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MESOTHELIAL DIFFERENTIATION, MESOTHELIOMA AND TUMOR MARKERS IN SEROUS CAVITIES

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ABSTRACT

Mesothelial cells covering the serous membranes are of mesodermal origin although they acquire several characteristics of epithelial cells. The cytological and histological diagnosis of malignant tumors in serous cavities is sometimes a major challenge: the main diagnostic alternatives are reactive benign mesothelium, mesothelioma and adenocarcinoma. The aims of this thesis were to improve the differential diagnosis of these conditions and to study mechanisms related to mesothelial differentiation.

None of the antibodies recommended for distinguishing between mesothelioma and adenocarcinoma are entirely specific. Using logistic regression analysis the tissue immunoreactivities of 10 antibodies were ranked, 2 rejected and a panel of the best 8 retained. The optimized panel should preferably be applied in two steps, starting with the 4 diagnostically most valuable parameters.

The additional diagnostic value of two biochemical assays in ascitic fluid was assessed in cases having inconclusive effusion cytology. An increased concentration of CEA indicated a carcinoma. A higher cholesterol level indicated carcinomatosis, but was not entirely specific for this condition. These two chemical assays measure different effects of tumors (CEA production versus cell disintegration), and when they were combined, increases in both parameters were specific for carcinomatosis and improved the sensitivity of "single" effusion cytology.

Mesothelial cells obtained from benign effusions are not terminally differentiated and grow uniformly with a fibroblastic or epithelioid morphology *in vitro*. To investigate how this differentiation correlated with Wilms' tumor susceptibility gene 1 (WT1) and proteoglycans (PGs), their expressions were studied by semiquantitative RT-PCR. mRNAs for WT1 and cell surface PGs syndecans-1, -2, -4 and glypican-1 were more abundant in epithelioid cells, while fibroblastic cells expressed more of the matrix PGs biglycan and versican.

To assess the involvement of other molecules in mesothelial differentiation, suppression subtractive hybridization was used. This showed a pattern of genes coordinately regulated during this process. The genes overexpressed in fibroblastic cells were either matrix-associated or related to proliferation, while those upregulated in the epithelioid phenotype were related to a more differentiated cell type. Immunohistochemistry with available antibodies confirmed that the differences were also present *in vivo* at sites of mild mesothelial activation and regeneration.

Since PGs and WT1 were related to phenotypic differentiation in benign mesothelial cells, their expression was also studied in adenocarcinomas and mesotheliomas. Adenocarcinoma cells produced more mRNA for syndecan-1, but mesothelially derived cells expressed WT1, biglycan and larger amounts of syndecan-2. Syndecan-4 was highly expressed in all malignant cell lines, as compared to benign mesothelial ones. Versican expression was associated with a high rate of proliferation. Pilot experiments suggest that the reciprocal appearance of syndecan-1 and syndecan-2 may be a useful diagnostic marker with dominance of syndecan-2 reactivity in mesothelial tissues.

In summary, these data show the role of PGs in mesothelial differentiation, and suggest that they can be used as markers to distinguish malignant mesothelioma from other conditions. The findings also indicate that epithelioid mesothelial cells may be derived from subserosal fibroblastic cells, which would then correspond to a less differentiated mesothelial phenotype.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text with their roman numerals (**I-V**).

I. Brockstedt U, **Gulyás M**, Dobra K, Dejmek A, Hjerpe A
An optimized battery of eight antibodies that can distinguish most cases of epithelial mesothelioma from adenocarcinoma
Am J Clin Pathol 2000, 114: 203-209

II. **Gulyás M**, Kaposi AD, Elek G, Szollár LG, Hjerpe A
Value of carcinoembryonic antigen (CEA) and cholesterol assays of ascitic fluid in cases of inconclusive cytology
J Clin Pathol 2001, 54: 831-835

III. **Gulyás M**, Dobra K, Hjerpe A
Expression of genes coding for proteoglycans and Wilms' tumour susceptibility gene 1 (WT1) by variously differentiated benign human mesothelial cells
Differentiation 1999, 65: 89-96

IV. Sun X*, **Gulyás M***, Hjerpe A
Differentiation of benign mesothelial cells as reflected by differential gene expression in fibroblastic and epithelioid phenotypes
Submitted

V. **Gulyás M** and Hjerpe A
Proteoglycans and WT1 as markers for distinguishing adenocarcinoma, epithelioid mesothelioma, and bening mesothelium
J Pathol 2003, 199: 479-487

* Equal first authorship (Paper **IV**)

CONTENTS

LIST OF ABBREVIATIONS

AR	Aldose reductase
BSA	Bovine serum albumin
CBP2	Collagen binding protein 2
CDK	Cyclin-dependent kinase
CEA	Carcinoembryonic antigen
CK	Cytokeratin
CS	Chondroitin sulfate
DIG	Digoxigenin
DS	Dermatan sulfate
EGF	Epidermal Growth Factor
EMA	Epithelial membrane antigen
FACS	Fluorescence-activated cell sorter
FGF-2	Fibroblast Growth Factor-2
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
HA	Hyaluronan
HAS	Hyaluronan synthase
HB-EGF	Heparin-binding Epidermal Growth Factor
HCgp39	Human cartilage glycoprotein 39
HexUA	Hexuronic acid
HGF	Hepatocyte Growth Factor
HPLC	High-performance liquid chromatography
HS	Heparan sulfate
KS	Keratan sulfate
LCAT	Lecithin:cholesterol acyltransferase
NADP	Nicotinic adenine dinucleotide phosphate
PAS	Periodic acid-Schiff
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDGF-β	Platelet-derived Growth Factor-β
PG	Proteoglycan
PKC	Protein kinase C
pRb	Retinoblastoma protein
RF	Relative fluorescence
ROC	Receiver operating characteristics
ROS	Reactive oxygen species
RT- PCR	Reverse Transcriptase Polymerase Chain Reaction

SDS	Sodium dodecyl sulfate
SSH	Suppression Subtractive Hybridization
SV40	Simian virus 40
Tag	Large T-antigen
TGF-β	Transforming Growth Factor-β
TM	Thrombomodulin
TSG	Tumor suppressor gene
TTF-1	Thyroid Transcription Factor-1
VEGF	Vascular Endothelial Growth Factor
WT1	Wilms' tumor susceptibility gene 1

1 BACKGROUND

Malignancies in serous cavities are mainly due to metastatic tumors, while the primary malignant tumor of the serous membrane, the malignant mesothelioma, is much less common. The benign mesothelium, a transitional tissue, exhibits a mixture of mesenchymal and epithelial characteristics. This feature is retained in malignant tumors arising from these cells, and causes severe problems for pathologists in differential diagnosis. The various phenotypic growth patterns of malignant mesotheliomas may also be related to their prognosis. However, little is known about how mesothelial cells differentiate. A better comprehension of these differentiation mechanisms might therefore be of value in finding potential mesothelioma markers and improve understanding of the biology of such tumors.

1.1 SEROUS CAVITIES

Serous cavities are the body cavities lined by serous membranes. The visceral sheet of these membranes covers the internal organs, while the parietal sheet lines the body wall. Apart from the pleural, pericardial and peritoneal cavities, containing the lungs, heart, and abdominal organs, respectively, in the male, the tunica vaginalis testis lines the sac that partly surrounds the testes.

All serous membranes consist of a monolayer of flattened mesothelial cells resting on a basement membrane, and a submesothelial connective tissue with scattered fibroblastic cells underneath. The mesothelial cells, joined by tight or gap junctions and desmosomes, have long (up to 3 μm), slender microvilli on their surface. They secrete an effusion rich in hyaluronan [5] and phosphatidylcholine [46], both suggested as having a lubricant function.

Although mesothelial cells actively secrete a fluid, much of the serous effusion is a transudate of blood plasma, with a protein concentration of less than 20 g/L. The submesothelial layer contains a plexus of blood vessels and lymphatic channels, demarcated above and below by a thin elastic lamina. The lymphatic lacunae open to the mesothelial surface via narrow gaps, so-called stomata, which provide a direct exit from the serous space. The effusion is largely absorbed by the parietal serous membrane via these stomata.

1.2 MESOTHELIAL DIFFERENTIATION

The serous membranes develop from the mesodermal tissue bordering the intra-embryonic coelomic cavity [136]. The mesothelial cells are therefore of mesodermal origin, although they continuously differentiate towards an epithelioid morphology throughout adult life.

This process partly resembles embryonic nephrogenesis and formation of the surface (coelomic) epithelium of the ovaries. During the transitions of mesodermal cells into cells having an epithelial growth pattern, these three tissues (kidney, ovary, mesothelium) express the Wilms' tumor susceptibility gene 1 (WT1). In contrast to the transient embryonic expression of this gene during renal epithelialization, continuous

expression of WT1 during adult life has been described only in gonadal cells and mesothelial tissues [63, 117].

Unlike the mesothelium, these other mesodermal-epithelial transitions (kidney, ovary) result in tissues with adhesion mechanisms and ultrastructure of epithelial type. The mesothelium acquires only some of these epithelial characteristics, such as polygonal cell shape, the ability to form cytokeratin intermediate filaments, desmosomes and a basement membrane; but others, such as E-cadherin adhesions [1, 35, 120, 147], glycocalyx and microvillus-associated core rootlets, are less typical or not seen [11].

On the other hand, surface mesothelial cells also retain some mesenchymal attributes, such as containing vimentin intermediate cytoskeletal filaments, and N-cadherin [1, 35, 120, 147] as the main adhesion molecule. Moreover, the preserved mesenchymal nature is also reflected by the production of connective tissue matrix constituents, such as fibronectin, laminin, [128] and type I and III collagens [36, 57, 128, 153]. In resting serosal tissue, there are scattered fibroblast-like cells beneath the basement membrane that express vimentin as the main intermediate filament and have no microvilli on their surface. As the tissue becomes stimulated to proliferate, these submesothelial fibroblastic cells also coexpress vimentin and cytokeratins and develop surface microvilli [15, 16].

1.3 MESOTHELIAL REGENERATION

If the mesothelium is injured, two principally different mechanisms have been suggested for regeneration of the tissue, although these views are still disputed. When the fragile epithelioid mesothelium is damaged, the denuded surface may be covered rapidly by flattened superficial cells. These are not terminally differentiated, and they temporarily develop a spindle-shaped morphology while proliferating during repair. These cells may be derived from the surrounding surface mesothelium and migrate to the defect as well as, from the free-floating mesothelial cells exfoliated into the effusion fluid, and resettle on the denuded area [51, 175].

