

Expression of Cancer-Associated Molecules in Malignant Mesothelioma

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Abstract: Malignant mesothelioma (MM) is a malignant tumor derived from mesothelial cells, native cells of the body cavities. Exposure to asbestos is the most strongly established etiologic factor, predominantly for the most common disease form, pleural mesothelioma. The pathogenesis of MM involves the accumulation of extensive cytogenetic changes, as well as cancer-related phenotypic alterations that facilitate tumor cell survival, invasion and metastasis. This review presents current knowledge regarding the biological characteristics of this disease that are linked to the so-called hallmarks of cancer. In addition, data suggesting that the anatomic site (solid tumor vs. effusion) affects the expression of metastasis-associated and regulatory molecules in MM are presented. Finally, recent work in which high-throughput methodology has been applied to MM research is reviewed. The data obtained in the reviewed research may aid in defining new prognostic markers and therapeutic targets for this aggressive disease in the future.

Keywords: malignant mesothelioma, hallmarks of cancer, metastasis, high throughput

Introduction

Malignant mesothelioma (MM) is a tumor derived from mesothelial cells, native cells of the body cavities. The pleural cavity is the most common site, with a present ratio of 9:1 with peritoneal tumors. Exposure to asbestos can be documented in about 80% of the cases (Britton, 2002). The incidence of MM appears to be rising steeply in western countries, a trend that is likely to continue (Parker, 2003; van Ruth, 2003). MM is an aggressive and rapidly fatal disease, with a median survival of 8 months if untreated, although selected patients achieve survival of 2 years and more when surgery is combined with adjuvant therapy (Parker, 2003; van Ruth, 2003). Validated clinicopathologic prognostic factors of poor survival are non-epithelioid tumor type, poor performance status, male gender, high white blood cell count and low hemoglobin levels (Burgers, 2004). The disease develops for decades, during which the neoplastic cells accumulate a variety of chromosomal aberrations. Extensive studies using traditional cytogenetics, analysis of microsatellite instability/loss of heterozygosity and fluorescent in situ hybridization (FISH) have shown that gains or losses of entire chromosomes or chromosome fragments most frequently involve chromosomes 1, 3, 6, 7, 9, 14, 18 and 22 (reviewed in Sandberg, 2001; Musti, 2006). Use of gene-specific molecular probes has identified loss of the cyclin-dependent kinase-4 inhibitor (CDKN2A) (Prins, 1998; Illei, 2003), neurofibromatosis type 2 (NF2) (Bianchi, 1995; Sekido, 1995), p15 and p16 (Xio, 1995), p53 (Cote, 1991), and Fhit (Pylkkanen, 2004) genes in MM, supporting the role of cell cycle deregulation and inactivation of tumor suppressor genes in its pathogenesis.

Simian virus 40 (SV40) is an additional postulated contributing factor in the development of MM. Some of the Polio vaccines prepared during the 50's and early 60's have been shown to be contaminated with SV40, a polyomavirus that produces an asymptomatic infection in macaque rhesus monkeys, its natural host. Earlier studies have reported high levels of expression of SV40 DNA or large T antigen protein in MM (reviewed in Shah, 2006), although not all studies reproduced this observation (Simsir, 2001; Manfredi, 2005). The presence of SV40 has been associated with expression of several cancer-associated molecules, the majority of which are discussed below. The presence of SV40 in MM was shown to be associated with more frequent methylation of the *RASSF1* gene, a putative tumor suppressor gene in lung cancer (Toyooka, 2001). SV40 replication was associated with autocrine activation via

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hepatocyte growth factor/scatter factor (HGF/SF) and its receptor Met (Cacciotti, 2001). The presence of SV40 has been shown to be associated with higher levels of vascular endothelial growth factor (VEGF) (Cacciotti, 2002), telomerase activity (Foddiss, 2002), expression of the cell cycle inhibitor p21^{WAF1/CIP1} (Baldi, 2002), p53 inactivation (Carbone, 2003) and activation of the phosphatidylinositol 3-kinase/AKT signaling pathway (Cacciotti, 2001; Ramos-Nino, 2005).

The role of SV40 in the pathogenesis of MM has been growingly questioned in recent years. Twenty-one of 31 papers published in the time period from 2002 until the publication of the above-mentioned review have failed to find any evidence supporting the presence of SV40 in MM or found only rare DNA or protein expression of the virus (Shah, 2006). Furthermore, contamination with laboratory plasmids has been shown to be a frequent source of false-positive results in studies analyzing the presence of SV40 (Lopez-Rios, 2004), and serological studies have shown rare seroreactivity for the virus among the general population and MM patients, as well as cross-reactivity with the more prevalent BKV and JCV polyomaviruses (Shah, 2006). These data suggest a need to re-evaluate the role of SV40 in MM.

