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STUDIES ON MESOTHELIAL DIFFERENTIATION

PROGNOSTIC AND THERAPEUTIC APPROACHES TO MALIGNANT MESOTHELIOMA

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HOPE

"Hope is the companion of power, and the mother of success; for who so hopes strongly, has within him the gift of miracles."

-- *Samuel Smile*

ABSTRACT

Malignant mesothelioma (MM), a tumor with a poor prognosis, causes the death of most patients within a year of diagnosis since various treatments seem to have little effect on its outcome. The biological behavior of MM varies greatly, and this heterogeneity is related to its morphology, which may be epithelioid, sarcomatoid (fibroblastoid) or biphasic, the sarcomatoid phenotype being associated with a shorter survival. The mechanisms by which the MM cells differentiate into epithelioid or fibroblastoid phenotypes are largely unknown.

We used a MM cell line that, depending on the serum supplementation, differentiates into sublines with two phenotypes, the epithelioid STAV-AB and fibroblastoid STAV-FCS. A similar variability in growth phenotype can also be seen in benign mesothelial cells that have exfoliated into effusions. In short-term cultures, such cells may grow with a fibroblastoid or epithelioid morphology. The growth patterns obtained remain stable during several passages and the benign and malignant cells can be used as *in vitro* models when studying mechanisms of mesothelial differentiation.

The aims of the present study were to determine the candidate genes involved in the differentiation of benign and malignant mesothelium. This was done by using Suppression Subtractive Hybridization (SSH) and Microarray techniques, and the findings were confirmed *in vitro* and *in vivo*. Such screening of gene expressions may be of value in finding diagnostic markers and targets for novel treatments of MM.

Both the benign and malignant epithelioid cells expressed more genes related to specialized functions associated with metabolism, cellular defense, apoptosis and differentiation, while those related to growth factors and their receptors were more abundantly expressed in the sarcomatoid phenotype. These findings accord with the view that the fibroblastoid cell type represents a less differentiated stage than the epithelioid ones.

Our data indicate that the different tumor phenotypes use different driving mechanisms, and the heterogeneity of the tumor may therefore be one explanation for the poor response to chemotherapy. A better response might be expected if targets were selected according to the individual phenotypes. The targeting of two differentiation-associated functions induced apoptosis *in vitro*.

The thioredoxin/thioredoxin reductase (Trx/TR) system was differentially expressed, although extremely large amounts were present in both cell sublines. This may make the cells particularly sensitive to selenite, and this salt reduced the cell viability of MM cells, especially in those with fibroblast-like phenotype. A combination with doxorubicin was synergistic, and markedly affected on both cell phenotypes. A similar induction of apoptosis occurred in the epithelioid cells, after giving proteasome inhibitor (PSI). Here the dose- and time- dependent sensitivity were mainly confined to cells with an epithelioid morphology. Selenite and proteasome inhibitors are potentially new contributions for the treatment of MM.

LIST OF PUBLICATIONS

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

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

II. **Sun Xj***, Gulyás M*, and Hjerpe A.
Differentiation of benign mesothelial cells as reflected by differential gene expression in fibroblastic and epithelioid phenotypes.
Am J Respir Cell Mol Biol, 2004; 30:510-518.

III. **Sun Xj**, Wei L, Liden J, Hui G, Dahlman-Wright K, Hjerpe A, and Dobra K.
Molecular characterization of tumor heterogeneity and malignant mesothelioma cell differentiation by gene profiling.
J Pathol. 2005; 207(1):91-101.

IV. **Sun Xj**, Gulyás M, Hjerpe A, and Dobra K.
Proteasome inhibitors induce apoptosis in human mesothelioma cells.
Cancer Letter, in press, 2005.

V. Nilsonne G, **Sun Xj**, Nyström C, Rundlöf A-K, Björnstedt M, and Dobra K.
Doxorubicin-resistant sarcomatoid malignant mesothelioma cells are highly sensitive to selenite – a novel therapeutic approach related to tumor cell differentiation and the thioredoxin system.
Int J Cancer, submitted for publication.

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LIST OF ABBREVIATIONS

Adp16INK4A	p16INK4A in adenoviral vectors
AIF	Apoptosis-inducing factor
Annexin V-FITC	Fluorescein isothiocyanate conjugated antibody to Annexin V
AOEs	Antioxidant enzymes
Apaf-1	Apoptotic protease activating factor-1
APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1
AR	Aldose reductase
Bax	Proapoptotic protein
BIN1	Bridging integrator 1
BMP4	Bone morphogenetic protein 4
BSA	Bovine serum albumin
CBP2	Collagen binding protein 2
CCNB1	Cyclin B1
CCND1	Cyclin D1
CD24	CD24 antigen
CDH2	Cadherin 2, type 1, N-cadherin
CDK2	cyclin-dependent kinase 2
CDK4	cyclin-dependent kinase 4
CDKN3	Cyclin-dependent kinase inhibitor 3
CDKs	cyclin dependent kinases
CENPA	Centromere protein A, 17kDa
CK7	Cytokeratin 7
CK8	Cytokeratin 8
CKs	Cytokeratins
CTSD	Cathepsin D
CYP1B1	Cytochrome P450, subfamily I
DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein
DIG	Digoxigenin
DP1	Likely ortholog of mouse deleted in polyposis 1
DTNB	5´dithiobis(nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EIF2S1	Eukaryotic translation initiation factor 2, subunit 1 alpha
EIF5	Eukaryotic translation initiation factor 5
EPS8	Epidermal growth factor pathway substrate 8
FACS	Fluorescence-Activated Cell sorter
FasL	Fas ligand
FGF	Fibroblast growth factor
FGFR1	Fibroblast growth factor receptor 1
gamma GCS	Gamma-glutamylcysteine synthetase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPx	Glutathione peroxidase
Gran	Granulin
GSH	Glutathione
GSSG	Oxidized GSH

GST	Glutathione S-transferases
H2AFO	H2A histone family, member O
HA	hyaluronan
HCgp39	Human cartilage glycoprotein 39
HGF	hepatocyte growth factor
HIF1A	Hypoxia-inducible factor 1, alpha subunit
HPCE	High-performance capillary electrophoresis
HPLC	High-performance liquid chromatography
IκB	Inhibitor of nuclear factor kB
IAP-1	Human inhibitor of apoptosis protein-1 gene
IC_{50}	Inhibitory concentration 50%
IER3	Immediate early response 3
IFI16	Interferon, gamma-inducible protein 16
IFI27	Interferon alpha-inducible protein 27
IGF-1	Insulin-like growth factor-1
IGFBP3	Insulin-like growth factor binding protein 3
IGFBP6	Insulin-like growth factor binding protein 6
IGFBP7	Insulin-like growth factor binding protein 7
IL18	Interleukin 18
IL1R1	Interleukin 1 receptor, type I
Int α5	Integrin α5
ITGB5	Integrin, beta 5
ITPR3	Inositol 1,4,5-triphosphate receptor, type 3
KRT5	Keratin 5
LCAT	Lecithin:cholesterol acyltransferase
LLnL	N-acetyl-leucyl-leucyl-norleucinal
LTBP1	Latent Transforming growth factor beta binding protein1
MCM7	MCM7 minichromosome maintenance deficient 7
MDK	Midkine
MDM2	Mouse double minute 2 homolog isoform, p53-binding protein
MDR-1	Multi-drug-resistance gene
MM	Malignant mesothelioma
Mn-SOD	Manganese superoxide dismutase
MRP	Multidrug resistance-associated protein
MRP1 & 2	Multidrug resistance-associated protein 1 and 2
MT3	Metallothionein 3 (growth inhibitory factor)
MUC1	Mucin 1, transmembrane
MYCBP	C-myc binding protein
N33	Tumor suppressor candidate 3
NaDOC	Sodium deoxycholate
NaN3	Sodium azide
NAPD	Nicotinamide Adenine Dinucleotide Phosphate
NAPDH	Nicotinamide Adenine Dinucleotide Phosphate, reduced form
NF2	Neurofibromatosis gene 2
NFkB	Nuclear factor kappa B
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha
NPC1	Niemann-Pick disease, type C1

