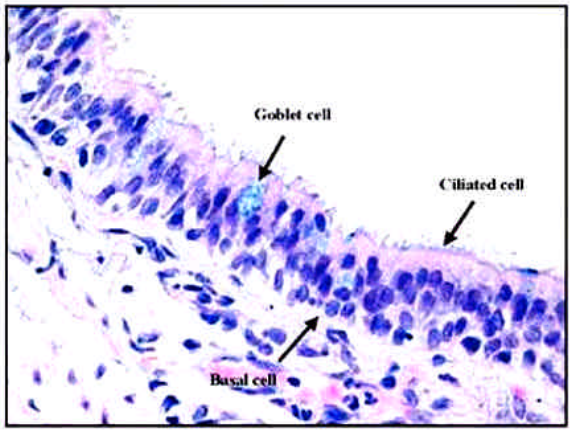

Figure 1. Normal respiratory mucosa. HES (Hematoxylin Eosin Saffron). Magnification, x400.

shown that these cells contain secretory granules of the mucous type and electron-dense cores ressembling serous granules. Mucous cells have various functions: they produce mucus to protect the lining mucosa from dehydration and damage from inhaled particles, pathogens and chemical irritants (4) and they act as progenitors of ciliated and other epithelial cell types in lung development and repair process (5,6,7). Studying the histogenesis of metaplastic change in human and hamster respiratory epithelium *in vitro*, Trump *et al.* (2) showed that mucous cell hyperplasia bears close relationship to squamous metaplasia, which is a frequent change following injury, arising subsequently by division of mucous cells called small-mucous-granules cells. These results were conflicting in regard to previous hypothesis applied to tracheobronchial epithelium and uterine cervix where basal cells were thought to be the stem cells. Moreover, during lung development, ciliated cells and secretory cells differentiate prior to basal cells (8).

The submucosal glands form a major source of tracheobronchial mucus. They are numerous in cartilaginous bronchi. The gland unit is of tubuloalveolar-type composed of a narrow ciliated duct, a collecting duct of intermediate morphology, mucous tubules and mucous and serous acini.

The Clara cells are located in terminal bronchioles acting as the stem cell of small airways where basal cells and mucous cells are normally sparse. Ciliated cells, mucous cells and also type II pneumocytes may develop from the Clara cell subsequent to its division and differentiation (9,10).

The mature alveolar epithelium is simple, composed of two cell types, type I and type II pneumocytes. Type I cells cover the surface of alveoli by very thin cytoplasmic sheets whereas type II cells are sparse and located in the corners of alveoli. Autoradiographic studies have demonstrated that during repair of lung injury, alveolar type II cells proliferate and differentiate into alveolar type I cells (11,12,13).

2.2. Lung Non-Neuroendocrine Carcinomas: Pathology and Histogenesis

Lung cancer is one of the most commonly diagnosed malignancies in Europe and United States and the first cause of cancer death in the developing world. Despite more accurate tumour staging and treatment planning, the prognosis of non-small-cell carcinomas (NSCLC) is poor (5-year survival rates less than 20%) (14,15). Small-cell carcinoma accounts for 25 percent of cases of lung cancer. The remaining 75 percent of lung cancer fall into NSCLC that include several histologic subtypes such as squamous cell carcinoma, adenocarcinoma and large-cell undifferentiated carcinoma (16). In Europe, squamous cell carcinoma is the most frequent NSCLC subtype accounting for about 40 percent of all lung cancers while adenocarcinoma represents 15 to 25 percent of these malignancies. Several large studies have demonstrated a change in the incidence of the different histologic subtypes of lung cancer with adenocarcinoma dramatically increasing particularly in the United States where it is now the most common subtype (15,17,18). The reasons for this increase are unclear but may be related to changes in types of cigarette smoked. Smoker of filter cigarettes tends to inhale the smoke more deeply than the smoker of plain cigarettes. Thus, the peripheral lung is exposed to higher amounts of nitrogen oxides, nitrosated compounds and lung-specific smoke carcinogens (19). A higher smoke delivery of the organ-specific lung carcinogen NNK to the peripheral lung is a major contributor to the increased risk of cigarette smokers for lung adenocarcinoma (20). The increase in bronchioloalveolar carcinoma's (BAC) incidence contributes also to the higher incidence of adenocarcinoma (21,22).

Lung adenocarcinomas are known to be very heterogeneous clinically and histologically both within the different subtypes (acinar, papillary, bronchioloalveolar, solid with mucin formation and mixed) according to the World Health Organization (WHO) classification (16) and within a same tumour. BAC is a particular subset of adenocarcinoma associated with different histological subtypes (mucinous, nonmucinous and mixed) and a better prognosis. The definition of bronchioloalveolar carcinoma is now restricted to only noninvasive tumours without stromal, vascular, or pleural invasion. Moreover, adenocarcinomas with an invasive acinar, solid or papillary component are classified as nonBACs (mixed subtype) even in case of predominant bronchioloalveolar pattern (16). Therefore, classification of lung carcinomas is of great importance and the most meaningful classification will likely to have both biological basis and prognostic implications. Histologically, adenocarcinoma is characterized by glandular formation with mucin secretion while squamous cell carcinoma is characterized by keratinization and desmosomes (16).