Patients with the more common pleural MM typically present with cough, dyspnea and chest pain, reflecting the presence of pleural plaques and a characteristically unilateral malignant hemorrhagic effusion (Pass, 1993; Moskal, 1998). Patients with peritoneal disease present with abdominal distention and pain that are caused by the presence of malignant cells in ascites and in solid peritoneal lesions, a clinical picture that may be indistinguishable from that observed in ovarian or peritoneal carcinoma (Eltabbakh, 1999; Sugarbaker, 2003). Although present in a large number of cases at autopsy, clinically detectable distant metastases are rare at presentation.

The main differential diagnosis of epithelioid MM, the most common histologic type, is with metastatic adenocarcinoma, most frequently of ovarian, breast or lung origin, and reactive mesothelium (RM). Benign mesothelial cells react to a wide variety of stimuli and injuries by proliferation and reactive cellular changes that may mimic the morphology of malignant cells (Bedrossian, 1994). These changes may be especially pronounced following radiation or chemotherapy, common adjuncts to surgery in the treatment of various

malignancies (Bedrossian, 1994). The overlapping morphology of these cell types does not allow for a reliable diagnosis of MM without the use of ancillary techniques. In recent years, immunohistochemistry has largely taken over the role of electron microscopy in this field. While the possibility to diagnose MM in cytological material is still not universally accepted, improved antibody panels and the use of formalin-fixed paraffin-embedded cell block material for effusion diagnosis have led to results that are at least comparable to those observed in surgical specimens, with lesser morbidity and cost (Figures 1-A, 1-B) (Bedrossian, 1998; Davidson, 2001). In fact, the diagnosis of MM can be reliably obtained within less than one day using flow cytometry, a procedure that is optimal for effusion specimens (Davidson, 2002a; Sigstad, 2005). Recently, molecular methods such as comparative genomic hybridization (CGH) and FISH have been successfully used for the diagnosis of MM in cytological material (Nagel, 2002; Illei, 2003).

Cancer cells show a spectrum of phenotypic aberrations, all reflecting the loss of host regulation and the ability to override checkpoints that allow normal tissue to remain in homeostasis. These consist of altered cellular adhesion, abnormal response to growth-promoting signals, cell cycle deregulation and evasion of apoptosis, and enhanced proteolysis and angiogenesis (Hanahan, 2000). Although the majority of translational correlates that are related to chromosomal changes in MM and the mechanisms of epigenetic regulation in this disease are still undefined, recent research has defined many of the phenotypic characteristics of MM that are related to the so-called hallmarks of cancer. This review presents currently available data regarding the cancer-related biology of MM, with focus mainly on research performed on clinical specimens. Data suggesting that the anatomic site (pleura vs. peritoneum) and growth conditions (solid tumor vs. effusion) may affect the phenotype of MM cells are presented. Finally, recent work in which high-throughput methodology has been applied to MM research is reviewed.

Proteolytic Enzymes

Matrix metalloproteinases (MMP), a family of zinc- and calcium-dependent enzymes, are central mediators of the biology of tumor invasion and metastasis, due to their ability to degrade the basement membrane

and essentially all extracellular matrix (ECM) components (Egeblad, 2002). Two members of the family, MMP-2 and MMP-9, are the proteases involved in basement membrane degradation, a major event in the dissemination of epithelial cancer. MM cell lines have been shown to express and activate MMP-2 and MMP-9, and express and secrete several other members of this family, including MMP-1, MMP-3, MMP-7 and MMP-10, as well as the MMP inhibitors TIMP1-3 (Liu, 2001). MMP expression and secretion by MM cells *in vitro* is increased by several growth factors, including HGF/SF (Harvey, 2000; Liu, 2003), epidermal growth factor (EGF), acidic and basic fibroblast growth factor (aFGF, bFGF), insulin-like growth factors I and II (IGF-I,II) and transforming growth factor- α (TGF- α) (Liu, 2003). MMP-2 and MMP-9 activity, as measured by gelatin zymography, was documented in clinical MM specimens, with predominant activation of the former enzyme. MMP-9 activity was significantly higher than in benign pleura, and MMP-2 was an independent predictor of poor survival (Edwards, 2003). Our group recently found expression of MMP-2, MMP-9, MMP-14 (MT1-MMP) and TIMP-2 and activation of MMP-2 and MMP-9 in MM effusions (Sivertsen, 2006). MMP-2 and TIMP-2 mRNA expression was significantly higher in peritoneal compared to pleural MM effusions (Sivertsen, 2006). The data in the above studies suggest a central role for MMP in the pathogenesis of MM.

