Pathogenesis of Malignant Pleural Effusions and Talc Pleurodesis[1]

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In this review I hope to cover two critical areas, namely the mechanism of formation of malignant pleural effusions and the treatment of malignant pleural effusions via pleurodesis. The pathogenesis of malignant pleural effusions involves several steps. These allow the malignant cell to separate from the primary tumor, dock onto the area of the metastatic site, transmigrate through the pleural membrane and initiate autocrine proliferation. The role of several factors including integrins and hyaluronan will be described. I will use the role of the glycosaminoglycan, hyaluronan, and its interaction with the CD44 receptor to illustrate the mechanisms of metastatic tumor formation on the pleural surface.

Since talc pleurodesis remains the mainstay of treatment for malignant pleural effusions, this review will discuss the inflammatory responses induced by talc and the mechanisms of talc induced fibrosis. The concept that the normal mesothelium plays a critical role in the development of pleural fibrosis will be introduced. The possibility that talc may serve other functions besides the induction of pleural fibrosis will be discussed.

Pathogenesis of Malignant Pleural Effusions

The ability of a tumor to establish successful colonies at a point distant from the original site (metastasis) is one of its most important hallmarks. The theoretical steps of this process are known as the metastatic cascade [1,2] (Fig. **1**). This cascade of events leads to the eventual seeding of the pleural surface, and independent growth of the tumor on its pleural metastatic site. The cascade consists of: (1) loss of contact of the primary malignant cell with the surrounding tumor or neighboring cells; (2) adhesion and penetration through a vessel wall; (3) movement via migration through the vascular surface onto the pleural surface moving from the basilar surface to the apical surface of the pleura; (4) constitutive production of autocrine growth factors; (5) induction of angiogenesis allowing for local spread and growth of the tumor.

Loss of adhesion and dislodgement of neoplastic cells from the primary tumor site

Movement or dislodgement of the neoplastic cell from its in situ position requires diminishing of adherence between cells

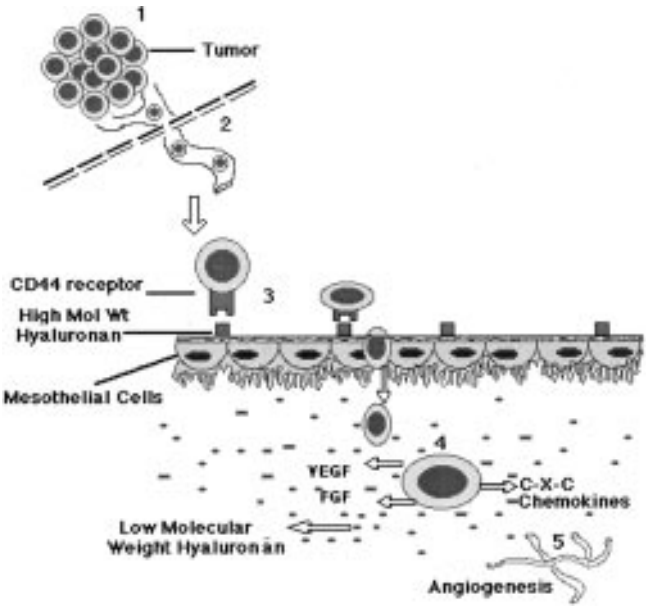

Fig. 1 Pathogenesis of malignant pleural effusions.
The metastatic cascade involves several steps:
1. Loss of adherence of malignant cell from primary tumor.
2. Adherence and penetration of blood vessel wall.
3. Migration through the pleura.
4. Constitutive production of autocrine growth factors.
5. Induction of angiogenesis.

as well as between cell and extracellular matrices [3,4]. Several studies have discussed the role of adhesion molecules expressed by malignant cells and their role in the dislodgement of neoplastic cells. For a tumor cell to lose contact with its neighboring cells, its adhesive properties must change. For example, breast cancer cells are known to express CD44 receptor which binds to hyaluronan, a high molecular weight, extracellular matrix component. Changing the breast cancer cell CD44 profile can achieve this. Increased expression of CD44 can enhance the binding of the cell to the local high molecular weight hyaluronan and increase production of low molecular weight hyaluronan. A matrix of hyaluronan in the pericellular area of the breast cancer cell would decrease the affinity of the cell for the surrounding hyaluronan deficient cells by interfering with adhesive processes, thus leading to detachment [5,6]. This CD44-ligand complex activates the

Pneumologie 53 (1999) 493 – 498

[1] Herrn Prof. Dr. R. Loddenkemper, Berlin, zum 60. Geburtstag gewidmet

actin cytoskeletal structure via signal transduction causing a rise in intracellular calcium and cyclic AMP, which is associated with protein kinase A activity inside the cell. This response then leads to movement of the malignant cell via haptotaxis along hyaluronan rich surfaces. In a variety of human tumors, expression of the surface glycoprotein CD44 correlates with advanced stages of tumor growth and metastatic potential [7,8].