Multiple sequential morphologic changes are described in the development of squamous carcinoma in the large airways, particularly in smokers and reflect sequential steps in carcinogenesis. It is now commonly accepted that

squamous cell carcinoma arises from squamous metaplastic epithelium with progressive dysplasia (23). The WHO uses the term "preinvasive" for these"epithelial abnormalities that are cytologically neoplastic but do not penetrate the basement membrane" (16). Mucosal changes in the large airways that may precede or accompany invasive squamous carcinoma include hyperplasia (goblet cell or basal/reserve cell hyperplasia), metaplasia, which are generally thought to be reversible. These changes are followed by varying degrees of squamous dysplasia and squamous carcinoma *in situ*, from which an invasive carcinoma may develop (24,25). Molecular abnormalities correlate with morphologic changes in keeping with a multistage model of carcinogenesis. For instance, several studies have demonstrate increasing p53 immunoreactivity in increasing grades of dysplasia (26,27,28) or increasing frequency of p16 methylation during disease progression from basal cell hyperplasia (17%) to squamous metaplasia (24%) to carcinoma *in situ* (50%) lesions (29). According to the "field-cancerization theory", the chronic exposure of the entire bronchial epithelium to carcinogens (i.e. cigarette smoke) leads to an increased risk for multistep cancer development.

2.3. Mucin Genes

Mucins constitute a heterogeneous group of highly O-glycosylated macromolecules synthesized by a wide variety of epithelial tissues and thought to promote tumor-cell invasion and metastasis. They have a characteristic aminoacid composition, with a high content of threonine and serine carrying O-linked glycan chains and distributed in tandemly repeated motifs in the central part of the protein backbone. There are secretory or membrane-associated forms of mucins. Secretory mucins form the mucus gel and contain almost 80% carbohydrates by weight. To date, 8 human mucin genes (which encode the apomucin backbone): *MUC1* to *MUC4, MUC5AC, MUC5B, MUC6* and *MUC7* have been well characterized from various epithelial tissues or mucosae including airways though not all have been completely sequenced (30,31,32). More recently, additionnal cDNA clones have been proposed as *MUC8* (33), *MUC9* (34), *MUC11* and *MUC12* (35) but their expression are not so well defined. The family of four genes *MUC2, MUC5AC, MUC5B, MUC6* maps to 11p15.5 (36) and encodes large secretory gel forming mucins containing cystein-rich subdomains. The other genes *MUC1, MUC3, MUC4, MUC7, MUC8* are scattered on different chromosomes (37) and encode membrane-bound or secreted mucins having other peptide organizations, the first and the best known of them being *MUC1*.

Several arguments suggest that mucins play a role in tumour-cell invasion and metastasis, resulting in prognostic implications. MUC1 is a transmembrane molecule with a large extracellular domain protruding high above the cell surface thought to reduce cell-cell and extracellular matrix (ECM)-cell adhesion in cancer cells (38,39) but direct evidence for a role of specific mucin genes in tumor progression is lacking. One study shows that splenic-portal inoculation in athymic mice of MUC2 antisense construct in highly metastatic human colon cancer cells resulted in a reduction in MUC2 levels and a marked decrease in liver colonization (40). Sialomucin complex (SMC), a rat homologue of the human mucin MUC4 isolated from highly metastatic ascites 13762 mammary adenocarcinoma cells is thought to potentiate metastasis by sterically disruption of molecular interactions for cell-cell and cell-ECM adhesions and by suppression of anti-tumor immunity by inhibition of interactions between cytotoxic lymphocytes and target tumor cells (41). One recent study shows that *in vivo*, subcutaneous injection of SMC-overexpressing cells results in substantially greater lung metastasis than injection of SMC-repressed cells. Moreover, injection of A375 human melanoma cells followed by in vivo induction of SMC overexpression within the solid tumor resulted in spontaneous distant metastasis (42).

Physiological expression patterns of most of these genes have been studied in different laboratories using various techniques including northern blot, dot blot, reverse dot blot, RT-PCR or *in situ* hybridization. With the differentiation state of epithelial cells and with malignant transformation, the tissue and cell-specific expression of mucin genes becomes dysregulated (43,44). The pattern of mucin mRNA and glycoproteins are frequently altered in adenocarcinomas compared to normal tissues. Alterations include increased, decreased, lost or aberrant expression of mucin mRNA. Immunohistochemical studies using antibodies that recognize the tandem repeat sequences of apomucin have shown an increased immunoreactivity in carcinomas compared with normal tissues, most likely due to incomplete glycosylation and exposure of peptide epitopes (45). So, studies of apomucin expression necessitate antibodies whose epitopes are not obscured by glycosylation. There is now a great interest in mucins as markers of differentiation and tumor progression.