NPCAP	Nuclear pore complex-associated protein TPR
p14(ARF)	ARF tumor suppressor protein p14
p15(INK4b)	Tumor suppressor protein p15(INK4b), cyclin-dependent kinase inhibitor 2B, inhibits CDK4
p16(INK4a)	Tumor suppressor protein p16(INK4a), cyclin-dependent kinase inhibitor 2A, inhibits CDK4
p21WAF1/CIP1	Cyclin-dependent kinase inhibitor 1A
p27(kip1)	Cyclin-dependent kinase inhibitor 1B
PA28 beta	Proteasome activator subunit 2
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF-A & -B	Platelet derived growth factors-A & B
PDGFRB	Platelet-derived growth factor receptor, beta polypeptide
P-GP	Phosphoglycoprotein
PI	Propidium iodide
PIGF	Phosphatidylinositolglycan, class F
PKC	Protein kinase C
PMS2L9	Postmeiotic segregation increased 2-like 9
pRb	Protein of retinoblastoma tumor suppressor gene
PRKCL1	Protein kinase C-like 1
PSI	N-carbobenzoxy-L-isoleucyl-L-γ-t-butyl-L-gultamyl-alanyl-L-leucinal
PSMA3	Proteasome subunit, alpha type, 3
PSMA4	Proteasome subunit, alpha type, 4
PTPRK	Protein tyrosine phosphatase receptor type, K
PTPRM	Protein tyrosine phosphatase, receptor type, M
RARRES1	Retinoic acid receptor responder 1
RARS	Arginyl-tRNA synthetase
Real-time RT-PCR	Real-Time Reverse Transcriptase Polymerase Chain Reaction
RFC4	Replication factor C 4, 37kDa
ROS	Reactive oxygen species
RRAS	Related RAS viral (r-ras) oncogene homolog
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSH	Suppression subtractive hybridization
STMN1	Stathmin 1/oncoprotein
SV40	Simian virus 40
SV40 Tag	SV40 early region T antigen
TAF9	TAF9 RNA polymerase II, TATA box binding protein-associated factor, 32 kDa
TCEA2	Transcription elongation factor A (SII), 2
TGF-α	Transforming growth factor-α
TGFB1	Transforming growth factor, beta 1
THBS2	Thrombospondin 2
TIMP3	Tissue inhibitor of metalloproteinase 3
TM	Thrombomodulin

TM4SF1	Transmembrane 4 superfamily member 1
TNF-α	Tumor necrosis factor-α
TrxR/TR	Thioredoxin reductase
TRA1	Tumor rejection antigen (gp96) 1
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Trx	Thioredoxin
UBE2C	Ubiquitin-conjugating enzyme E2C
UPP	Ubiquitin/proteasome pathway
VEGF	Vascular endothelial growth factor
WT1	Wilms' tumor suppressor gene 1
YES1	V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1

1 BACKGROUND

Benign mesothelial cells may grow as fibroblast-like cells in a stroma or as flattened epithelioid cells covering the surfaces of the serosal cavities- i.e., in the cavities surrounding the lungs, heart and abdominal organs and the tissue forming the tunica vaginalis testis. Both phenotypes are closely related. Malignant mesothelioma (MM) is a type of cancer in which mesothelial cells become malignant. It may affect any mesothelium (Bignon J, et al, 1993), the vast majority of cases being found in the pleura. MM usually retains the same growth pattern as the original benign mesothelium, growing with either of epithelioid or fibroblastoid morphology or mixtures thereof. The mechanisms underlying regulation of differentiation of mesothelial and mesothelioma cells are still poorly understood.

The development of MM is related to exposure to fibrous minerals, such as asbestos. Although rare in unexposed subjects, the common use of asbestos has increased the incidence of MM in industrialized and developing countries. On the basis of known exposures, this incidence is expected to increase (Nicholson WJ, 1982; Britton M, 2002) over the next 30 years and to cause 250,000 deaths in Western Europe (Peto J, et al, 1999). MM is frequently difficult to diagnose, inaccessible to curative surgery, and responds poorly to radiotherapy or chemotherapy (Sterman DH, et al, 1999). With the exception of a smaller number of slow growing tumors, the prognosis of MM is extremely bad with few patients surviving more than one year, regardless of treatment (Sugarbaker DJ, et al, 2002).

In view of this, management of MM needs to be improved by acquiring more knowledge about the pathogenesis and molecular biology of MM. Gene expression profiling, especially differential gene expression analysis using microarray, has permitted the molecular characterization of different cancers, including MM (Mohr S, et al, 2002; 2004). Applied to cell lines, microarray analysis has shown that several genes may control MM progression (Kettunen E, et al, 2001).

1.1 MESOTHELIUM: DIFFERENTIATION AND REGENERATION

1.1.1 DIFFERENTIATION

The mesothelium develops from the mesodermal plate and its coelomic cavity (Sadler TW, 1985). The two cell types in this tissue have different morphologies: (1) a monolayer of epithelioid cells on the surface, resting on a basement membrane that separates them from the underlying stroma and (2) fibroblast-like cells embedded in an extracellular matrix. The former cells express both epithelial markers, such as cytokeratins (CKs), and mesenchymal markers, such as vimentin, while the CKs are less apparent in the scattered quiescent fibroblast-like cells. When the latter cells are exposed to certain stimuli, they may acquire epithelial markers and a less marked expression of vimentin (Bolen JW, et al, 1986; Hammer and McNutt SP, 1987). It has been debated whether these cells should be classified as epithelial or mesenchymal, but they are now considered mesenchymal, since both cell types use N-Cadherin as the main adhesion molecule (Peralta Soler A, et al, 1995; Simsir A, et al. 1999; Davidson B, et al, 2001).

Functionally, the mesothelium is a dynamic cellular membrane with many important functions. It has long been known that the production of a special lubricating fluid

enables the organs to slide without friction against each other. These cells are also ascribed some important roles including the transport and movement of fluid, and the secretion of bioactive substances, such as pro-inflammatory cytokines and growth factors. They also synthesize the components of the extracellular matrix, whereby they maintain the integrity of the tissue in a way that also favors serosal repair (Mutsaers SE, 2002).

1.1.2 REGENERATION

The regulatory mechanisms, which underly the rapid regeneration and healing of mesothelial injury are disputed. It has long been assumed that such an injury should stimulate the resident fibroblast-like cells in the underlying subserosal stroma to proliferate, differentiate, and migrate to the surface where they cover the defect, and acquire epithelioid characteristics (Ellis H, et al, 1965; Raftery AF, 1973). However, much of the regenerating mesothelium originates directly from the surrounding epithelioid surface cells, and less often from fibroblast-like cells (Mutsaers SE, 2000) although both these cell types can be the source of regeneration, depending on whether the basement membrane is intact. If a deeper injury penetrates this membrane, the fibroblast-like cells participate in healing and may differentiate into epithelioid ones. The close relationship between these two phenotypes is also obvious during growth of these cells *in vitro*, when cells with an epithelioid phenotype may also change to a fibroblast-like growth pattern (Klominek J, et al, 1989).

Mesothelial regeneration requires the recruitment of inflammatory cells to the wound surface and the release of mitogenic cytokines to activate and stimulate proliferation around the wound (Fotev Z, et al, 1987; Mutsaers SE, et al, 1997). Activated mesothelial cells then break their cell-to-cell contacts and migrate onto the wound's surface (Whitaker D and Papadimitriou JM, 1985). Recent evidence suggests that hepatocyte growth factor (HGF) plays an important role in this process, since it is secreted by the epithelioid surface (Warn R, et al, 2001) and adjacent fibroblast-like cells (Yashiro M, et al, 1996).

1.2 MALIGNANT MESOTHELIOMA

1.2.1 DIFFERENTIATION AND MORPHOLOGY

Malignant mesotheliomas (MMs) have many histological features and the malignant mesothelium may show two main types of growth pattern: epithelioid or fibroblast-like, reflecting the benign precursor tissue. MMs may consist of only one of these cell types, - i.e., an epithelioid or sarcomatoid MMs - or both kinds of cells are present in the same tumor, which is common – i.e., a mixed or biphasic tumor. Although a malignant tumor is frequently assumed to have a monoclonal origin, many mutations or deletions have occurred before the tissue is available for diagnosis. By then the genetic material in a MM is very heterogeneous. The finding that MM cells *in vitro* may change their morphology because of external factors, however, suggests that the difference in phenotype is not necessarily an expression of additional mutations (Klominek J, et al, 1989).