Adhesion Molecules and Other Membrane Receptors

Cell-cell adhesion is essential for normal cellular architecture and homeostasis. Cadherins, a family of Ca^{2+} -dependent integral membrane glycoproteins, are located at the cell-cell adherens junctions, where they mediate homophilic contact with neighboring cells (Takeichi, 1991). Early data suggested that MM and AC cells exclusively express the epithelial and neural type of this molecule (E-cadherin and N-cadherin), respectively (Han, 1997). These data have been shown to be erroneous by other investigators and us (Davidson, 2002; Abutaily, 2003; Sivertsen, 2006a; reviewed in Ordonez, 2003a). In fact, MM and serous AC are the two tumors that most strikingly exhibit co-expression of different cadherins, including N-cadherin, P-cadherin and E-cadherin (Sivertsen, 2006a). Furthermore, MM cells in effusions upregulate the expression of these three cadherin

molecules, suggesting that they play a role in metastasis and tumor progression (Sivertsen, 2006b). This profile may aid MM cells in combining conserved adhesion between tumor cells with the aggressive behavior of epithelial/epithelioid cells undergoing epithelial-mesenchymal transition (EMT), a process that is characterized by loss of E-cadherin and acquisition of N-cadherin (reviewed in De Wever, 2003). Interestingly, MM cells, including those of the epithelioid type, frequently express neural cell adhesion molecules (NCAM), members of a different family of adhesion molecules that are expressed by neural and neuroendocrine cells and biphasic tumors (e.g. synovial sarcoma) (Lantuejoul, 2000). Seen together with the observation that MM express nerve growth factor receptors (Davidson, 2004), it is evident that this tumor is able to produce a remarkable array of molecules that have been previously thought to be lineage-specific.

Integrins, a second family of adhesion molecules, are heterodimers consisting of noncovalently linked α and β subunits (Hynes, 1992). Integrins are fundamental regulators of cell growth, migration, survival and differentiation, and transduce signals from the extracellular environment to the gene expression machinery, thereby modulating signaling events initiated by growth factor receptors (Howe, 1998; Dedhar, 2000). The extracellular matrix (ECM) integrin ligands include laminin, fibronectin, collagen, vitronectin, entactin, tenascin, and fibrinogen (Ruoslahti, 1991).

Klominek and co-workers previously showed that integrins are expressed in MM cell lines and that they mediate migration towards ECM proteins, including collagen type IV, fibronectin and laminin (Klominek, 1997). Koukoulis et al. studied 22 clinical MM specimens and found that the expression of most integrins, including the $\alpha 6 \beta 4$ integrin, in epithelioid MM is similar to previously reported patterns in AC of the lung, and often of other origins (Koukoulis, 1997). We recently found that MM cells frequently express the αv and $\beta 1$ integrin subunits, and that the $\alpha 6$ subunit, part of the $\alpha 6 \beta 4$ and $\alpha 6 \beta 1$ laminin receptors, is more frequently expressed in MM compared to ovarian and breast AC (Sigstad, 2005). In contrast, the 67kDa laminin receptor, a non-integrin receptor that is frequently expressed in AC at all sites, is only rarely expressed in MM (Figure 1-C) (Reich, 2005). In view of the recent

data of our group regarding the role of laminin receptors in tumor invasion and metastasis via activation of MMP-2 synthesis in malignant melanoma (Givant-Horwitz, 2004), it is conceivable that integrins, rather than the 67kDa receptor, may contribute to MMP production following binding to laminin in MM.

Angiogenic Molecules and Other Growth Factors and Growth Factor Receptors

MM cells synthesize a large number of angiogenic molecules and other growth factors that may provide autocrine stimulation and relative independence from the stromal and endothelial production of these factors. The most extensively studied molecule in this context is VEGF. Expression of VEGF and its receptors flt-1 and KDR has been shown in several studies, supporting the existence of this autocrine pathway in MM (Konig, 1999; Kumar-Singh, 1999; Ohta, 1999a; Konig, 2000; Strizzi, 2001a; Catalano, 2002; Davidson, 2004). A similar autocrine loop may exist for PDGF and its receptor PDGFR (Ascoli, 1995; Langerak, 1996; Klominek, 1998a). PDGFR is of special interest since the α subunit of the receptor is the predominant one expressed in benign mesothelial cells, whereas expression of the β subunit is found in MM cell lines and clinical specimens (Versnel, 1991; Ramael, 1992). Inhibition of PDGF- β using ribozyme technique results in reduced cell growth concomitantly to reduction in PDGF- β levels in the VAMT-1 cell line (Dorai, 1994).