3. MUCIN GENE EXPRESSION IN NORMAL RESPIRATORY TISSUES

Four of the mucin genes (*MUC4, MUC5AC, MUC5B* and *MUC* 8) were isolated by screening a human tracheobronchial cDNA library, *MUC4, MUC5AC* and *MUC5B* in our laboratory with a polyclonal antiserum raised against deglycosylated glycopeptides purified from human sputum (46,47). *MUC4, MUC5AC* and *MUC5B* demonstrate to encode major airway mucins by northern blot analysis and *in situ* hybridization performed using normal specimens. The expression of seven mucin genes (*MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC7* and *MUC8*) has been demonstrated in normal tracheobronchial mucosa by northern blot (32,33,46,47,48,49). In contrast to northern or slot blot analysis, *in situ* hybridization which is a sensitive technique allows the cellular localization of the signal and the determination of the expression pattern of mucin genes in human adult mucosae. The tandem repeat organization and the choice of 'consensus' oligonucleotide probes specific for the tandem repeat of each gene is a convenient way of obtaining the amplification of the signal by hybridizing a maximum number of small probes all along the same mRNA molecule (50) (Table 1). Recent studies have characterized the pattern of mucin gene

Table 1. Oligonucleotide probe sequences for *in situ* hybridization

Gene	Oligonucleotide sequence
MUC1	5′ GTC CGG GGC CGA GGT GAC ACC GTG GGC TGG GGG GGC GGT GGA GCC CGG 3′
MUC2	5′ GGT CTG TGT GCC GGT GGG TGT TGG GGT TGG GGT CAC CGT GGT GGT GGT 3′ T
MUC3	5′ GGT GGT CTC GGT GGT GGT GAT <GA AGA AGT GAA GCT GGG AGT ACT GTG 3′ G
MUC4	5′ GTC GGT GAC AGG AAG AGG GGT GGC GTG ACC TGT GGA TGC TGA GGA AGT 3′
MUC5AC	5′ AGG GGC AGA AGT TGT GCT CGT TGT GGG AGC AGA GGT TGT GCT GGT TGT 3′
MUC5B	5′ TGT GGT CAG CTC TGT GAG GAT CCA GGT CGT CCC CGG AGT GGA GGA GGG 3′
MUC6	5′ TTC AGG ATG GTG TGT GGA GGA AGC ATG TGA GTG GAG AGA TGT AGA AGT 3′
MUC7	5′ CGG TGG AGC TGG TGT AGT TGC AGA AGG TGT GGG TGG GGC AGC TGT GGT 3′

Table 2. Cytodifferentiation during lung development. Major events

Gestation	Major events	
To 6 weeks	Lung buds form	J22-J26
	Lung primordia	J28
6-16 weeks	Neuroendocrine cells	8 wk
	Ciliated cells	10-13 wk
	Mucous cells	13-14 wk
	Submucosal glands	15-16 wk
17-24 weeks	Basal cells	17 wk
	Clara cells	19 wk
	Alveolar cells	22 wk
25 weeks to term	Complete division of conducting airways	28 wk
	Acini formation	
	Surfactant maturation	

expression in normal adult and fetal lung with regard of their cellular localization and variations along the respiratory tract (51-54).

3.1. Fœtal tissues

The primitive lung is a small epithelial tubule originating as a diverticulum from the foregut 22-26 days after fertilization. This bud then grows on either side of the foregut as the embryonic lungs, with a striking multiplication of branches by sequential budding and segmentation and progressive elongation giving rise to airways and distal alveoli. As branching proceeds, the lung epithelium undergoes cytodifferentiation (Table 2). In fetal respiratory epithelium, secretion of mucus begins by 13 wk of gestation, although the biologic role of fetal derived mucus *in utero* remains largely unknown (1).

Mucin gene expression is subjected to profound differential regulation during human fetal development from 6.5 to 27 wk of gestation (53). *MUC4* is the first mucin gene to be expressed, as early as 6.5 wk after gestation, initially occurring in the embryonic foregut before organogenesis. *MUC1* and *MUC2* are expressed from 9.5 wk of gestation in trachea, bronchi, epithelial tubules and terminal sacs. At this stage of gestation, the tracheal epithelium is poorly differentiated, with ciliated cells only appearing between 11 and 12 wk and secretory cells appearing by 13 wk. These cells develop at later stages of gestation in bronchi as differentiation proceeds peripherally through the tissue. For *MUC2*, the labeling was located in all surface epithelial cells and was distributed throughout the cytoplasm up to 13 wk of gestation. After this time, the labeling was confined to basal cells of the pseudostratified epithelium, up to 23wk. Thus *MUC1*, *MUC2*, and *MUC4* are expressed in fetal lung before epithelial cytodifferentiation into ciliated or secretory cells (53). In contrast, *MUC5AC* and *MUC5B* expression is initiated concomitantly with epithelial cell differentiation around 13 wk of gestation in epithelial folds and gland ducts (53). In addition, *MUC5B* is detected in mucous acini as soon as they form, from the 18th week (53). Finally, the late onset of *MUC7* expression is confined to serous cells at 23 wk of gestation and appears to be associated with the cytodifferentiation of this cell type (51, 53). MUC3 and MUC6 mRNA are not detected in trachea, bronchi, bronchioles, terminal sacs or alveoli (51,53).