The second possible mechanism for regeneration has been assumed to be from fibroblast-like multipotential subserosal cells, which would serve as a source of mesothelial repair. Especially when the basement membrane is affected, these cells are thought to migrate to the surface, and gradually develop epithelial characteristics [126]. On stimulation, these proliferating fibroblastic cells begin to express several cytokeratins together with vimentin, as compared to the expression of vimentin alone in resting submesothelial fibroblasts [15, 16, 36]. However, much evidence has accumulated showing that the surface mesothelium can act as a source of precursor cells in serosal repair, but far less proof is available that fibroblastic subserosal cells play a similar role [101].

1.4 PROLIFERATIVE MESOTHELIAL REACTION

In cases of prolonged stimulation ─ e.g., because of an underlying disease process or persistent injury ─ the mesothelium will exhibit a chronic response in the form of mesothelial hyperplasia. In this reactive process, both the surface and submesothelial layers show signs of proliferation ─ increase in the number of cells (hyperplasia) ─ and, activation, resulting in larger cell volume (hypertrophy). Common causes of this

process, are liver cirrhosis, pulmonary infarct, collagen disease, chronic inflammation, irradiation, chemotherapy, traumatic irritation, foreign substance or underlying neoplasm [10].

Microscopically the flattened epithelioid mesothelial cells show swelling and may become cuboidal; they also increase in number, which leads to formation of papillary projections on the serosal surface. These papillae can be covered occasionally by several layers of mesothelial cells. The submesothelial connective tissue is thickened because of enlargement and an increase in the number of fibroblastic cells, as well as, the deposition of extracellular matrix [9, 98]. In cytologic preparations from effusions, reactive mesothelial cells may show many changes: enlargement, variations in size, cytoplasmic vacuolation, increase in the nuclear-cytoplasmic ratio, nuclear chromatin irregularities, pseudoglandular clustering, binucleation and occasional mitoses. These cells sometimes show nuclear features that mimic those of neoplastic transformation. In the histologic and cytologic nomenclature, apart from “mesothelial hyperplasia”, the terms “mesotheliosis” or “reactive mesothelial cells/tissue” are used to describe the proliferative mesothelial reaction.

2 MALIGNANCIES IN SEROUS CAVITIES

2.1 MALIGNANT MESOTHELIOMA

Malignant mesothelioma, the primary malignant tumor of the serous membrane, arises from the mesothelial cells. Mesotheliomas usually develop in the pleura, but also in the peritoneum and occasionally in the pericardium or tunica vaginalis testis. They start as a localized tumor, but spread rapidly to involve most of the visceral and parietal surfaces. Mesotheliomas are always malignant and the terms “benign fibrous mesothelioma” and “multicystic mesothelioma” have recently been regarded as misnomers. The former is considered not to be of mesothelial origin, but a “localized fibrous tumor” derived from true fibroblasts, while the neoplastic nature of the latter has been questioned, and the term “multicystic mesothelial proliferation” suggested.

2.1.1 Etiology and pathogenesis

2.1.1.1 Asbestos induced tumorigenesis

Most malignant mesotheliomas are associated with a previous long-term exposure to fibrous minerals of which asbestos is the most important. This indicates an indisputable role of such fibers in the etiology of the tumor, which has also been shown in animal experiments [89, 148, 170]. Only a small percentage of persons exposed to these fibrous minerals develop mesothelioma, which suggests that other factors are involved.

Most commercial asbestos is a mixture of various fibers. The two subgroups of these asbestos fibers are the serpentine and amphibole families. The serpentine-like chrysotile fibers are long and curly, while the amphiboles, represented by crocidolite, amosite and tremolite among others, are shorter and rod-like.

Chrysotile fibers are less oncogenic, if at all. They undergo fragmentation, can be partly digested by cellular enzymes, and they are efficiently removed from the lung by alveolar macrophages. The rod-like amphibole fibers, especially crocidolite, are particularly carcinogenic, especially if the fiber is longer than 8 μm [92]. These fibers can not be removed effectively and accumulate in the lung. They may reach the pleura through the lymphatics, or by direct penetration, and cause fibrosis, pleural plaques, and sometimes mesothelioma [17, 96].

The same oncogenic effect on mesothelium is seen with erionite, a mineral which has a structure similar to that of rod-like amphiboles. Erionite has a high mortality rate due to mesothelioma in central Anatolia, Turkey.

Mesothelioma is characterized by a long latency of about 20-40 years from the time of initial asbestos exposure to diagnosis, which suggests that multiple cumulative cytotoxic, genetic and proliferative events occur during the malignant transformation of a mesothelial cell.

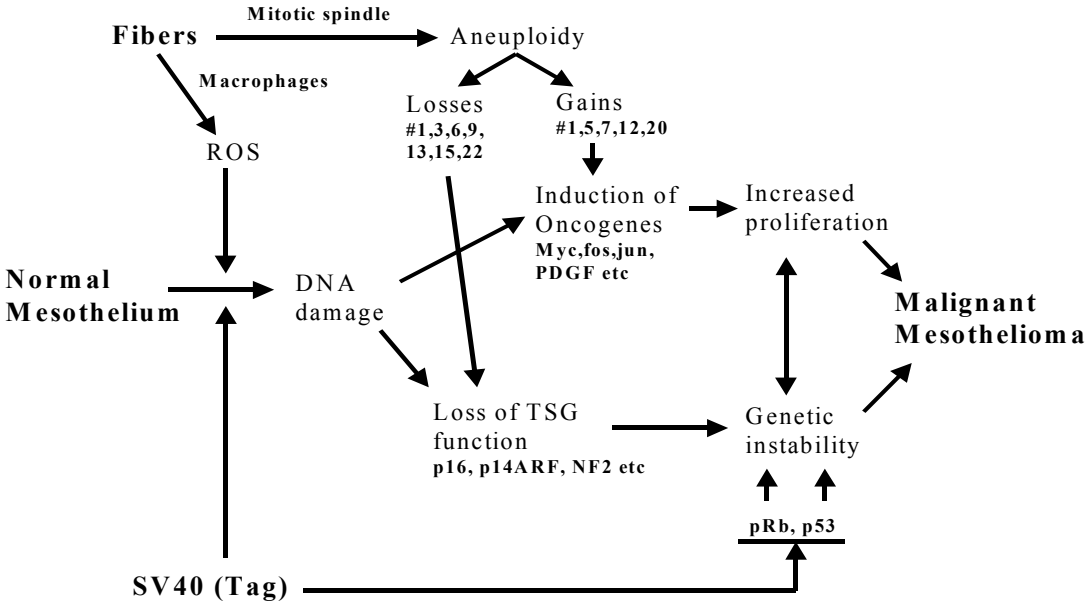

Fig 1. *Schematic model of mesothelioma pathogenesis. Asbestos fibers induce reactive oxygen species (ROS) that cause mutations or deletions. This leads to the inactivation of tumor suppressor genes (TSGs) as an early event. ROS may also stimulate the formation of oncogenes, which promotes proliferation. Such effects can also be the result of losses and gains due to asbestos-mediated chromosome missegregation. Presence of SV40 Tag may contribute by inhibiting TSGs. Modified from [99].*

Asbestos fibers may physically interact with the mitotic spindle apparatus of mesothelial cells, causing chromosome missegregation, which leads to aneuploidy [8]. On the other hand, asbestos can also generate reactive oxygen species (ROS), which may be due to a direct mechanism, catalyzed by ferrous iron on the fiber surface, or indirectly, during attempts by macrophages and neutrophil granulocytes to phagocytose. These ROS may interact with the genome, inducing mutations via DNA strand breaks and deletions [96]. Cytogenetic analyses of mesotheliomas have shown frequent deletions of specific sites in chromosome arms 1p, 3p, 6q, 9p, 13p, 15q, 22q [81, 118].

The accumulation of numerous chromosomal deletions suggests a multistep process of tumorigenesis, characterized by the loss and/or inactivation of several tumor suppressor genes (TSGs). Three of these TSGs change frequently, probably early in this process; they seem to play a critical role in the pathogenesis of mesothelioma. Two of them are located at 9p21 chromosomal region.

The gene **p16 (INK4a)** encodes for a protein, which binds to cyclin-dependent kinases 4 and 6 (CDK4/6) and thereby inhibits the catalytic activity of CDK4/6-cyclin D. This inhibition reduces phosphorylation of the retinoblastoma protein (pRb), and consequently causes cell cycle arrest in the G1 phase [143]. The other TSG of this region is **p14ARF**, which encodes for a protein that is essential for the activation of p53 [116, 152]. The simultaneous loss of p16 and p14ARF functions

will then perturb both pRb- and p53-dependent regulatory pathways, thereby reducing the genetic stability of the cell and increasing proliferation.

The neurofibromatosis type 2 TSG, **NF2**, resides on chromosomal region 22q12, which is frequently mutated and/or has undergone allelic loss in mesothelioma. The NF2 product, called merlin, is a negative growth regulator, which has been assumed to function by modulating growth factor and extracellular matrix signals that trigger cytoskeleton-associated processes [145].

The genetic instability caused by inactivation of TSGs increases the rate of DNA rearrangements and amplifications. This, together with the increases in number of chromosomes (polysomy), may activate growth-promoting proto-oncogenes. The latter genes encode transcription factors and growth factors, such as platelet-derived growth factor PDGF-β [55, 78], that activate cellular proliferation, which, in turn, render the tissue even more susceptible to subsequent mutations. Although these oncogenic effects are considered to be later events during carcinogenesis, it has also been shown that the ROS created by asbestos may activate proto-oncogenes c-fos and c-jun directly [59].

2.1.1.2 Simian virus 40

Not all mesotheliomas can be associated with exposure to asbestos. Because of this and the long delay in the development of the tumor, the question has been raised about other carcinogens and/or cofactors. Simian virus 40 (SV40) is a tumor virus that can induce mesotheliomas and other tumors in hamsters, and also transform human cells *in vitro* [20, 29].

Apart from viral coat proteins, its circular DNA encodes three other proteins, including the large T-antigen (Tag) responsible for the potent transforming ability of the virus. Tag binds and inhibits cellular p53, pRb, and several other TSG products [124, 162]. The Tag-mediated inactivation of these TSGs may make mesothelial cells more susceptible to the transforming effects of asbestos, and SV40 may act as a cocarcinogen. Several authors have found SV40-like sequences in human mesotheliomas, using PCR technology and immunohistochemistry, but their prevalence shows geographic differences. This virus was detected in 40-70% of these tumors in Italy and USA [39, 119], but in fewer than 10% in Finland and Sweden [61, 125].

One way in which SV40 may have been transferred to humans is via contaminated polio vaccines. The use of different vaccines could be a plausible explanation of the differences in prevalence of SV40 in various populations. Although Tag has also been shown to damage DNA directly [2], the role of this virus in the carcinogenesis of mesothelioma remains controversial [105].

2.1.1.3 Familial mesothelioma

Clustering of mesothelioma in families has also been reported, but the specific genetic factors implicated in the etiology of these familial mesotheliomas are not known. A genetic susceptibility has been thought to be a contributory factor [6, 37]. This inherited characteristic seems to play a role in the development of mesotheliomas induced by exposure to erionite in certain villages in Turkey [133].

