Cherubini E, et al., J Cytol Tissue Biol 2016, 3: 011
DOI: 10.24966/CTB-9107/100011

HSOA Journal of Cytology and Tissue Biology

Review Article

Inducers of Epithelial Mesenchymal Transiton and Cancer Stem Cells in Malignant Pleural Effusions

Emanuela Cherubini[1]*, Enrico Giarnieri[1,2], Alberto Ricci[1,2], Salvatore Mariotta[1,2], Maria Rosaria Giovagnoli[1,2] and Rita Mancini[1,3]

[1]Department of Clinical and Molecular Medicine, Sapienza University of Rome, Rome, Italy

[2]Azienda Ospedaliera Sant'Andrea, Rome, Italy

[3]Laboratory of Research and Diagnostics, Department of Surgery "P.Valdoni", Sapienza University of Rome, Rome, Italy

Abstract

The Epithelial to Mesenchymal Transition (EMT) plays a role not only in tumor metastasis but also in tumor recurrence. This process is believed to be tightly linked to the presence of Cancer Stem Cells (CSCs) however, it is still not clear which factors could induce EMT and how it could be a source for CSCs. It has been demonstrated that Malignant Pleural Effusion (MPEs) may represent an excellent source to identify markers and molecular mechanisms involved in EMT and CSCs development. Growth factors, cell differentiation markers and molecular adhesion are involved in some of the crucial neoplastic cell events such as proliferation, metastasis, resistance to chemotherapy and EMT. In this review, we summarize the current understanding of which molecular markers can orchestrate EMT and CSCs in MPEs.

Keywords: Cancer stem cell; Epithelial mesenchymal transition; Malignant pleural effusion; Tumor markers

Introduction

Lung cancer has produced the highest mortality rate in the world, current therapy is relatively ineffective and the survival rate at 5 years is still only 15% for the advanced disease. The presence of neoplastic cells in the pleural fluid represents a common medical problem in cancer patients with advanced neoplastic disease and it leads to poor survival [1-8]. Lung and breast cancers cause approximately 75% of all MPE. However, for around 10% of MPE cases, the primary tumor is unknown [9-13]. In MPEs it has been observed that neoplastic cells produce factors that contribute to overcoming the protective mesothelial layer. For example, neoplastic cells are capable of internalizing the CD44-hyaluronan complex and hydrolyzing it in oligosaccharides showing increased permeability in the mesothelial layer and angiogenic chemotactic ability [14]. Furthermore, VEGF and bFGF produced by neoplastic cells increase the permeability of the pleural surface [15]; a low level of endostatin observed in patients with malignant pleural effusion increases endothelial cell migration, angiogenesis and tumor growth [16]. Microenvironment, hypoxia and chemokines can modify the mesothelial cell phenotype. The ability of these cells to switch dynamically between different phenotypic states led to a series of studies in which different authors demonstrated that MPEs could be an excellent source in cancer biology investigation and the identification of potential target therapy solutions. Following this, studies identified the presence of small sub-populations of cells, also named cancer stem cells or cancer initiating cells, within the tumor cells, causing the aggressive behaviour of cancer cells [17-19]. The presence of these sub-populations, capable of self-renewal and multipotent differentiation, could add a new element in cancer research, explain the concept of heterogeneity, relapse after treatment and resistance to conventional chemotherapies.