Adhesion and penetration of vessel wall

Several studies indicate the role of endothelial cell adhesion molecules in the adherence and penetration of the blood vessel wall by tumor cells allowing them to disseminate. Once the tumor cell penetrates the vessel wall using various adhesion molecules such as E-cadherin and integrins, the cells reach the microvascular beds and are able to colonize metastatic sites. Malignant cells express endothelial cell adhesion molecules and this expression is modulated by cytokines. Integrins on tumor cells, for example, bind receptors on endothelial cells such as intercellular adhesion molecule-1 (I-CAM) and vascular cell adhesion molecule (V-CAM). Importantly, tumor cell proteoglycans recognize and bind platelet endothelial cell adhesion molecule (PECAM-1). The metastatic cell or group of cells may cluster with leukocytes and this increases their rate of metastasis, particularly in diseases such as melanoma [8,9].

Adhesion and migration of the malignant cell through the pleura

The docking of a malignant cell to a specific site is not an accidental phenomenon. Several factors are involved in the process of the malignant cells slowing down in the microcirculation allowing for the phenomenon of transmigration similar to that seen with inflammatory cells. The malignant cell moves towards the vessel wall and out of the main flow through the capillary. It is seen to "roll" against the vessel wall. This phenomenon is then halted by integrin mediated adhesion [10]. Hyaluronan is a high molecular weight polymer composed of the repeating disaccharide unit β-1, 3N-acetyl glucosaminyl-β-1, 4 glucuronide and is present in the pericellular and extracellular matrix of many tissues. It is produced in significant quantities by mesothelial cells and this production is augmented in certain situations associated particularly with malignant cell migration, invasion, and proliferation. The high molecular weight hyaluronan is a relatively inactive extracellular matrix component but when it undergoes hydrolysis, it produces fragments of low molecular weight which mediate many of the processes described. Hyaluronan interacts with specific malignant cell surface receptors such as CD44 and receptor for hyaluronan mediated motility (RHAMM). CD44 is a broadly expressed integral membrane glycoprotein whose diverse structure is determined by variable usage of at least ten exons encoding a segment of the membrane proximal domain and by cell type specific glycosylation and glycosaminoglycan substitution [11]. Interactions between CD44 and hyaluronan have been shown to mediate critical aspects of tumor cell adherence. In particular, we have demonstrated that the standard isoform, CD44s, which does not contain variant exon products and is highly expressed in malignant breast cancer cells is a specific mechanism whereby breast cancer cells adhere to pleural

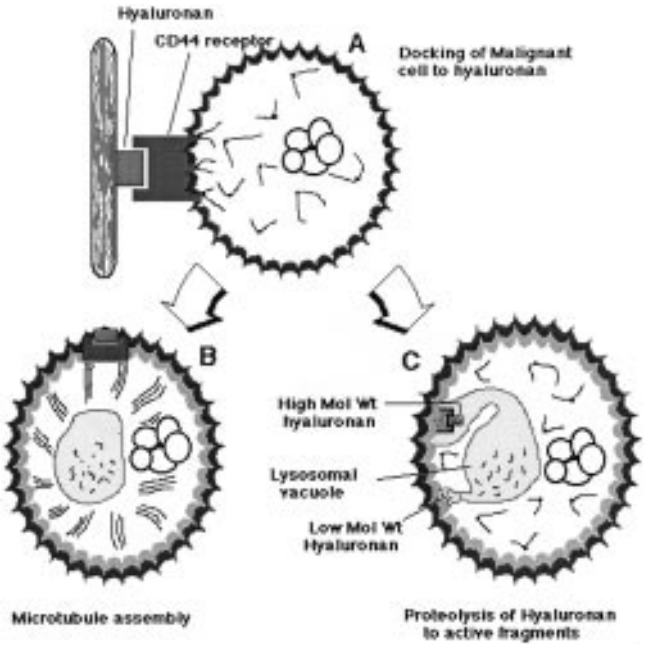

Fig. 2 The effects of docking of the CD44 receptor on a malignant cell with hyaluronan.
A. Malignant cell CD44 receptor connects with hyaluronan, an extracellular matrix protein produced by mesothelial cells.
B. The CD44 receptor is connected to the actin filaments. Occupation of the CD44 receptor on the cell surface leads to microtubule assembly allowing the malignant cell to become motile.
C. The CD44-hyaluronan complex is internalized into a lysosomal vacuole where lysosomal proteases degrade the high molecular weight hyaluronan into low molecular weight fragments. These low molecular weight fragments function as growth factors for malignant cells and are angiogenic.