The epithelioid tumor cell type is usually recognized on histological examination, while the fibroblastoid tumor cells can be difficult to distinguish from reactive stromal cells. In some studies, the entirely epithelioid tumor has been reported to be the commonest (up to 70% of the cases), but the percentage of mixed type increases with the size of the

tumor tissue on which the diagnosis is based, and entirely sarcomatoid MMs are less common (7-20%) (Roberts GH, 1970; Johansson L and Linden CJ, 1996).

The growth patterns of well-differentiated epithelioid forms vary from papillary, solid, vacuolated, or tubular. The cells are more or less pleomorphic with large nuclei, prominent nucleoli and eosinophilic cytoplasm. Other features commonly include frequent mitoses, an irregular chromatin pattern, and multinucleated tumor cells (Kawai T, et al, 1981; Kwee WS, et al, 1982; Adams VI, et al, 1986). At the ultrastructural level, these cells have a characteristic appearance with long slender microvilli that lack a glycocalyx and may occupy cytoplasmic or intercellular neolumina.

The sarcomatous form of MM most often resembles a fibrosarcoma mainly with spindle- or ovoid-shaped cells, creating a storiform. Multinucleated atypical cells may be seen. The cells of the well-differentiated forms often mimic reactive fibroblasts and can be difficult to distinguish from fibromatoses. Ultrastructurally, these fibroblast-like cells are less characteristic. They may express sparse microvilli, whose close relation to collagen fibers may be diagnostic.

A mixed or biphasic growth pattern with epithelial and mesenchymal elements is commonest, although the percentages of the two subtypes usually differ and their distribution is not uniform. The identification of both these elements, which are malignant, is virtually diagnostic of malignant mesothelioma. However, an adenocarcinoma frequently stimulates its stroma, sometimes to such an extent that the tissue imitates this biphasic growth pattern. In many cases, it then becomes necessary to use other diagnostic methods – e.g., histochemistry, immnohistochemistry, and electron microscopy to distinguish between MMs and adenocarcinomas (Warhol MJ, 1982).

1.2.2 CYTOGENETIC AND MOLECULAR CHANGES IN MESOTHELIOMA

1.2.2.1 Cytogenetic changes induced by asbestos

The main known causative factor for MMs is exposure to asbestos fibers (Wagner JC, et al, 1960; Craighead JE and Mossman BT, 1982). Asbestos consists of a group of minerals that occur naturally as masses of strong, flexible fibers, which can be separated, into thin woven threads. It has been widely used in many industrial products. These tiny asbestos particles, especially the thin, rod-like amphiboles float in air and they may be inhaled or swallowed. When inhaled, these small fibers reach the alveoli, where they may penetrate into the pleural cavity, and collect near the mesothelial cells (Boutin C, et al, 1996).

Various oncogenic mechanisms have been suggested. First, the asbestos fibers may act directly on the mesothelioma cells and mechanically disturb the mitotic spindle apparatus, which can lead to karyotypic aberrations (Ault JG, et al, 1995). On the other hand, they may initiate the formation of reactive oxygen species (ROS) that cause mutations. The inflammatory response to asbestos particles may also be involved including the generation of cytokines, which affect cell replication and differentiation (Mossman BT, et al, 1996). The induction of the proto-oncogenes c-fos and c-jun, which encode transcription factors, that activate various genes important in the initiation of DNA synthesis (Heintz NH, et al, 1993) also seems to play an important role in the development of MM.

In vitro studies have shown that exposure to asbestos, especially amphibole fibers > 10 microns, may activate oncogenes and /or loss of suppressor genes (Barrett JC, et al,

1989). Indeed, karyotypic analyses of human mesotheliomas have demonstrated that frequent abnormalities are common, especially those involving chromosomes 1, 3, 6, 9, 13, 15, and 22 (Barrett JC, et al, 1989; Gibas Z, et al, 1986; Mossman BT, et al, 1990; Popescu NC, et al, 1988). One of the most common nonrandom changes is a deletion of the short arm of chromosome 3 in the region between p14 and p21 (Popescu NC, et al, 1988; Petursdottir TE, et al, 2004). In these patients a significant correlation exists between chromosomal aberrations and the pulmonary asbestos fiber burden, and an inverse correlation between the survival time and the number of copies of chromosome 7 short arms (Tiainen M, et al. 1989). Recent comparative genomic hybridization in MMs has also shown the loss of chromosomes 14, 15, and 22, and a gain of chromosome 7 (Baser ME, 2002). Karyotyping, such as with chromosome-specific FISH probes, can therefore be of a diagnostic value in distinguishing mesotheliomas from benign, proliferating mesothelium, which typically has no cytogenetic abnormalities.

1.2.2.2 Molecular changes

The protein p16INK4a (p16) is the product of the p16/CDKN2A gene at 9p21, which can bind to the cyclin-dependent kinase CDK4 and inhibits the catalytic activity of the CDK4/cyclin D enzymes (Serrano M, et al, 1993), and CDK4-mediated phosphorylation of pRB (Geradts J, et al, 1995). The genomic polymerase chain reaction has shown that a homozygous deletion of p16 is a frequent finding in mesothelioma cell lines and MMs. At the transcript level, the expression of p16 is markedly downregulated also in MM cell lines that do not have such homozygous deletions of this gene (Cheng JQ, et al. 1994) and immunohistochemistry shows that the synthesized p16INK4a protein is abnormal (Kratzke RA, et al, 1995). Moreover, re-expression of p16INK4a in MM cells causes cell cycle arrest and cell death, inhibits the formation and growth of a tumor, reduces the size of a tumor and its spread in MM xenograft experiments (Frizelle SP, et al, 1998), which would suggest that p16INK4a gene transfer might be a specific target for the novel treatment of mesotheliomas.

The ARF tumor suppressor protein p14 (ARF), encoded by the INK4a/ARF locus, is a major mediator of p53 activation in response to oncogenic stress (Stott FJ, et al, 1998; Palmero I, et al, 1998; 2002). It promotes degradation of the MDM2 protein and therefore prevents the MDM2-mediated inhibition of p53. Homozygous deletions of the INK4a/ARF locus may therefore cause inactivation of p53 and the loss of p14(ARF), such deletions are common in MMs (Yang CT, et al, 2000). Since p14(ARF) and p16(INK4a) are found in the same locus, they can be lost simultaneously. The elimination of these two tumor suppressor proteins would then affect the regulation of the pRb and p53 pathways. The p53 and pRb proteins are essential regulators of the genes involved in cell cycle arrest (like p21WAF1/CIP) at the G1-S checkpoint, a result of DNA damage. The p53 may also initiate apoptosis by activating genes, such as Bax. The loss of both p53 and pRb functions will therefore severely affect the genetic stability of the cell. Mutations of the p53 gene resulting in nonfunctional protein products are sometimes found in MMs, although this is considered to be a late mutation in these tumors (Cote RJ, et al, 1991; Metcalf RA, et al, 1992).

WT1 (Wilms' tumor suppressor gene) is normally expressed in mesothelium and has been found to be mutated in some MMs (Park S, et al, 1993; Walker C, et al, 1994). It is noteworthy that the WT1 protein can form a complex with p53 (Maheswaran S, et al,

1993) and downregulates the cyclin/CDK-complexes that interact with the protein products of pRB and p53 (Kudoh T, et al, 1995).

Merlin, the protein product of neurofibromatosis gene 2 (NF2), belongs to a family of proteins that link cytoskeletal elements to the plasma membrane of the cell (Trofatter JA, 1993; Haase VH, 1994). This protein may play a role in signal transduction from the cell membrane to the nucleus. NF2, a tumor suppressor gene on chromosome 22q, has been associated with the development of familial and spontaneous tumors of neuroectodermal origin. Mutations of NF2 cause neurofibromatosis and these are also common in MMs. They include deletions, insertions, and a nonsense mutation, all of which predict truncation of the protein product of NF2 (Bianchi AB, et al, 1995).

High expressions of platelet-derived growth factors (PDGF-A and -B chains), insulin-like growth factor-1 (IGF-1), IGF-binding protein 3, IGF-1 receptor, and the low expression of transforming growth factor-a (TGF-α) have all been shown in MM, which would suggest that PDGF and IGF-1 may have an autocrine effect on these tumors (Gerwin BI, et al, 1987; Gabrielson EW, et al, 1987; Lee TC, et al, 1993). Recent studies concerning the expression of EGFR have shown that one (3%) of 30 reactive mesothelial proliferations and 17 (45%) of 39 mesotheliomas expressed this receptor (Cai YC, et al, 2004). The use of EGFR as a diagnostic marker for MM has been suggested, and the findings may also indicate an additional therapeutic option.