The features shown by advanced mesotheliomas suggest complex and diverse patterns of changes in growth regulation during carcinogenesis, which involve both inactivation

of TSGs and activation of proto-oncogenes. This process may be facilitated by SV40 large T-antigen, which inhibits p53 leading to impaired DNA repair and apoptosis in asbestos-damaged cells (Fig. 1).

2.1.2 Classification and morphology

Mesotheliomas show a wide range of growth patterns, which is reflected in the traditional histological classification as epithelioid, sarcomatoid (or fibrous) and biphasic (or mixed) [7]. The epithelioid type is said to be the most common both in the pleura and peritoneum [157], but widespread sampling of the tumor increases the percentage of tumors diagnosed as biphasic [69, 167].

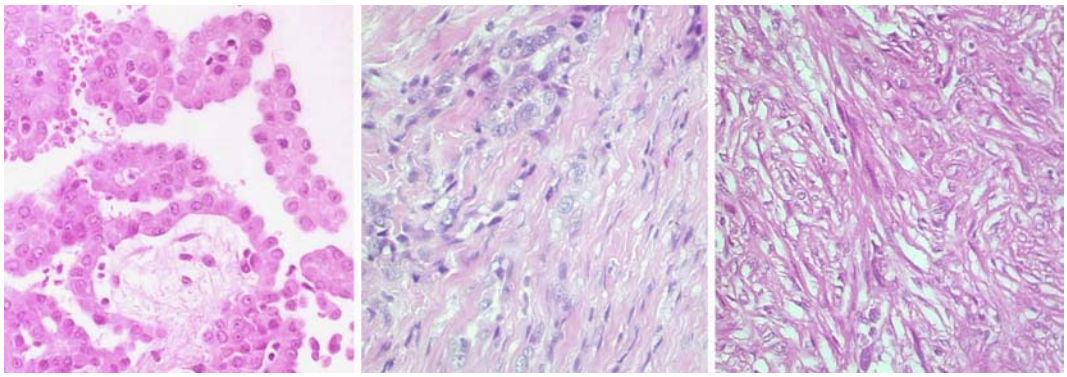

Fig 2. *Malignant mesothelioma with epitheloid differentiation (left), biphasic phenotype (middle) and sarcomatoid growth pattern (right)*

Epithelioid mesotheliomas or tumor components often show well-differentiated tubulopapillary or tubular patterns. The cells with eosinophilic cytoplasm are usually cuboidal, but columnar or flattened cells are occasionally seen. The nucleus appears round and vesicular under the light microscope and contains a prominent nucleolus, but sometimes multiple nucleoli. Mitotic figures are rare, except in poorly differentiated tumors. A solid (non-glandular) type of epithelioid mesothelioma exists, consisting of sheets of polygonal cells. If well-differentiated, these cells resemble hyperplastic mesothelium, and pose a difficult problem in differential diagnosis.

In the **sarcomatoid** (fibrous) type of mesothelioma or such tumor components of biphasic mesotheliomas, the cells are spindle-shaped or oval and found in varying amounts of collagenized stroma. The cellularity and cellular morphology may vary greatly, and differentiation into specialized types of tissues, such as fibrosarcomatous, chondrosarcomatous, osteosarcomatous [183], liposarcomatous [74] and myosarcomatous [90] tissues may be seen. In the desmoplastic form of diffuse mesothelioma, the spindle cells are widely scattered in a densely collagenized stroma, which may make it difficult to distinguish from scar tissue. At the other end of the spectrum, the tumor may be markedly pleiomorphic and indistinguishable from other types of undifferentiated sarcoma.

Biphasic (mixed) mesotheliomas show malignant elements with epithelial and mesenchymal features. The two subtypes commonly occur in different parts of the tumor, and the proportions between them are usually unequal. Sometimes the two

phenotypes are admixed in small areas, or the cells are transitional in type [156]. Previously, a biphasic morphology has been described in only about ¼ of mesotheliomas, but recent studies show a higher percentage (46-63%), correlating with the amount of tissue on which the diagnosis is based [69, 167].

2.1.3 Clinical course and prognosis

Typically, mesotheliomas develop after a long period of 20-40 years from the time of initial exposure to asbestos. The commonest early symptoms are dyspnea, cough, chest pain and development of an effusion fluid. In pleural mesotheliomas, the lung may be directly invaded, and at autopsy, metastases have frequently spread to the hilar lymph nodes and even to the liver and other distant organs [65].

The tumor is very aggressive, with a median survival of 4 to 12 months, depending on the histological type, epithelioid tumors being slightly less aggressive, regardless of the therapy used [134]. However, the prognosis varies greatly and some patients live for more than 5 years. Mesothelioma is usually resistant to chemotherapy, and expresses several enzymes involved in multidrug resistance [42, 155]. These detoxication proteins and the anti-apoptotic products of the bcl-2 gene family, that are also expressed in mesotheliomas [149, 150], have both been associated with a more aggressive behavior of tumors [50, 163].

2.2 SECONDARY TUMORS OF THE SEROUS CAVITIES

Metastatic involvement of the serous membranes is much commoner than a primary malignant mesothelioma [142, 151]. This ratio is usually in the order of 7-20:1, but lower ratios are found in areas with endemic exposure to asbestos. Many types of tumors, especially carcinomas, may spread to serous membranes. These metastases extend directly from adjacent tissues or underlying lymphatics, and once in the serous cavity, they become disseminated with the effusion.

The commonest primary malignant tumors causing metastases to the **pleura** in descending order are adenocarcinomas of the breast, lung, ovary, stomach, large intestine, pancreas, thyroid and kidney [28]. Adenocarcinoma of the lung, because of its peripheral location, is the commonest cause of pleural carcinomatosis in males. Some peripheral pulmonary adenocarcinomas of bronchioloalveolar type spread over the pleural surfaces in a manner similar to that of mesotheliomas. Since this tumor also mimics epithelioid mesothelioma histologically, it may be difficult to determine the origin of such tumors, and the term “pseudomesotheliomatous adenocarcinoma” has been suggested for these conditions [44, 58].

Apart from adenocarcinomas, any histologic type of lung cancer may involve the pleura, although the less frequent large cell carcinoma and the more centrally located squamous cell and small cell carcinomas are less common causes of carcinomatosis. Occasionally, a metastatic squamous cell carcinoma of the pleura or pericardium originates in other mucosae, such as in the esophagus.

Pericardial metastases are usually due to carcinomas of the lung, breast and thyroid, but sarcomas and malignant thymomas may also involve the pericardium.

In the **peritoneum,** the ovary, stomach, large intestine, pancreas and breast are the commonest sources of metastatic carcinoma, followed by the lung, kidney, uterus and prostate. The most frequent primary tumor that causes peritoneal carcinomatosis and neoplastic ascites in females is papillary serous adenocarcinoma of the ovary, arising from the ovarian surface. This surface epithelium develops from the coelomic membrane. Although of the same mesodermal origin as the mesothelium, the ovarian surface acquires more epithelial characteristics. Because of the developmental similarities of the tissues, the ovarian papillary serous adenocarcinoma may resemble well-differentiated tubulopapillary mesothelioma. This is also reflected by the partial overlap of the two immunophenotypes.

The primary serous papillary carcinoma of the peritoneum is an extraovarian serous tumor, which closely resembles its ovarian counterpart histologically, and may occur in women without an ovarian tumor. Despite the primary peritoneal location of extraovarian serous tumors, which may mimic the spread and morphology of peritoneal mesotheliomas, they should be considered as originating from ectopic coelomic epithelium, similar to that in the ovaries. This also accords with their biologic behavior. The mucinous type of ovarian adenocarcinoma is less frequent and resembles a mesothelioma less, than serous tumors do.

Non-epithelial malignancies — i.e., lymphomas, various sarcomas and melanoma **—** may also infiltrate or give rise to metastases on the serous membranes. Lymphomas and leukemias can spread to the serous cavities without involving the pleura or peritoneum directly. As regards the histological types of malignant lymphomas, serous effusion and pleural/peritoneal infiltration seem to be commoner with lymphoblastic types [104] and malignant lymphoma of peripheral T-lymphocyte origin [172]. Multiple myeloma may also be associated with serous involvement [64].

The histological findings in sarcomas arising from subserosal tissues can vary greatly and it may be difficult to determine the origin and histogenesis of these masses, especially when they are composed of undifferentiated spindle cells. Immunohistochemistry is necessary to distinguish such tumors from sarcomatoid mesotheliomas.

3 SEROUS EFFUSIONS

Any pathological condition, benign or malignant, affecting the serous membranes or the balance between the hydrostatic and osmotic pressures, may result in an effusion into serous cavities. This fluid is either a transudate with low protein content or a protein-rich exudate.

Transudates develop because of changes in fluid pressures, — i.e., in cardiac failure, hypoproteinaemia (e.g., liver cirrhosis, nephrosis syndrome, exudative enteropathy and severe starvation) and ovarial fibroma (Meigs' syndrome).

Exudates, on the other hand, result from serosal irritation of any cause leading to an increase in permeability of the serous membranes. The irritation is usually due to primary inflammatory conditions or neoplastic involvement of the serous membranes. Such an effusion contains various amounts of inflammatory cells (granulocytes, lymphocytes, histiocytes) and exfoliated cells from the mesothelium and from possible primary or metastatic tumor.

4 DIFFERENTIAL DIAGNOSTIC CHALLENGES IN HISTOLOGY AND CYTOLOGY

The distinction between malignant mesotheliomas, metastatic tumors and benign proliferative or fibrotic conditions of the serous membranes is essential in **diagnostic histopathology** and for later therapy.

In most cases, a mesothelioma has an epithelioid growth pattern or tumor component with adenomatous morphology, and it is therefore easily misdiagnosed with routine histology as a metastatic adenocarcinoma of the lung or other origin. The sarcomatoid (fibrous) pattern of mesothelioma, on the other hand, may mimic extensive pleural fibrosis, as well as other connective tissue tumors with a similar pattern of growth. Each histological type of mesothelioma thus has its own diagnostic problems, and a distinction must be drawn between:

1. epithelioid mesothelioma and adenocarcinoma;
2. epithelioid mesothelioma and reactive mesothelial hyperplasia;
3. fibrous mesothelioma and other connective tissue tumors;
4. fibrous mesothelioma and fibrosis;
5. biphasic mesothelioma and other biphasic tumors.

An effusion is frequently the first material available for **cytologic diagnosis** in disorders affecting the serous membranes. Sarcomatous tumor cells, such as those from fibrous mesothelioma components, are usually not identified as elongated cells in the effusion fluid, presumably because only a few are shed, and if detected, they probably round up to look epithelioid.

