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MALIGNANT MESOTHELIOMA

an experimental study with emphasis on proteoglycans in mesothelial cell growth and differentiation

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Separabis terram ab igne
subtile a spisso
suaviter
cum magno
ingenio

(Hermes Trismegistos)

ABSTRACT

Malignant mesothelioma is a highly aggressive tumor with median survival ranging from 4 to 12 months and, despite intense therapeutical efforts, it is invariably fatal. Mesothelioma cells are unique in the sense that they possess a biphasic growth potential and can be stimulated by serum growth factors to differentiate into stable epithelial or fibrous phenotypes. The prognosis of this tumor varies greatly depending on the differences in growth pattern, the most important predictor of poor prognosis being a fibrous phenotype.

To study the molecular basis of mesothelial differentiation, we used benign and malignant mesothelial cells in various stages of phenotypic differentiation. In order to evaluate the impact of proteoglycans (PG) on this process, a series of PGs were analyzed by semiquantitative reversed transcriptase polymerase chain reaction. The cells with epithelial phenotype showed increased expression of syndecan-2, syndecan-4 and hyaluronan synthase, and fibroblast-like cells expressed more matrix PGs: versican, decorin and biglycan. The PG profile may serve as a ”fingerprint”, and reflect the maturation of mesothelial cells. The functional importance of syndecans in mesothelial differentiation was further shown by antisense targeting; down-regulation of each particular syndecan caused a loss of epithelial morphology. Syndecans -1 and -4 are also needed for cell adhesion.

The differentiation of mesothelioma cells was influenced by treatment with various growth factors (TGF-β2, EGF, FGF-2, IGF-I and PDGF-BB). These factors affected the proliferation and morphology of mesothelioma cells to various extents, and the PG profile changed, in parallel, with an induced epithelial-mesenchymal transition. Exposure to EGF and IGF-I caused a fibroblast-like morphology simultaneously with a reduction in the syndecan expression levels. At the same time, the levels of shed syndecan-1 increased in the culture medium.

The involvement of other regulatory molecules in mesothelioma differentiation was assessed by subtractive hybridization, which has revealed a limited number of genes being differentially expressed between cells of epithelial or fibrous phenotypes. Most of these genes were recovered from the epithelial cells, which may indicate a more mature phenotype. The expression level of thioredoxin reductase, a small redox-active protein involved in drug resistance, was extremely high in both cell sub-lines, and may reflect the generic insensitivity of mesotheliomas to chemotherapy.

Although syndecans play a major role in regulating cell morphology, little is known about their subcellular distribution. Using confocal laser microscopy we found a substantial proportion of syndecans at intracellular locations, and syndecan-1 accumulated in the nucleus in a time-dependent manner. There was a close spatial relation of syndecans to tubulin in both interphase and mitotic cells. Vinblastine treatment interfered with the nuclear transport, and syndecan-1 and tubulin co-polymerize in paracrystalline occlusion bodies, in parallel with impaired nuclear transport. These findings suggest a tubulin-mediated transport mechanism. TGF-β2 reduced the proliferation rate of mesothelioma cells, concomitantly with a delay in nuclear transport of syndecan-1.

These data show that all syndecans are involved in maintaining the epithelial morphology, and that various amounts and translocation of syndecans may participate in molecular switches that regulate cell differentiation and proliferation. The above mechanisms may represent crucial steps, and possible future targets for therapy, that can be used to improve the management of patients with malignant mesothelioma.

LIST OF PUBLICATIONS

This thesis is based on the following articles which will be referred to in the text by their Roman numerals (I-V).

I. **Dobra K**, Andäng M, Syrokou, A., Karamanos NK and Hjerpe A
Differentiation of mesothelioma cells is influenced by the expression of proteoglycans
Exptl Cell Res 258:12-22, 2000

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

III. Sun X*, **Dobra K***, Björnstedt M and Hjerpe A
Upregulation of 9 genes, including that for thioredoxin, during epithelial differentiation of mesothelioma cells
Differentiation 66:181-188, 2000

IV. Brockstedt U*, **Dobra K***, Nurminen M and Hjerpe A
Immunoreactivity to cell surface syndecans in cytoplasm and nucleus: tubulin dependent rearrangements
Exptl Cell Res 274:235-245, 2002

V. **Dobra K**, Nurminen M and Hjerpe A
Effects of growth factors on mesothelioma cells. Changes in growth pattern and syndecan levels
Int J Cancer Submitted for publication.

* The first two authors contributed equally to these articles.

CONTENTS

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CS	Chondroitin sulfate
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
FACS	Fluorescence-Activated Cell Sorter
FGF-2	Fibroblast Growth Factor-2
GAG	Glycosaminoglycan
GalAG	Galactosaminoglycan
HA	Hyaluronan
HAS-1	Hyaluronan synthase 1
HS	Heparan sulfate
IGF-I	Insulin-Like Growth Factor-I
MAP	Microtubule Associated Protein
MM	Malignant mesothelioma
PBS	Phosphate-buffered saline
PDGF-BB	Platelet-derived Growth Factor-BB
PG	Proteoglycan
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TGF-ß	Transforming Growth Factor- ß
Trx	Thioredoxin
TSG	Tumor suppressor gene
TR	Thioredoxin reductase
WT-1	Wilms' tumor susceptibility gene 1

BACKGROUND

Malignant mesothelioma is an aggressive neoplasm, derived from the lining cells of a serous cavity, most frequently from the pleura, but also from the peritoneum, pericardium and tunica vaginalis testis .
The earliest detailed descriptions of primary serosal tumors date back to 1767, by Lieutaud, followed, in1819, by Laennec [quoted by Wagner 1993]. The term mesothelium was first used in 1890 by Minot. For the description of the malignant tumor derived from mesothelium Adami used the term mesothelioma in 1909 [quoted by Whitaker 1992]. Despite an increase in the frequency of this tumor during the nineteenth century, skepticism concerning the existence of a primary serosal tumor as a separate entity, persisted until the mid twentieth century, when the tumor was closely associated with a particular carcinogen — asbestos. The role of the serosal lining cells — i.e., mesothelium — in tumor histogenesis was extensively debated and questioned. Mesotheliomas have distinctive growth characteristics and show highly specific microscopic features with epithelial or mesenchymal characteristics, or a mixture of both. This biphasic growth potential of the tumor indicates an origin in the mesodermally-derived mesothelial cell.

SEROSAL MEMBRANES

MESOTHELIAL INJURY AND REGENERATION

The pleural and peritoneal cavities are lined by a continuous serous membrane, which consists of a single layer of flat mesothelial cells resting on a basement membrane, and with a submesothelial layer of connective tissue of variable thickness. The basic mechanism of surface mesothelium renewal, such as following injury, however, remains obscure and controversial. Some authors have presented evidence that the mesothelium is formed by macrophages deposited on the denuded surface [*Ryan et al. 1973*]; others have proposed that desquamated mesothelial cells reattach to the denuded surfaces [*Papadimitriou 1985, Mutsaers et al. 2000*]. On the basis of electron microscopic studies of the healing of the mesothelium, it has also been suggested that the mesothelial cell develops from subserosal mesenchymal cells [*Bolenet et al 1986*].

MESOTHELIAL DIFFERENTIATION

Ultrastructural [*Slater et al. 1989*] and immunhistochemical [*Carter et al. 1992*] studies, using monoclonal antibodies to intermediate filaments, show that the subserosal mesenchymal cells possess unique characteristics, and may be regarded as multipotential cells. Fibroblast-like submesothelial cells express only vimentin when at rest; when proliferating, they coexpress low molecular weight cytokeratins as well. When surface differentiation occurs, they add high molecular weight cytokeratins to their intermediate filament phenotype, at the same time as the production of vimentin is reduced [*Hammar & McNutt 1987*].

DIFFUSE MALIGNANT MESOTHELIOMA

ETIOLOGY AND PATHOGENESIS

Asbestos exposure

Epidemiological studies have established exposure to asbestos fibers as a main primary cause of malignant mesothelioma [*Wagner et al. 1960*, *Craighead & Mossman 1982*]. Other studies confirmed this relationship on the basis of exposure histories and the finding of asbestos bodies in the lung [*McCaughey et al. 1962*]. An association between asbestos exposure and diffuse mesothelioma was also shown experimentally, by injecting these fibers directly into the serosal cavities of experimental animals [*Wagner 1962*, *Smith et al. 1965*].

Most commercial asbestos is a mixture of different types of fibers. There are two main types of asbestos: long, curly, serpentine-like chrysotile (”white asbestos”), and the shorter, rod-like amphibole fibers, which include crocidolite (”blue asbestos”), amosite (”brown asbestos”) among others. A similar fiber structure is seen in erionite, a mineral regarded as the major cause of malignant mesothelioma in certain areas, such as in central Anatolia, Turkey.

Asbestos-induced oncogenesis

Experimental studies suggest that the carcinogenicity of asbestos in serous membranes is related to their fibrous structure rather than to their chemical characteristics [*Stanton 1973*]. Furthermore, differences in the dimensions of various types of asbestos fibers may explain the variations in their carcinogenicity [*Timbrell 1973*]. The curly white asbestos is therefore less oncogenic than other types of fibers, and fibers less than 0.25μm in diameter and more than 8μm in length are more potent than shorter, thicker ones [*Stanton 1973*]. Asbestos fibers deform the cytoskeleton in mesothelial cells more efficiently than in airway epithelial cells [*Lechner et al. 1985*]. In tissue culture, asbestos physically interacts with the mitotic spindle apparatus [*Ault et al. 1995*] and can interfere with normal chromosome segregation leading to aneuploidy [*Hesterberg & Barrett, 1985*]. Crocidolite asbestos has been shown to induce the expression and enzymatic activity of the mammalian DNA repair enzyme, apurinic/apyrimidic (AP)-endonuclease, suggesting that release of reactive oxygen species (ROS) generated by asbestos can damage DNA [*Fung et al. 1998*]. Asbestos can also induce the proto-oncogenes *c-fos* and *c-jun* which encode transcription factors that activate various genes critical in the initiation of DNA synthesis [*Heintz et al. 1993*]. The induction of these transcription activators may enhance cellular proliferation and could render cells more susceptible to subsequent mutations.