3.2. Adult tissues

We have analyzed by *in situ* hybridization the expression patterns of *MUC1-MUC4*, *MUC5AC*, *MUC5B*, *MUC6*, *MUC7* in normal airways (50,53,54), completed by immunohistochemistry for the secreted mucins (MUC2, MUC5AC, MUC5B and MUC6) with specific antibodies for MUC2, MUC5AC, MUC5B and MUC6 apomucins (antibodies features are listed in Table 3). We showed that the mucin gene expression pattern is complex in normal airways and lung involving six genes *MUC1*, *MUC2*, *MUC4*, *MUC5AC*, *MUC5B* and *MUC7* expressed in an array of epithelial cells exhibiting various phenotypes: *MUC1*, *MUC2*, *MUC5B* and *MUC7* in the submucosal glands and *MUC1*, *MUC2*, *MUC4* (Figure 2), *MUC5AC* and *MUC5B* in the surface epithelium. In distal bronchioles, Clara cells express *MUC1* and *MUC4* (data unpublished). The alveolar type II epithelial cells express the MUC1 glycoprotein detected by immunohistochemistry (59) but any other mucin genes are detectable (53,54). In our experience, the two types of alveolar cells express MUC1 by immunohistochemistry (data unpublished) using M8 antibody which recognizes the tandem repeat sequences (60,61).

The two major sites of mucus production in airways are goblet cells in surface epithelium and mucous cells in submucosal glands. MUC5AC and MUC5B are two secreted mucins, encoded by the 11p15.5 mucin gene cluster, which are associated with mucus secretion in airways, MUC5AC in surface epithelium and MUC5B in submucosal glands (52-54). MUC5AC mRNA and protein are located in the more specialized cells for mucus secretion, i.e., the goblet cells. *MUC5B* mRNA and protein

Table 3. Characteristics of the antibodies used

Antibody	Specificity	Type	Peptide Amino Acid Sequence	Ref
LUM2-3	MUC2 N-terminal to the D4 domain	Rabbit polyclonal	NGLQPVRVEDPDGC	55
CLH2	MUC5AC TR	IgG1-MoAb	TTSTTSAP SAPTTSTTSAPT CTTSTTSAPTTSTTSAPTTS	56
LUM5B-2	MUC5B TR	Rabbit polyclonal	RNREQVGKFKMC	57
CLH5	MUC6 TR	IgG1-MoAb	SFQTTTTYPTPSHPQTTLPC	58

TR : Tandem Repeat

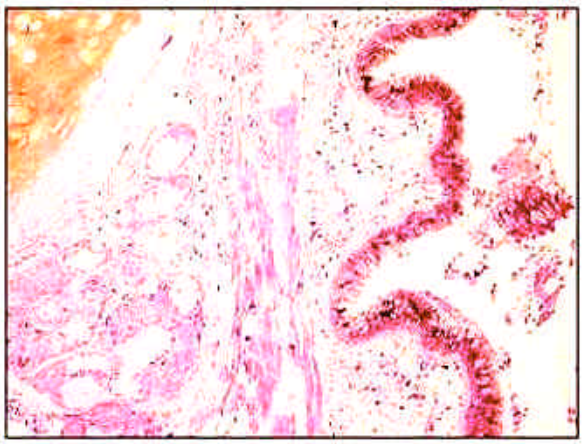

Figure 2. Normal intrapulmonary bronchus. Expression of *MUC4* gene mRNAs by *in situ* hybridization. The signal is intense in the surface epithelium and undetectable in submucosal glands. Magnification, x100.