Both the epithelioid fractions of mesotheliomas and adenocarcinomas often exfoliate tumor cells in large numbers. Their similar morphology makes the distinction between these conditions a common diagnostic problem. It is also difficult to distinguish between these two types of tumor and reactive mesothelium, not only because the tumor cells resemble each other, but also because benign mesothelial cells may show signs of growth stimulation that mimic malignancy.

In this benign proliferative mesothelial reaction, so-called “mesotheliosis”, these hyperplastic (reactive) mesothelial cells may show nuclear features and occasional mitoses resembling differentiated mesothelioma or carcinoma cells. In conventional cytology, these cells are often denoted as “suspicious for malignancy”. Diagnosing primary or secondary tumors based on effusion cytology thus involves not only the problem of distinguishing between the two types of tumor cells, but also between well-differentiated tumors from cases of mesotheliosis that have cytomorphological findings suggestive of malignancy (Fig. 3).

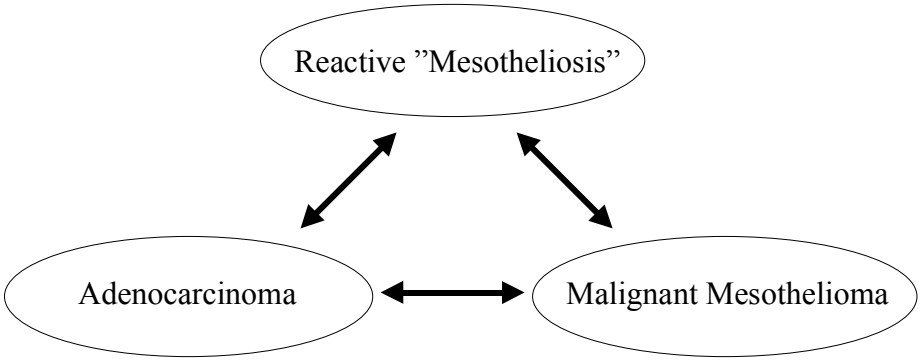

Fig 3. *Differential diagnoses representing main diagnostic dilemmas in effusion cytology.*

Moreover, the cytology is sometimes falsely negative because too few neoplastic cells are present in the fluid, or because the processing of specimens was suboptimal with lysis of tumor cells. These cases with signs of poor preservation of cells and/or cellularity, together with cases having some cellular atypia (“suspicious for malignancy”) form a group of “inconclusive” cytological reports. For these reasons, a specific cytological diagnosis of malignancy in effusions has a sensitivity of only 40-80% [4, 24, 123].

5 ADJUVANT DIAGNOSTIC ANALYSES

The correct diagnosis of a tumor involving the serous cavities is frequently a difficult challenge for pathologists, the difficulties sometimes even preventing the detection of a malignant condition. One main problem is to distinguish between an epithelioid mesothelioma and a metastatic adenocarcinoma. Since this usually can not be done with conventional histological staining alone, ancillary morphological and chemical analyses are useful in order to increase the diagnostic accuracy.

5.1 HISTOCHEMISTRY

Histochemical reactions have long been used to distinguish between the mesothelial and epithelial lineage of tumor cells, based on their production of specific carbohydrate compounds.

Most mesotheliomas secrete large amounts of hyaluronan, a glycosaminoglycan (GAG), while adenocarcinomas are more likely to produce mucinous glycoproteins. Hyaluronan can be demonstrated by staining with Alcian blue, and comparing the reactions with and without hyaluronidase pretreatment [171]. Since hyaluronan is water-soluble, much of it will be lost during routine processing in aqueous fixatives, such as formalin, and during staining with aqueous solutions. Moreover, alcianophilic staining of the connective tissue can be found in some adenocarcinomas with a markedly proliferative stromal reaction [83].

The mucins produced by adenocarcinomas carry oligosaccharides that give the periodic acid-Schiff (PAS) reaction even after diastase pretreatment. A positive reaction strongly favors the diagnosis of an adenocarcinoma, rather than a mesothelioma, but since about half of the former tumors do not produce such mucins, the result may be inconclusive [129]. The diagnostic value of the Alcian blue and PAS reactions thus leaves many cases unresolved, and in most laboratories these histochemical tests have now been replaced by immunocytochemistry.

5.2 IMMUNOCYTOCHEMISTRY

Numerous (more than 200) immunohistochemical markers have been described as useful in the diagnosis of mesothelioma. This was previously based primarily on antibodies that reacted with adenocarcinomas, but not with mesotheliomas — i.e., so-called "negative" markers [19, 107].

"Positive" markers, usually expressed in epithelioid mesotheliomas, but not in adenocarcinomas, became commercially available only from the mid-1990s. However, none of these mesothelioma markers are expressed exclusively in mesotheliomas, and neither the "positive" nor the "negative" markers have an absolute specificity and sensitivity.

5.2.1 Markers favoring adenocarcinoma

Carcinoembryonic antigen (CEA) is a glycoprotein component of the glycocalyx of epithelial cells. It is the most widely used "negative" marker for mesothelioma, and has a high specificity. This tumor is almost invariably non-reactive for at least one CEA epitope, while a high percentage of adenocarcinomas (more than 80% of lung adenocarcinomas) gives a positive reaction pattern [19, 178].

Since polyclonal anti-CEA sera also contain antibodies that bind, for example, the cross-reactive antigen of granulocytes, these preparations are less specific and label a proportion of mesotheliomas. One commercial monoclonal antibody, however, has a high specificity in this respect [40]. Not all adenocarcinomas produce CEA, and this marker has only limited value in detecting metastases from serous carcinomas of the ovary, as well as carcinomas of the thyroid and kidney.

CD15 (Leu-M1) is a monocyte-granulocyte related marker also expressed by Reed-Sternberg cells in Hodgkin's disease. This antibody reacts with some adenocarcinomas, but has only moderate sensitivity (62-82%) [108, 110]. One advantage of this antibody is a better sensitivity for serous ovarian carcinomas than that of CEA. It has been regarded as very specific in excluding mesotheliomas and most other sarcomas [144]. However, when using consecutive clinical biopsies, its reaction pattern is clearly positive in a group of mesotheliomas [41].

BerEp4 is a monoclonal antibody that recognizes a glycoprotein epitope present in most epithelial cells, but absent in mesothelial cells [79]. The typical positive reaction pattern is common in lateral cell membranes. This marker is very sensitive mainly in pulmonary adenocarcinomas, but its specificity is poor for excluding mesotheliomas, since in larger clinical series, it may show reactivity in up to 26% of such tumors [113].

Sialyl-Tn (B72.3 or **TAG-72)** is a monoclonal antibody raised against human breast cancer cells. It is reactive with most adenocarcinomas, but is usually negative in mesothelioma cells. Despite the fairly high sensitivity of Sialyl-Tn for adenocarcinomas, it has also been reported in some mesotheliomas, and its specificity in excluding mesotheliomas varies considerably in different studies (52-98%) [41, 158].

Thyroid Transcription Factor-1 (TTF-1) is a highly specific marker for lung and thyroid adenocarcinomas, and seems to be useful in cases of neoplastic pleural involvement [114, 115]. **CA19-9** is commonly present in serous carcinomas of the ovary, but not in mesotheliomas, and thus can be helpful in distinguishing between these malignancies [110].

5.2.2 Markers favoring mesothelioma

Calretinin is a calcium-binding protein strongly expressed in neural tissues, and in certain nonneural cells, such as adipocytes, kidney tubules, Leydig and Sertoli cells, eccrine glands, and mesothelial cells [48]. Numerous immunohistochemical studies have shown that anti-calretinin is a relatively specific marker of mesothelial proliferations, with reactivity in adenocarcinomas in only rare cases. Mesotheliomas typically show strong nuclear and cytoplasmic immunoreactivity for calretinin, and

hitherto this antigen has been regarded to be the most sensitive (90-100%) of the "positive" mesothelioma markers [48, 111, 165, 176].

Epithelial membrane antigen (EMA) is commonly used as a "positive" mesothelioma marker, when showing an intense membranous staining pattern. The mere presence of this antigen is of no value, since adenocarcinomas also show EMA reactivity, but this is mainly cytoplasmic. The presence of EMA reactivity accentuated in cell membranes suggests a mesothelioma, while a cytoplasmic pattern is more indicative of an adenocarcinoma [82]. The occurrence of cases with overlapping staining patterns limits the value of this antigen as the sole criterion in distinguishing between these malignancies [52].

Thrombomodulin (TM) is a transmembrane proteoglycan present in endothelial cells, syncytiotrophoblasts, synovial cells, keratinocytes, transitional epithelial cells, and mesothelial cells. The reactivity for TM is associated with the cell membrane, and it is commonly found in epithelioid mesotheliomas, but not in most adenocarcinomas [31]. Unlike adenocarcinomas, squamous cell carcinomas often express TM [109]. In areas of tumor degeneration and necrosis there may be a granular cytoplasmic staining, that is difficult to interpret correctly. Furthermore, the delicate membranous staining pattern of TM in formalin-fixed and paraffin-embedded tissues tends to be focal, which could result in a false-negative report on small biopsy specimens.

HBME-1 is a monoclonal antibody that was generated by using a suspension of human mesothelial cells. It is thought to recognize an antigen present on the villous surface of mesothelial and mesothelioma cells. Like EMA, the typical reactivity to HBME-1 is greater towards the periphery of the cell in mesotheliomas, while the staining pattern is cytoplasmic in adenocarcinomas. Since some adenocarcinomas also show a membranous staining pattern, overlapping with that of mesotheliomas, its specificity for excluding adenocarcinomas is relatively low [94].

Vimentin and various **cytokeratin (CK)** intermediate filaments are typically co-expressed in benign and malignant mesothelial cells, which reflects the transitional phenotypic character of these cells. A wide range of CKs has been described in mesothelial cells, including those of low molecular weight (40-46 kDa: CKs 17, 18 and 19), intermediate molecular weight (50-54 kDa: CKs 7, 8 and 14), and high molecular weight (56-64 kDa: CKs 3, 4, 5, 6) [91, 169, 174]. When antibodies reacting with a broad spectrum of CKs (pan-CK antibodies) are used, we typically see a positive reaction also in fibroblast-like mesothelioma cells, but in adenocarcinomas, a similar reactivity in stromal fibroblasts is much less common.

At the same time, the typical diagnostic reaction pattern for vimentin is staining of the epithelioid mesothelioma cells. Although coexpression of cytokeratin and vimentin was previously thought to be specific for mesotheliomas, it also occurs in adenocarcinomas — e.g., in serous ovarian carcinomas, adenocarcinomas of the lung, breast and endometrium. A monoclonal antibody reacting only with CKs 5 and 6 (anti-cytokeratin 5/6) has recently become commercially available. This epitope is claimed to give reactivity in most epithelioid mesotheliomas and squamous cell carcinomas, but only occasionally in adenocarcinomas. It has therefore been suggested as a sensitive "positive" mesothelioma marker, although its specificity is not very good [21, 30, 112].