Inducers of EMT

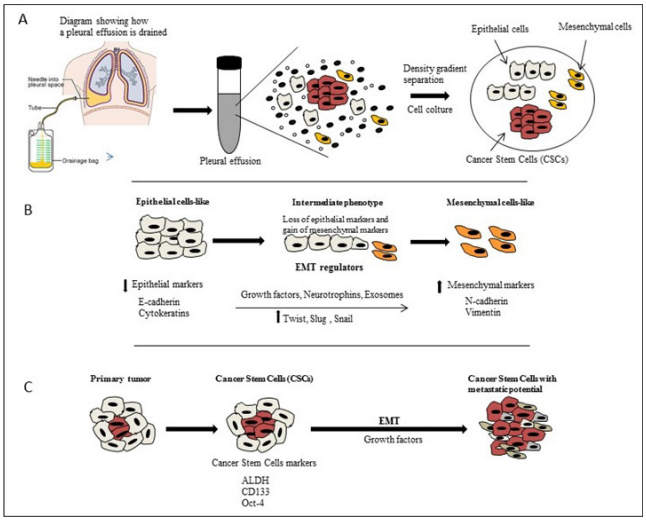
The expression of EMT markers and of their related transcription factors has often been studied in stabilized cell lines. So far, very limited analysis has been performed in fresh cultures from primary tumors. It is widely accepted that cancer stem cells are represented by a distinct subset of cancer cells. In fact, in comparison with cancer cells, CSCs are distinct in their ability to self-renewal, form tumors in immune-deficient mice and differentiate in other cell types. This distinct population was initially identified in leukemias, but subsequently identified in several solid malignancies (breast, lung, prostate, colon, brain, head and neck, liver) as well as in MPEs [20-25]. In pleural effusions, cancer cells are susceptible to anoikis and apoptotic triggers, grow in suspension and develop compact multicellular spheroid aggregates. These cellular aggregates can resist against anoikis and apoptosis, and probably chemotherapeutic agents. It has been observed that the presence of elevated integrin attachments, available inside the tridimensional aggregate configuration, favors the protection and survival of cells [26]. In patients with ovarian cancer, peritoneal fluids and ascites, frequently exhibit spheroid aggregates, raising interest in their formation and function [27]. An association between contractile behavior, compact spheroid-forming ability, and the invasive capacity of cancer cells in 3D has been detected [26]. Cancer cells are able to form spheroids [28-30]. It has been suggested that there could be a positive correlation between spheroid formation and tumorigenity [30], such as the possibility that spheroid cancer cells gain invasive properties by undergoing EMT [26,31]. Epithelial plasticity is the ability of the cells to switch from a different state of phenotype, and the EMT is considered a transition from epithelial to mesenchymal phenotype (Figure 1 A,B,C). On the base of function and pathways, EMT shows different subtypes: embryonic development, tissue repair, inflammation and cancer [32]. The progression of most carcinomas

***Corresponding author:** Emanuela Cherubini, Department of Clinical and Molecular Medicine, Sapienza University of Rome, Rome, Italy, Tel: +39 0633775314; E-mail: emanuela.cherubini@uniroma1.it

Citation: Cherubini E, Giarnieri E, Ricci A, Mariotta S, Giovagnoli MR et al. (2016) Inducers of Epithelial Mesenchymal Transiton and Cancer Stem Cells in Malignant Pleural Effusions. J Cytol Tissue Biol 3: 011.

Received: Septembert 16, 2016; **Accepted:** November 01, 2016; **Published:** November 14, 2016

toward malignancy is associated with the loss of epithelial differentiation and by switching towards mesenchymal phenotype with increasing cell motility and invasion. Recent studies have demonstrated that EMT plays a critical role not only in tumor metastasis but also in tumor recurrence, which is believed to be tightly linked to the presence of CSCs [33-35]. It is still not clear which factors could induce EMT and how the EMT could be a resource for CSCs [36]. Hypoxia but also cytokines can be factors inducing EMT activation but also transcription factors and adhesion molecules are differentially expressed [37].



Figures A and B: The EMT mechanism transform MPE cells like from epithelial to mesenchymal cell like with suppression of epithelial and activation of mesenchymal regulator markers.

Figure C: A subset of MPEs cells, known as CSCs, are tumorigenic and these cells are defined by their capacity for self renewal and differentiation plasticity. Furthermore these cells show CSCs and EMT markers suggesting that EMT is also thought to be a mechanism by which CSCs form.

Exosome

Recently, studies have evaluated the biology and composition of exosomes in cancer development has been observed that exosomes derived from tumor cells, communicate with stromal cells and vice-versa to promote tumor growth [38]. Exosomes released from cancer cells, may affect normal cells through the intercellular transfer of oncogenic materials such as DNA, mRNA, regulatory miRNA, oncoproteins and MHC class I and II proteins [39,40]. Furthermore, tumor and stromal cells can regulate the invasiveness of cancer cells through exosome-mediated delivery of protein and miRNA in the regulation of EMT-related pathways. In lung cancer cells and in late stage of lung cancer serum, an involvment of exosome as drivers of epithelial to mesenchymal transition has been recently reported, furthermore Lin et al., demonstrated that contents exosomes miR-205-5p and miR-200b were markedly increased in malignant pleural effusion [41,42].