Chromosomal damage induced by asbestos

A hallmark of mesotheliomas is the large number of nonrandom cytogenic alterations [*Lechner et al. 1985, Knuutila et al. 1993, Lu et al. 1994, Pelin et al. 1995*]. For human tumors, these include monosomy or deletion of some of chromosomes 1, 3, 4, 6, 9, 14, 15, 18, 19, 22, and trisomies or polysomies of chromosomes 1, 5, 7, 11, 12, 20, 22.

This array of nonrandom chromosome deletions in human mesotheliomas suggests that several tumor suppressor genes (TSGs) and oncogenes may be involved in the

genesis of this disease. However, only the involvement of five TSGs — i.e., p16 (INK 4a), P15 (INK4b), p53, NF2, and WT1 — has been confirmed so far [*Lechner et al. 1997*].

Karyotypic studies show multiple clonal chromosomal abnormalities in most human malignant mesothelioma specimens [*Gibas et al. 1986, Popescu et al.1988, Tainen et al. 1988, Flejter et al. 1989, Hagememeijer et al. 1990, Taguchi et al. 1993*]. Deletions of specific chromosomal sites in the short (p) arms of chromosomes 1, 3, and 9 and long (q) arm of chromosome 6 occur frequently, and loss of a copy of chromosome 22 was the single most consistent numerical cytogenetic change [*Taguchi et al. 1993*]. It is noteworthy that most of the changes described above occur in combination in a given malignant mesothelioma.
Comparative genomic hybridization also reveals multiple genomic imbalances [*Balsara et al. 1998*]. In accord with previous karyotypic data, chromosomal losses were more frequent than gains with this approach.

Induction of proto-oncogenes

The nonrandom rearrangements and polysomy of chromosomes 1, 7 and 22 may generate growth-promoting oncogenes. Oncogenes often cause inappropriate expression of growth factors (GFs), growth factor receptors and other compounds involved in the signaling mechanisms. In consequence, normal growth control mechanisms are abrogated. It has also been suggested that autocrine production of PDGF-B chain may stimulate autoreplication of tumor cells, also when there are no or low levels of β receptors [*Langerak 1993*, *Langerak 1996, Gerwin 1996*]. Such activation of proto-oncogenes together with inactivation of tumor-suppressor genes may cooperate in a multistep series of critical events in the development of malignant mesothelioma.

Inactivation of tumor suppressor genes

The accumulated loss and/or inactivation of multiple TSGs in chromosomes 1p, 3p, 6q, 9p and 22q appear to play a critical role in the pathogenesis of malignant mesothelioma. TSGs within two of these regions, — i.e., $p16^{INK4A}$-$p14^{ARF}$ at 9p21 and *NF2* at 22q12 — are frequently altered in malignant mesotheliomas.

P16 (INK4a)

The gene coding for p16 (INK4a) was identified as the 9p21 putative TSG [*Kamb et al. 1994a, Nobori et al. 1994*]. It is particularly interesting because of its location in the region that is often deleted in malignant mesotheliomas. The protein encoded by p16 (INK4a) binds to cyclin-dependent kinase CDK4 and thereby inhibits the catalytic activity of the CDK4/cyclin D enzymes [*Serrano et al. 1993*]. Abnormal p16 protein levels were observed in most, if not all, malignant mesothelioma and malignant mesothelioma derived cell-lines [*Kratzke et al. 1995*]**.** The product of the p16 (INK4a) gene induces a G1 cell cycle arrest by inhibiting the phosphorylation of the retinoblastoma protein, pRb. Thus, homozygous loss of p16 (INK4a) and $p14^{ARF}$ would together affect both Rb- and p53 dependent growth regulatory pathways.

NF2

The neurofibromatosis type 2 (NF2) autosomal dominant tumor-suppressor gene resides on chromosome 22, which is frequently abnormal in mesotheliomas. Malignant mesotheliomas show mutation and/or allelic loss of NF2, suggesting that inactivation of this gene occurs via a two-hit mechanism [*Cheng et al. 1999*]. NF2 codes for a protein called merlin, which may play a role in cell surface dynamics and structure by linking the cytoskeleton to the plasma membrane [*Bianchi 1995*].

Presence of Simian Virus 40 sequences

Not all mesotheliomas are associated with asbestos exposure, and there latency for development of this tumor is long. Other factors or cofactors, that render certain individuals more susceptible, are therefore of considerable interest. A second way in which p53 may be inactivated, is the expression of the large T antigen (Tag) of Simian Virus 40 (SV40). Tag mediated inactivation of TSG products, such as pRb and p53, may make mesothelial cells more susceptible to the transforming effects of asbestos, and asbestos and SV40 may then act as cocarcinogens in mesotheliomas. Alternatively, SV40 may contribute to the fully transformed phenotype of cells previously damaged by asbestos. Although, several authors found SV40-like DNA sequences in mesotheliomas (*Carbone 1997a-b, Testa 1998)*, the role of SV40 in mesothelioma carcinogenesis is still controversial [*Nelson 2001*]. SV40-like sequences have been detected not only in mesotheliomas, but also in pleural plaques, lung tumors, and in lung parenchyma of individuals without cancer [*Galateau-Salle et al. 1998*]. One way in which SV40 can be transferred to humans is via contaminated polio vaccine. This offers a plausible explanation for the geographic differences in the prevalence of SV40 in various populations. SV40 was present in only 10% of Swedish malignant mesotheliomas, as compared to 40-69% in Italy and USA [*Priftakis et al. 2001*, *Pepper et al. 1996, De Luca et al. 1997*].

Familial malignant mesothelioma

Familial mesotheliomas have also been reported, although the specific genetic factors involved in the etiology of the tumor are not known [*Dawson et al.1992*, *Attanoos & Gibbs 1997*]. Clustering of malignant mesothelioma in families suggests that genetic susceptibility is a contributory factor [*Ascoli 2001*], and there is also an inherited component in the development of mesothelioma in the erionite exposed Turkish patients [*Roushdy-Hammady et al. 2001*] .

Taken together, malignant mesothelioma results from the accumulation of numerous acquired genetic events, mainly chromosome deletions, indicating a multistep cascade involving the inactivation of multiple TSGs. This process may be facilitated in cells containing SV40 large T antigen (Tag), because the Tag-mediated inhibition of p53 would impair DNA repair and apoptosis in cells damaged by asbestos [Fig. 1].

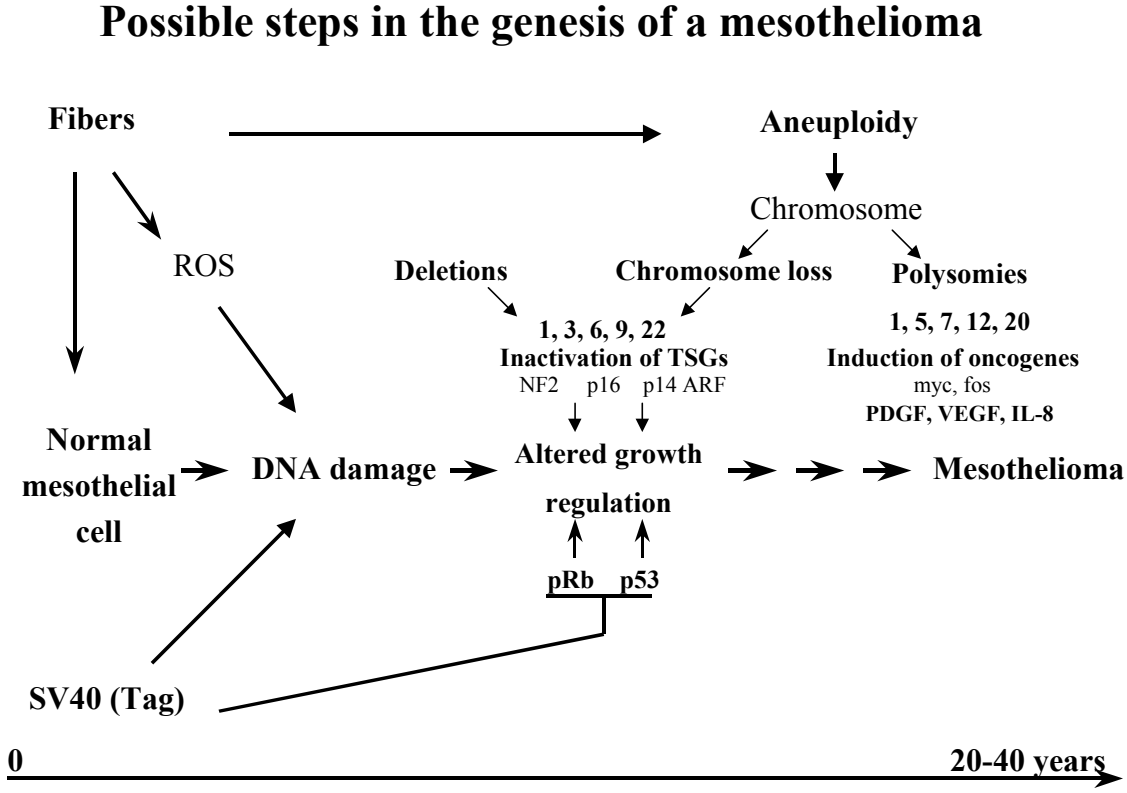


Fig. 1. *Working model depicting the pathogenesis of malignant mesothelioma. Asbestos fibers cause genomic instability directly or indirectly by inducing reactive oxygen species (ROS) that cause mutations or other DNA damage. This leads to genetic instability and subsequent chromosomal rearrangements, and a subset of genomic alterations; i.e., activation of proto-oncogenes myc, fos, and inactivation of tumor-suppressor genes (TSGs). Chromosome deletion or loss is more common, but chromosome gains may also occur. Altered methylation and autocrine upregulation of growth factors and cytokines leads to the invasive growth and production of IL-8, a major angiogenesis factor, that gives the malignant mesothelioma metastatic potential. Modified after [Lechner et al. 1997, Murthy A and Testa JR. 1999].*