The constant development of new immunological reagents has also yielded additional mesothelioma markers that were not available for evaluation in **Paper I**. Since **WT1** is continuously expressed in cells of mesothelial lineage, it has also been suggested as a marker for mesothelioma [117]. Although newer antibody preparations have improved the signal-noise ratio, a nuclear reactivity to WT1 is not seen in mesotheliomas alone. This reactivity can also be found in other coelomic tumors, such as serous carcinomas of the ovary and peritoneum, and occasionally other carcinomas may give a positive reaction pattern [3, 115].

Mesothelin is a surface glycoprotein strongly expressed in mesotheliomas and serous carcinomas of the ovary, as well as in pancreatic and squamous carcinomas [27]. A promising commercial anti-mesothelin monoclonal antibody became available recently, although its practical utility has not yet been evaluated in larger series.

5.3 BIOCHEMICAL ANALYSES

Apart from routine diagnostic cytology with use of adjuvant morphological techniques, effusions can also be used for chemical analyses. Many substances, measured by means of their biochemical or antigenic characteristics in effusions, have been regarded as indicators of malignancy, but none fulfills the criteria of an ideal tumor marker [168]. Some of these markers are related to a specific tumor or certain types of tumors, while others hallmark a wide range of malignancies. In principle, the same tumor-associated antigens used to monitor tumor progression by analyses of serum, can also be measured in effusions. However, their utility as single diagnostic marker is limited, usually because of insufficient specificity.

5.3.1 Hyaluronan

Hyaluronan, a glycosaminoglycan, that is frequently produced in excess by many mesotheliomas, perhaps because of the mesenchymal nature of the tumor. The determination of the hyaluronan concentration in effusions has proved useful as an ancillary diagnostic method. The measurement can be made in many ways, but high-performance liquid chromatography (HPLC) seems to provide the best specificity. This analysis specifically detects a mesothelioma in about 60% of the cases, distinguishing the primary serosal tumor from reactive benign mesotheliosis and metastatic adenocarcinoma [106].

5.3.2 CEA

CEA is an oncofetal antigen synthesized by many carcinomas, especially adenocarcinomas of the gastrointestinal tract, breast, lung and mucinous carcinomas of the ovary. This antigen has been found at raised titre in the serum of patients with carcinoma arising from various primary sites, and can also be detected in effusions. About 50% to 60% of malignant effusions are associated with elevated CEA levels.

It has been suggested that a high concentration of this marker in the effusion indicates serosal metastases from a CEA-producing tumor [4, 54], but this has also been found in carcinomas without serosal involvement [24, 122], probably as a result of elevated serum levels. An increase in the CEA concentration has also occasionally been

reported in benign effusions associated with gastrointestinal perforations and inflammatory conditions; however, consistently low levels are found in mesotheliomas [122, 123].

5.3.3 Cholesterol

Cholesterol is a lipoid, important in intermediary metabolism. This steroid structure is a precursor of steroid hormones and a major component of cell membranes. The cholesterol concentration is commonly raised in exudates. This has partly been associated with the disintegration of many cells, which usually occurs in primary or metastatic tumors of the serous membranes, but also in serosal inflammations [25, 26].

These causes of serosal irritation, on the other hand, increase the permeability of the affected serous membranes, and it has also been suggested that cholesterol may enter the cavity from the lymphatic space [53, 95]. High cholesterol concentrations in effusions are found most frequently in malignant processes, and cholesterol has therefore been regarded as a potential tumor marker. Similar findings, however, may also occur in benign conditions, such as pleuritis, peritonitis, and occasionally in chronic cardiac congestion [95, 135], which makes this analysis nonspecific, when used as a single tumor marker.

5.4 ELECTRON MICROSCOPY

Electron microscopy, a very useful technique, is sometimes even considered as the gold standard in the diagnosis of mesothelioma, although it needs optimal availability and fixation of tissue biopsies or cell clusters in effusions [11, 156, 157].

Both transmission electron microscopy and scanning electron microscopy have been used to distinguish between epithelioid mesothelioma and adenocarcinoma. Mesotheliomas tend to possess longer, slender, often branching microvilli without glycocalyx, as compared to the short, blunt forms with core rootlets and glycocaliceal coat, as seen in adenocarcinomas. Intermediate filaments are abundant, showing a perinuclear condensation in mesothelioma while they are less prominent in adenocarcinoma.

It may, however, be diffucult to distinguish mesothelioma from reactive mesothelium on the basis of the above mentioned cellular criteria alone. For this, one must also recognize the malignant features, which may include nuclear polymorphism, macronuclei, deep cytoplasmic indentations in the nucleus and presence of cytoplasmic or intercellular neolumina, signs that are sometimes seen only with electron microscopy [70]. Since this type of microscopy is time-consuming and costly, it is frequently done as a third stage in the investigation in routine practice.

6 MESOTHELIUM AS A MODEL FOR CELL DIFFERENTIATION

Due to the transitional nature of mesothelium, this tissue contains cells that have both mesenchymal and epithelial characteristics, and this is seen during conditions of rest and activation. The ability to divide is retained by cells in the mesothelial surface, and these cells can be grown in cultures. This can be achieved after enzymic digestion of surgically removed intact omental or pleural tissues.

The resulting confluent cultures, grown on an ordinary, plastic surface [153, 164] or on a collagenous matrix, form a monolayer of cobblestone-like cells. When the same mesothelial cells are cultured within a collagen matrix, they take on a spindle-shaped morphology and then migrate towards the surface. On reaching the surface, they change their morphology and spread to cover it with polygonally-shaped epithelioid cells. The cells that remain in the matrix retain their spindle shape. Such an experimental model clearly shows the importance of the extracellular environment in mesothelial differentiation [14].

Mesothelial cell cultures can also be obtained from benign effusions, and both fibroblastic [77] and epithelioid [97] growth patterns have been reported. Under standard culture conditions, such benign mesothelial cells acquire a uniform phenotype — either fibroblastic or epithelioid — even in early passages, and the pattern obtained is stably retained through subsequent passages (**III**). Since both of these fibroblastic and epithelioid cultures are generated from exfoliated mesothelial cells, their mesothelial origin is certain, as also shown by their ultrastructural and immunocytochemical characteristics. These cultures can therefore be used to study differences between the two phenotypes as a model for mesothelial cell differentiation.

Mesotheliomas retain the ability to change phenotype also. In biphasic mesotheliomas the malignant epithelioid and fibrous morphologies occur side by side. This bivalent growth potential of cells is sometimes also preserved in mesothelioma cell lines, where the addition of different serum substituents induces differentiation into either epithelioid or fibrous phenotypes. The effect of serum factors on these tumor cell lines, however, is reversible, and the cultures can be shifted back and forth between the two growth patterns [72].

7 FACTORS ASSOCIATED WITH MESOTHELIAL DIFFERENTIATION

7.1 WILMS' TUMOR SUSCEPTIBILITY GENE 1 (WT1)

WT1 gene encodes a zinc finger DNA-binding protein, which acts as a transcriptional regulator and is essential for mesenchymal-epithelial transition. WT1 is expressed in a dynamic and tissue-specific pattern during embryogenesis in the kidney, gonad and mesothelium [63, 117]. All of these tissues undergo a mesenchyme-to-epithelial switch during development. Knockout experiments showed that the mice lacking WT1 failed to develop these tissues [73].

In contrast to the condensing mesenchyme of the fetal kidney, which loses WT1 expression after epithelization, the gonadal cells and mesothelium retain this expression throughout adult life. WT1 expression has therefore been recommended as a marker for mesothelial lineage and is present to a great extent in mesotheliomas [3, 117, 173]. The WT1 gene is found on chromosome 11p13, and alternative splicing at two independent splice sites results in four mRNA transcripts [56]. However, the WT1 gene generates at least 24 slightly different protein isoforms through alternative splicing, RNA editing, and alternative translational start sites [139, 140]. A balanced presence of the protein isoforms seems to be necessary for adequate WT1 function.

The WT1 acts as repressor for a variety of target genes, including growth factors and growth factor receptors [140]. On the other hand, it has also been shown that WT1 functions as a transcriptional activator for genes like syndecan-1 [32] and E-cadherin [62]. The effects of syndecan-1 [66] and cell adhesion receptor E-cadherin [160] have both been associated with epithelial differentiation.

7.2 PROTEOGLYCANS

Proteoglycans (PGs) are a heterogeneous group of macromolecules that are ubiquitously present in the body. They form important components of the extracellular matrix and fulfill a variety of functions on the cell surface and in the cytoplasm.

These molecules contain a core protein to which at least one glycosaminoglycan (GAG) side-chain is covalently attached. Although most of these proteins occur solely in a PG form (full-time PGs), certain proteins occur both in a PG form and in a non-PG form without GAG chains (part-time PGs). A PG is defined on the basis of the protein structure derived from its gene and, in only a few cases, do we see splice variants.

The protein core is thus identical for each PG or splice variant. However, the attached GAG chains provide considerable variability. This results in sophisticated structures of various size and characteristics, which play an important role in biological processes, and involve more or less regulatory functions in cell differentiation.

7.2.1 Glycosaminoglycans

GAGs are long unbranched polysaccharides consisting of specific disaccharide repeats. The one sugar of these disaccharides is a hexosamine — either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) — and the other is a hexuronic acid (HexUA) — either D-glucuronic acid or L-iduronic acid — or galactose.

Following the formation of a tetrasaccharide linkage region on the protein core, the GAG chain is synthesized in the Golgi by the alternate addition of the hexosamine and glucuronic acid at the nonreducing end of the chain [85]. Once the chain elongation is complete, several modification reactions may occur that cause marked variations between chains. Thus some of the glucuronic acids are epimerized to iduronic acid and in some GAGs, the hexosamines may also be deacetylated. A sulfate may then be transferred to any free hydroxyl group or to a deacetylated amino group, usually to positions in the hexosamine. This sulfation results in one or two O-sulfates per disaccharide, and in heparan sulfate (HS) and heparin also presence of N-sulfate. The final GAG is thus highly polyanionic, the charge density correlating with the sulfation.

Depending on the basic structure of the repeating disaccharide, the GAGs are named HS, heparin, chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronan (HA).