mesothelial cells. Addition of CD44s antibodies to the media blocks the adherence of malignant breast cancer MCF7 cells to mesothelial monolayers [12–14]. The ability of CD44 to bind hyaluronan is very tightly regulated by the presence or absence of variant exon products. Thus, just the mere expression of CD44 by a tumor cell does not imply that it will bind to hyaluronan [15].

This process of binding of CD44 to hyaluronan is associated with a critically important step in the process of movement of malignant cells through the pleural membrane. The hyaluronan CD44 complex is internalized and then joined to a lysosomal compartment where acid hydrolases act upon it [16] (Fig. **2**). Thus, hyaluronan, a large molecular weight component, is broken up into several small, activated, low molecular weight fragments which now can further activate and participate in the process of transmigration of the malignant cell through the pleura. This degradation of extracellular hyaluronan by lysosomal hyaluronidase has profound implications. The cleavage of the N-acetyl hexosaminidic linkages allows for a series of saccharides to be formed. Upon exhaustive digestion, a tetrasaccharide is the major product followed closely by hexasaccharides and a smaller amount of higher oligosaccharides. These oligosaccharides are chemoattractant for tumor cells. They are produced in significantly greater quantities on the apical

surface of the mesothelial cell than on the basilar surface. This allows for migration of the tumor cell along the low molecular weight hyaluronan gradient through intercellular spaces into the pleural space [17]. Since binding of CD44 to hyaluronan is associated with activation of the mesothelial and malignant cell cytoskeleton, it is possible that mesothelial cell monolayers allow for intercellular transmigration of malignant cells, allowing the malignant cell to appear on the apical surface of the mesothelium in an hyaluronan rich environment where it can now establish itself and proliferate.

Constitutive production of autocrine growth factors

Tumor cells expressing CD44 may gain a selective advantage by binding growth factors and presenting them either to themselves or the neighboring cell in the process of metastatic colony formation. This binding is mediated by either covalently bound heparin sulfate side chains or by recruitment of glycosaminoglycans such as heparin or chondroitin sulfate by certain CD44 receptors. Tumor cells present growth factors in a CD44 dependent manner to mesothelial cells thus stimulating them to produce competence growth factors which are then presented to high affinity receptors on their own surfaces. For example, inhibition of growth on the target pleural surface where mesothelial cells produce growth factors can be demonstrated in tumors such as lymphoma, where intravenously injected pan CD44 antibody selectively inhibited lymphoma growth on the metastatic pleural site, but had no influence on other sites. Other studies [18–20] have shown that CD44 v6 specific antibodies inhibit in vivo tumor cell growth and metastasis. An example of growth factors which can affect tumor growth is the fibroblast growth factor (FGF) family including FGF 2, 4 and 8. FGF is a ligand for the CD44 receptor and binds specifically to heparin sulfate modifications located on the v3 exon. Addition of CD44 v6 specific antibody to a co-culture assay abrogates mesenchymal growth proliferation. In other studies [16], investigators found that suppression of metastasis formation in pleural colonization by melanoma cells occurred with blokkage of CD44. There appear to be two ways of interfering with adhesion and growth of the metastasizing tumor cells. One is blockage of adhesion molecules on the metastasizing cell by antibody or occupancy of the ligand by soluble adhesion molecules, i.e. receptor globulins [19]. In this study, the investigators analyzed metastasis and tumor growth by using both methods of blockage and found that they could indeed decrease lung colonization by melanoma by blockade of CD44. These experiments are fully in line with the hypothesis of "seed and fertile soil" and its extension towards metastasis formation based on a disturbance of the micro-environment. They point to a significant contribution of CD44 ligands in facilitating metastatic growth. We have demonstrated that interleukin-8 (IL-8) is an autocrine growth factor for mesothelioma both in vivo and in vitro.