CLASSIFICATION AND MORPHOLOGY

The mesothelioma may be restricted to a small area as a localized tumor, or it may involve the serosal membrane multifocally or grow in a diffuse manner. Benign adenomatoid or papillary tumors of mesothelial origin have been described, but most mesothelial tumors definitely follows a malignant course also when gowing as a localized nodule, which may be an early stage of diffuse malignant mesothelioma.

Four main histologic categories of diffuse malignant mesothelioma can be recognized: epithelial (tubulopapillary and nonglandular or epithelioid), sarcomatous (including desmoplastic), biphasic (mixed), and poorly differentiated (or undifferentiated) [Fig. 2]. It has been stated that 50% of pleural, and 75% of peritoneal diffuse malignant mesotheliomas are of epithelial type [*Kannerstein & Churg 1980*]; 25% and 15%, respectively, are of biphasic or sarcomatous type, and the remaining cases are poorly differentiated or unclassifiable. Sufficient sampling, however, often shows both epithelial and fibroblastic components, consequently the proportion of mixed type increases with the amount of tissue available for diagnosis [*Johansson & Linden 1996*].

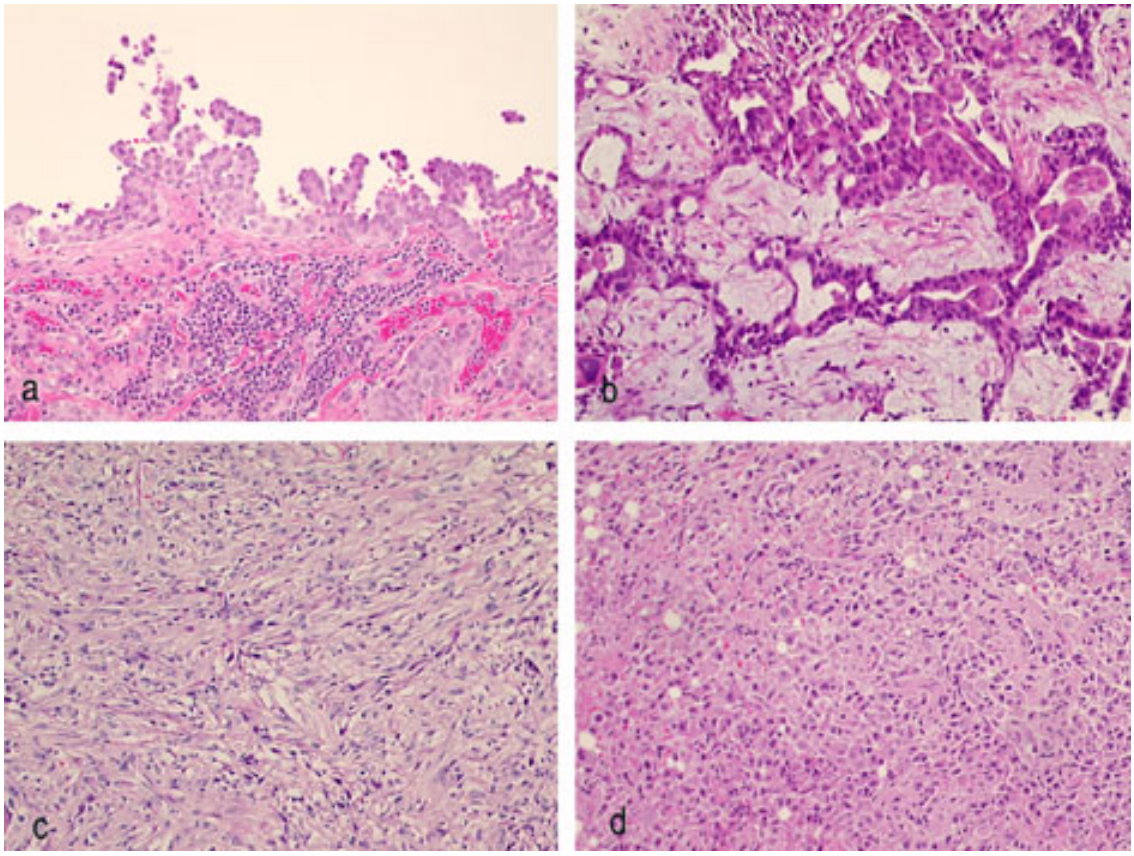
In **well differentiated tubulopapillary** neoplasms, the tumor cells often have an acidofilic cytoplasm; they are usually cuboidal or flattened, and sometimes possess uniform vesicular nuclei with prominent nucleoli. The solid or **epithelioid** form is composed of sheets of polygonal cells, which, when well differentiated may closely resemble hyperplastic mesothelium and pose a difficult diagnostic problem. In tumors that are entirely or partly **sarcomatous**, the neoplastic mesenchymal tissue consists of spindle-shaped or oval cells and may have a various histologic patterns, and giant cells are occasionally prominent. In the **desmoplastic** form of diffuse mesothelioma, much of the tumor is fibrous and it may be difficult to distinguish from reactive pleural fibrosis. In **biphasic tumors**, malignant elements of both epithelial and mesenchymal appearance are present, although the latter may be difficult to recognize as malignant. The two phenotypes frequently occur in different parts of the same tumor, but sometimes they are intimately admixed.

Fig. 2. *Different histological phenotypes of diffuse malignant mesothelioma: (a) papillary growth, (b) biphasic with coexistence of epithelioid and myxosarcomatous components, (c) sarcomatous and (d) poorly differentiated*

BIOLOGICAL BEHAVIOR

Malignant mesotheliomas characteristically have a variable biological behavior, and prognosis. The development of malignant mesothelioma occurs after a long latency, typically 20-40 years from the time of initial asbestos exposure to diagnosis [Fig. 1], suggesting that multiple genetic events are required for tumorigenic conversion of mesothelial cells [*Selikoff et al. 1980*]. Malignant mesothelioma, frequently produces large amounts of hyaluronan (HA). The analysis of this glycosaminoglycan (GAG) in effusions has proved to be a powerful ancillary method in the diagnosis of malignant mesothelioma [*Nurminen et al. 1994*].

Another typical feature of malignant mesothelioma is the tendency to give local recurrences in the biopsy area or following thoracoscopy. A plausible explanation for this phenomenon is the high local concentration of growth factors in the wound fluid, which may act as a chemoattractant for mesothelioma cells, stimulating them to migrate into the biopsy area. Such an effect has been shown for PDGF-BB, which together with PDGF receptor beta and integrin $\alpha 3\beta 1$, is necessary for the motile response of malignant mesothelioma cells [*Klominek et al. 1998*].



CLINICAL FEATURES

The typical clinical findings in malignant mesothelioma are dyspnea, chest pain, or pleural effusion. The first material available for diagnosis is often an effusion taken for therapeutic purposes. The distinction between pleural epithelial mesothelioma and metastatic adenocarcinoma involving the pleura is an important diagnostic problem for pathologists. Diagnostic accuracy can be improved by the use of ancillary methods, such as optimized immunhistochemical panels [*Dejmek & Hjerpe 1994, Ordonez 1999, Brockstedt et al. 2000, Carella et al. 2001*], determination of HA levels of pleural effusions [*Nurminen et al. 1994*] and ultrastructural analysis [*Warhol et al. 1982, Stoebner & Brambilla 1982*].
Many cases of malignant mesothelioma are diagnosed late in the development of the disease and treatment is often ineffective. Malignant mesothelioma are often highly resistant to a number of chemotherapeutic agents [*McLaren et al. 2001*], which may be related to overexpression of detoxication proteins associated with drug resistance [*Segers et al. 1996, Dejmek et al. 1998*], yet the mechanisms by which resistance occurs are still poorly understood.

Traditionally, strategies for the treatment of malignant mesothelioma have included supportive care, surgery, radiotherapy and chemotherapy. Single modality therapy using traditional approaches alone has failed to improve patient survival compared to supportive care. Multimodality approaches, in particular, cytoreductive surgery (pleuropneumonectomy) followed by sequential chemotherapy and radiotherapy are more promising [*Jaklitsch et al. 2001*], especially for patients with epithelial histology, negative resection margins, and no metastases to extrapleural lymph nodes. Innovative therapies, such as the use of photodynamic therapy, targeted cytokines and gene therapy, are currently being investigated for management of malignant mesothelioma [*Davidson et al. 1998, Molnar-Kimber et al. 1998, Caminschi et al. 1999, Sterman et al. 1999, McLaren et al. 2000, Schouwink et al. 2001*].