E-cadherin

Down-regulation of E-cadherin and up-regulation of N-cadherin has been reported in tumor cells suggesting that EMT can occur heterogeneously and/or transiently within an invasive tumor [43]. On the contrary Zhao et al., observed that tumor cells in the pleural effusions mainly expressed an epithelial E-cadherin phenotype marker. The high expression of E-cadherin was associated with EGFR mutation predicting better outcome [44].

Vimentin and LASP-1

Vimentin, an intermediate filament protein normally expressed in mesenchymal cells, can be expressed in epithelial cells undergoing EMT, in both physiological and pathological conditions [45]. Vimentin and N-cadherin up-regulation and reduction of E-cadherin protein expression are representative of EMT markers in several tumor types. In breast and lung cancer, up-regulation of EMT markers like Vimentin, N-cadherin, cadherin-11, smooth- muscle-actin, and the reduction of characteristic epithelial markers like E-cadherin and cytokeratin has been frequently observed and associated with high aggressiveness and metastatic activity [37]. In papillary thyroid cancer *in vitro* studies demonstrated that Vimentin was required for the development and maintenance of a mesenchymal morphology and invasiveness, concluding that EMT is a common event and that Vimentin regulates EMT in thyroid cancer [46]. EMT is regulated by the activation of a cascade of transcription factors among which the most frequently involved are Snail, Slug and Twist. Twist is a highly conserved basic helix-loop-helix transcriptional factor. Its expression induces mesenchymal markers such as N-cadherin, Vimentin and fibronectin [47]. However the loss of E-cadherin expression is necessary but not sufficient to trigger EMT in cells over-expressing Twist. In addition, restoration of E-cadherin expression does not revert the mesenchymal phenotype [48]. Recently, has been reported a relationship between vimentin and LASP 1 in undifferentiated HCC cell lines. Authors sustained that Vimentin (VIM) is a new molecular partner of LASP-1, an important hallmark of the epithelial-mesenchymal transition [49]. Further studies will be needed to verify a possible relationship between LASP-1 and cancer cells derived from malignant pleural effusion.

Twist

Twist, a highly conservative basic Helix-Loop-Helix (bHLH) transcription factor, is overexpressed in a variety of human tumors and associated to cancer invasion, metastasis and poor prognosis. Also it plays an important role in multiply processes including angiogenesis, resistance to apoptosis, multidrug resistance and EMT [50- 53]. Twist expression enhanced cell migration, tumor-sphere formation and stem-like markers [54] and up-regulation has been reported in invasive breast cancer, esophageal, parathyroid, hepatocellular and human bladder carcinoma [46,55-57]. In MPEs, Twist in adherent and spheroid cells, showed an extremely variable pattern of expression from sample to sample, which under scores the heterogeneity of malignant pleural effusion cells.

Snail and slug

Snail (Snai1) and Slug (Snai2), evolutionarily conserved members of the Snail family of zinc finger transcription factors, play an important role in embryonic development. They regulate the process of EMT, characterized by loss of intercellular adhesions and acquisition of a migratory phenotype [58]. Snail and Slug have been shown to be involved in pancreatic cancer development [59] and their effects were observed to be most active at the invasive front of tumors in invasive human breast cancers [60].

TGF-β

Inflammatory cells are the main source of growth factors. In MPEs, neoplastic cells are frequently associated to a variable presence of inflammatory cells. TGF-β1 is an inductor of pleurodes and regulate proliferation, migration and differentiation cell, and a potent chemoattractant for fibroblasts. Authors observed that mesothelial cells

stimulated by TGF-β leads to collagen synthesis, matrix proteins, matrix metalloproteinase-1 and 9, and tissue inhibitor of matrix metalloproteinases-2 [61,62]. EMT can be induced or regulate by TGF-β not only during embryogenesis but also in fibrosis and cancer. TGF-β is considered a key effector of EMT during cancer progression and metastasis. Studies showed that transgenic expression of activated TGF-β1 correlates with invasive spindle cell carcinomas. TGF-β1 production by cancer cell triggers EMT, enhances angiogenesis providing an exit route for migratory mesenchymal cells [63].