Additional pro-angiogenic molecules and growth factors that are synthesized by MM cell lines and clinical specimens are interleukin-8 (IL-8) (Galffy, 1999; Davidson, 2004), bFGF (Strizzi, 2001b; Davidson, 2004), TGF- α (Langerak, 1996), IGF (Hoang, 2004a), heparanase (Davidson, 2004), thrombospondin (Ohta, 1999b), HGF/SF (Klominek, 1998b; Thirkettle, 2000) and several members of the Syndecan proteoglycan family (Kumar-Singh, 1998; Gulyas, 2003). MM cells express both EGFR (Dazzi, 1990; Pache, 1998; Manning, 2002; Cai, 2004; Destro, 2006) and its family member erbB-2 (Thirkettle, 2000), and EGFR expression has been shown to be closely related to the pathogenesis of asbestos injury (Pache, 1998; Manning, 2002). However, EGFR mutations were not detected in pleural MM (Destro, 2006).

Microvessel density is an independent prognostic marker in MM (Edwards, 2001). The clinical significance of angiogenic molecule expression is suggested by the observed correlation between VEGF and bFGF and poor survival, with an opposite finding for Syndecan-1 (Kumar-Singh, 1999). EGFR expression correlates with worse survival in MM, but this finding loses significance when histologic type (epithelioid vs. sarcomatoid) is taken into account (Dazzi, 1990). EGFR expression did not correlate with survival in a recent study (Destro, 2006).

We recently found that the activated nerve growth factor (NGF) receptor p-TrkA is more frequently expressed compared to p75, another NGF receptor that belongs to the tumor necrosis factor family, in MM, and that p-TrkA expression is significantly higher in peritoneal MM compared to their pleural counterparts (Figures 1-D, 1-E). In addition, p-TrkA expression was marginally higher in effusions, while p75 expression was significantly higher in solid MM (Davidson, 2004). This suggests that p-TrkA plays a significant role in the biology of this disease and may be a relevant therapeutic target, especially for MM cells in effusions.

We subsequently studied the anatomic site-related expression of angiogenic molecules in MM, including VEGF, IL-8, bFGF and heparanase. Heparanase is an endoglycosidase that degrades heparan sulfate in the extracellular matrix (ECM) and cell surfaces and has a role in cancer metastasis and angiogenesis (Edovitsky, 2004). We found significantly lower heparanase and bFGF expression in effusions compared with solid tumors (Davidson, 2004) (Figures 1-F, 1-G). This finding is comparable to our previous data in ovarian and breast carcinoma (Davidson, 2002b; Konstantinovskiy, 2005) and suggests a reduced need for pro-angiogenic stimuli in effusions.

The above data suggest that multiple growth factor-initiated survival pathways may be activated in MM, although expression of some of these molecules may differ as function of the anatomic site and microenvironment. Studies investigating the therapeutic value of different receptor tyrosine kinase inhibitors are at advanced phase for many tumors, and inclusion of these agents in treatment protocols may be relevant for at least a subset of MM patients (Kindler, 2004).

Apoptosis and Cell Cycle Molecules

As is the case for the majority of cancers, MM cells have deregulated response to pro-apoptotic signals

and activate or deactivate cell cycle molecules that mediate sustained proliferation (reviewed in Fennell, 2004). Earlier work has shown that higher protein expression of the cell cycle inhibitor p27^{kip1} predicts longer survival in MM (Beer, 2001; Bongiovanni, 2001; Baldi, 2004), although this correlation was lost in multivariate analysis (Baldi, 2004). Protein expression of p21, another cell cycle inhibitor, correlated with better survival in univariate analysis in the latter study (Baldi, 2004), but showed no correlation with survival in an additional report (Isik, 2001). Interestingly, two independent studies found that both high proliferation and high apoptosis correlate with worse survival in MM (Beer, 2000; Kahlos, 2000), the latter report showing that higher levels of the anti-oxidant enzyme manganese superoxide dismutase are associated with low proliferation (Kahlos, 2000). Data from the same group show that the anti-apoptotic proteins Bcl-X and Mcl-1 and the pro-apoptotic protein Bax are co-expressed in MM, with less frequent expression of another anti-apoptotic protein, Bcl-2 (Soini, 1999). A recent study similarly showed co-expression of the anti-apoptotic proteins Bcl-2, Bcl-XL and Mcl-1, with variable loss of expression of the anti-apoptotic Bad, Bak, Bax, Bid and Bim (O’Kane, 2006). Expression of Fas ligand and loss of Bax were recently shown to correlate with shorter survival (Kokturk, 2005). Silencing methylation of TNF-related apoptosis-inducing ligand (TRAIL) receptors has been found in various cancers, including clinical specimens and MM cell lines, documenting an important mechanism for the evasion of apoptosis (Shivapurkar, 2004).