PROGNOSIS

The prognosis of malignant mesothelioma varies greatly because of differences in growth potential and difficulties in obtaining an early diagnosis. Mesotheliomas are highly aggressive tumors, with median survival ranging from 4 to 12 months, depending on the histological subtype [*Ruffie et al. 1989, Tammilehto et al. 1992, Fusco et al. 1993*]. Independent indicators of poor prognosis in multivariate analysis included non-epithelial cell type, performance status and increased angiogenesis, assessed by microvessel density (MVD) [*Bongiovanni et al. 2001, Edwards et al. 2001, Thylen et al. 2001*]. Regardless of the type of therapy used, malignant mesothelioma is invariably a fatal disease. Mesotheliomas are rarely cured, and the 5-year survival rate is estimated at less than 5% [*Achatzy et al. 1989*]. However, some patients survive several years without treatment [*Law et al. 1984*].

MESOTHELIOMA AS A MODEL FOR CELL DIFFERENTIATION

Most tumors gradually lose their epithelial character in a process termed the epithelial-mesenchymal transition. These phenotypic changes often occur as epithelial cells become tumorigenic. Loss of specific differentiation markers, adoption of a migrating morphology and progressive replacement of the cytokeratin network by vimentin intermediate filaments characterize this transition. Invasive growth of these cells also involves loss of epithelial polarity, and they undergo changes referred to as dedifferentiation.

Mesothelioma cells obtained from a pleural effusion show diverging differentiation potential and inducible growth pattern. These cells possess a characteristic biphasic growth potential, and can be induced by serum factors to differentiate into stable epithelial or fibrous phenotypes [*Klominek et al. 1989*] similar to the *in vivo* situation. Epithelial cells of mesothelial origin undergo a reversible morphological transition after exposure to several growth factors. This epithelial-mesenchymal transition involves transient cytoskeleton remodeling , and it is accompanied by changes in the adhesive status of these cells. In this way, the unique properties of mesothelioma cells provide an excellent model for identifying the critical changes in the regulation of cell differentiation and tumor cell progression.

PROTEOGLYCANS

Various components of the extracellular environment control cellular processes, such as proliferation, differentiation and migration. Proteoglycans (PGs), a heterogeneous group of such complex molecules, constitute complex cell-surface and matrix components with great degree of structural diversity. The term PG refers to a dichotomous structure, consisting of a protein core to which glycosaminoglycan GAG side chains are attached. Both the protein core and its side chains undergo a series of modifications, finally resulting in more or less sophisticated compounds of varying size, fine structure and ability to interact in biological processes.

GLYCOSAMINOGLYCAN SIDE CHAINS

Glycosaminoglycans (GAGs) are linear polysaccharides consisting of repeating disaccharide units. They include heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate, keratan sulfate and hyaluronan (HA). HS has the most variable structure, with a complexity that easily surpasses that of common nucleic acid [*Iozzo 2002*]. It consists of alternating uronic acid (D-glucuronic acid [GlcA] or L-iduronic acid [IdoA]) and D-glucosamine (GlcN) units [Fig. 3]. The length of the HS chains can vary more than 10-fold with cell type and core protein.

Synthesis and modification of the GAG chains

The biosynthesis of protein-bound GAGs, including HS, occurs in the Golgi apparatus with chain initiation at defined Ser-Gly amino-acid sequences on the core protein. The first step is the transfer of xylose to the hydroxyl group of these serine residues, followed by stepwise attachment of three more sugars, to form the linkage tetrasaccharide, the basis for subsequent GAG chain elongation [*Lindahl et al. 1998*]. The presence of xylose is noteworthy, the PGs being the only mammalian compounds carrying this sugar, otherwise found in plants. When the HS chain is assembled, the individual saccharide units are subjected to a series of modification reactions that superimpose uronic acid C5 epimerization and complex patterns of sulfation at selective positions [*Lindahl et al. 1998; Lander & Selleck 2000; Perrimon & Bernfield 2000, Selleck 2000*]. These reactions create clusters of highly sulfated disaccharides alternating with larger, unmodified domains [*Maccarana et al. 1996*]. The sulfated domains of HS are the main regions involved in the recognition of growth factors and other proteins [*Gallagher 2001*]. It seems that the system is not template-driven and these reactions do not go to completion, which results in a high degree of structural diversity [*Turnbull 2001*]. The overall size of the HS chain can vary from 20-150 disaccharides, which adds another level of complexity.

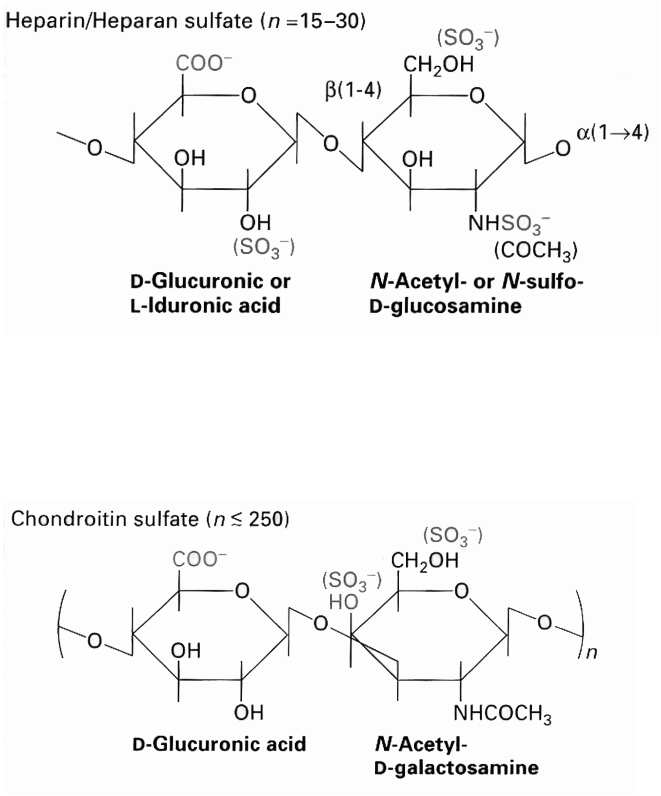


Fig. 3. Repeating disaccharide structure of heparan sulfate and chondroitin sulfate.

PROTEOGLYCAN CORE PROTEINS

The proteoglycan (PG) superfamily now contains more than 30 full-time molecules that fulfil a variety of biological functions. The PG nomenclature has undergone rapid conceptual changes. Initially, PGs were given names which reflected the source and nature of their glycosylation, — i.e., basement membrane heparan sulfate PG or large aggregating chondroitin sulfate PG of cartilage, else they were arbitrarily named as fractions from the isolation procedure. In recent years, molecular biological techniques to isolate and sequence DNA have lead to a naming convention, which ignores any posttranslational modification. As more PGs are studied in detail, and their functions and properties determined, there has been a tendency to name them on the basis of these features, such as decorin, which decorates collagen fibrils, and fibromodulin, which modulates the formation of collagen fibrils.

When possible, PGs have been grouped into distinct gene families and subfamilies thereby providing a simplified nomenclature based on their core protein design [Fig. 4].

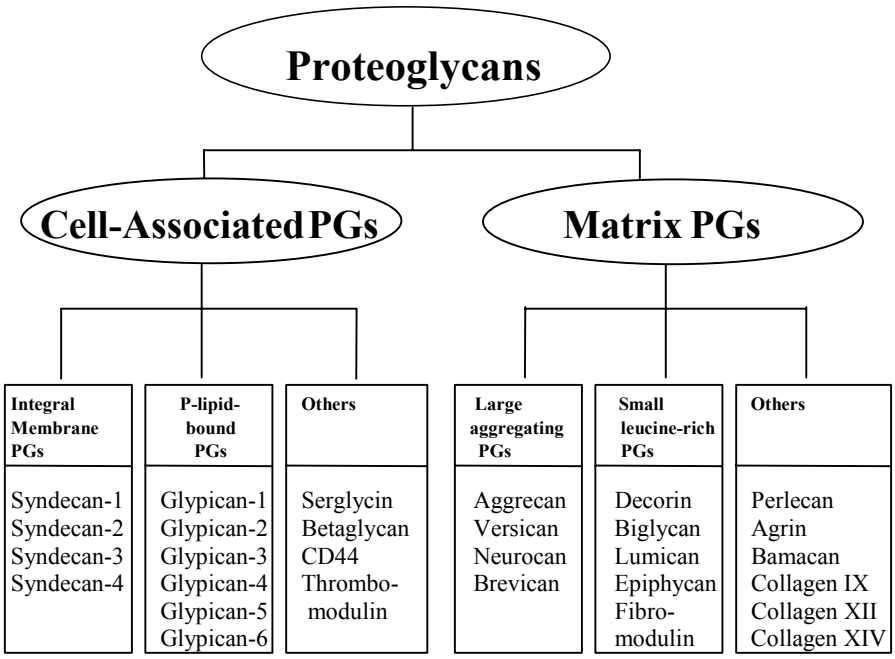


Fig. 4. *Simplified diagram showing the major families of the ever increasing number of PG molecules. The first divider in this categorization reflects the location of the PG; whether found mainly in association with the cell or the extracellular matrix.*

CELL-ASSOCIATED PROTEOGLYCANS

The HS chains at the cell surface are mostly attached to syndecan or glypican core proteins. These protein cores determine the proximity of the HS chains to the cell surface, when, where and to what extent the HS chains are expressed, and the rate and mechanism of HS turnover [*Bernfield et al. 1999*]. The members of the syndecan and glypican families account for most of the cell-associated HS. The remaining HS found on the cell surface belongs to so-called part-time or ”amateur” HSPGs, like betaglycan (TGFβ type III receptor), CD44E (epican) and CD44H (lymphocyte-homing receptor).