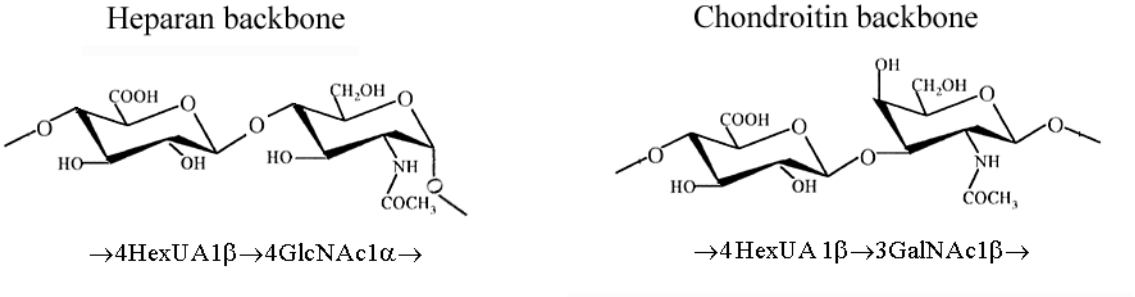

Fig 4. *Repeating disaccharide structure in heparan and chondroitin*

7.2.1.1 Hyaluronan (HA)

HA, a GAG present extracellularly in all tissues, consists of N-acetylglucosamine and glucuronic acid repeats and has exceptional characteristics. This polysaccharide is synthesized on the cell surface by one of the three known hyaluronan synthases (HASs) by elongating the chain at the reducing end. The resulting polysaccharide is never sulfated, but may be extremely long (consisting of up to 25 000 disaccharides), and is the only GAG that is never covalently linked to a protein.

A significant characteristic of HA is its capacity to bind very large amounts of water. A single HA chain may form the core for aggregates with matrix PGs, such as versican and aggrecan, creating huge hydrated complexes [80]. The production of HA by mesothelioma cells *in vitro* seem to be greater in the epithelioid phenotype than in the fibroblastic one [72, 166]. This may accord with the role of the benign surface

mesothelial cells forming a lubricant film containing this GAG. HA has also been ascribed a role in cell proliferation and migration [161].

7.2.2 Proteoglycan classification

The PGs are commonly grouped according to their main location — i.e., whether they are cell-associated or found in the extracellular matrix. They are thus further classified into families, on the basis of similarities in their core protein structure.

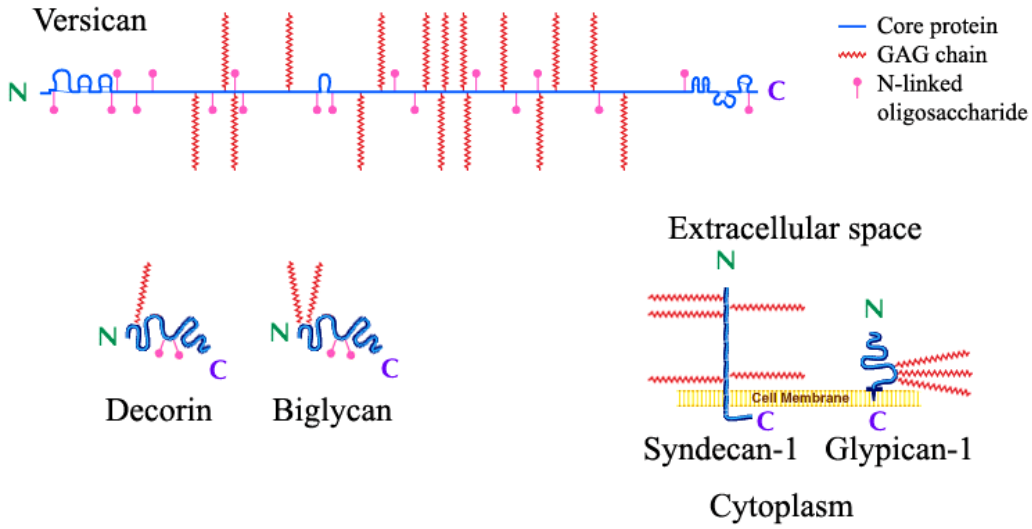

FIG 5. *The PGs are a structurally heterogenous group of substances present in the cell membrane and in the extracellular matrix*

7.2.2.1 Cell associated proteoglycans

The PGs in cells are mainly located on the cell membrane, although some of them occur in the cytoplasm and may even be translocated to the nucleus [18]. The core protein of the cell surface PGs is integrated either in the cell membrane, such as in the transmembrane syndecan family, or covalently attached to the membrane, such as in the glypicans.

7.2.2.1.1 Syndecans

Syndecans are the most common integral membrane PGs, and the family includes four members — i.e., syndecan-1, syndecan-2 (fibroglycan), syndecan-3 (N-syndecan) and syndecan-4 (amphiglycan). Their core protein spans the plasma membrane and contains a short cytosolic domain, as well as a long extracellular domain to which 2-3 HS chains are attached close to the N-terminus [12]. The syndecans may also carry CS side-chains close to the plasma membrane [127, 146]. The cytoplasmic domain of core protein binds to the cytoskeleton via actin filaments. The transmembrane and cytoplasmic domains are highly conserved in all syndecans, but the ectodomains show only limited amino-acid-sequence similarity. Syndecans-1 and –3 and syndecans-2 and –4 can be considered to form subfamilies, based on sequence comparisons in these regions [22].

All members of the syndecan family are involved in growth factor signaling, mediation of cell adhesion and regulation of cellular differentiation [22, 47], and the various functions ascribed to individual syndecans are based on similarities and differences in their structure. A number of growth factors — e.g., fibroblast growth factor 2 (FGF-2), heparin-binding epidermal growth factor (HB-EGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) — bind to HS chains of cell surface PGs, mainly those of syndecans but also of glypicans.

These PGs serve as co-receptors, the ligation being necessary for a functioning growth factor-receptor complex. It has also been suggested that the HS protects the ligand from degradation and concentrates it on the cell surface. The HS chains of syndecans also bind other extracellular matrix components, such as collagen, fibronectin, laminin, tenascin, etc. [22].

Syndecan-1 is the major syndecan in epithelial cells while it is less markedly present in mesenchymal tissues [86], although plasmocytes abundantly contain this PG [177]. This transmembrane PG is restricted to the baso-lateral surface of polarized cells, where it participates in cell-cell contacts and has been associated with epithelial differentiation [66].

Antisense syndecan-1 cDNA transfection causes a loss of epithelial character and the development of a fusiform cell shape [71]. The epithelial-mesenchymal transition, seen in the embryonic palate, is closely associated with the synchronous downregulation of syndecan-1 and epithelial cell adhesion molecule E-cadherin [154].

A higher expression of syndecan-1 in carcinomas has been suggested as a marker for less aggressive behavior, perhaps by promoting tight cell-cell adhesions [67]. Interaction of syndecan-1 and proteases associated with invasion may reduce enzymic activity and therefore the ability of the tumor to invade [84, 87, 138].

Syndecan-2, also called fibroglycan, accumulates in mesenchyme during embryogenesis; it is later expressed more in connective tissues, than in epithelial ones. It has therefore been assumed to hallmark the connective tissue origin of the cells. Syndecan-2, in this respect, differs from syndecan-1, the latter rather being characteristic of epithelial tissues [34, 86, 87].

Syndecan-3 is mainly found in neural tissues and associated with oligodendrocyte differentiation and myelin formation [23].

Syndecan-4 is a major PG expressed in almost all tissues and it occurs in focal adhesions of anchorage dependent cells. These sites are essential to cellular signaling and they mediate cell-cell and cell-matrix adhesions via actin intracellular filaments that terminate at these points in the membrane.

During signaling, the cytoplasmic domain of syndecan-4 stimulates protein kinase C (PKC) activity at the cell membrane [93, 179]. Higher expression of syndecan-4 has been described in proliferative conditions [49, 76, 184], and enhanced cytoplasmic immunostaining has been found in malignant primary tumors of the liver [132].

7.2.2.1.2 Glypicans

Glypicans constitute another family of cell membrane HSPGs, the core proteins of which are covalently anchored to phosphatidyl-inositol. These PGs are mainly located on the apical surfaces of cells integrated in tissues. Six members of this family have been identified so far [38], and they are widely distributed in both mesenchymal and epithelial cells [33, 131]. Glypican-3, which is mainly expressed in mesodermal tissues, is thought to be a negative regulator of proliferation. Mutation of its gene causes overgrowth of several organs and tissues [121], and downregulation via methylation of the DNA promoter region has been found in rat and human mesotheliomas [100].

7.2.2.2 Matrix proteoglycans

Apart from collagens, PGs also constitute important components of the extracellular matrices and, as such, they are more abundantly present in mesenchymal tissues. Most matrix PGs contain CS and/or DS side-chains.

They can be classified into two groups according to their size and functional properties. The large matrix PGs are extremely large macromolecules, which can form aggregates with HA and therefore cause a swelling pressure in the tissue. They involve versican, found mainly in soft connective tissues, and aggrecan, which is present in cartilage.

The other group is the small matrix PGs (decorin, biglycan, fibromodulin), the core proteins of which characteristically have repeated leucine-rich motifs. These PGs carry only one or two GAG chains and one function ascribed to them is the organization of the fibrillar structure of collagens.

7.2.2.2.1 Large matrix PGs

Versican is a large aggregating PG, containing 12-16 CS chains and a number of N- and O-linked oligosaccharide residues. Its core protein binds to HA via the globular N-terminal domain. Alternating splicing generates four versican isoforms.

Apart from presence of versican as a structural component in the extracellular matrix in connective tissues, it has been shown that an abundance of versican may modulate cell proliferation and migration [75, 185]. Its growth stimulatory effect may be partly due to a destabilizing effect of versican on the focal cell contacts, which then repress cell adhesion [180, 182]. Larger amounts of versican have been found in highly malignant mesenchymal tumors [68] and in proliferating stromal cells of invasive carcinomas [103, 137]. It has been suggested that prostatic carcinoma cells produce factors which regulate the stromal production of versican [137].

7.2.2.2.2 Small leucine-rich matrix PGs

Decorin has one CS or DS chain attached to its protein core. It is relatively abundant in mesenchymal tissues [130], where it interacts with collagen fibrils, helping to stabilize them and orient fibrillogenesis [141]. The core protein of decorin also binds transforming growth factor beta (TGF-β) and neutralizes its growth-stimulating effect [181].

Biglycan contains two chains of CS or DS. This is found mainly in mesenchymal tissues, often in the pericellular matrix of developing cells [13]. Biglycan functions as a modulator of cell adhesion and organizer of collagenous network and, like decorin, it also interacts with TGF-β [60].

8 AIMS OF PRESENT STUDIES

- To determine whether recently available immunohistochemical markers can improve the ability of a panel to distinguish epithelioid mesotheliomas from adenocarcinomas and evaluate the efficacy of such a panel

- To investigate whether two chemical markers add diagnostic value to effusion cytology

- To determine conditions for *in vitro* culture of exfoliated benign mesothelial cells and study their expression of PGs and WT1

- To find factors involved in the differentiation of benign mesothelial cells

- To study the expression of PGs and WT1 in adenocarcinomas, mesotheliomas and benign mesothelial cells, and find new diagnostic parameters for distinguishing between these conditions

9 COMMENTS ON METHODOLOGY

All methods used in this thesis are presented in detail in the respective papers. Three of these deserve particular attention.