1.2.2.3 Simian virus 40 (SV40)

SV40, a DNA tumor virus of the papovavirus family, is oncogenic in animal species, but it has not been thought to infect human cells. When injected intrapleurally in hamsters, it induced mesotheliomas (Carbone M, et al, 1999; Cicala C, et al, 1993). The SV40 DNA oncogenicity is associated with its large T antigen (Pass H, et al, 1998; Testa JR, et al, 1998). The protein coded by this gene inhibits p53 and pRb (Carbone M, et al, 1997; De Luca A, et al, 1997), and it has been suggested that SV40 may act as a cofactor promoting asbestos-related oncogenesis. Recent studies have shown the presence and expression of SV40 in human MMs, and it has been suspected that the source of such an infection was contaminated polio vaccines (Carbone M, et al, 1994). This is still debated; the reported percentages of MMs carrying SV40 DNA differ considerably, and they seem to be very low in some areas, including the Nordic countries (Priftakis P, et al, 2002).

1.2.3 DIAGNOSIS AND THERAPY – THE PROBLEM OF DRUG RESISTANCE

1.2.3.1 Diagnosis

In many cases, the diagnosis of mesothelioma is difficult, the two main differential diagnoses being metastatic adenocarcinoma and reactive pleuritis. The feasibility of treatment is determined on the basis of the histological or cytological examination of tissue samples, which can be obtained in various ways (Stamat JC, et al, 1999). The diagnosis cannot be regarded as complete without about the histological findings with identification of a malignant mesothelium and its histological type- i.e., epithelioid, sarcomatous or mixed.

An effusion (pleural, pericardial, ascitic) is commonly the first sign of a mesothelioma. It is usually sent for cytological examination, which makes cytology the first step in

making the diagnosis (Kho-Duffin J, et al, 1999; Reis-Filho JS, et al, 2002). Pleural mesothelioma is difficult to distinguish from metastatic adenocarcinoma, but the diagnosis greatly affects the treatment and an incorrect diagnosis of a metastatic condition may avert a long and costly search for a nonexistent primary lesion. However, the cytological material rarely permits a specific diagnosis of a MM at routine examination. To distinguish mesotheliomas from metastatic cancer, other methods, such as immunohistochemistry (Wirth PR, et al, 1991; Dejmek A and Hjerpe A, 1994; Brockstedt U, et al, 2000; Carella R, et al, 2001) and electron microscopy (Suzuki Y, 1980; Warhol MJ, et al, 1982), may be of value. The levels of hyaluronan (HA) in effusions is often high in MMs, pathognomonic values being found in about 60% of the cases, and the analysis of this carbohydrate, using HPLC (Hjerpe A, 1986; Nurminen M, et al, 1994) or HPCE (Karamanos NK and Hjerpe A, 1997), can also provide ancillary diagnostic information.

1.2.3.2 Therapy

At present, MM cannot be cured. Various treatment protocols have been evaluated, most of which require combinations of chemotherapy. The commonest methods include combinations of chemotherapeutic agents with response rates of about 30% (Ryan CW, et al, 1998, Green MR, 2002). A multimodality approach has been reported- e.g., radical surgery (Treasure T, 2004) with chemotherapy, and radiotherapy (Sugarbaker DJ, 1999; Jaklitch MT, et al, 2001). Radical surgery (pleuropulmectomy) is sometimes considered when the morphological phenotype is an epithelioid mesothelioma. When surgery is combined with extensive radiotherapy that includes the lowest parts of the pleura, considerable improvements have been obtained as regards the risk of a local recurrence (Ahamad A, et al, 2003). The latter approach may increase the importance of early detection. The ability to diagnose MM with cytology on the basis of the first effusion is therefore very important.

1.2.3.2.1 Innovative and experimental treatments

Various types of gene therapy have also been proposed. These include “suicide genes” which make the tumor cells susceptible to antiviral agents, and genes that stimulate natural defense mechanisms, such as cytokine genes to stimulate natural killer cell activity and heat shock protein genes to increase presentation of tumor antigens (Albelda SM, 1997). Moreover, the overexpression of p14 (ARF) causes G (1)-phase arrest and apoptotic cell death, which suggests that such gene therapy-based approach may be useful in the treatment of MMs (Yang CT, et al, 2000). Another possible target for novel therapy is the vascular endothelial growth factor and its receptor (VEGF/VEGFR), which is essential for the tumor to exceed a certain size. It has recently been shown that VEGF is also needed for growth of these tumors, and that this growth can be inhibited by treatment with the VEGF receptor tyrosine kinase inhibitors, such as SU5416 (Litz J, et al, 2004) and PTK787 (Qian DZ, et al, 2004). All of these studies are at a very early stage and not yet realistic options for treatment.

1.2.3.2.2 Conventional therapies

Several properties of MM render it resistant to conventional therapies. MM is usually wide spread rather than localized, first affecting the parietal and then the visceral pleurae. It rapidly infiltrates neighboring structures, such as the lungs, diaphragm, chest wall, and mediastinum. These features often make complete surgical resection

impossible. MM is also resistant to radiotherapy and successful radiation requires large doses (Ahamad A, et al, 2003). Despite numerous trials using various chemotherapeutic agents, none has been found to be effective (Ryan CW, 1998). More importantly, the effect on survival is limited (Ong ST, et al, 1996; Ryan CW, 1998; Baas P, et al, 1998).

1.2.3.3 Drug resistance

MMs are largely chemoresistant and have upregulated several systems involved in multidrug resistance. Therefore new agents are needed to improve the results of treatment. This multidrug resistance is poorly understood and it may be due to a number of factors.

1.2.3.3.1 Free radicals and antioxidant enzymes (AOEs)

AOEs may play a critical role in the resistance of malignant cells to cytotoxic drugs and radiation. Recent studies have shown that the levels of manganese superoxide dismutase (Mn-SOD, a mitochondrial superoxide scavenging AOE), and catalase (an important hydrogen peroxide scavenging AOE) are higher in MM tissues and cell lines than in benign mesothelial cells. The most resistant cell line has the highest protein expression of catalase (Kahlos K, et al, 1998; 2001). The formation of Mn-SOD can be induced *in vivo* by asbestos fibers (Kinnula VL, et al, 1996), multiple cytokines such as TNF-α (Wong GH and Goeddel DV, 1988; Kinnula VL and Crapo JD, 2003), and oxidative stress (Kinnula VL and Crapo JD, 2004). MM cells with lower levels of Mn-SOD (M14K) are more sensitive to epirubicin-induced toxicity and apoptosis than cells with high Mn-SOD (M38K) (Kahlos K, 2000), which indicates that the high resistance of MM cells to cytotoxic drugs is associated with resistance to apoptosis and possibly with the high antioxidant capacity and Mn-SOD of the cell (Kahlos K, 2000).

1.2.3.3.2 Membrane pump extrusion of chemotherapeutic agents

P-glycoprotein-mediated drug efflux may be involved in the resistance of some MMs (Isobe H, et al, 1994). The multidrug resistance (MDR-1) gene encodes this phosphoglycoprotein, P-GP, which serves as an energy-dependent drug candidate pump that reduces intracellular drug accumulation and cytotoxicity (Borst P, et al, 2000).

1.2.3.3.3 Expression of multidrug resistance genes and multidrug resistance-associated proteins

Drug resistance commonly occurs with overexpression of other genes associated with multidrug resistance. Some studies have shown that the expression of glutathione and glutathione S-transferases (GST) is high in MM (Kinnula K, et al, 1998; Dejmek A, et al, 1998), which accords with the low responsiveness of mesotheliomas to chemotherapy. Oxidants and cytotoxic drugs induce Glutathione (GSH) -associated pathways, and they are also involved in the progression and resistance of some tumor cells *in vitro* (Jarvinen K, 2002). Gamma-glutamylcysteine synthetase (gamma GCS) is also highly expressed in most cases of MM and may play an important role in the primary drug resistance of MM *in vivo* (Jarvinen K, 2002). The mRNAs of the multidrug resistance-associated protein (MRP) and gamma GCS heavy subunit genes are highly expressed in MM cell lines *in vitro* (Ogretmen B, et al, 1998). The

expression levels of MRP1 and MRP2 are also increased in MM *in vivo* (Soini Y, et al, 2001).