SYNDECANS: INTEGRAL MEMBRANE HSPGS

The syndecan family consists of structurally-related cell surface PGs, which hitherto have included four members: syndecan-1, syndecan-2 (fibroglycan), syndecan-3 (neurocan) and syndecan-4 (amphiglycan). Their core proteins span the membrane with very well preserved transmembrane and cytoplasmic domains [reviewed by *Bernfield et al. 1992, David 1993*]. The chromosomal locations, exon organization, and sequence relationship of syndecans suggest that the gene family arose by gene duplication and divergent evolution from a single ancestral gene, and that syndecans -1 and -3 and syndecans 2- and - 4 represent subfamilies [reviewed by *Bernfield et al. 1999*]. Each gene product is a single type-I membrane-spanning protein, showing a variable N-terminal extracellular domain, but with preserved GAG attachment and proteolytic cleavage sites. This contrasts with the highly preserved cytoplasmic C-terminal and transmembrane domains [Fig. 5]. The extracellular domain of syndecan-1 contains two regions of GAG attachment, one near the N terminus, that consistently has three HS chains, and another near the plasma membrane, that serves as an attachment site for CS. Multiple HS chains are needed for optimal syndecan-1 function, since mutation of one or more Ser-Gly attachment sites, reduces its function [*Langford et al. 1998*].

The turnover of syndecans mainly occurs by proteolytic cleavage of core proteins near the cell surface, releasing the intact ectodomain with all the side chains. The transmembrane and cytoplasmic domains may localize the syndecans into discrete membrane microdomains. Therefore syndecan-1 polarizes to the baso-lateral cell surface of epithelia [*Rapraeger et al. 1987*], but syndecan-4 is ubiquitous. The cytoplasmic domains contain two invariable regions (C1and C2) separated by a variable one (V). The C1 region of syndecan-3 binds Src family kinases, the actin-binding proteins cortactin and tubulin. The preserved C2 regions contain an EFYA sequence that can bind PDZ domain-containing proteins, such as syntenin or CASK [reviewed by *Bernfield et al. 1998*]. Tyrosine residues on the cytoplasmic domain of all syndecans can be phosphorylated *in vivo*. These relations suggest that each syndecan family member interacts with the same proteins (via the transmembrane domain and C regions), but also has unique interactions (involving the ectodomain and V region).

Syndecans are transiently expressed during morphogenesis

Syndecan-1 appears in the developing mouse embryo as early as the 4-cell blastula stage, continues its expression in ectoderm and becomes localized in mesodermal cells following gastrulation [*Sutherland et al. 1991*]. After being transiently induced in condensing mesenchyme in some tissues, it

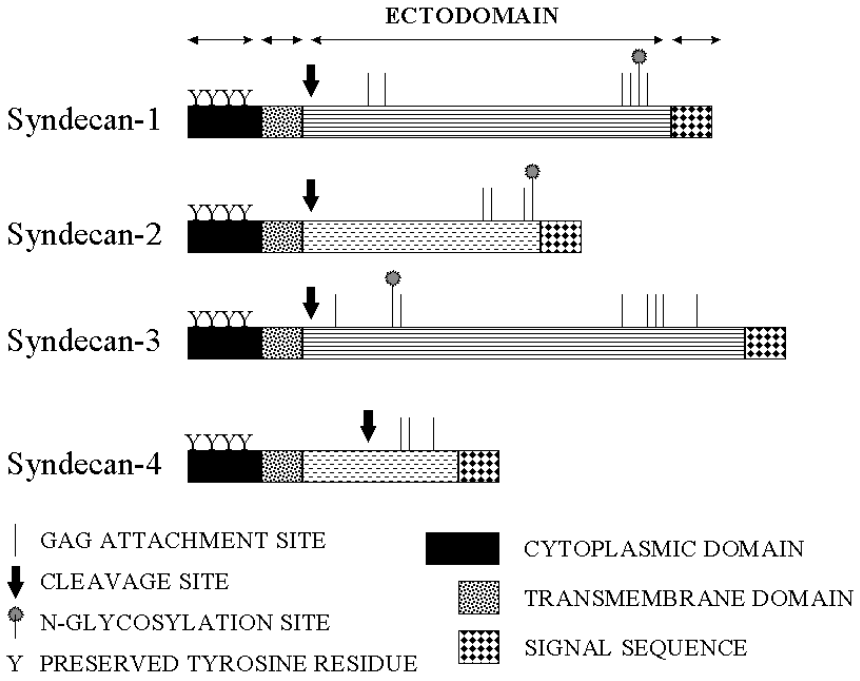


Fig. 5. *Schematic illustration of structurally-related syndecan genes, showing the two subfamilies of syndecans: syndecan-1 and -3, and syndecans -2 and –4, respectively. The extracellular domain is highly variable with the exception of the GAG attachment sites and the proteolytic cleavage site near the plasma membrane. In contrast the endo- and transmembrane domains are well preserved.*

becomes localized in epithelial cells, where it is expressed throughout maturity [*Kim et al. 1994*]. Syndecans-2 and -4 are expressed later [*Gallo et al. 1993*]. In mouse embryos, syndecan-2 accumulates in mesenchyme derived from the lateral plate mesoderm as well as the neural crest, and on cells that later form connective and skeletal tissues [*David et al. 1993*].
In adults, syndecan-1 is a major PG in epithelial cells, but only a minor component in fibroblasts. Apparently, confluent human fetal fibroblasts can also synthesize syndecan-1, indicating that the expression of this PG may be related to or regulated by the formation of cell-cell contacts [*Lories et al. 1992*]. Syndecan-2 abounds in mesenchymal tissues, its expression contrasting with that of syndecan-1 [*Mali et al. 1990, David et al. 1993*]. Syndecan-3 is mainly found in neural tissues, while syndecan-4 is an ubiquitous PG acting as a focal adhesion component [*Woods and Couchman, 1992*].

Syndecan interactions and functions

The differences and similarities in structure largely account for the various syndecan functions. Much of our present knowledge about how syndecans interact is based on studies of syndecan-1. These PGs seem to have partly overlapping functions, but other functions are mainly ascribed to particular members of the syndecan family [*Sanderson 2001*]. Syndecans bind a variety of molecules in the cellular microenvironment. In this way syndecans can interact with growth factors, extracellular matrix components, enzymes, protease inhibitors and chemokines among other extracellular constituents [Fig.6], and play role in signal transduction. Many of these ligand-binding reactions, depend on defined structures or sequences in the HS side chains, but they may also involve the protein cores.

Coreceptor for soluble ligands

The most extensively studied ligand, — i.e., fibroblast growth factor (FGF) — forms a complex with HS, which concentrates the factors on the cell surface before presenting them to their specific receptors, and at the same time protects them from degradation [*Esko 1991*, *Carey 1997*]. HS also binds to the specific growth factor receptors, and syndecan seems to be needed to form tight functional complexes between the growth factors and their receptors. For a number of growth factors, e.g. FGFs, vascular endothelial growth factor (VEGF), heparin-binding epidermal growth factor (HB-EGF) and others, the interaction has been shown to be critical for growth factor signaling [*Rapraeger 1995, Bernfield 1999*].

Coreceptor for insoluble ligands in the extracellular matrix

The extracellular matrix (ECM) consists of many large multidomain proteins most of which bind, via discrete domains, to both integrins, a large family of heterodimeric adhesion receptors, and to cell surface HS. Syndecans often act as coreceptors in these interactions, modifying the cytoskeletal organization and the adhesive phenotype [for review see *Woods & Couchman 1998, Ridley et al 1993*]. While syndecan-2 controls the assembly of laminin or fibronectin into a fibrillar matrix [*Klass et al. 2000*], syndecan-4 is required for the development of focal contact sites [*Woods & Couchman 1992*], and the extracellular matrix adhesion being a prominent role for this latter syndecan [*Saunders et al. 1989*, *Kato & Bernfield 1990, Inki et al. 1994*, *Tumova et al 2000*]. Moreover, syndecan-4

signals cooperatively with integrins in a Rho dependent manner in the assembly of focal adhesions and actin stress fibers [*Saoncella et al. 1999*].

HSPGs inhibit invasion by promoting tight cell-cell and cell-ECM adhesion.

Interactions of HSPGs with the pericellular matrix also affect the activity of proteases secreted by tumor cells and therefore the ability of these cells to invade. Examination of a panel of cell lines showed that lines not expressing syndecan-1 readily invade and migrate in collagen gel, while those expressing this PG do not [*Liebersbach & Sanderson 1994, Mali et al. 1994, Sanderson et al. 1994*]. The strongest evidence that HSPGs inhibit invasion comes from studies on heparanase [*Nakajima et al. 1988, Nicolson et al. 1998*]. However, during recent years, more and more contradictory data have accumulated regarding the role of syndecan-1 in tumor cell invasion, and it seems that under certain circumstances this PG may also promote invasion [*Hirabayashi et al. 1998, Conejo et al. 2000*].