Induction of angiogenesis

Angiogenesis is the process whereby new vessels emerge from existing endothelial lined vessels. This is an invasive process that requires proteolysis of the extracellular matrix, proliferation and migration of endothelial cells as well as the synthesis of new matrix components. It is therefore a critically important modality whereby tumor growth and further metastasis occurs [20]. The process of tumor induced angiogenesis itself results in an increased number of vessels within the tumor tissue. The vessel surface inside the tumor is a putative target of tumor cell adhesion and invasion as well as consecutive local and systemic spread. The implanted tumor cell and its expression of CD44 has been correlated to an advanced stage of tumor growth. Breast cancer has a well defined stromal vascular component and produces angiogenic factors. Recent investigations demonstrate that metastatic breast cancer cells express CD44 receptor and that inhibition of the CD44 receptor inhibits the binding to low molecular weight fragments of hyaluronan which are known angiogenic factors. Thus, the process of binding CD44 to hyaluronan allows for degradation of the high molecular weight hyaluronan into its low molecular weight fragments which are angiogenic and allows for rapid growth of new endothelial vascular sprouts and proliferation of endothelial cells both from existing vessels and differentiation of endothelial cells from stem cells. Importantly, malignant cells release several other factors that have the capacity to induce angiogenesis. These include the matrix metalloproteinases (MMP's) [21]. These further degrade components of the extracellular matrix including hyaluronan allowing for active forms of these products to be formed which then stimulate angiogenesis. Among other angiogenic factors are integrins, particularly the α-v integrins [22] and vascular endothelial growth factor (VEGF) [23]. Fibroblast growth factor-1, fibroblast growth factor-2 and hepatocyte growth factor are also angiogenic factors.

In reviewing the process of metastasis, I have tried to demonstrate the process using the interaction between a single molecule, namely hyaluronan, and its ligand, CD44, as an example. However, it is clear that there is an overlay of several different factors which come into play at different stages of the metastatic cascade. Importantly, different tumors may produce different growth factors, permeability factors and adhesion related factors. Adding to the complexity of the mechanisms during the metastasis of a malignant cell to the pleura, a single factor may have multiple functions. VEGF not only causes new vessel formation, but also alters the permeability of the mesothelial monolayer [24]. IL-8, a well described inflammatory cell chemokine, also functions as an independent growth factor for malignant melanoma cells as well as mesothelioma cells [25]. Thus, the interaction of malignant and mesothelial cells and their extracellular matrix is an example of one of the multiple pathways that allow the malignant cells to elude control by the host organism and allow its independent growth and function on the pleural surface.

Pleurodesis

Pleurodesis involves the introduction of sclerosing agents into the pleural space to achieve symphysis between the visceral and parietal pleural layers in order to prevent accumulation of either air or fluid in the pleural space. A large number of agents have been introduced into the pleural space in an attempt to achieve sclerosis. In 1935, [26] Bethune reported that when talc was introduced into the pleural space, significant sclerosis was achieved, leading to disappearance of the pleural effusion. Since that time, the installation of talc via medical thoracoscopy has been refined by several inve-

stigators. An elegant review of the process was recently published by Dr. Loddenkemper [27]. Among the sclerosing agents used are tetracycline hydrochloride, doxycycline, minocycline, bleomycin and Corynebacterium parvum. More than 30 agents have been used achieving different rates of success [28,29]. Since talc has emerged as the most effective agent and has been studied both in vivo and in vitro in some detail, I will illustrate the pathogenesis of pleurodesis using talc as the index agent.

Talc

Talc is a hydrated magnesium silicate (3 MgO 4 SiO_2 H_2O) and is produced and sold by several companies both in Europa and in the United States. It is available in a variety of particle sizes and configurations either from the biochemial companies or from the original mining source. Talc provided by the US Pharmacopeia (USP) is asbestos free, but not necessarily sterile [30]. Talc has been shown to contain bacillus species which are generally nonpathogenic. Talc requires sterilization prior to use either by dry heat exposure, ethylene oxide gas or gamma irradiation. Talc particle size when prepared in my laboratory is 2.1 ± 0.89 μ as determined with a Sony CCD-IRIS/RGB video camera (Sony Corporation, Tokyo, Japan) attached to an Olympus IMT-2 microscope (Olympus Corporation, Tokyo, Japan) interfaced with a DataStar 486-66 computer containing Image Pro Plus software (Media Cybernetics, Siversprings, MD).