1.2.4 PROGNOSIS

There is a considerable delay between exposure to asbestos and the development of invasive mesothelioma. For example, those exposed in the 1940s to 1970s are only now being diagnosed as having this tumor since the normal latency period is 15-50 years. By the time the tumor is diagnosed, curative treatment is usually not feasible. The median survival rate varies between 5 and 16 months according to the histological type and clinical stage at diagnosis (Chahinian AP, et al, 1982; Schildge J, et al, 1989; Fusco V, et al, 1993). The epithelioid type has the most favorable prognosis, while the fibrosarcomatous growth pattern implies a shorter survival. In a few cases, the course is slow and survival long whatever the therapy (Dejmek A, 1992); indeed, single cases have survived more than 15 years regardless of the therapy (Law MR, et al, 1984).

1.3 APOPTOSIS

The programmed cell death called apoptosis differs in many respects from less specific necrosis. This is an orderly predetermined process, whereby cells, that are no longer needed or detrimental to the organism or tissue, are removed in an orderly manner. There are three pathways that lead to apoptosis: (1) an "extrinsic" pathway initiated by external signals – e.g., from FasL, TNF and caspase-8, (2) an "intrinsic"/mitochondrial pathway triggered by internal signals – i.e., Bcl-2, Apaf-1 (apoptotic protease activating factor-1) and caspase-9, – and (3) a third, caspase-independent pathway. The first two are therefore mediated by caspases, a family of cysteine proteases, and both pathways merge at caspase-3. The third caspase-independent one requires a molecule called apoptosis-inducing factor (AIF) that controls early morphogenesis for triggering apoptosis (Joza N, et al, 2001).

Apoptosis is characterized by specific morphological changes including cell membrane blebbing, cytoplasmic and nuclear condensation, DNA fragmentation, and the formation of apoptotic bodies. This specific type of cell death occurs in physiological and pathological conditions. Timely regulated cell death is very important for embryogenesis and tissue homeostasis (Schutte B, et al, 2000). Dysregulation of apoptosis is associated with a number of diseases. Failure to induce apoptosis can result in lymphoproliferative disease or cancer (Fadeel et al, 1999). In tumorigenesis, dysregulation of apoptosis together with deregulation of the cell cycle are considered necessary for tumor progression (Hanahan D and Weinberg RA, 2000). Such dysregulation might be useful as a therapeutic target to restore or even induce apoptotic signaling.

1.3.1 INDUCTION OF APOPTOSIS

Tumor suppressor genes encode critical cell cycle regulatory proteins that are frequently mutated or deleted in mesothelioma and, therefore, are important candidates for the treatment of cancer on the basis of gene replacement strategies. For example, the restoration of p53 function can induce the arrest of MM cell growth and apoptosis by the overexpression of p53 (Giuliano M, et al, 2000), by the expression of regulators of

p53 levels, such as $p14^{ARF}$ (Yang CT, et al, 2000), or by the expression of antisense transcripts to SV40 Tag (Waheed I, et al, 1999). The greater sensitivity of MM to apoptosis-inducing agents, such as most anticancer drugs, makes this an apparent paradox. Tumors with such upregulation may have pro- and anti-apoptotic mechanisms in equilibrium and they may therefore be more dependent on this than other tumors that have developed without a particular defense against apoptosis. Although this defense makes the cells resistant, interference with the protective mechanism itself may even increase the effect of pro-apoptotic events.

Adenoviral gene-based therapy, such as the expression of p16INK4A in adenovirus (Adp16INK4A) inhibits cell proliferation significantly, which suggests that Adp16INK4A may be useful in the treatment of human mesotheliomas (Yang CT, et al, 2003).

Apoptotic resistance of cancer cells can be overcome by combinating the treatments that activate the two major apoptotic pathways: (i) the death receptor pathway activated by death ligands and (ii) the DNA damage pathway activated by chemotherapy. A recent study has shown that mesothelioma cells, resistant to most treatments, respond to the combination of the death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) and chemotherapy (Liu W, et al, 2001).

1.3.2 RESISTANCE TO APOPTOSIS

The resistance to apoptosis of MM is another mechanism by which a tumor can reduce the effect of chemotherapy. Most drugs used for chemotherapy induce apoptosis by affecting the mitochondrion. The BCL-2 family of proteins and related phosphorylating enzymes are the major regulators of mitochondrial apoptotic homeostasis. Some studies have shown that the resistance to apoptosis of MM cells is associated with a change in the expression of pro-apoptotic Bax and anti-apoptotic protein BCL-XL, but rarely with the anti-apoptotic protein Bcl-2 (Narasimhan SR, et al, 1998; Soini Y, et al, 1999). This BCL-XL overexpression may prevent apoptosis in MM, while downregulation of the expression of the BCL-XL protein using antisense oligonucleotides (Smythe WR, et al, 2002) or histone deacetylase inhibitor (Cao XX, et al, 2001) causes apoptosis in MM cell lines and mesothelioma, and can sensitize human MM cells to the effects of conventional chemotherapy agents, such as cisplatin (Ozvaran MK, et al, 2004). Therefore, acquired resistance of MM to chemotherapy can result from a decrease in the induction or occurrence of apoptosis.

The human inhibitor of apoptosis protein-1 gene (IAP-1), which has a protein product that facilitates resistance to apoptosis in many types of cancer, is highly expressed in MM, as shown by differential display. It may also favor a drug-resistant phenotype in these tumors (Gordon GJ, et al, 2002).

1.4 POSSIBLE NEW TARGETS

1.4.1 THE THIOREDOXIN (Trx) SYSTEM

The Trx system is composed of TrxR (previously abbreviated TR), Trx and NAPDH. TrxR is a homodimer of 56 kDa subunits - a member of a pyridine nucleotide disulfide oxidoreductase family. Trx is a group of 12-kDa thiol/disulfide oxidoreductases. They are reduced by NADPH, a reaction catalyzed by TrxR. The Trx system is an efficient general protein disulfide reductase system (Holmgren A and Björnstedt M, 1995), which plays an important role in the redox regulation of signal transduction, transcription, cell growth, and apoptosis. Three members of the Trx group, Trx-1 (Grogan TM, et al, 2000), mitochondrial Trx-2 (Spyrou G, et al, 1997), and a novel Golgi apparatus-associated Trx-3 (Jimenez A, et al, 2004), have been identified. The activity of Trx depends on the redox status of TrxR, the activity of which in turn is dependent on a selenocysteine residue. There are three separate mammalian thioredoxin reductases. These include widely expressed cytosolic TrxR1, mitochondrial TrxR2, and TrxR3, which is located mainly in testis (Sun QA, et al, 1999; 2001). TrxR has broad substrate specificity and selenium is needed for cell proliferation (Björnstedt M, et al, 1997).

Some studies have shown that the levels of Trx-1 protein are significantly increased in several types of cancers that affect humans - i.e., in the stomach (50% of cases) (Grogan TM, et al, 2000), colon (55%) (Gallegos A, et al, 2000), and pancreas (41%) (Nakamura H, et al, 2000). In gastric cancer, a highly significant correlation has been found between an increase in Trx-1 expression and inhibition of apoptosis (Grogan TM, et al, 2000). Moreover, the increase in Trx-1 expression is an independent prognostic factor in non-small cell lung cancer patient, which suggests a shorter survival rate (Kakolyris S, et al, 2001). Reduced thioredoxin acts as an autocrine growth factor in various tumors, as a chemoattractant, and it synergizes with interleukins-1 and -2. The effects of anti-tumor drugs, such as carmustine, cisplatin, and oxaliplatin, can be explained in part by the inhibition of TrxR (Arnér ES, et al, 2001; Witte AB, 2005). Accordingly, high levels of the enzyme can promote drug resistance (Becker K, et al, 2000).

The high expression of Trx/TrxR described in MM [**paper I**] may play an important role in the drug resistance of malignant mesotheliomas (Kahlos K, et al, 2001). These findings firmly establish Trx-1 as an important target for drugs to treat MMs.

1.4.2 PROTEASOME

The proteasome system is an intracellular complex with protease activity, the detection of which resulted in the 2004 Nobel prize in chemistry. Selected intracellular proteins to be degraded are first tagged with ubiquitin, which makes them enter the degrading proteasome, thereby constituting the Proteasome-Ubiquitin Pathway (UPP). The ubiquitination is a highly specific process that determines which proteins should be degraded.