Recently, there has been growing focus on the role of the inhibitor of apoptosis (IAP) family in cancer. IAPs are caspase inhibitors that prevent apoptosis by specifically inhibiting caspases 3, 7 and 9. To date, eight human IAPs have been identified: cellular IAP1 (c-IAP1), cellular IAP2 (c-IAP2), neuronal apoptosis inhibitory protein (NAIP), Survivin, X-linked IAP (XIAP), Apollon, testis-specific IAP (Ts-IAP), and Livin (Nachmias, 2004). Frequent Survivin expression was found in MM cell lines and solid tumors, and blocking of Survivin by antisense oligonucleotides induced apoptosis in the MS-1 and H28 MM cell lines (Xia, 2002). Survivin levels decreased following treatment with Cisplatin, and treatment with anti-Survivin oligonucleotides resulted in p53 activation and sensitization to apoptosis in the ZL34 cell

line (Hopkins-Donaldson, 2006). Survivin mRNA expression was found to be elevated in pleural MM and inflammatory pleuritis compared to normal pleura using real-time PCR (Falleni, 2005). c-IAP1 expression was reported to be elevated in pleural MM compared to normal pleura and lung tissue, and antisense targeting of cIAP-1 resulted in caspase 9 cleavage and sensitization to apoptosis in the 94-589 cell line (Gordon, 2002a).

We recently analyzed the expression of XIAP, Survivin and Livin in MM (Kleinberg, 2007). Using immunoblotting, we detected expression of XIAP and Survivin, but no expression of Livin, in effusion specimens. Immunohistochemical analysis of 112 MM showed significantly higher XIAP expression in peritoneal compared to pleural MM and in effusions compared to solid lesions, with reduced expression of nuclear (postulated proliferation-related) Survivin and the proliferation marker Ki-67 in effusions compared to solid tumors. These data suggest that XIAP and Survivin, but not Livin, are frequently expressed in MM, and that the reduced nuclear Survivin expression in effusions may be related to lesser degree of proliferation. The upregulation of XIAP in MM effusions and in peritoneal mesothelioma suggests a major pro-survival role for this IAP member at these anatomic sites.

These studies suggest that deregulation of cell death and survival by different mechanisms is frequent in MM, and that modulation of these pathways may be considered as a therapeutic modality in this cancer.

Differentiation Markers

Two molecules that have received attention with respect to MM diagnosis and prognosis in recent years are the Wilms’ tumor 1 (WT1) gene and mesothelin. WT1 is a tumor suppressor gene localized to chromosome 11 that is inactivated through mutation in 10% of Wilms’ tumors, a pediatric malignancy of renal origin. However, it is expressed in other tumors, such as leukemia, desmoplastic small round cell tumor and MM, and may function as both tumor suppressor and survival factor (Scharnhorst, 2001). WT1 expression in MM correlates with expression of Syndecan-1 (Kumar-Singh 1998), but not of EGFR and IGFR, two transcriptional targets of WT1, or survival (Kumar-Singh, 1997). WT1 does seem to have a role in differentiating MM from lung and other AC, though not from the closely-related serous AC of

the ovary, the main differential diagnosis of MM within the peritoneal cavity (Kumar-Singh, 1997).

Mesothelin is a 40-kDa cell surface glycoprotein that is synthesized from a 69-kDa precursor (Chang, 1996). Mesothelin binds to phosphatidylinositol, and dissociates from this signaling-related protein following treatment with phospholipase C (Chang, 1996). The biological role of mesothelin is uncertain at present, although it may have a role in cell adhesion (Chang, 1996). Mesothelin is frequently expressed on RM and MM, but despite earlier claims that its expression is limited to cells of mesothelial lineage (Urwin, 2000; Ordonez, 2003b), has been shown to be expressed in several tumor types, including non-mucinous ovarian, lung and pancreatic AC (Miettinen, 2003; Ordonez, 2003b). In view of the fact that lung and ovarian AC are two of the main differential diagnoses for MM, mesothelin appears to have limited value as a diagnostic marker. Mesothelin may yet have a role in other contexts. Serum mesothelin levels are elevated in MM, suggesting a role in early diagnosis of this tumor in high-risk populations (Robinson, 2003), a role as marker for monitoring treatment response (Hassan, 2006), and a potential target for tumor-related therapy (Hassan, 2004). A role in disease monitoring in MM was recently suggested also for megakaryocyte potentiating factor, a protein that is cleaved from a mesothelin precursor (Onda, 2006).