Soluble paracrine effector

Proteolytic cleavage near the plasma membrane instantly releases and converts membrane-anchored molecules into soluble effectors, in a process known as shedding [*Hooper et al 1997, Werb 1997*]. This changes the function of the HSPG and can reduce the level of cell surface HSPGs. Syndecan shedding leaves a potentially interactive peptide containing the transmembrane and cytoplasmic domains. The shed fragment retains its HS chains and may compete with the cell membrane-anchored syndecans for interactions, acting either as agonists or antagonists to the ligand activity. Shedding of syndecans-1 and -4 is accelerated by direct proteolytic cleavage (thrombin, plasmin) by cellular stress (mechanical, heat shock, hyperosmolarity) via activation of multiple intracellular signaling pathways. The proteolytic cleavage seems to be mediated by a metalloproteinase that can be blocked by a tissue inhibitor of metalloproteinase (TIMP)-3. Shedding is stimulated by tissue injury and syndecan ectodomains are found in inflammatory fluids where they modulate the activities of growth factors and proteinases [*Kainulainen et al. 1998, Kato et al. 1998*].

Internalization receptor

Ligand bound to the cell surface may be internalized, perhaps best exemplified by antithrombin (AT III) and lipoprotein lipase. These ligands can be internalized along with the HSPG, as part of a possible turnover and recycling. It is noteworthy that, FGF-2 can be internalized on both syndecans and glypicans via caveolae through an FGF receptor-independent mechanism [*Gleizes et al. 1996*] which, together with regulated nuclear translocation of the GFs [*Maher 1996*], may indicate more specific biological roles of these HSPGs in growth factor-mediated cell signaling.

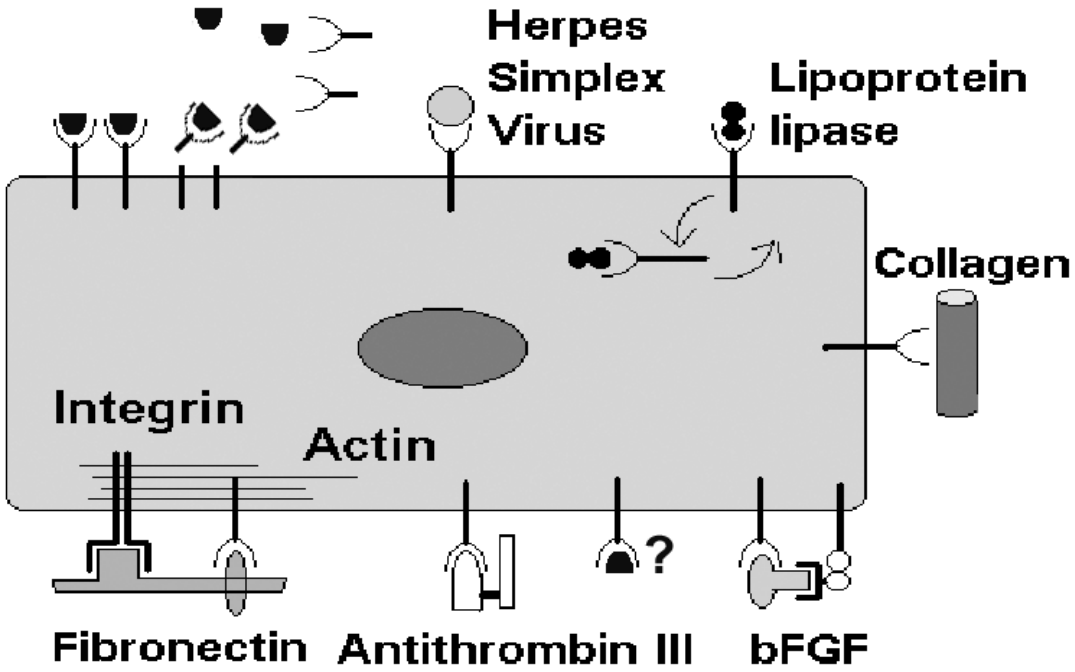


Fig. 6. *The syndecans participate in various ligand-binding reactions in the cell membrane. These include binding to insoluble matrix components and receptor functions. Shedding of syndecan creates soluble fragments with preserved binding capacity, that may act competitively .*

GLYPICANS: GLYCOSYL-LINKED HSPGS

The second family of cell membrane HSPGs constitute the glypican-related PGs covalently linked to the cell surface via glycosylphosphatidylinositol. The modular structure of the glypicans has been highly preserved throughout evolution, showing more than 90% sequence similarity, as compared to glypicans from different vertebrate species. Six members of this family have hitherto been identified [*David et al. 1990, De Cat & David 2001*].

In humans, mutations have been found only in glypican-3, causing overgrowth of several tissues and organs [*Pilia et al. 1996*]. This observation supports the idea that glypican- 3 (OCI-5/GPC3) is a negative regulator of cell proliferation. GPC3 is mainly expressed in mesoderm and mesoderm-derived tissues, and to a much lesser extent in endodermal and ectodermal tissues. A recent differential mRNA display study on normal rat mesothelial cells and mesothelioma cell lines reveals, that GPC3 is downregulated in the tumor cell lines [*Murthy et al. 2000*]. Moreover, a similar downregulation was found in primary rat mesotheliomas and cell lines derived from human mesotheliomas. Most of these cell lines displayed aberrant methylation in the GPC3 promoter region, and ectopic expression of GPC3 inhibited their colony-forming ability.

It sould be noted that, in cancers originating in tissues that are GPC3 positive in adults, the expression of GPC3 is silenced or reduced during tumor progression, but in tumors originated from tissues that only express GPC3 in the embryo, GPC3 expression tends to reappear on malignant transformation, and in these organs it behaves as an oncofetal protein. In this regard, GPC3 is even more frequently upregulated in hepatocarcinomas than α-feto-protein [*Hsu et al. 1997*].

As HS-carrying molecules, glypicans were first thought to be potential regulators of heparin-binding growth factors. However, the involvement of glypicans in the *in vivo* regulation of this binding remains to be determined.

Glypican has also been shown to occur intracellularly, i.e., in the nuclei of neurons and glioma cells, together with biglycan, a small leucine-rich chondroitin sulfate PG, which raises the possibility that they may modulate nuclear processes, such as the control of cell division [*Liang et al. 1997*].

MATRIX PROTEOGLYCANS

The matrix PGs are structural components of the extracellular matrix and associate tightly with hyaluronan (HA) and other proteins. Matrix PGs frequently carry CS and/or DS chains. Most of them are recovered in two groups: one group of large PGs that can aggregate to HA and one group of small PGs with a leucine-rich core protein. The large PGs generally dominate the matrix PGs and comprise versicans found in soft connective tissues and aggrecan typically expressed in cartilage. The small matrix PGs (decorin, biglycan and fibromodulin) have been associated with various ligand-binding reactions of a more specific nature. The derived protein sequence of decorin and biglycan shows high homology, strongly suggesting that these two proteins are the result of a gene duplication [*Fisher et al. 1989*].

LARGE AGGREGATING PGS

Versican

The extracellular matrix of a tumor stroma includes components, which have a biologically active role, that affect the tumor cells rather than just being a passive mechanical support. Moreover, it has been shown that extracellular matrix rich in versican and HA may modulate cell proliferation and migration [*Dours-Zimmermann et al. 1994, Iozzo 1998*]. Versican is synthesized in a variety of embryonal tissues, such as by immature mesenchymal cells [*Nakashima et al.1990*]. A strong reaction to versican has also been found in the proliferating mesenchymal cells located in the peripheral invasive areas of carcinoma tissues [*Nara et al. 1997*]. Poorly differentiated prostate carcinomas have significantly greater levels of stromal versican than well and moderately differentiated ones [*Ricciardelli et al 1998*].

Of particular interest is the observation that versican is abundant in the subcellular interstitium of the cultured fibroblasts, but is selectively excluded from focal contacts where integrins and fibronectin co-localize with cytoskeletal components [*Yamagata et al. 1993*]. This suggests that the role of versican may be to destabilize the focal cell contacts.

SMALL LEUCINE-RICH PROTEOGLYCANS

The family of small leucine-rich PGs are associated with certain functions, best known is the interaction between decorin and collagen [reviewed *by Iozzo 1998*]. The binding, involving both the protein core and the GAG chains, is important for the assembly of the collagen fibrils [*Danielson et al. 1997*]. Another important characteristic of decorin is its high affinity for TGF-β , allowing decorin to function as a reservoir for this growth factor in the extracellular milieu [*Danielson et al. 1997*]. It appears that in certain cellular systems, decorin blocks the activity of TGF-β, whereas in others its binding augments the bioactivity of the cytokine. Emerging results support the idea that a function of the secreted decorin is its ability to inhibit cellular proliferation, which may represent a biological response of the host to counterbalance the effects of tumor cells [*Iozzo 1995*].

BASEMENT MEMBRANE PROTEOGLYCANS

The main PGs found in basement membranes are perlecan, agrin, and bamacan [*reviewed by Iozzo 1998*]. The first two of these carry HS side chains, whereas the latter is a chondroitin sulfate PG. These proteins are involved in the control of lipoprotein metabolism, the adhesion of cells to the substratum, the interactions between cells and matrix, and in the control of cellular growth [*Iozzo et al.1994*]. Perlecan seems to be involved directly in promoting the growth and invasion of tumor cells by its ability to capture and store growth factors [*Folkman et al. 1988*], by entrapping them within the basement membrane [*Vigny et al.1988*] or in the tumor stroma [*Ohtani et al. 1993*]. Downregulation of perlecan expression suppresses the invasive behavior of melanoma cells *in vitro* [*Adatia et al. 1997*] and inhibits tumor growth and angiogenesis of colonic carcinoma cells *in vivo* [*Sharma et al. 1998*].