Mesothelial cell responses to talc

Mesothelial cells are metabolically active cells that continuously line the pleura as a monolayer. They are the first cell type to encounter talc when instilled into the pleural space either in the form of talc insufflation or talc slurry. They are, therefore, likely candidates for initiating and propagating the acute inflammatory response seen in the pleural space mediated by talc. Recent work [31] demonstrates that talc induces a rapid neutrophil influx followed by an accumulation of macrophages in the pleural space. The investigators studied 13 consecutive patients with spontaneous pneumothorax and 8 patients with malignant pleural effusions who had received talc poudrage. The investigators found that talc administration induced rapid accumulation of neutrophils, reaching its maximum after 3 – 24 hours. In patients who had pneumothoraces, the neutrophils were seen in the pleural space after only 3 hours. In patients with malignant pleural effusions, neutrophils were seen to accumulate 24 – 48 hours following talc instillation. Among the most important findings described include the fact that the chemokine IL-8 correlated with the level of neutrophils seen in the pleural space indicating that talc induced the release of neutrophil chemokines into the pleural space. Though the authors did not find a correlation between a mononuclear cell influx and monocyte chemoattractant protein-1 (MCP-1), they suggest that the lack of association was perhaps because of the high background concentration of MCP-1 which made statistical analysis more difficult. In other in vivo studies [32], it was demonstrated that fibrinolytic activity as expressed by D-dimer levels showed a decline 24 hours after talc poudrage in patients with successful pleurodesis as apposed to those patients who had unsuccessful pleurodesis. It appears, therefore, that increased pleural fibrinolytic activity is associated with failure of pleurodesis while increased pleural coagulation and decrease in fibrinolytic activity is associated with a successful pleurodesis attempt. These in vivo studies demonstrate that an acute inflammatory process is initiated in the pleural space following insufflation of talc. In in vivo studies [33], we recently evaluated the concept that the mesothelial cell was an important component of the early pleural response to talc. We investigated whether talc stimulated pleural mesothelial cells to release C-X-C and/or C-C chemokines and express adhesion molecules that initiate and amplify the inflammatory process in the pleural space. IL-8 is a C-X-C chemokine that is an 8.5 kd peptide which preferentially attracts and activates neutrophils. MCP-1 is an 8.7 kd protein that belongs to the C-C sub family of chemokines supergene family and has specific chemoattractant and activating capacity for monocytes. Monocytes themselves can be a source of IL-8 and MCP-1 once recruited to the pleural space. Adhesion molecule expression on the surface of mesothelial cells is important both in cell-cell interaction and plays a key role in cellular traffic through the pleural mesothelium particularly during an inflammatory response where leukocytes migrate from the peripheral circulation into the pleural space. Intercellular adhesion molecule-1 (ICAM-1) is an adhesion molecule that binds with its natural ligand (CD11A-CD18) receptors on leukocytes which then transmigrate across the inflamed mesothelium into the pleural space in response to talc. We found that stimulation with talc, though not glass beads (SiO_2) of similar size, caused a rapid increase in release of both IL-8 and MCP-1. Expression of ICAM-1 on mesothelial cells was examined by flow cytometry and found to increase significantly. These responses were seen to be related to dose and time of exposure to talc [33].

By definition, the achievement of pleural sclerosis involves the development of an exuberant fibrotic response on the surface of the visceral and parietal pleura connecting the two and obliterating differentiating margins between them. This process requires the presence of a large number of fibroblasts which proliferate locally, connecting the two surfaces. Fibroblast growth is dependent on the presence of growth factors such as FGF and platelet derived growth factor (PDGF). We evaluated pleural fluids obtained from patients who had both successful or unsuccessful pleurodesis following talc insufflation [34]. We found that patients with successful pleurodesis had a rapid and marked increase in the amount of basic fibroblast growth factor (bFGF) in their pleural fluids. Patients with unsuccessful pleurodesis had a significantly lower amount of fibroblast growth factor in the pleural fluid. We also evaluated the release of bFGF in vitro from talc stimulated mesothelial cells. We found that talc induced mesothelial cell production of bFGF. This production was inhibited by the use of cycloheximide which prevents protein synthesis or by the use of colchicine which prevents adherence and/or phagocytosis of talc. Glass beads of similar size as talc did not cause release of bFGF from mesothelial cells indicating that this was a specific response to talc. Another interesting and important finding in the patient studies was that there was a significant inverse correlation between the release of bFGF into the pleural fluids and the tumor size as evaluated by an objective grading scale during thoracoscopy. This implied that a large tumor volume on the surface of the pleura was associated with a low level of bFGF and a failure of pleurodesis. On the other hand, a small tumor volume and by

implication a larger surface area of normal mesothelial cells was associated with a high bFGF level and successful pleurodesis. This indicates that a vigorous response by normal pleural mesothelial cells is critical for achieving sclerosis. However, if the pleural surface is covered with metastatic deposits there is a smaller surface area of normal mesothelium exposed to talc with subsequent decrease in release of growth factors for fibroblasts and eventual failure of pleurodesis.