Intracellular Signaling

Signals originating from cell surface receptors are relayed to the nucleus via intracellular networks that are predominantly regulated through activation of kinases and phosphatases. Molecular events that occur following exposure to asbestos *in vitro* have been shown to affect mitogen-activated protein kinase (MAPK) signaling (Zanella, 1996; Ramos-Nino, 2002; Shukla, 2003; Swain, 2004). Our group recently investigated protein expression (level) and phosphorylation status (activation) of the three MAPK members- extracellular-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and high osmolarity glycerol response kinase (p38) in RM and MM specimens. Expression and activation of p38 (phospho-p38, p-p38) was found in the majority of specimens, with less frequent phosphorylation of ERK and JNK (Figure 1-H). MM and RM cells showed similar MAPK expression,

activation and activation ratio. These data are the first evidence of *in vivo* activation of MAPK in clinical MM and RM. The similar values in these two groups suggest that MAPK may not be involved in the transformation of benign to malignant mesothelium, and therefore question the validity of MAPK as molecular therapeutic targets in MM (Vintman, 2005).

Recent work has shown that Wnt-1, member of the Wnt family, is involved in inhibition of apoptosis in MM *in vitro*, and that inhibition of Wnt-1 or its receptor Dishevelled induces apoptosis via JNK activation (You, 2004). These data are supported by the frequent inactivation through methylation of several secreted frizzled-related proteins, inhibitors of the Wnt pathway, in MM cell lines and clinical specimens (Lee, 2004).

Although these studies provide important data regarding intracellular signaling in MM, our understanding of these pathways in clinical MM is minimal at present, and their complexity warrants further research.

The Immune Response

The host response in cancer involves the production of an array of molecules designed to inhibit cancer cell growth and metastasis. In reality, this process often achieves the opposite effect due to the ability of tumor cells to utilize host-produced molecules as growth promoters and to evade immune response-related mechanisms (Elenbaas, 2001).

Asbestos inhibits the activity of lymphocyte-activated killer cells (LAK) *in vitro* (Manning, 1991). Tumor-infiltrating lymphocytes show reduced expression of activation markers and the production of cytokines is dysregulated in a murine MM model (Bielefeldt-Ohmann, 1994). IL-12 and IL-2 have been shown to induce an anti-tumor effect in experimental models (Caminschi, 1998; Porta, 2000), while patient treatment with recombinant GM-CSF does not induce disease regression (Powell, 2006). However, anti-tumor effect was seen with use of soluble type II TGF- β receptor and interferon- β in experimental models (Odaka, 2001; Suzuki, 2004). Improved survival was observed following treatment of MM patients with Adenovirus-mediated herpes simplex virus thymidine kinase/ganciclovir gene therapy (Serman, 2005).

We recently studied two aspects of the immune response in MM. In the first study, we analyzed the expression of HLA-G, a non-classical major

histocompatibility complex (MHC) class I that has been hypothesized to mediate cancer cell evasion of the host immune response (Algarra, 2004). We found that MM cells only rarely express HLA-G, but that expression is significantly higher in effusions (Figure 1-I), suggesting that cells at this site may utilize this surface molecule to evade the immune response in effusions (Kleinberg, 2006).

Chemokines are a family of small molecules that regulate the immune response and mediate several cancer-related events via specific receptors (Homey, 2002; Wilson, 2002). Chemokines produced by cancer and stromal cells attract lymphocytes and monocytes expressing their receptors to the tumor site. However, chemokine receptors have also been reported to be expressed on tumor cells, thereby creating an autocrine loop that mediates pro-growth signals (Homey, 2002). A recent study using a cytokine array system showed that the supernatant of cultured MM cells and the effusion fluid contain 25 different chemokines (Hegmans, 2006). We analyzed the characteristics of the leukocyte infiltrate expression and the expression of 5 chemokine receptors in MM and RM effusions. Chemokine receptors were frequently expressed on leukocytes in MM and RM effusions, but were infrequently present in MM cells and universally absent in RM cells. This finding suggests a major role for an autocrine chemokine pathway in leukocytes, but not in MM cells. We additionally found increased monocyte infiltration and monocyte chemokine receptor expression in MM compared to RM effusions, suggesting these cells may have a tumor-promoting rather than inhibiting effect (Davidson, 2007).

High-Throughput Methods

Advances in molecular techniques allow for multi-parameter analysis of genes (comparative genomic hybridization, CGH), mRNA transcripts (cDNA arrays) and proteins (proteomics). Among these methods, cDNA analysis is the one that has been most often used in studies of MM. Comparative analysis of clinical specimens and MM cell lines identified candidate gene products that are upregulated in the former group, such as matriptase (Hoang, 2004b).