are essentially detected in the mucous cells of submucosal glands and in gland ducts, serous cells being negative. Therefore, MUC5AC expression is stronger in trachea and main bronchi where goblet cells are numerous, compared with intrapulmonary bronchi. The intensity of the expression decreases in the epithelium of segmental bronchi but remains strong at the joining up of the proximal ciliated duct and the surface epithelium. *MUC5AC* is undetectable in bronchioles of less than 1 mm diameter and alveolar epithelial cells. For the same area of normal bronchial mucosa, the intensity of *MUC5AC* expression decreases from the surface epithelium to the submucosal glands where it is absent (54). MUC5B is expressed in some of the goblet cells of the surface epithelium while each goblet cell express MUC5AC. These two subtypes of goblet cells respectively MUC5AC+, MUC5B+ and MUC5AC+, MUC5B- could reflect cells at varying stages of mucin synthesis (54). Both biochemical studies and MUC gene expression by *in situ* hybridization show that MUC5AC is the major component of mucus in normal airways and is produced mainly by goblet cells (50, 53, 54, 62, 63). *MUC2* mRNA and protein are weakly expressed in the surface epithelium, in some of the goblets cells and within submucosal glands, in mucous cells and in some of the serous cells. So, MUC2 is not a prominent mucin in respiratory secretions as already suggested by biochemical analysis of respiratory tract secretions (62). Consequently, several studies have suggested a role for MUC2 in the pathogenesis of inflammatory airway disorders (63,64). Moreover, transcriptional activation of *MUC2* by *Pseudomonas aeruginosa* lipopolysaccharide may play a role in the pathogenesis of cystic fibrosis (65). We have observed (unpublished data) in one case of cystic fibrosis an intense expression of *MUC2* in basal cells by *in situ* hybridization, similar to *MUC2* expression in fetuses up to 23wk, in contrast to the weak and diffuse expression in normal adult mucosa. Transcriptional activation of *MUC2* and its location in pluripotential stem cells or basal cells suggest a role of *MUC2* in epithelial renewal in airways. Moreover, the expression pattern of MUC2, MUC5AC and MUC5B is in agreement with a previous report that investigated the mucociliary differentiation-dependent mucin gene expression in bronchial cells cultured in presence of retinoic acid (66). Following retinoic acid treatment of retinoid-deficient human tracheobronchial epithelial cell cultures, induction of mucin gene expression occurs sequentially : *MUC2*, *MUC5AC*, *MUC5B* were upregulated at 24, 48 and 72h, respectively. This study indicates that *MUC2* mRNA expression is an early marker of mucous differentiation whereas *MUC5AC* and *MUC5B* mRNA are expressed later, during more advanced stages of mucous differentiation (66).

MUC5B and *MUC7* define mutually exclusive cellular compartments within submucosal glands, respectively mucous acini and a subpopulation of serous acini (52). This differential expression of mucin genes in submucosal glands might reflect differences in the differentiation state of these two cell types, serous cells being replaced by mucous cells in response to a variety of airway insults (4).

In contrast to *MUC5AC* and *MUC5B* which are expressed in specialized cells, *MUC1*, *MUC2* and *MUC4* are found in all epithelial cells of the surface epithelium, both in ciliated cells, goblet cells and Clara cells suggesting various functions of mucin gene besides mucus production (54,67). Moreover, *MUC4* is the first mucin gene to be expressed in the foregut (53). The primitive epithelial cells

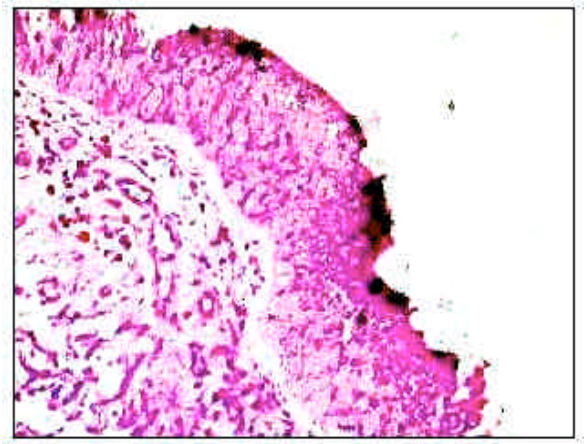

Figure 3. Dysplasia. Expression of *MUC5AC* gene mRNAs by *in situ* hybridization. The signal is strong in the upper part of the epithelium. Magnification, x400.

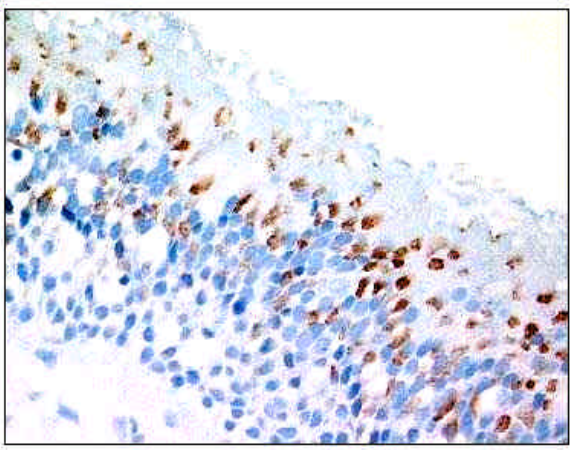

Figure 4. Normal bronchus adjacent to epidermoid carcinoma. Expression of MUC5AC protein by immunohistochemistry. The staining is strong in many suprabasal cells called small-mucous-granule cells. Magnification, x400.

have the potential to differentiate in all the epithelial cell types of the conducting airways and alveolar epithelium, suggesting that *MUC4* has a great importance in lung development and cell differentiation.

MUC3 and *MUC6* have never been detected in normal adult and fetal respiratory tract by in situ hybridization and immunohistochemistry (50,51,53, 54,67-69). Only one study by northern blot has reported low levels of MUC3 mRNA in nonneoplastic tissue adjacent to carcinomas (70). MUC8 expression has been reported in normal bronchial epithelium by RT-PCR and immunohistochemistry in ciliated and mucus-secreting cells (67).