VEGF

Vascular Endothelial Growth Factor (VEGF) is a permeability and angiogenic factor mediating neovascularization [64]. Its expression is up-regulated in activated pleural mesothelial cells and produced in large amount in inflammatory and malignant effusions [65-67]. VEGF control mesothelial cells permeability throught phosphorylation of adherens junction proteins and dynamic interactions between molecular adhesions. Exposure to noxious stimuli, the interaction of surface ligands with intercellular molecules expressed on mesothelial cells can cause cell migration and dispersion of high molecular weight proteins across the pleural membrane leading pleural effusion creation VEGF stimulation of normal epithelial cells and differentiated carcinoma cells can induce EMT [68,69], at the same time, hypoxia induces EMT in mesothelial cells by activation of HIF-1α, the major driver of VEGF expression in tumors [70,71].

PDGF

Mesothelial cells are able to produce Platelet-Derived Growth Factor (PDGF) a mitogenic cytochine that stimulate hyaluronan production and stimulate growth of fibroblast. Among PDGF family polypeptide chains encoded by four genes known as PDGF-A, B, C, D, PDGF factor D (PDGF-D) overexpression has observed in a variety of cancers. Overexpression of PDGF-D cells showed loss or relocation of E-cadherin, increased expression levels of vimentin that taken together contributes to EMT in human cancer [72].

Neurotrophins and TrK receptors

Recently, particular interest is given to the neurotrophin growth factor family. The term Neurotrophins (NTs), including Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), NT-4/5 and NT-6, refers to a family of related polypeptide growth factors whose activities were originally related to a variety of neural cell types. The biological effects of NTs are mediated through two unrelated classes of cell-surface membrane receptors characterized by different binding affinities and molecular weights [73,74]. All the NTs interact with a transmembrane glycol-protein without a direct catalytic function, the 75 kDa low-affinity p75 receptor [75]. p75 receptor belongs to the tumor necrosis factor receptor family. NTs also bind distinct members of a superfamily of 140 - 145 kDa high affinity transmembrane tyrosine kinase receptors known as Trks [76]. NGF interacts with TrkA, BDNF and NT-4/5 with TrkB and NT-3 with TrkC receptor. Nts are involved in the transformation and tumor progression of many types of solid tumors and hematological malignancies [77]. NTs and their receptors are widely expressed in many lung cancers [78] and this expression suggests that NTs may be involved in controlling growth and differentiation of human lung cancer. In particular, the TrkB/ BDNF axis has a very important role in the proliferation, differentiation and tumor invasion and, furthermore, has been reported that TrkB is a negative prognostic factor in lung cancer [79]. Despite the growing interest in the NT system in several lung diseases and in lung cancer [80-84], their role in malignant pleural effusion has been investigated only sparsely [85]. *In vitro* cell cultures obtained from lung adenocarcinoma pleural effusion showed that TrkB is required for the maintenance of cells with slow proliferation that are still capable of growing in non-adherent conditions, rather than for the survival of progenitor and terminally differentiated cells [86]. TrkB is considered a promoter of EMT and anoikis resistance. Authors reported that TrkB is an inducer of EMT in head and neck, colorectal cancer and, recently, in human salivary adenoid cystic and endometrial carcinoma [87-89]. In metastatic breast cancers, the acquisition of metastatic ability, which leads to clinically incurable disease and poor survival, has been associated with acquisition of EMT program and CSCs via activation of PI3K/AKT and IL6/JAK2/STAT3 signaling pathways [90].