Comparative analysis of lung AC and pleural MM identified several markers that are differentially expressed by these two tumors, including calretinin and thyroid transcription factor-1 (TTF-

1), that are commonly used in the diagnostic setting (Gordon, 2002b). Two studies in which MM specimens were compared to benign pleura and/or RM-derived cell lines have revealed a large number of genes that are differentially expressed in these two conditions (Singhal, 2003; Kettunen, 2005). Singhal et al. found 166 genes that were upregulated and 26 that were downregulated in MM, including cytoskeletal elements (e.g. annexins, integrins, keratins), molecules involved in protein synthesis and in metabolic pathways and gene products defined as having therapeutic and prognostic implications (Singhal, 2003). Kettunen et al. analyzed the expression of 588 gene products and found several that were over-expressed in MM compared to benign mesothelium (the collagen 1A2 chain and the β 4 integrin subunit) and RM-derived cell lines (e.g. ezrin, bFGF, the tPA and uPA plasminogen activators, N-cadherin, cytokeratin 7), and others that were reduced in malignant cells (TRAIL, cytokeratin 19, α 3 integrin subunit) (Kettunen, 2005). The latter study also reported histologic type-specific upregulation of gene products (e.g. P-cadherin in epithelioid MM; MMP-9 and tPA in sarcomatoid MM), thus providing molecular correlates for these morphologic differences (Kettunen, 2005). Two recent studies focused on the prognostic value of different molecules in MM (Gordon, 2003; Pass, 2004). Gordon et al. found several mRNAs that are expressed in higher levels in MM cases with better (e.g. hyaluronan synthase) and worse (e.g. insulin-like growth factor-binding protein-3; IGFBP-3) outcome (Gordon, 2003). Pass et al. found 27 genes with predictive value using two different statistical tests (dChip and SAM), including the α 6 integrin subunit, the metastasis suppressor nm23, fibroblast growth factor 7 and IGFBP5 (Pass, 2004). A recent study by Lopez-Rios et al. identified several new genes that aid in the differentiation between sarcomatoid and epithelioid MM, including Uroplakin 1B and 3B, kallikrein 11, claudin 15 and Annexin A9, all more highly expressed in epithelioid MM (Lopez-Rios, 2006). Of note, the authors report relatively low predictive value for survival for this method, with no significant additive power to data obtained by standard clinicopathologic variables and p16/CDNK2A status, suggesting that global gene expression profiling may have a greater role as a research tool than in clinical practice (Lopez-Rios, 2006).

Along this line, our group recently performed a cDNA analysis comparing diffuse malignant peritoneal mesothelioma (DMPM) and ovarian carcinoma cells in effusions, two tumors with common histogenesis that share expression of

many diagnostic and differentiation markers. In this analysis, we identified 189 genes that are differentially expressed in these cancers, including higher gene expression of calretinin, vitronectin, claudin 15, $\alpha 4$ laminin and hyaluronan synthase 1