The active catalytic site in the proteasome is located in a 20S multisubunit structure consisting of four stacked rings arranged around an inner channel. When capped by the

19S regulatory complex at each end, the 20S complex forms the core of the 26S proteasome, the major extralysosomal mediator of protein degradation in the cell. The UPP, which consists of the ubiquitin-conjugating system and the proteasome, is a major pathway of proteolysis in eukaryotic cells that controls intracellular protein degradation (Hochstrasser M, 1995; Ciechanover A, 1994; Jentsch S, 1995).

The selectivity of the ubiquitination enables the cell to control many important processes – e.g., cell cycle progression, DNA repair, and gene transcription**.** The deregulation of various UPP functions is thought to play a role in malignant transformation (Masdehors P, et al, 2000) and is related to a worse prognosis (Chiarle R, et al, 2000; Li B and Dou QP, 2000). On the other hand, inhibition of the proteasome would lead to arrest of the cell cycle and ultimately to apoptosis and tumor regression as the single agent in a broad spectrum of tumor cell lines and in *in vivo* xenograft models (Orlowski RZ, et al, 1998; Adams J, et al, 1999; Soligo D, et al, 2001).

The association between proteasome activity and malignancy may be related to the turnover of oncoproteins and transcriptional regulators. Proteins that are degraded by the UPP include the inhibitor of nuclear factor κB (NF) - ubiquitinated IκB (Ni H, et al, 2001; Orlowski RZ and Baldwin AS, 2002), the tumor suppressor p53 (Salvat C, et al, 1999), the cyclin-dependent kinase inhibitors p21 and p27 (Kudo Y, et al, 2000; Hideshima T, et al, 2001), and the pro-apoptotic protein Bax (Li B and Dou QP, 2000). The accumulation of these substrates, which results from proteasome inhibition, causes inactivation of NFκB, a reduction in NFκB-dependent transcription of genes which are essential to the promotion of tumorigenesis, an increase in p53-mediated transcription of genes which are important for apoptosis and negative regulation of the cell cycle, p21- and p27-mediated induction of cell cycle arrest. The inducible activation of the nuclear transcription factor NF-κB seems to inhibit the apoptotic response to chemotherapy and irradiation (Wang CY, et al, 1996).

This makes the proteasome system a possible target for new drugs, particularly for tumors with a deregulated UPP. Several studies suggest that tumor cells are more susceptible to proteasome inhibitors than normal cells (Soligo D, et al, 2001; Hideshima T, et al, 2001; Qin JZ, et al, 2005), but the molecular basis of this differential susceptibility remains to be elucidated. The resistance to chemotherapy and radiation therapy limits the effectiveness of many treatments currently available to patients, and proteasome inhibitors may counteract such effects. The inhibition of proteasome function blocks the activation of chemotherapy-induced NFκB by stabilizing IκB, which remarkably increases chemosensitivity and apoptosis (Orlowski RZ and Baldwin AS, 2002). In combination of a proteasome inhibitor, such as bortezomib with other forms of chemotherapy (SN-38/CPT-11, TRAIL) seems likely to overcome the usual poor response to chemotherapy (Cusack JC, et al, 2001; Ganten TM, et al, 2005). Proteasome inhibitors may also increase radiosensitivity (Russo SM, et al, 2001).

The UPP is therefore necessary for the degradation of proteins involved in control of the cell cycle and the growth of tumor. Proteasome inhibitors arrest or retard progression of cancer, by interfering with the degradation of regulatory molecules. The

development of proteasome inhibitors and the necessary *in vitro* and *in vivo* studies of proteasome inhibition, as regards to cancer therapy, have already been started (Orlowski RZ, et al, 2002; Richardson PG, et al, 2003; 2004; Papandreou CN, et al, 2004; Lee KW, et al, 2005).

2 PRESENT INVESTIGATION

2.1 AIMS

The main aim of this thesis was to study the differences between epithelioid and fibroblast-like differentiation in malignant and benign mesothelial cells.

The STAV mesothelioma cell line was originally obtained from a pleural effusion, and it was found to differentiate into sublines with either of the two phenotypes, depending on the serum supplementation (Klominek J, et al, 1989).

A similar variation in growth pattern can also be obtained in cultures of benign mesothelial cells, which then grow with a fibroblastic or epithelioid morphology *in vitro* (Lanfrancone L, et al, 1992; Mouriquand J, et al, 1977). The growth patterns are stable during several passages, as shown by their ultrastructural and immunocytochemical characteristics (Gulyas M, et al, 1999).

Therefore, the STAV cell sublines and benign mesothelial cell cultures provide models for tracing molecular changes involved in mesothelial cell differentiation. Better understanding of the mechanisms underlying this differentiation may provide clues to improve the diagnostic methods, and even suggest some targets for novel therapies.

2.2 SCREENING APPROACHES FOR MESOTHELIAL CELL DIFFERENTIATION

2.2.1 SUPPRESSION SUBTRACTIVE HYBRIDIZATION (SSH)

The PCR-based cDNA subtraction method called suppression subtractive hybridization (SSH) is a simple, fast, efficient method that minimizes the loss of low abundance molecules (Diatchenko L, et al, 1996; Gurskaya NG, 1996). We used this strategy to isolate several cDNA clones representing transcripts of genes that are differentially expressed in one of the phenotypes. A high level of enrichment of rare transcripts has been achieved by including a normalization step in the subtraction procedure. Under our standard conditions, the driver cDNA removes most of the sequences common to both the tester and driver cDNA samples. This method also eliminates any intermediate step(s) for physical separation of ss and ds cDNAs, needs only a small amount of the starting material (0.2-2μg of poly A^+ RNA), requires only one subtractive hybridization round, and obtains more than 1,000-fold enrichment for differentially expressed cDNAs. The SSH method was used in papers [**I**] and [**II**] to show the differentially expressed genes, comparing the epithelioid and fibroblastoid phenotypes in benign and malignant mesothelial cells.

2.2.2 MICROARRAY

Monitoring gene expression is widely used in medical and biological research, including the classification of diseases, understanding of basic biological processes, and identifying new drug targets. Until recently, comparison of the expression levels in various tissues or cell lines was limited to tracking a few genes at a time. The

microarray procedures enable one to monitor the activities of thousands of genes simultaneously. With this procedure, RNA or DNA is prepared from the biological material to be analyzed. In this study, we used the Affymetrix' technique, in which the RNA is subjected to reverse transcriptase reactions, eventually generating cRNA with biotin tags incorporated. This then hybridizes to the array probe sequences attached to the chip, and the hybrids formed can be detected by adding the avidin-labeled fluorochrome and subsequent laser excitation. The Affymetrix system used [**paper III**] also allows the detection of splice variants by probing various parts of an expressed gene, and the discrimination of specific and nonspecific signals, by comparing the probe sequences to single base mismatches.

Affymetrix chips are now available that are hybridized to more than 10,000 genes. One problem in acquiring useful information with this method, however, in the enormous amount of information yielded. Chips with particular "assortments" of genes, such as the Human Cancer G110 oligonucleotide array chip used by us (Schaefer KL, 2002; **paper III**), somewhat reduce these problems.

In clinical studies, expression profiling using the Affymetrix system may do more than just provide a better understanding of biological and pathobiological processes and even the function of genes and proteins. A molecular phenotype can be established in this way, which might lead to optimization and specific tailoring of therapies on the basis of the individual tumor.

2.2.3 REAL-TIME RT-PCR

Real-time RT–PCR is a sensitive, quantitative, and reliable method for RNA quantification (for theoretical basis of this method, see Gibson UE, et al, 1996 and Bieche I, et al, 1999). The simplest detection technique for newly synthesized PCR products in real-time PCR uses SYBR Green I fluorescence dye that binds specifically to the minor groove double-stranded DNA (Morrison T, et al, 1998). Two quantification types of real-time RT-PCR are usually employed: (1) A relative quantification based on the relative expression of a target gene versus a reference gene was used in paper [**III**]. To study the physiological changes in gene expression, the relative expression ratio is adequate for most purposes. (2) An absolute quantification of target DNA can also be done, based either on an internal or an external calibration curve (Morrison T, 1998; Pfaffl MW, 2001). Unregulated genes or housekeeping genes, like glyceraldehyde-3-phosphate dehydrogenase (GAPDH), albumin, actins, tubulins, cyclophilin, 18S rRNA or 28S rRNA (Marten NW, et al, 1994), have mainly been used as reference genes. The mRNA synthesis of these genes is considered to be stable in various tissues, even during experimental treatments (Foss DL, et al, 1998; Thellin O, et al, 1999). Relative expression is sufficient for the most relevant physiological expression changes. For purposes of the differential display of mRNA, the relative expression ratio is an ideal and simple method for the verification of RNA or DNA array chip technology results.