Cancer Stem Cell Markers

It has been recently demonstrated that MPEs could be an excellent source for growing *in vitro* and *in vivo* cell cultures reproducing the natural heterogeneity of primary lung adenocarcinomas. Furthermore, the presence and proliferation of cancer cells in pleural effusion may be mediated by cancer stem cells, which also risk undergoing Epithelial Mesenchymal Transition (EMT). In the case of MPEs, fluids contain a population with enormous self-renewal and regeneration capacity, ability to escape apoptosis and resistance to anoikis. In MPEs, putative CSCs have been identified using a variety of markers. The identification of CSCs is carried out on the basis of molecular expression markers. However, these markers are not always and uniformly expressed across tumor types, for this reason the topic is still under discussion.

ALDH

Aldehyde Dehydrogenase (ALDH) is a marker frequently used to distinguish normal and malignant stem/progenitor cells. Through oxidation of retinol to retinoic acid, ALDH is involved in early stem cell development [91]. Initially used to sort haematopoietic stem cells [92], ALDH contributes to drug resistance through the detoxification of many cytotoxic agents and has been reported as a reliable CSC marker in several tumor types [92-94]. Isolated ALDH1- positive cells from stable cell lines show features of CSC. This has been correlated with poor prognosis for patients with early-stage NSCLC [95]. Recently, through primary cultures obtained from MPEs, authors have described the existence of cells with ALDH1 activity by FACS analysis both in adherent and spheroid culture [96]. Results showed that in the majority of samples analyzed, the percentage of ALDHbr (ALDH-bright) cells increased upon culturing in spheroid conditions, providing information about the presence of putative CSC in MPE primary cultures [29].

CD133

Another marker used to identify CSCs is the CD133, a member of transmembrane glycoproteins. CD133, also known as prominin-1, is a cell-surface transmembrane glycoprotein that has been used in the identification of putative CSCs in several malignant tumors. These cells have shown increased tumorigenic potential in transplantation studies *in vitro* and *in vivo* and, in clinical studies, an association with poor prognoses and distant metastases [97-99] however, the exact function of CD133 has not yet been established. In NSCLC patients, significant increase in CD133 cells has been observed suggesting the involvment of this cell population in tumor growth vasculogenesis. Eramo et al., were able to isolate small niche of CD133 from SCLC

and NSCLC observing that CD133 cell population showed ability to self-renew but were not tumorigenic [100]. In a study of CSCs markers in MPEs, authors observed three different combination patterns of positive or negative protein expression of Nanog, OCT-4 and CD133 observing that the rates of immunoreactivity for these three CSC-representative markers range were from 15% to 90% associated to variations and combinations of their expression probably due to pleural effusion intratumoral heterogeneity [101].

OCT-4

OCT-4 and Nanog are both transcription factors essential for normal pluripotent cell development. Nanog is chiefly responsible for differentiation during embryogenesis whereas OCT-4 may have long-term influences on both tissue proliferation and differentiation [102]. OCT-4 is especially expressed in embryonic stem cells and germ cells, and has been detected in specific types of testicular germ-cell tumors. Moreover, it is also preferentially expressed in undifferentiated human ESCs, pancreatic islets, and diffuse-type gastric cancers. Therefore, it has been suggested that OCT-4 contributes to maintaining stem cell properties. However, several recent reports have suggested that as many as 25% of the cancer cells within certain tumors have the properties of CSCs [102]. Data suggested that cancer cells show various cell surface markers and the use of more than one marker to isolate CSCs might increase the possibility to detect cell sub-population. Therefore more investigation should be done in order to detect more markers to identify CSCs in MPEs.

Conclusion

The identification of more efficient therapies for the treatment of malignant pleural effusion in patients with metastatic lung cancer is crucial to understand the mechanisms that cause current fail treatment. Characterization of CSCs in malignant pleural effusion and recent understanding of EMT contributed to better know about environment, behavior and prognosis of this tumor. Advances have been made towards elucidating causes and mechanisms of EMT in malignant pleural effusion considering EMT process as one of possible mechanisms through which CSCs are generated. However, beyond identification and characterization of cell surface markers, there is still much that remains unknown about CSCs and EMT interaction including the mechanisms they utilize to maintain their chemoresistance.

Acknowledgement

This work was supported by AIRC Grant IG 17009 to R. Mancini.

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