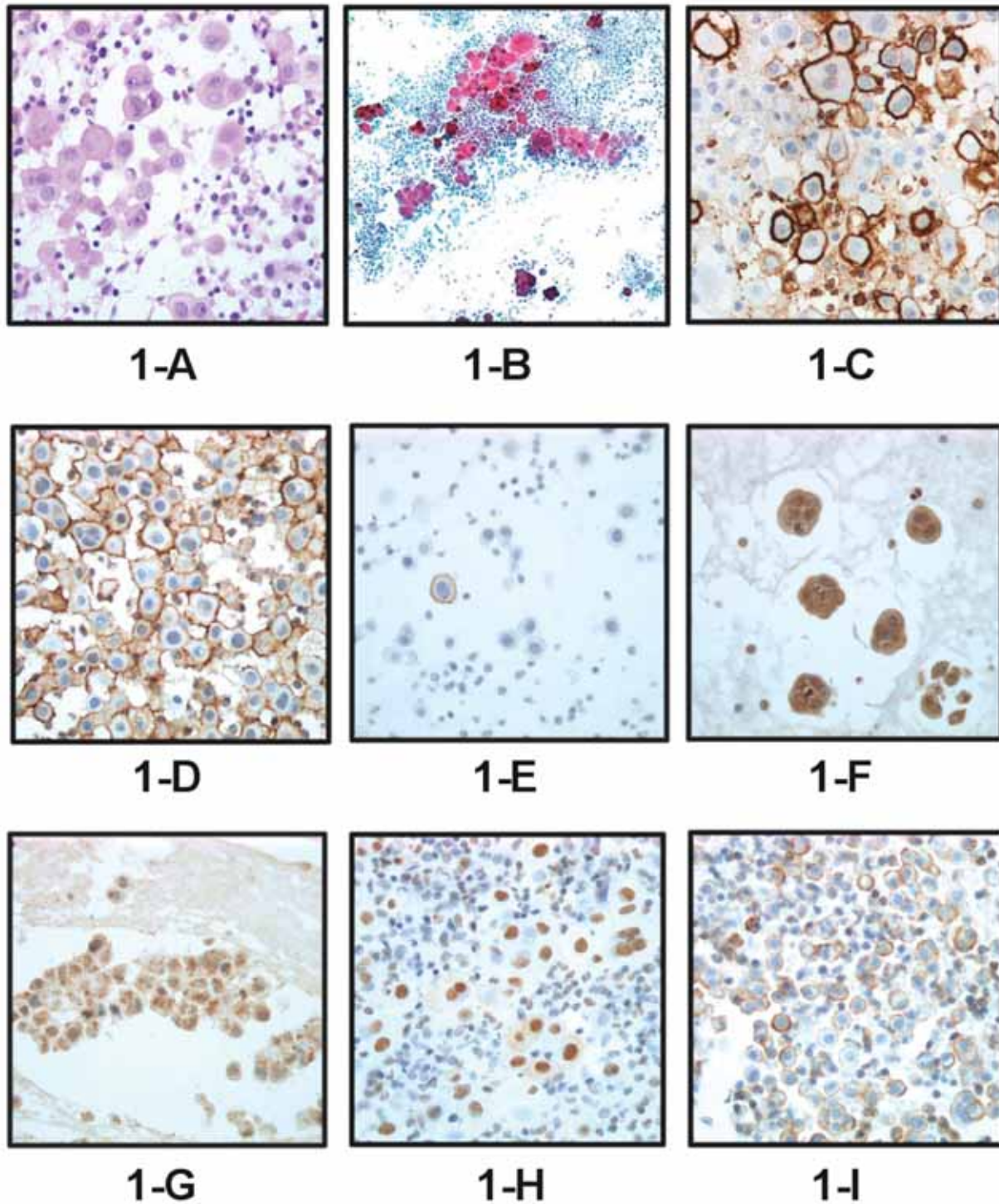


Figure 1. **A:** H&E staining of a cell block section from a pleural mesothelioma; **B:** PAP-stained smear from the same specimen; **C:** Protein expression of the 67kDa laminin receptor, a rare event in mesothelioma; **D:** p-TrkA membrane expression in a pleural mesothelioma; **E:** Focal p75 expression in the same specimen. Only one cell expresses this receptor; **F-G:** Heparanase (F) and bFGF (G) cytoplasmic expression in two pleural mesotheliomas; **H:** Nuclear expression of activated ERK (p-ERK) in the pleural mesothelioma shown in figures A-B; **I:** membrane expression of HLA-G in a pleural mesothelioma effusion.

in DMPM, and higher IGF-II, IGFBP-3, cyclin E1, folate receptors 1 and 3, RAB25, MUC4, endo-thelin-1, CD24, kallikreins 6/7/8, claudins 3/4/6, Notch3 and MMP-7 expression in ovarian carcinoma (Davidson, 2006).

How this novel knowledge will impact on the diagnosis and clinical management of MM is yet to be seen. One area that has not yet received sufficient attention is large-scale protein analysis. Although data on mRNA level reflect the transcriptional activity of MM cells, protein analysis is more directly related to the biological activity of these molecules. Hegmans et al. recently studied the protein profile of exosomes, small membrane vesicles that are secreted into the ECM, in MM cell lines using the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). The authors identified several proteins that are secreted by MM cells *in vitro*, including MHC class I antigens, heat shock proteins (HSC70, HSP90), and cytoskeletal proteins (ezrin, actinin-4) (Hegmans, 2004). If confirmed in clinical material, these proteins may teach us more about the microenvironment of MM and provide new molecular targets for directed therapy.

Concluding Remarks

The rise in the incidence of MM presents a growing health problem and emphasizes the need for improved diagnosis, prognostication and treatment in this cancer. The body of work reviewed in this paper documents the growing effort to understand the biology of this disease and its genetic make-up, and to define the biological characteristics that favor the survival of MM cells. Our own data reveal differences between peritoneal and pleural MM, and between MM cells in solid lesions and effusions, suggesting that the tumor microenvironment in part regulates the synthesis of cancer-associated molecules in MM.

To date, our understanding of MM biology and the efforts to target key-molecules in this disease have not significantly altered the poor survival associated with this cancer type. However, knowledge regarding the biology of MM is being more frequently translated into new treatment approaches. Vogelzang et al. recently reviewed new agents that under evaluation for the treatment of MM, including EGFR, PDGFR, VEGF and HGF inhibitors, inhibitors of mTOR, a downstream molecule of the PI3K/AKT pathway, and inhibitors of the proteasome/ubiquitin pathway (Vogelzang, 2005).

Although recent results in clinical trials using the tyrosine kinase receptor inhibitor imatinib mesylate, which targets PDGFR and c-Kit, have not been encouraging (Mathy, 2005; Porta, 2007), it is to be hoped that the common effort of medical and research disciplines will in the future allow us to achieve more success in treating this highly lethal tumor.

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