Microarrays permit the parallel analysis of thousands of genes in two-differentially labeled RNA populations (Schena M, et al, 1995), while real-time RT-PCR simultaneously measures gene expression in many different samples of a limited

number of genes, and is especially suitable when only a small number of cells are available (Heid CA, et al, 1996; Fink L, et al, 1998). Both methods have the advantages of speed, output and a high degree of potential automation as compared to conventional quantification methods; such as Northern blot analysis and competitive RT-PCR.

2.3 APOPTOSIS ASSAYS

Apoptosis was determined by combinating morphology, caspase-3 activation, and FACS analysis.

2.3.1 MORPHOLOGICAL ANALYSIS

The cell morphology was routinely monitored under a light microscope during treatment. After 24 h of treatment, the cells on the chamber slides were fixed with ethanol and stained according to Papanicolaou [**paper IV**]. Alternatively, cells in suspension were prepared by cytospin, air-dried and stained according to May-Grunwald-Giemsa [**paper V**]. All slides were randomized and analyzed morphologically in a double-blind manner. Apoptotic cells were assessed by morphological criteria, such as cytoplasmic shrinkage, membrane blebbing, and disintegrated cell bodies, increase in nuclei density, and nuclear fragmentation. Cells with more than one nucleus or without cytoplasm were not counted. We found no signs of necrosis.

2.3.2 CASPASE-3 ACTIVATION

The caspase family of proteases plays an essential role in the execution of apoptosis. They are synthesized as catalytically inactive proenzymes in normal cells and are activated by proteolytic cleavage during apoptosis (Budihardjo et al, 1999). Therefore, activation (by cleavage) of caspases, such as caspase-3, is often used as an indicator of caspase-dependent apoptotic cell death. After treatment, the cell lysates were subjected to SDS-PAGE. Cleavage of caspase-3 was detected by Western blot analysis using antibody that recognized both procaspase-3 and cleaved caspase-3. There was no cleavage of caspase-3 in cells treated with DMSO, while the cleaved caspase-3 was detectable in cells treated with proteasome inhibitor [**paper IV**].

2.3.3 FACS ANALYSIS

After treatment with proteasome inhibitors or selenite, adherent cells were pooled including floating cells, and stained with fluorescein isothiocyanate conjugated antibodies to Annexin V (Annexin V-FITC) and propidium iodide (PI). Annexin V labels cells that demonstrate externalization of phosphatidylserine on the cytoplasm membrane, an early sign of apoptosis. The parallel staining with PI labels those cells that have an increase in permeability of the cell membrane, a later sign of apoptosis. When the dying cells begin to disintegrate, one cannot distinguish between apoptotic and necrotic cells using this method. Early apoptotic cells were defined as those being Annexin V-positive and PI-negative. A FACScan flow cytometer equipped with CellQuest and ModFit LT software was used for the analyses [**papers IV** and **V**].

2.4 SSH EXPERIMENT (PAPERS I AND II)

To understand better the regulatory mechanisms by which the malignant mesotheliomas and benign mesothelial cells differentiate into an epithelial or a fibroblast-like phenotype, we used a mesothelioma cell line that can differentiate into sublines with epithelial or fibroblast-like phenotype depending on their serum supplementation in paper [**I**]**,** and mesothelial short-term cultures that were obtained from benign effusions with epithelial or fibroblast-like phenotype in paper [**II**], as *in vitro* models. Genes found to be differentially expressed in these *in vitro* models were identified by suppression subtractive hybridization. Differential cDNA was amplified by PCR, and the amplimers were cloned and sequenced, confirming the differential expression by a separate dot blot test, Western blot and immunohistochemistry.

A limited number of differentially expressed cDNAs were isolated in this way in the two sublines: nine genes obtained from the epithelial subline, as compared to only two genes in the fibroblast-like phenotype. One of these differentially expressed genes codes for Trx. The higher expression of Trx by the epithelioid phenotype was also verified on the protein level by Western blot analysis. At the same time, the activity of TrxR was found to be exceptionally high in both sublines, but twofold greater in the epithelioid phenotype [**paper I**].

In benign fibroblastic mesothelial cells, nine overexpressed genes were found, using SSH [**paper II**]. Six of these genes are related to a proliferative cell type, and three are matrix-associated. Eight genes were similarly upregulated in the epithelioid phenotype; seven of which were related to specialized functions of a differentiated cell (metabolism, cytoskeletal composition, and regulation of differentiation), while one was associated with nuclear transport. These findings indicate that fibrous and epithelioid mesothelial cells in culture are closely related, the former representing a more activated and proliferative form. Immunocytochemistry studies also showed the difference *in vivo* by using commercial antibodies. Thus 14-3-3 ε, integrin α5 and collagen-binding protein 2 were found mainly in submesothelial fibroblast cells in which the tissue showed morphological signs of mild activation, while stronger staining of annexin II, cytokeratin 7 and cytokertin 8 were seen only in the flattened mesothelial surface cells.

2.5 MICROARRAY (PAPER III)

To further explore the molecular mechanisms responsible for this differentiation of mesothelioma cells, and understand better the factors that are important for progression of MM, we also used oligonucleotide arrays in our *in vitro* model of mesothelioma differentiation.

In this study, we identified 102 genes that were consistently deregulated in nine comparisons from the array containing 2059 human cancer genes. Of these, 56 genes were up-, and 46 were down- regulated, when comparing the epithelioid phenotype with the fibroblastic one. Some of the overexpressed genes were identical to those identified by suppression subtractive hybridization. Clustering of these genes in functional categories, such as signal transduction, receptors/ligands, cell growth and proliferation, cell adhesion, transcription, metabolism, and defense response, shows distinct patterns of the two phenotypes.

The molecular fingerprint of the sarcomatous tumor component indicates an over-representation of survival-promoting genes, growth factor receptors and other growth factor-binding proteins. Epithelioid mesothelioma cells express other tumor-promoting factors, concomitantly with differentiation, metabolism, and regulation of apoptosis. We selected 10 overexpressed genes of each phenotype for verification with Real-time RT-PCR, and they were all differentially expressed also by this latter analysis. Similarly, Western blot and immunohistochemical staining confirmed the overexpression of some of the selected proteins.

2.6 POSSIBLE NEW TARGETS FOR MESOTHELIOMA THERAPY [PAPERS IV AND V]

The Affymetrix analyses [**paper III**] suggest that there are major differences in tumor driving mechanisms in the two phenotypic sublines, although they originate in the same tumor. A tailored therapy may be more effective, if this heterogeneity is taken into consideration. Two systems that can provide such specific targets are the Trx system and the proteasome, which both were highly upregulated in STAV cell lines, especially in the epithelioid STAV-AB cells [**papers I** and **III**]. Such upregulation may indicate that these cells have an advantage as regards growth, and that targeting them might be of value for therapy. In papers [**IV**] and [**V**] we evaluated the differential apoptotic effect of the inhibitors of proteasome and selenite on the two mesothelioma sublines.

The proteasome inhibitor (PSI) shows substantial anti-tumor activity in several tumor cell lines. The mesothelioma cell viability is reduced in a dose- and time- dependent manner by both PSI and calpain inhibitor I, the latter another proteasome inhibitor necessitating higher concentrations. The STAV-AB subline is more sensitive to PSI than STAV-FCS, with a mean 24 hours IC_{50} of 4 µM and 16 µM for the two cell sub-lines, respectively. PSI seems to reduce mesothelioma viability by inducing apoptosis, as verified by cell morphology, Western blotting of caspase-3 cleavage, and flow cytometric analysis of apoptosis. Thus, analogs of PSI may be of value in the treatment of patients with mesothelioma, especially those with the epithelioid phenotype [**paper IV**].