9.1 LOGISTIC REGRESSION ANALYSIS

Immunohistochemical reactions show the presence and distribution of epitopes, and the patterns obtained may have information of diagnostic value. In the practical diagnostic situation, however, a variable number of reactions are "atypical" — i.e., they are difficult to interpret.

To resolve this problem we frequently need more information and perform a second, third or fourth, etc. analysis. The diagnostic accuracy may suffice for a diagnosis in cases with all parameters pointing in the same direction, but as the number of parameters increases, the percentage of cases without any "atypical" reaction decreases. Difficulties in handling such multifactorial data cause confusion in such cases.

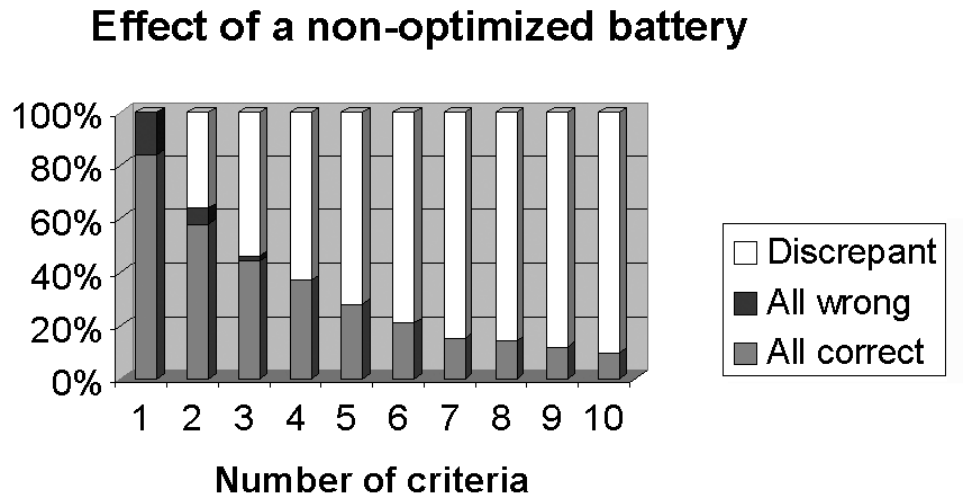

Fig 6. *In* **Paper I** *, we studied 10 criteria, all of which provided diagnostic information. When adding one parameter at a time, it will be found that the validity improves, but fewer than 10% of the cases then show that all reactions are in the same direction, and most of them become "divergent".*

To evaluate a larger number of parameters, as in Fig. 6, statistical analyses will be necessary. One way to do this is to use logistic regression analysis (JMP statistical discovery software, SAS Institute, Cary, NC, USA). With this method the data obtained are related to:

$$p(y=1)=e^{(b0+\Sigma bi*xi)}/(1+ e^{(b0+\Sigma bi*xi)})$$

where p is the probability of belonging to one diagnostic group, b_0 is a correction factor, b_i is an estimate expressing the diagnostic value of parameter i, while x_i is the outcome of the reactivity (0 or 1) of parameter i. The regression gives the best estimates

of b_0 and b_i and, when performed stepwise, also statistics for evaluating the additional diagnostic importance of each parameter added.

These estimates of b_0 and b_i can then be applied in individual cases to calculate the probability of belonging to one or the other diagnostic group. The calculation may thus be helpful for interpreting "atypical" and "confusing" reactivities.

9.2 SEMIQUANTITATIVE RT-PCR

Complementary DNA (cDNA) is synthesized from total RNA isolates of cells by reverse-transcriptase followed by subsequent PCR reactions with primer pairs for analyte genes. To make semiquantitative analyses of various analyte cDNA sequences, these are amplified along with primer pairs for the "housekeeping gene", glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as reference [159].

Since this internal standard is expressed more, the analyte cDNAs are PCR-amplified for 9-14 cycles before the GAPDH primers are added. The number of cycles for analyte and reference sequences must be individually optimized to obtain comparable amounts of amplimer, selecting cycle numbers to avoid saturation of any of them.

The PCR products are separated by electrophoresis in a 2% agarose gel containing ethidium bromide and the fluorescence of the bands is measured. Within a certain amplification interval, the ratio of the analyte fluorescence to that of the reference — i.e., the relative fluorescence (RF) — does not depend on the exact number of cycles, and has been shown to reflect differences in gene expression, which also correlate with the rate of synthesis of the corresponding protein [47, 88; **V**].

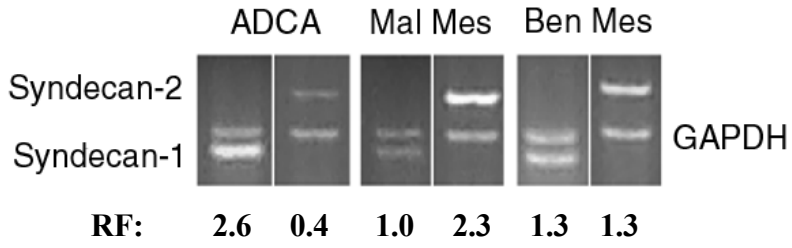

Fig 7. *The relative fluorescence (RF) is obtained by dividing the fluorescence of the analyte band (here syndecan-2, upper or syndecan-1, lower) by that of the reference band (here GAPDH, middle). The RF values not only allow the study of how one gene is expressed in different samples but also how the ratio between the expression of two different genes (e.g., the syndecan-2 / syndecan-1 ratio) may vary.*

The analytical window with stoichiometric relations to absolute amounts of cDNA, however, is limited, and the RF ratios are therefore best used semiquantitatively (Fig. 7). The advantage of using the primer pairs for the analyte and internal standard genes simultaneously in the same PCR reaction mix, as compared to doing them in separate vials, is the greater similarity in amplification conditions for the two amplimers involved [88].

9.3 SUPPRESSION SUBTRACTIVE HYBRIDIZATION (SSH)

Subtractive hybridization is a powerful technique that enables to compare two populations of mRNA and obtain clones of genes that are overexpressed in one population, but not in the other. Although there are several methods, their basic principles are similar. First, both mRNA isolates are converted into cDNA. The analyte cDNA is referred to as the "tester", while the cDNA that is to be compared as the reference is called the "driver". Tester and driver cDNAs are crosshybridized, frequently with the latter in excess, and the hybrid sequences are removed. Consequently, the remaining cDNAs that are not crosshybridized represent genes that are differentially expressed in the tester — i.e., they are absent or underrepresented in the driver mRNA.

SSH is an effective modification of this principle [45]. Using this method, the cDNA becomes fragmented, after which adaptor sequences are ligated with the fragments of the tester. These adaptors enable selective PCR amplification of differentially expressed genes while the amplification of other (nonspecific) sequences is suppressed. The sensitivity of the procedure is thus cosiderably increased. When the driver cDNA is added in large excess (more than 20-fold), then a limited number of highly overexpressed genes will be recovered from the tester mRNA isolate.

Amplimers from such genes are then cloned and sequenced to determine the origin of the gene. Other techniques, such as DNA microarray, may detect larger numbers of differentially expressed genes, while on the other hand, the differentially expressed genes obtained by SSH represent the largest differences, and the number of genes acquired is sufficient to discern patterns of gene expression.

10 RESULTS

SUMMARY OF PAPERS

10.1 PAPER I

The histological distinction between epithelioid mesotheliomas and adenocarcinomas requires an optimized immunohistochemical panel. All antibodies recommended for this purpose today may give atypical reaction patterns, none of which are completely specific.

Evaluating immunoreactivities of 10 antibodies in 119 mesothelioma and 57 adenocarcinoma tissues, we were able to optimize a panel consisting of 8 immunological parameters. The calculation of the discriminatory power of each antibody by logistic regression analysis gave an algorithm for interpreting discrepant reaction patterns.

CEA, CD15, BerEp4 and Sialyl TN, in order of declining efficacy, favor the diagnosis of an adenocarcinoma and similarly, calretinin, membranous EMA, thrombomodulin and HBME-1 suggest a mesothelioma. The panel should preferably be done in two steps, starting with 4 of the diagnostically most valuable antibodies (CEA, calretinin, CD15, EMA). The diagnosis of an adenocarcinoma can be made by having a reactivity pattern of at least 3 of the 4 favoring an adenocarcinoma. The second step uses the rest of the panel in the remaining unresolved cases.

10.2 PAPER II

This study concerns the differential diagnostic problem of cases with inconclusive ascites cytology. Many of these samples have been obtained from patients with serosal carcinomatosis, but this condition can not be reliably distinguished from benign conditions because of inadequate effusion volumes, small cellular yield or poor preservation of cells. Another problem is that benign mesothelial cells sometimes show signs of growth stimulation (mesotheliosis) that can mimic a malignancy.

By measuring the CEA and cholesterol concentrations in the ascitic fluid of 130 patients, of whom 57 had peritoneal carcinomatosis as verified by autopsy and/or histology, the additional diagnostic value of these biochemical assays to cytology was assessed.

CEA concentrations exceeding 5 ng/mL indicated carcinomas, occasionally without peritoneal involvement, which gave a sensitivity of 51% and specificity of 97% for carcinomatosis. A cholesterol level exceeding 1.21 mmol/L was found in 93% of cancers with peritoneal involvement, but it was not entirely specific (96%) for carcinomatosis.

Simultaneous increases in the concentrations of CEA and cholesterol were specific for carcinomatosis and this combination increased the sensitivity for diagnosing carcinomatosis from 77% with cytology alone to 88%. The correct diagnosis could

thus be established in a number (5/12) of carcinomatosis cases with inconclusive cytology. This indicates that the two biochemical markers in combination provide additional specific information about peritoneal carcinomatosis, and can therefore be used as an adjunct to cytology.

10.3 PAPER III

A benign effusion, being either an exudate or a transudate, contains mesothelial cells shed into the fluid in various amounts. I found that mesothelial cells, obtained from benign effusions and grown in short-term cultures, spontaneously develop uniformly with either a fibroblast-like or epithelioid morphology, and this pattern is stable through the early passages.

Several factors have been associated with the differentiation of mesothelial cells, such as hyaluronan, some proteoglycans (PGs) and WT1, which is an upstream regulator also of the PG syndecan-1. To determine the way in which the epithelioid and fibroblast-like differentiation of benign mesothelial cells correlated to WT1, PGs and hyaluronan synthase, their expression were studied by means of semiquantitative RT-PCR.

The expression of all these genes was associated with a variation in phenotypic differentiation. The gene expression of WT1 and cell surface PGs was greater in the benign mesothelial cells with epithelioid morphology than in the fibroblast-like ones, the difference being greatest for syndecan-4 and glypican-1, while that for syndecans-1 and -2 was less. The increase in WT1-associated mRNA was about as great as that of syndecans.