4. MUCIN GENE EXPRESSION IN LUNG CARCINOMAS AND PRECANCEROUS LESIONS

The alteration of the level and the expression pattern of mucin mRNA in lung adenocarcinoma or carcinoma cell lines compared with normal tissue has been suggested using dot blot, northern blot or immunochemistry techniques but without distinction of histologic subtypes (70-72). Yu *et al.* (71) found overexpression of *MUC5AC* in thirteen carcinomas, eight adenocarcinomas and five squamous cell carcinomas (21,7 %) and overexpression of *MUC5B* in 10 carcinomas, six adenocarcinomas and four squamous cell carcinomas (16,7 %) by slot blot analysis. Seregni *et al.* (72) studying *MUC1-MUC4* expression by northern blot analysis showed that the highest reactivity was observed for *MUC2* and *MUC4* mainly in the mucus-secreting adenocarcinoma type. *MUC3* and *MUC5B* were undetectable in the cancer specimens studied. Nguyen *et al.* (70) using the same technique found that squamous carcinoma overexpressed only *MUC4* compared to nonneoplastic tissue adjacent to carcinomas. High levels of *MUC3* and *MUC4* expression could be detected in well differentiated adenocarcinomas.

In our laboratory, we have analyzed and compared the qualitative and semiquantitative pattern of expression of the different mucin genes (*MUC1-MUC7*) by *in situ* hybridization in normal mucosa, preneoplastic lesions of the bronchus, squamous cell carcinomas and adenocarcinomas of the lung. Additionally, the results were analyzed for a relationship between the expression of any particular mucin gene and the histologic subtype (54,73).

4.1 Precursor Lesions of Squamous Cell Carcinoma, Invasive Squamous Cell carcinoma

In hyperplasia (basal cell/goblet cell), metaplasia and dysplasia, the pattern of qualitative expression of mucin genes is similar to that determined for normal mucosae. Nevertheless quantitative variations of the *MUC5AC* and *MUC4* expression levels are observed. In goblet cell hyperplasia, *MUC5AC* is overexpressed compared to normal mucosae, reflecting the increased number of goblet cells. *MUC5AC* is overexpressed in the upper part of the epithelium in basal cell hyperplasia, metaplasia and dysplasia (Figure 3) while in basal cells, *MUC5AC* is absent. Moreover, in our experience *MUC5AC* is overexpressed in normal bronchus epithelium adjacent to squamous cell carcinoma in comparison to the normal lobe (Figure 4) (54). The overexpression of this gene which is clearly related to mucous goblet cells in normal airways might be associated with mucous cell hyperplasia involving cell division and/or differentiation of small-granules mucous cells in precursor squamous lesions. These findings provide additionnal arguments in favor of a mucous cell origin of precursor lesions of squamous cell carcinoma, already suggested by experimental studies in hamsters (74). Proliferative changes were induced experimentally, by damaging the bronchial lining epithelium with a variety of physical or toxic agents. The earlier phase consisted of an increased number of small, basally located cells. These metaplastic cells in turn might be derived from proliferating mucous cells as suggested by the presence of many small mucous granules ultrastructurally in these metaplastic cells (2). It may also be true in humans because mucous cells can persist in preinvasive carcinoma and detection of traces of mucins is common in carcinomas of various types. Moreover, focal expression of cytokeratins that are usually

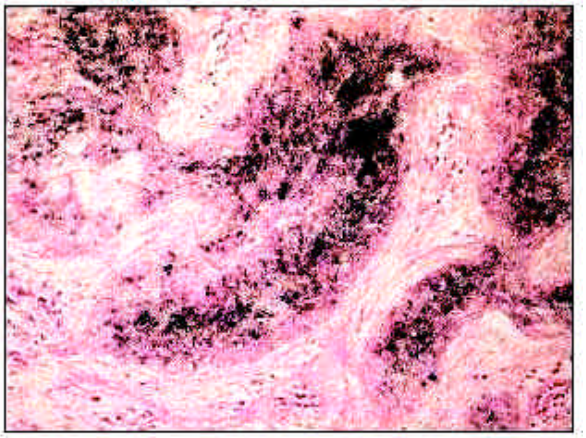

Figure 5. Well differentiated invasive squamous cell carcinoma. Expression of *MUC4* gene mRNAs by *in situ* hybridization. The signal is intense in the carcinomatous strands. Magnification, x200.

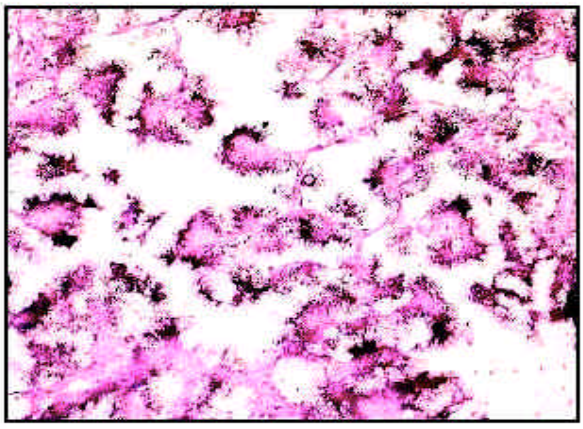

Figure 6. Mucinous type of bronchioloalveolar carcinoma. Expression of *MUC5AC* gene mRNAs by *in situ* hybridization. The signal is intense and diffuse in all carcinomatous cells. Magnification, x200.