The Trx system is indispensable in the defense against oxidative stress. It produces a reduced state in the cell that resists oxidation. The weakness of this cellular defense strategy is that all the thiol groups present may catalyze a nonstochiometric production of ROS when exposed to selenite. In Paper [**V**], we evaluated the effects of treatment with selenite alone on the redox balance in malignant mesothelioma cells – i.e., on viability, the induction of apoptosis, and the activity of the Trx- and GPx-systems. We also studied the combined effects of selenite and doxorubicin to evaluate possible synergism.

Selenite inhibited the growth of mesothelioma phenotypes in a dose- and time-dependent manner. The sarcomatoid cells are significantly more sensitive, with a mean 28 hours IC_{50} of 7.5 µM for the sarcomatous cell line and 21 µM for the epithelioid cells. This reduction in viability of mesothelioma cells was associated with apoptosis, as shown on morphological examination and FACS analysis of AnnexinV-positive

cells. Three Mesothelioma cell lines, and two adenocarcinoma cell lines also responded well to treatment with selenite, while benign mesothelium was less sensitive. The activity of TrxR1 and GPx increased markedly at low concentrations of selenite (2.5 μM), however, higher concentrations (5-30 μM) decreased the expression and activity of the two enzymes further.

Doxorubicin, on the other hand, caused a dose- and time-dependent inhibition of growth of epithelioid MM cells, but had no effect on the sarcomatoid cells; they proliferated at a similar rate regardless of the doxorubicin concentrations. When selenite was combined with doxorubicin, this drastically reduced the viability and induced apoptosis in both MM phenotypes, and the effect appeared to be synergistic. Therefore such a combination might be useful in the treatment of patients with mesothelioma, especially of mixed and sarcomatoid phenotypes.

3 DISCUSSION

Using suppression subtractive hybridization (SSH), we found two cDNAs that were overexpressed in the STAV-FCS subline, while nine genes were higher in the STAV-AB subline [**paper I**]. Some of these phenotype-specific transcripts may be factors that regulate the differentiation of the two cell sublines. One of the differentially-expressed cDNAs in the STAV-FCS subline was human 28S ribosomal RNA gene, homologous with the gene isolated from the Barstead stromal cell that may produce rapid and drastic changes in the phenotype (Gonzalez IL, et al, 1985). This is interesting from the point of view that the fibroblast-like mesothelial cells in culture correspond to a mesenchymal cell that gradually adapts epithelioid characteristics [**paper II**].

In the STAV-AB subline, one of the identified gene sequences is homologous with the human calcyclin gene [**paper I**] and therefore associated with epithelial differentiation of the mesodermally-derived mesothelioma cells. This gene has also been implicated in resistance to therapy (Shi Y, et al, 2004), but may have various biological functions and be involved in carcinogenesis (Rehman I, et al, 2004).

Other STAV-AB-derived sequences showed homology with the human mRNA for the proteasome subunit Y and for Trx. These two differences in STAV cell sublines found in papers [**I**] and [**III**] were evaluated as possible therapies in papers [**IV**] and [**V**].

Of the genes overexpressed in benign fibroblastic mesothelial cells, some are matrix-associated and therefore related to a mesenchymal phenotype, while most are associated with a less differentiated and highly proliferative cell. In contrast to this, the upregulated genes in the epithelioid phenotype are related to specialized functions, such as metabolism, cytoskeletal composition and the regulation of differentiation. The growth pattern as mesothelial cells from a less differentiated fibroblastic to a more specialized epithelioid phenotype may be regulated by the differentially expressed genes. Our findings in the benign model also accord with those in MM, in which the sarcomatous phenotype is more proliferative and has a worse prognosis.

Using SSH, there only seem to be a few differentially expressed genes found in mesothelioma cells that have different phenotypic growth patterns. We therefore utilized these two mesothelioma cell sublines as a model of the morphological changes *in vitro,* and oligonucleitide array to isolate more of the genes involved in the phenotypic transitions between epithelioid and fibroblast-like mesothelioma cells [**paper III**]. Some of the findings were in agreement with the previous study [**paper I**], but with the lower threshold, more genes were identified as deregulated (104 of 2059 genes), as compared with the 11 genes isolated by SSH.

The pattern of deregulated genes associated with metabolism accords with the findings in paper [**I**]. The fibroblast-like mesothelioma subline overexpresses genes that are associated with growth factors, their receptors, and the proteins involved in signal transduction, as well as signaling and the other two genes related to invasion, while the

genes related to metabolism, differentiation, defense response, and apoptosis were identified from the epithelioid one [**paper III**].

These data are important because they describe a functional heterogeneity related to the well-described morphological heterogeneity of MM. The less differentiated sarcomatoid MM cells produce more factors associated with a more aggressive behavior (Ceresoli GL, et al, 2001). This particular heterogeneity may play an important role in the ability of the tumor to respond to therapy. It may therefore explain why a drug will be only partly effective, and allow other tumor clones to expand.

In summary, these studies indicate that the differentiation of mesothelial cells is associated with the expression of certain patterns of genes. Although the epithelioid and fibroblastic phenotypes are closely related, the fibrous cells seem to represent a less differentiated phenotype, which may act as a precursor to the epithelioid counterpart.

Some of the differencially expressed genes were tested as possible targets for therapy. The catalytic 20S proteasome subunit was differentially expressed [**papers I** and **III**], and immunocytochemistry demonstrated that the proteasome system is abundantly present in MM tissue [**papers III** and **IV**]. Treatment with the proteasome inhibitor PSI reduced the viability of various MM cell lines in a time- and concentration- dependent manner, the epithelioid cells being more sensitive than the sarcomatoid ones [**paper IV**].

Trx/TrxR was highly expressed in both MM phenotypes, the highest levels being seen in the epithelioid cells [**paper I**]. However, the sarcomatoid phenotype was more sensitive to the effects of selenite than the epithelioid cells. Both cell phenotypes had extremely high levels of Trx/TrxR. They were probably in such a reduced state so as to permit selenite to induce the uncontrolled production of ROS species and apoptosis in the sarcomatoid cells, while the even higher levels of Trx/TrxR in the epithelioid ones seemed protective. However, greater selenite concentration or longer exposure times induced similar changes also in the epithelioid cells.

Chemotherapy of MM often gives only partial responses, which may correlate to our finding that the two main phenotypes utilize different tumor driving mechanisms. Therefore, it seems likely that better therapeutic effects can be obtained by combining drugs to combat both phenotypes. On the basis of our studies with sublines from one MM, we hypothesize that the combination of selenite and proteasome inhibitors can be an effective therapy for MM. The value of such tailored combinations, however, must be further tested in other cell lines and tumor tissue before clinical trials can be justified.

4 CONCLUSIONS

- Two mesothelioma sublines with epithelial (STAV-AB) or fibroblast-like phenotype (STAV-FCS) derived from a mesothelioma cell line is a useful *in vitro* model to study the differentiation of mesothelioma cell phenotypes.

- Suppression subtractive hybridization, comparing the two sublines, identified a few differentially expressed genes, which showed a pattern, suggesting that fibroblastic mesothelioma cells represent a less mature stage, from which epithelial cells can be derived depending on the stimulus.

- Oligonucleotide microarray revealed additional differentially expressed genes in the two sublines. These had similar distinctive patterns - i.e., survival-promoting genes, growth factor receptors and other growth factor binding proteins overexpressed in the sarcomatous tumor, and other tumor-promoting factors, together with differentiation, metabolism, and regulation of apoptosis in the epithelioid mesothelioma cells.

- The mesothelial cells obtained from benign effusions are not terminally differentiated and stable during early passages. The cultures with a fibroblastic or epithelioid phenotype *in vitro* provide a model for the study of mesothelial differentiation.

- Analysis of benign mesothelial cells by suppression subtractive hybridization show a pattern similar to that in mesothelioma cells, suggesting that the benign fibroblastic cell is more proliferative and less differentiated than the epithelioid one.

- MM cells showed overexpression of possible targets for therapy such as proteasome components and Trx/TR.

- Proteasome inhibitors such as PSI, effectively induce apoptosis of mesothelioma cells, and this or other analog drugs may be useful in the treatment of patients with mesothelioma, especially of epithelioid phenotype, while selenite reduced the viability of the mesothelioma cells by inducing apoptosis, showed synergistic effects when combined with doxorubicin, and can probably be used to the patients with mesothelioma, especially of the sarcomatoid phenotype.

- We have identified significant differences in the growth regulation of the two MM phenotypes. The use of such molecular phenotyping may therefore improve our ability to design therapies that are effective against both phenotypes.

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