HYALURONAN IN TUMORS

Most malignant solid tumors contain elevated levels of hyaluronan (HA). HA is otherwise ubiquitously present in most tissues. This GAG is synthesized by one of the three known hyaluronan synthases (HAS) located in the cell membrane [*Asplund et al. 1998, Jacobson et al. 2000,*]. The synthase activity fluctuates with the cell cycle, peaking at mitosis. It is functionally important not only in mitosis, but also for cell proliferation in general and the migration of cells [*Tammi & Tammi 1991,* reviewed by *Toole 2001*].

This enrichment of HA in tumors can be due to increased production by tumor cells themselves or to interactions between tumor cells and surrounding stromal cells that induce increased production by stromal cells. Particularly high levels of HA are seen in many mesotheliomas, and the detection of this GAG in effusions can be used diagnostically [*Nurminen et al. 1994*].

The molecular functions of HA fall into three categories. First, HA occupies an enormous hydrodynamic domain that greatly influences the hydration and physical properties of tissues. Second, it interacts with other ECM macromolecules, particularly with versican and aggrecan. The binding of these PGs creates huge aggregates, that increase the charge density and thereby the water-binding capacity of the tissue [*Laurent & Fraser 1992*]. The aggregation also anchors the PGs, preventing them from extraction away from the tissue. These HA interactions are essential to the structure and assembly of several tissues. Finally, HA interacts with cell surface receptors, notably CD44, and thereby influences cell behavior.

WT1

WT1 is a gene expressed in a number of cell types, such as in the kidney, gonads and mesothelium, at a time which corresponds to mesenchymal-to-epithelial transition and this gene has been ascribed a pivotal role in this developmental step. Further evidence for the importance of this gene in morphogenesis has been provided by WT1 knock-out mice, that lack the correct mesenchyme-to-epithelium transition of the mesothelium [*Kreidberg et al. 1993*]. In contrast to the transient embryonic expression observed in other tissues, a continuous expression of WT1 throughout adult life has mainly been described in mesothelium and in the gonads, although such expression has also been found in other mesoderm-derived tissues [*Park et al. 1993*]. Expression of WT1 has therefore been proposed as a marker for mesothelial lineage [*Walker et al. 1994*], and it is highly expressed and occasionally mutated in malignant mesothelioma [*Park et al. 1993, Amin et al. 1995*].

WT1 gene encodes a zinc finger protein that acts as transcriptional repressor for a variety of target genes, including growth factors and growth factor receptors [reviewed in *Scharnhorst et al. 2001*]. The complexity of this protein may depend on a large number of splice variants, at least 24 different forms being known. It is of note, that E-cadherin [*Hosono et al. 2000*] and syndecan-1 [*Cook et al. 1996*], two genes important for epithelial differentiation, both represent downstream target genes for WT1. This is further supported by the observation, that expression of syndecan-1 and E-cadherin are simultaneously lost during epithelial-mesenchymal transformation [*Sun et al. 1998*].

THE THIOREDOXIN AND THIOREDOXIN REDUCTASE SYSTEM

Thioredoxin (trx) is a small ubiquitous redox-active protein [*Holmgren & Björnstedt 1995*] originally discovered as a hydrogen donor to ribonucleotide reductase essential for DNA synthesis. Trx is thought to have important functions in the regulation of cell growth [*Ericsson et al. 1992, Rosén et al. 1995*]. This protein, present in human plasma, is secreted by normal and neoplastic cells via a leaderless secretory pathway [*Rubartelli et al. 1992*]. It operates with the FAD-selenoenzyme thioredoxin reductase (TR) and NADPH (the Trx-system) as an efficient general protein disulfide reductase system [*Holmgren & Björnstedt 1995*]. Mammalian TR is a homodimeric flavoenzyme with a selenocysteine, a FAD and a functional dithiol/disulfide in each subunit [*Williams 1992, Tamura & Stadtman 1996*]. The enzyme has a broad substrate specificity and reduces not only Trx from distant species, but also a wide variety of low molecular weight substrates, like selenium compounds and hydroperoxides [*Holmgren & Björnstedt 1995*, *Björnstedt et al. 1997*].

Trx binds to a variety of proteins and selectively activates the DNA binding of certain transcription factors, such as NF-κB and AP-1 [*Hayashi et al. 1993, Schenk et al. 1994*]. Trx stimulates cell growth and is an inhibitor of apoptosis [*Saitoh et al. 1998*]. An increase in thioredoxin levels seen in many human primary cancers, unlike in normal tissues, appears to contribute to an increase in cancer cell growth and resistance to chemotherapy [*Baker et al. 1997*].

AIMS

The major objective of this thesis was to understand the molecular basis of mesothelial differentiation, and to identify the positive and negative regulators involved in this process.

The studies were designed to explore the differences between mesothelial cells in various stages of normal and cancer cell differentiation. To address this question, benign and malignant mesothelial cells in various stages of phenotypic differentiation were used as model systems. The first aim was to see whether or not proteoglycans are involved in mesothelial differentiation, and to highlight the subcellular distribution of syndecans. The second aim was to find other factors involved in mesothelial differentiation by screening approaches. As a consequence of findings during the progress of this work, we also focused on the intranuclear occurrence of syndecans.

RESULTS

SUMMARY OF PAPERS

Paper I

This study was undertaken to see whether PG expression influences the differentiation of a mesothelioma. Differentiation, migration and proliferation place ever changing demands on the synthesis machinery of the cell. This implies a continuous rearrangement of the adhesive molecules and a dynamic use of the cytoskeletal components. Proteoglycans (PGs) as cell-membrane and extracellular matrix components involved in specific ligand-binding reactions, participate in cell-cell and cell-matrix interactions. Qualitative and quantitative changes in PG expression may therefore be one mechanism used by differentiating cells to meet these complex requirements.

To assess this hypothesis, we studied a mesothelioma model, where the growth pattern of the cells can be reversibly modified by varying the serum substitutes. The cells differentiate into epithelial or fibroblast-like morphology, mimicking the characteristic biphasic growth of this tumor.

Using semi-quantitative RT-PCR, we analyzed in parallel a series of PGs, showing increased expression of syndecan-2, syndecan-4 and hyaluronan synthase (HAS-1) in the epithelial phenotype, and fibroblast-like cells expressing more matrix PGs: versican, decorin and biglycan. The conditions for semi-quantitative evaluation of RT-PCR reaction products were optimized by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene. Western blotting provides evidence of shedding and rapid turnover of cell-membrane PGs. Here we also show with antisense downregulation, that all syndecans are essential for the epithelial morphology. Syndecans-1 and -4 – but not syndecan-2 – can also be associated with cell aggregation, indicating distinct functions of different syndecans.

We concluded that the pattern of PG expression varied with the differentiation of the cells. Epithelial and fibroblast-like mesothelioma cells differ in this respect from each other and from that in true epithelial tumors. The PG profile seems to be a characteristic feature that distinguishes various cell phenotypes.

Paper II

Benign mesothelial cells show growth characteristics similar to those of their malignant counterparts, the cells differentiating into either epithelial or fibrous phenotypes. To study how the epithelial and fibroblast-like differentiation of benign mesothelial cells correlated to the upstream regulator of syndecan-1 — i.e., Wilms' tumor susceptibility gene 1 (WT1), PGs and HA synthase — we studied their expression with semi-quantitative RT-PCR analyses. The expressions of these genes were all associated with a variation in phenotypic differentiation. Cell lines with epithelial morphology expressed more mRNA coding for WT1 and cell surface PGs than did the fibroblastic ones, the difference being greatest for syndecan-4 and glypican. The increase in WT1-associated mRNA was about as great as that of syndecans. Fibroblast-like cells, on the other hand, expressed substantially more of the matrix PGs versican and biglycan. HA synthase varied individually between the cell isolates, although epithelial cells often expressed higher levels. The findings

indicate that the regulation of mesothelial differentiation involves several factors, among these also WT1 and various PGs.

Paper III

To learn more about the mechanisms underlying mesothelioma differentiation, we studied a mesothelioma cell line that can be influenced by serum factors to differentiate into sub-lines with epithelial or fibroblast-like phenotype. Differential gene expression of these sub-lines was studied by subtractive hybridization.

Nine genes associated with cell growth and differentiation were found to be substantially overexpressed in the epithelial sub-line, as compared to only two genes in the fibroblast-like phenotype, which would suggest differences in maturation. One of these proteins was thioredoxin (trx), a small redox-active protein associated with drug resistance. Western blot analysis showed an increase in protein levels intracellularly and in the medium. Trx is reduced by selenoprotein TR and NADPH. The activity of thioredoxin reductase was high in both cell sub-lines, but increased 2-fold in the epithelially differentiated cells. In fact, the obtained levels of both trx and TR found in these mesothelioma cell lines were among the highest published.

Paper IV

Although syndecans play a major role in regulating cell morphology, little is known about their subcellular distribution and *in vivo* association with the cytoskeleton. To address this question, we used confocal laser microscopy and a panel of antibodies against syndecans-1, -2 and -4. We also monitored the spatial relation of syndecans to tubulin in mitotic and interphase cells.

Initially, the reactivity to syndecans was confined to the cytoplasm, staining of the cell membranes appearing later. We also found that syndecan-1 translocates to the nucleus in a time-dependent manner. The mitotic spindle shows unexpectedly more syndecan reactivities than interphase cells. After vinblastine treatment, syndecan-1 and tubulin were both recovered as paracrystalline occlusion bodies, and the nuclear reactivity to syndecan-1 disappeared, suggesting a tubulin-mediated nuclear transport of this PG. The nuclear translocation mainly involved syndecan-1, but syndecans-2 and -4 remained in the cytoplasm and cell membrane. The cytoplasmic distribution and dynamic rearrangement were not confined to mesothelioma cells alone, but were also found in a series of cell lines.