associated with glandular phenotype as cytokeratin 7 can be found in squamous cell carcinoma (75). In *in situ* or invasive squamous cell carcinoma, when epidermoid differentiation processes are achieved, consisting of appearance of intracellular bridges and loss of ciliated and goblet cells, *MUC5AC* is downregulated and then labeling completely disappears, while *MUC4* expression is maintained in all carcinomatous cells. *MUC4* is expressed independently of mucus secretion both in normal airways or in various stages of epidermoid differentiation (54,67). In normal airways, it is expressed by basal cells and probably ciliated cells as well as collecting ducts and goblet cells. Its expression is associated with squamous metaplasia even with complete squamous cell differentiation, dysplasia and most of squamous cell carcinomas even well differentiated and keratinized (Figure 5). Moreover, we have shown (53) both that *MUC4* is the earlier mucin gene expressed in the foregut, before epithelial differentiation into ciliated or secretory cells and that *MUC5AC* is acquired concomitantly to epithelial cell differentiation. These findings in adult squamous lesions and embryonic respiratory tract might argue about the histogenetic theory of non-small-cell bronchogenic carcinoma originating from an immature precursor being perhaps a pluripotent mucous cell (2,76) and the distinctive role of *MUC4* in lung carcinoma histogenesis. The mucin phenotype of this pluripotent mucous cell might be *MUC5AC+*, *MUC4+*.

MUC6 has been focally detected in one study by immunohistochemistry in normal peritumoral epithelium using a polyclonal antibody that recognize the TR sequences of this apomucin (67). No expression of *MUC3*, *MUC6* and *MUC7* is found in squamous lesions as in the normal respiratory surface epithelium (54). *MUC8* mRNA and protein have been detected in squamous cell carcinoma (67).

4.2. Adenocarcinoma

Lung adenocarcinomas express mucin mRNA which are expressed in normal respiratory mucosa (*MUC1*, *MUC2*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC8*) and *MUC3* and/or *MUC6* mRNA which are not detected in normal lung by *in situ* hybridization (53,54,67,73). Interestingly, the expression pattern of mucin genes differs according to the histological subtypes of adenocarcinomas as defined by the WHO classification (16,73). Nonmucinous type of BACs and non BACs share the constant expression of *MUC1* and *MUC4*, the variable expression of *MUC2*, *MUC3*, *MUC5AC* and *MUC5B* and the absence of *MUC6* expression (73).

Among adenocarcinomas, the mucinous type of BAC has a particular pattern of mucin gene expression since all mucin genes are expressed except MUC7. The expression of *MUC5AC* (Figure 6) and *MUC5B* is the most intense and diffuse among all subtypes. Coexpression of *MUC1* and *MUC3* is constant. Coexpression of *MUC2*, *MUC4* and *MUC6* is very frequent. This complex but homogeneous expression pattern in mucinous BAC is in agreement with the great cellular homogeneity of this type of adenocarcinoma (73). There is now limited but convincing evidence that BAC is distinct from typical adenocarcinoma of the lung. When restrictive criteria for diagnosis of bronchioloalveolar carcinoma are used, this tumour exhibits a better prognosis than the other types (77). Whether the histological subtype is a prognostic factor in BAC, remains debated (78). A characteristic feature of the biology of BAC is the development of multifocal lesions within the lung parenchyma, the high frequency of diffuse pulmonary involvement with limited regional lymph node involvement and rare brain metastases (77). The different morphological and clinical patterns of the subtypes of BAC suggest that their biological behavior may differ. Clinically, the mucinous subtype is more strongly associated with diffuse pulmonary involvement than the nonmucinous subtype (79). There are already some molecular data that support the biological difference of mucinous type of BAC. A significantly higher frequency of K-*ras* mutations in the mucinous form of BAC than in the other subtypes has been demonstrated (80).

In lung, mucin gene expression may serve as a marker of cytodifferentiation. We showed that *MUC5AC* is expressed in goblet cells in normal respiratory mucosa and disappears when complete epidermoid differentiation is

achieved in metaplasia and squamous cell carcinoma (54). The great amount and the constant expression of this mucin in mucinous type of BAC might indicate that this subtype of adenocarcinoma had sustained a well-differentiated phenotype similar to the goblet cell, correlated with a noninvasive pattern and a better prognosis than nonBACs (73).

The expression of secretory mucins *MUC2*, *MUC5AC* and *MUC5B* is associated both with the different histological subtypes of adenocarcinomas and mucous secretion (73). Mucous-secreting adenocarcinomas maintain focal *MUC5AC* and *MUC5B* expression. Non-secreting and poor differentiated adenocarcinomas lost both *MUC5AC* and *MUC5B* expression and maintain *MUC2* mRNA expression. These results are in agreement with the previous report about MUC gene regulation in bronchial cells cultured in presence of retinoic acid already discussed about normal tissues (66). In this study, *MUC2* expression is an early marker of mucous differentiation whereas *MUC5AC* and *MUC5B* mRNA are expressed later, during more advanced stages of mucous differentiation.