Fibroblastic cells, on the other hand, made substantially more mRNA of the matrix PGs biglycan and versican, but decorin was detected in only trace amounts in both morphological phenotypes. The expression for hyaluronan synthase-1 varied in both cell lines, although some epithelioid cell isolates showed higher levels.

The results indicate that both WT1 and PGs are involved in the multifactorial regulation of mesothelial differentiation. The findings also suggest that WT1 as well as cell surface and matrix PGs can be used to characterize the mesothelial lineage.

10.4 PAPER IV

To assess the involvement in this differentiation of molecules other than those studied in **Paper III**, differentially expressed genes were identified by suppression subtractive hybridization, using the same *in vitro* model with benign mesothelial short- term cultures. To verify the results, we studied the distribution of the corresponding gene products in tissue sections that showed various degrees of activation and regeneration in the mesothelium.

Of the 9 genes found to be overexpressed in fibroblastic mesothelial cells, 3 are matrix-associated (integrin α5, collagen binding protein 2, human cartilage glycoprotein 39), while the others are associated with a proliferative cell type (14-3-3 ε, plexin B2, N33, and 3 genes encoding ribosomal elements). Seven of the 8 genes upregulated in the epithelioid phenotype are related instead to specialized functions, such as metabolism

(aldose reductase, lecithin:cholesterol acyltransferase, ATPase 6), cytoskeletal composition (cytokeratins 7 and 8) and regulation of differentiation (granulin, annexin II).

Immunohistochemistry with available antibodies to 6 of the differentially expressed gene products confirmed the differences also *in vivo*. Distinct reactivities for 14-3-3 ε, integrin α5 and collagen binding protein 2 were found mainly in submesothelial fibroblastic cells where the tissue showed morphological signs of mild activation. Antibodies to annexin II, cytokeratin 7 and cytokeratin 8 labeled only the flattened mesothelial cells of the surface at these sites.

The pattern of differentially expressed genes in the fibroblast-like cultures thus correlated best with the immunophenotype seen in activated subserosal fibroblastic cells. Taken as a whole, the study showed a series of genes coordinately regulated during mesothelial differentiation and suggests possible markers for differentiation and activation in the mesothelium.

10.5 PAPER V

Although numerous immunohistochemical markers have been recommended for diagnosing mesothelioma and metastatic adenocarcinoma, additional parameters are needed for better discrimination. Another problem is to distinguish these cells from reactive benign mesothelial cells, which often mimic a malignancy in cytological specimens. Since PGs and WT1 seem to be related to the phenotypic differentiation of cells, some of the mRNA analyses used in **Paper III** were done in adenocarcinoma and mesothelioma cell lines, and these were also compared to benign mesothelial cells in short-term culture.

The aim of the study was to determine the way in which the expression and synthesis of WT1, syndecans, matrix PGs and hyaluronan synthase-1 (HAS-1) could be used to characterize these groups of cells. The main differences were also evaluated on the protein level in paraffin-embedded tumor tissues and cells in routinely prepared effusion specimens. The principal goal was to determine whether any of these parameters might be of diagnostic value in distinguishing between the three conditions.

Adenocarcinoma cells produced more mRNA for syndecan-1, while mesothelially-derived cells expressed WT1, biglycan and larger amounts of syndecan-2. The difference between these two syndecans in gene expression was best monitored by the ratio between them. Syndecan-4 was highly expressed in all malignant cell lines, while lower levels were seen in benign mesothelial cells. Although hyaluronan HAS-1 and versican could not distinguish between the three conditions, versican expression was associated with a high rate of proliferation (Table 1).

These findings suggest that syndecan-1 and syndecan-2 may be useful diagnostic markers, with more intense staining of the latter epitope in mesothelial tissues. The reciprocal appearance of these two syndecans in mesothelioma and adenocarcinoma is

evident not only with RT-PCR and FACS *in vitro*, but also with immunocytochemistry in clinical material.

Table 1. *Changes in mRNA expression profiles generally found in cell lines from mesotheliomas and adenocarcinomas, as compared to benign mesothelium (Based on findings in* **Paper V***)*

	Mesothelioma	Adenocarcinoma
Syndecan-1	~	↑
Syndecan-2	↑	↓
Syndecan-4	↑	↑
Versican V_0 and V_1	↑ *	↑ *
Biglycan	↑	↓
HAS-1	~	~
WT1	↑	↓

* increased expression only in highly proliferative cell lines

11 DISCUSSION

The cells responsible for mesothelial regeneration have been thought to come from two sources — i.e., exfoliated epithelioid cells of the serosal surface and multipotential subserosal cells. However, the latter has long been disputed. Mesothelial cells from benign effusions spontaneously differentiate to grow with a homogenous fibroblastic or epithelioid morphology in culture (**III**). The growth pattern may depend on stimuli before the shedding of cells from the serosal surface, such as inflammatory mediators, factors involved in proliferative liver disease, etc. In mesothelial cultures obtained from transudates not associated with destruction of the basement membrane, the cells usually grow with a fibroblast-like morphology. It thus seems that both of these morphologies in effusion cultures derive from exfoliated superficial serosal cells that have an epithelioid phenotype before to shedding.

PGs and WT1 are involved in this differentiation of benign mesothelial cells and characterize the fibroblast-like and epithelioid phenotypes (**III**). The differences in PG expression between the fibrous and epithelioid growth patterns seen in these benign cells are similar, but not identical to those found in mesothelioma cell lines [47]. Moreover, the PG profile and WT1 expression are also distinctive in mesothelioma and adenocarcinoma cells, and to a lesser extent in benign and malignant mesothelium (**V**; see below), a reflection of their importance in phenotypic differentiation.

The mesothelial differentiation is coordinately regulated from a less differentiated fibroblastic to a more specialized epithelioid growth pattern, as shown by differential gene expression (**IV**). While epithelioid cells overexpressed genes related to specialized functions of a differentiated cell type, the upregulated genes in fibroblastic cells were related to a more proliferative and less differentiated cell. This also accords with the findings in malignant mesotheliomas, where the sarcomatous variant has a poorer prognosis than the purely epithelioid tumor.

The findings of differential gene expression were further studied by immunohistochemistry with available antibodies in mesothelial tissues (**IV**). The immunophenotype that best corresponded to the gene expression of the fibroblast-like mesothelial cells in culture was that of the submesothelial fibroblastic cells in mildly activated mesothelium. When there was a marked regenerative or proliferative stimulus of the tissue, both the epithelioid and fibroblastic mesothelial cells had an immunophenotype intermediate to that found in less activated cells of fibrous and epithelioid morphology. This indicates that mesothelial cells can shift between epithelioid and fibrous growth patterns, as was also suggested by Bittinger [14] and Mutsaers [102], and both of these cell phenotypes may therefore contribute to regeneration by also recruiting cells from the submesothelial layer.

In effusion cytology, the first challenge is to distinguish between a malignant condition and a reactive one (mesotheliosis), and the two biochemical analyses studied here may be of help (**II**). An increase in the concentration of CEA indicates a carcinoma, regardless of its possible serosal involvement. An elevated cholesterol level in the effusion, on the other hand, indicates neoplastic involvement of the serous cavity, but as a single assay it is not entirely specific for such a condition. These two chemical assays thus measure different effects of tumors (CEA production versus cell disintegration), and when they were combined, simultaneous increases in both

parameters were specific for carcinomatosis, improving the sensitivity of "single" effusion cytology. This is of value in cases of inconclusive cytology.

When optimizing a battery of immunohistochemical markers for diagnosing mesothelioma with logistic regression analysis, the diagnostic value of each parameter is evaluated separately (**I**). Thus conventional criteria, such as the coexpression of vimentin and cytokeratin as well as their typical occurrence in particular cells, were of no additional value when the regression analysis had selected the 8 most suitable parameters of 10. Calretinin proved to be the best positive direct mesothelioma marker, while CEA was the best negative one. The interpretation algorithm provided by the analysis specifically identified about 90% of the mesothelioma cases. This battery is optimized for use in histological material; however, when using a similar panel in effusion cytology, its sensitivity is considerably lower [43].

Therefore, there is a need to develop more markers, particularly when working with cytological material. Better markers may also reduce the number of parameters neded in the panel. When comparing benign and malignant cells *in vitro*, cell surface proteoglycans syndecan-2 and syndecan-1 showed reciprocal mRNA expression patterns in cells of mesothelial origin and adenocarcinoma cells (**V**). The same alternating appearance of these two syndecans was also seen immunocytochemically in histological tumor material and exfoliated cells from effusions with a predominant syndecan-2 reactivity in mesothelial tissues. On the basis of the RT-PCR findings, WT1 and biglycan indicate a mesothelial origin of the cells, while the expression of versican and syndecan-4 seem to correlate with proliferation and malignancy, respectively. The diagnostic value of the latter findings and their use alone or in combination with other diagnostic parameters, however, must be tested *in vivo*.

12 CONCLUSIONS

1. An optimized panel of immunohistochemical markers improves the diagnostic accuracy of distinguishing between mesothelioma and adenocarcinoma, and the present battery gives a specific mesothelioma diagnosis with close to 90% sensitivity.

2. The combination of adjuvant biochemical markers, such as CEA and cholesterol, which measure essentially different effects of tumors, adds diagnostic value to effusion cytology.

3. Benign mesothelial cells that have exfoliated into effusions are not terminally differentiated. *In vitro,* they spontaneously grow with a consistent fibroblastic or epithelioid morphology. The pattern of growth may be related to stimuli before the cells are shed from the serosal surface.

4. This *in vitro* model permits the study of mesothelial differentiation into fibroblastic or epithelioid morphology. The pattern of differentially expressed genes indicates that the fibroblastic cells represent a less differentiated and more proliferative phenotype than the more specialized epithelioid mesothelial cells.

5. The differential gene expression in the fibroblast-like mesothelial cultures corresponds best to the immunophenotype of activated subserosal fibroblastic cells *in vivo.* This supports the view that subserosal cells are involved in mesothelial regeneration.

6. Some of the products of the differentially expressed genes in fibroblastic or epithelioid mesothelial cells could serve as markers for differentiation and activation in serosal tissues.

7. WT1 and PGs characterize benign mesothelial cells and are related to their differentiation. WT1 and cell surface PGs (syndecans and glypican-1) predominate in epithelioid cells, while matrix PGs are more abundant in fibroblastic cells.

8. The PG profile and WT1 are also characteristic of adenocarcinoma and mesothelioma cells, respectively. They may prove useful for distinguishing these tumors from each other and from benign mesothelium.

9. The syndecan-2 / syndecan-1 ratio is potentially an additional marker for distinguishing between mesotheliomas and adenocarcinomas in clinical work. A predominance of syndecan-2 favors the diagnosis of a mesothelioma with high efficacy, similar to that of calretinin.

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