Paper V

Malignant mesothelioma may express features of an epithelial and/or a sarcomatous tumor, the latter indicating a poorer prognosis. To mimic the epithelial-mesenchymal transition, also called dedifferentiation, mesothelioma cells with an epithelial morphology were treated with various growth factors (TGF-β2, EGF, FGF-2, IGF-I and PDGF-BB) and the expression levels of syndecans-1, -2, and -4 were monitored at 30 min, 6h and 18h by semi-quantitative RT-PCR and FACS analysis. The PG profile correlated to the morphological appearance and proliferation rate of the treated cells.

An early response was obtained only with syndecan-4 mRNA expression levels. Changes in the differentiation pattern appeared later. Exposure to EGF and IGF-I induced a fibroblast-like morphology simultaneously with a reduction in the syndecan expression levels. At the same time, the levels of shed syndecan-1

increased. TGFβ2 increases the focal contacts and showed a marked upregulation of syndecan-4 and downregulation of syndecan-1. TGFβ2 also inhibited the nuclear transport of syndecan-1 simultaneously with an antiproliferative effect.

GENERAL DISCUSSION

During morphogenesis, PGs are expressed transiently in a tissue-type specific manner. This makes them particularly interesting in differentiation processes, such as the maturation of mesothelial cells. Mesothelial regeneration seems to occurs, at least partly, from the submesothelial mesenchymal cells, which are considered to be multipotential. Mesothelial cells possess unique properties and can be influenced to differentiate into stable epithelial or fibroblast-like phenotypes by serum components. These cells carry several PGs on their surface and in the surrounding matrix. We have shown that there is a close correlation between the morphological appearance and the PG expression profile of mesothelial and mesothelioma cells. It is the PG profile rather than the expression levels of individual PGs that reflects the differentiation of these intermediate cells [**Papers I-II**].

The complex PG coat surrounding the cells shear a common mechanism for growth factor binding, namely, the attached GAG side chains. However, this also adds another level of diversity to the system. The structural complexity of this PG coat probably affects the individual biological response of tumor cells to external stimuli. Mesothelial cells spontaneously differentiate towards an epithelial morphology, but this may also be triggered by inflammatory mediators [**Paper II**]. We have shown that this spontaneous differentiation may be reversed by serum growth factors [**Paper V**]. These factors seem to influence the synthesis and shedding of PGs which may generate potent stimulatory or inhibitory fragments. The effects of shed syndecan may differ considerably from those of the syndecans anchored to the cell membrane.

These seemingly paradoxical effects involving a delicate interplay between growth factors and HSPGs, especially syndecan-1, may be related to the following:

- Sequestration and/or concentration of GFs on defined regions within the matrix, resulting in ”GF reservoirs” and potential enhancement of growth factor effects [*Lyon & Gallagher 1994*].
- Fragments of degraded HS can bind and activate GFs and motility factors present in the tumor microenvironment [*Kato et al. 1998*].
- Competitive depletion of available GFs may inhibit GF effects.
- Internalization and nuclear transport of growth factors [*Roghani & Mortacelli 1992*, *Duverger et al. 1995Jans DA et al 1998, Adam 1999*] may have stimulatory or inhibitory effects [*Fedarko et al. 1989*, *Bush et al. 1992*, *Jans and Hassa 1998, Cheng et al.2001, Kovalszky et al. 1998,* Paper IV].

The main mechanism underlying growth-factor binding is regulated by the fine structure of HS. However, it is not known whether HS chains carried by the various syndecans differ in their fine structure or binding affinity. In the present thesis, we studied the interaction between PGs and various growth factors. In [**Paper V**]**,** we show that the effect of growth factors depends not only on, but also modifies the expression and synthesis of these cell surface PGs. This indicates a delicate

interplay and possible autoregulation of these substances.

Cancer cells differ from their normal counterparts in their pathophysiological state, particularly in the control of cell growth, differentiation and division. An exciting but relatively unexplored area, is the potential role of syndecans in intracellular signaling. As noted above, syndecans are integral membrane proteins. However, in [**Paper IV]** we describe a mechanistic model for the internalization of syndecans and tubulin-mediated nuclear transport of syndecan-1. The syndecans also seem to differ in their tubulin binding affinity. Syndecan-1 is the major nuclear component, and it may act as transporter for growth-factors and/or growth factor receptors. On the basis of the present findings, it can be speculated that the transport of protein GFs and their receptors into the nucleus occurs as a complex stabilised with syndecan and mediated by tubulin-syndecan interaction. Syndecan-1, which ligate to both these systems, may be involved not only in endocytosis and transport of GFs to the perinuclear area, but also in the precise nuclear targeting of GF-GFR complexes from the perinuclear cytoplasm. Syndecan-1 may also have a dual role of action, promoting or inhibiting direct nuclear targeting of genes [*Fedarko et al. 1992, Kovalszky et al. 1998, Cheng et al. 2001*]. However such a hypothesis necessitates further verification

Syndecan-1 blocks invasion, but once the tumor cells have entered the circulation, syndecan-1 overexpression may have effects opposite to those in the tissue of origin, and it may induce metastasis [*Hirabayashi et al. 1998*]. Downregulation of syndecans –1, -2 and -4 [**Paper I**], one by one using antisense targeting had no effect on the anti-invasive properties of the studied mesothelioma cells. This would suggest that other members of the syndecan family or other adhesive molecules may compensate for the loss of individual syndecans. In this respect syndecans seem to have partly overlapping functions.

FUTURE CHALLENGES

These observations underline the importance of identifying specific HSPGs in tumors and clarifying their functions. Core proteins and HS chains both participate in the above-mentioned biological processes. Many authors have evaluated the expression of protein cores in malignant cells without considering the possible changes in the fine structure of HS, which also could modulate PG functions. Likewise, changes in cell surface HS chains are usually described without taking in consideration the protein core expression [*Tumova et al. 2000*]. More must be learned about the concomitant expression of PGs in various tumors which, together with a better understanding of the overall HS sulfation pattern, might improve our knowledge of differentiation and susceptibility of tumor cells to external stimulatory or inhibitory agents.

New advances have been made in saccharide sequencing techniques [*Rhomberg et al 1998, Karamanos &Hjerpe 1999, Turnbull et al. 1999, Vives et al 1999*], which might widen our perspectives for PG biology. Decoding of messages in HS sequences would then be essential to explain how these HS structures work. This could provide a better basis for the understanding of their biological importance, and the concept named the ”heparanome” [*Turnbull et al. 2001*]. Further studies should also be done concerning the complex pattern of PGs synthesized. Such knowledge about the importance of a ”PG-profile” can preferably be obtained with DNA arrays that aim at evaluating the simultaneous expression of various PGs.

The importance of syndecans for the proliferation and differentiation of mesothelioma cells also makes these PGs possible targets for future chemotherapy. One possibility could be specifically tailored xylosides, which interfere with the synthesis of the HS and hamper cell proliferation. Another alternative is the specific HS sequences responsible for the specific binding of growth factors, these sequences may block the proliferation of cultured cells. Such prospects, however, necessitates extensive studies both *in vitro* and *in vivo*.

GENERAL SUMMARY

- ***Mesothelial differentiation correlates with the PG profile in benign and malignant cells. In particular, some cell-associated PGs — i.e., syndecans -2 and -4 — are more abundant in epithelial cells, whereas matrix PGs are overexpressed in fibroblast-like cells.***
- ***The PG profile may serve as a "fingerprint", and reflect the maturation of mesothelial cells.***
- ***The functional importance of syndecans in mesothelial differentiation was further demonstrated by antisense downregulation and growth factor induction. Epithelial differentiation, seems to be regulated at least partly, via syndecans, since antisense downregulation of each particular syndecan caused a loss of epithelial morphology and appearance of a fusiform growth pattern.***
- ***Syndecans -1 and -4 are both essential for cell adhesion. A reduction of their synthesis increased the dissociation of cells.***
- ***Syndecan-4 reacts as an immediate "early response gene" to the addition of growth factor.***
- ***Peptide growth factors affect the proliferation and morphology of mesothelioma cells to different extents, most pronounced effect being obtained by EGF and IGF-I. The PG profile changed, in parallel, with an induced epithelial-mesenchymal transition.***
- ***TGF-β2 reduced the proliferation rate of mesothelioma cells, concomitantly with a delay in nuclear transport of syndecan-1.***
- ***We found a substantial proportion of syndecans at intracellular locations, and a time-dependent accumulation of syndecan-1 in the nucleus, the mitotic spindle showing the highest intracellular concentration of syndecans.***
- ***Vinblastine treatment interferes with this nuclear transport. Syndecan-1 and tubulin were found to co-polymerize in paracrystalline occlusion bodies, in parallel with impaired nuclear transport. These findings suggest a tubulin-mediated transport mechanism.***
- ***Subtractive hybridization reveals only a limited number of genes that are differentially expressed in mesothelioma cells with various phenotypic differentiation. Most of them were recovered from the epithelial cells, which may indicate a more mature phenotype.***
- ***Activity of thioredoxin reductase was high in both cell sub-lines, but induced 2-fold in the epithelially-differentiated cells, and may contribute to the generic drug resistance of malignant mesothelioma .***

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