MUC3 and *MUC6*, which are not expressed in normal adult and fetal lung, are expressed in lung adenocarcinomas (73). The *MUC6* gene is clustered with *MUC2*, *MUC5AC*, and *MUC5B* (in that order) at chromosomal location 11p15.5 (36). All four of the mucin genes show different expression patterns *in vivo* both in normal tissues and carcinomas suggesting that the regulation of this gene cluster is complex. However, the pattern of expression in mucinous type of BAC (i.e *MUC2*, *MUC5AC*, *MUC5B* and *MUC6*) might support some co-regulatory features for the 11p15.5 mucin gene cluster. *MUC6* is with *MUC5AC* primarily expressed in the stomach where *MUC5AC* is associated with surface mucous epithelial cells and *MUC6* with pyloric mucous gland cells (68). Honda *et al.* (81) previously described a subtype of mucinous bronchioloalveolar carcinoma expressing gastric mucins by histochemistry so called «gastric-type pulmonary carcinoma», morphologically indistinct from the other subtypes of mucinous BAC. Histochemically, this subtype is characterized by carcinoma cells (located in the protruding portions of the papillary structures) that differentiate as gastric-type surface mucous cells and carcinoma cells in the indented portions that differentiate as pyloric gland-type mucous cells. Heterotopic gastric mucosa was considered to be the origin of these tumours especially since respiratory and digestive tracts are foregut derivatives. The expression of «gastric» mucins has already been described in various sites including mucous metaplasia of the pancreatic duct and pancreatic ductal adenocarcinoma (82). This particular phenotype seems to be associated with malignant progression. We believe as others (67) that the expression pattern of mucin genes in lung carcinomas, particularly *MUC3* and *MUC6* genes in mucinous BAC, supports that lung carcinomas originate from a common endodermal precursor cell with the potential for multicellular differentiation (76) including expression of mucins of gastric type.

Whereas we did not detect *MUC4* gene expression in normal type II pneumocytes (54) we found it in type II pneumocyte hyperplasia. The other mucin genes were not detectable in this cell type. Adenocarcinomas could be separated in two categories depending upon mucin gene expression (73). On one hand, mucinous type of BAC is a very differentiated adenocarcinoma with both constant expression of the 11p15 mucin genes and phenotypic characteristics of the goblet-cell type. On the other hand, nonmucinous type of BAC and nonBAC share constant expression of *MUC1* and *MUC4*, as type II pneumocyte hyperplasia and focal expression of *MUC5AC* and *MUC5B* only in mucous-secreting areas. Finally, histological subtyping of adenocarcinomas might be more related to the degree of glandular differentiation than to the cellular origin.

5. PERSPECTIVE

In contrast to northern or slot blot analysis, *in situ* hybridization and immunohistochemistry allow the cellular localization of the signal. These techniques showed both that many tumors contain numerous residual normal cells which could be readily identified by morphological examination and that mucin genes can be expressed strongly by these entrapped cells ; for example *MUC5B* in submucosal glands and *MUC4* in bronchioles. Moreover, a signal confined to one cellular type such as *MUC5AC* in normal tissue or a weak and diffuse signal in carcinoma such as *MUC2* message can be distinguished even if negative cells are the major component. These data explain probably some conflicting results about the pattern of expression in epidermoid carcinoma and adenocarcinoma using blot analysis and show that the labeling intensity obtained by these two techniques is difficult to compare.

For several years, studies that focus on changes in the expression of mucin genes have suggested that their role exceed mucus production. The altered expression of mucin genes in bronchogenic carcinoma especially in well differentiated and keratinized epidermoid carcinoma and preneoplastic lesions suggests a role for some of them in neoplastic transformation and tumour progression. Moreover, the distinct profile of mucinous type of BAC supports the biological difference of this type of lung adenocarcinoma and the involvement of mucin genes in the cellular pathway of mucinous differentiation. From a clinical point of view, mucin genes could serve as diagnostic and prognostic markers. For instance, in the management of patients with BAC, detection of tumour markers in sputum or bronchial lavage fluid and bronchoalveolar lavage by immunohistochemistry could be of great importance since this type of carcinoma does not invade the bronchi. Histological diagnosis of BAC is often made using surgical specimens or samples obtained by transbronchial or needle biopsies. Further investigations are necessary to evaluate the potential utility of the aberrant expression of *MUC3* and *MUC6* in bronchial lavage fluid as a diagnostic argument of BAC recurrence.

The expression of mucin genes reflect a precise state of differentiation more complex than morphologic

differentiation grade which indicates only the similarity to normal glands. The study of the expression of mucin genes contributes to the determination of molecular basis of histopathological classification of lung carcinomas and supports the concept of a common cell origin from an immature and pluripotent mucous cell.

In the future, both studies about the regulation of mucin genes and the functional approach with knock out mice will allow the understanding of all the data accumulated with expression studies in cancers.

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