An interesting finding in our in vitro studies demonstrates that adherence or phagocytosis of talc to the mesothelial cell is critical for the development of an acute inflammatory response. When phagocytosis of talc was inhibited by the use of colchicine, the release of both C-C, C-X-C chemokines and bFGF was inhibited. This in vitro finding may have some clinical implications. Some investigators have demonstrated that though there is moderate success achieved with talc slurry, talc insufflation gives better results [35]. At least in in vitro studies, it appears that intimate approximation, (either adherence or actual phagocytosis of talc by mesothelial cells) between talc particles and mesothelial cells is important for the development of the acute inflammatory response. When talc is administered as talc slurry, there is significantly greater difficulty in achieving this approximation uniformly over the surface of the pleura and may be in part responsible for the reticence with which experienced medical thoracoscopists view talc slurry pleurodesis.

Malignant cell responses to talc

To date, malignant cell responses to talc have not been an area of interest or investigation primarily because the role of talc insufflation into the pleural space has been palliative. However, the tumor deposits on the surface of the mesothelium come into intimate contact with the insufflated talc as well. We evaluated the responses of several malignant mesothelioma cell lines to talc. We found that talc induced apoptosis, or programmed cell death, in the malignant mesothelioma cell lines, but not in normal pleural mesothelial cells [36]. This response is interesting because several chemotherapeutic agents work by inducing apoptosis in tumor cells. This mechanism and form of cell death plays a major role in the treatment of malignancies. Talc, which is a non-toxic chemical and has a therapeutic role as a slerosing agent in patients with malignant mesothelioma has now been demonstrated to have properties that induce apoptosis in mesothelioma cells and may indeed result in containment or decrease of tumor bulk. In control experiments, mesothelioma cells and normal mesothelial cells were exposed to glass beads of the same size as talc. Three malignant cell lines CRL-2081, CRL-5820 and CRL-5915 were exposed to talc, glass beads or media alone. Apoptosis was evaluated by terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling (TUNEL). They were also evaluated by in situ apoptag labeling and by quantitation of DNA fragmentation by calorimetric DNA staining. Apoptosis was also evaluated by DNA ladder formation. Talc exposed mesothelioma cell lines revealed different steps of the apoptic process, namely chromatin condensation and fragmented nuclei. At a dose of 6 µg/cm, talc was able to induce significant apoptosis in cancer cell lines. Interestingly, other agents that have been used for sclerosis in the past such as doxorubicin, cisplatin and cyclophosphamide are capable of inducing apoptosis in malignant cells. Lovastatin also induces apoptosis in malignant mesothelioma cells. It is possible that at early stages of malignant pleural disease, the tumor surface area and tumor volume are small. The talc therefore covers a relatively larger tumor area. However, when tumor mass is large, talc may not effectively reach the deeper areas of the tumor and thus induction of apoptosis is less likely in deeper parts of the tumor since it only acts on the surface. An ideal locally acting chemotherapeutic agent would affect malignant cells and not harm the adjacent normal cells. Talc appears to fulfill this criterium since our studies demonstrate that normal pleural mesothelial cells do not undergo apoptosis when activated by talc [36].

Conclusion

It is a privilege to be able to review the topic of malignant pleural effusions and talc pleurodesis in a venue that honors Professor Loddenkemper. Dr. Loddenkemper's work illustrated that careful, scientific evaluation of data allows for the development of fresh concepts. His work has opened the window of medical thoracoscopy to the world. Using novel techniques and tools as described by him, we have begun to glimpse the mesothelial-malignant cell interactions and the involvement of specific pleural cytokines in the development of malignant pleural effusions and pleural fibrosis secondary to talc insufflation. Still, despite preliminary data generated with significant effort in many research laboratories, detailed articulation of the metastatic process remains incomplete. We know that the behavior of neoplasms in the pleural space is determined by many factors forming a complex network of molecule-receptors. It is abundantly clear that a single “magic marker” will not be available, to allow us to predict the course of the disease in the patients with metastatic pleural involvement. It is fervently hoped that the linking of clinicians and basic scientists across the world will allow us the privilege of continuing to examine the pathogenesis and treatment of malignant pleural effusions in depth.

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