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Malignant Mesothelioma: Mechanism of Carcinogenesis

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Keywords

Malignant mesothelioma • Mesothelial carcinogenesis • Asbestos • Pleural carcinogenesis • Genomic changes • Epigenetic changes • Signaling pathways.

Abstract

Almost 60 years ago, malignant mesothelioma (MM) was acknowledged as a specific cancer related to the inhalation of asbestos fibers (1). Its strong association with asbestos exposure triggered the development of researches. They consisted in epidemiological studies to know the risk factors that explain MM occurrence in the population, and of experimental studies to understand MM biological development as a neoplastic disease. Since that time, MM remains a rare and highly aggressive cancer that prompts researches to better manage patients with MM and to offer efficient therapies. To achieve this goal, a solid knowledge of the mechanisms of mesothelial carcinogenesis is needed and deserves basic researches to progress. So far, our knowledge is based on pathophysiological and toxicological researches, and from biological and molecular studies using MM tissue tumor samples and cell lines from humans and experimental animals. Most experimental studies have been based on the cellular and/or animal responses to asbestos fibers, and in genetically modified mice, demonstrating the genotoxic effect of asbestos and relationship with MM induction. The development of large-scale analyses allowing global integration of the molecular networks involved in mesothelial cell transformation should increase our understanding of mesothelial carcinogenesis. In human, MM tumors appeared as heterogeneous entities, based on morphological patterns and molecular specificities including gene mutations. The recent development of high throughput methods allowed classification of MM according to their histological type, genomic and epigenomic characteristics and deregulated pathways. The aim of the present review is to propose a potential mechanism of mesothelial carcinogenesis by integrating data, underlying the mechanisms that may be shared with other types of fibres that may pose current health issue.

Introduction

Our present knowledge of the mechanisms of mesothelial carcinogenesis originates from pathophysiological and toxicological research carried out *in vivo* in rodents and in mammalian cells in culture. The development of analytical tools allowed biological and molecular studies of malignant mesothelioma (MM) tissue tumor samples and cell lines from humans and experimental animals. Most experimental studies have been based on the cellular and/or animal including genetically modified mice responses to asbestos fibers. These investigations have provided a body of data on the cellular and molecular effects of asbestos fibers on mesothelial cells and the mesothelium, including genomic and genetic changes and alterations of regulatory and signaling pathways. Human MM has been characterized at the genomic, genetic, epigenetic, and physiological levels, with the development of large-scale analyses allowing global integration of the molecular networks involved in the transformation of the mesothelial cell.

As this volume is devoted to occupational cancer, the studies reported here will focus on asbestos, the only known human etiological factor widely used in the occupationnal environment associated with MM. Although epidemiological studies have clearly linked mesothelial carcinogenesis with both occupational and non-occupational asbestos exposure, no history of exposure can be found in about 10–20 % of MM cases (2-5). Some MM may be related to other fiber exposure or to other causes (6). Indeed, other types of natural fibers are associated with MM following environmental exposure, and other fibers used for industrial or commercial applications have been found to produce MM in animals, including man-made mineral fibers and more recently CNT. In 2014, IARC reviewed the classification of other fibrous materials, fluoro-edenite, silicon carbide fibres and whiskers, and CNT. Fluoro-edenite, a fibrous amphibole, was classified as carcinogenic (Group 1) as asbestos and erionite, silicon carbide whiskers as probably carcinogenis (Group 2A) and a type of CNT as possibly carcinogenic (Group 2B) (7).

CNT are of particular interest because of similarities with asbestos, which are discussed in several reviews (8-10). Recent studies investigating the effects of other elongated particles such as carbon nanotubes (CNT), and asbestos fibres as controls, have brought additional information on the mechanism of action of asbestos. In the field of investigation of the toxic potency of nanoparticles, the relation between the biological effects of asbestos and their properties led to the concept of high aspect-ratio nanoparticles (HARNs). In some parts of this review, we will mention CNT, which are a type of engineered HARNs that induces mesotheliomas and lung cancer in animal experiments (11). The aim of the present chapter is to update the data on potential mechanisms of mesothelial carcinogenesis by integrating data based on cellular and molecular effects of asbestos fibers on mesothelial cells with data obtained on altered physiological and molecular features of MM (12)

Deposition and Translocation of Asbestos Fibers

The initial route of entry of asbestos fibers is by inhalation. Fibers deposit in the tracheobronchial regions, distal airways, and alveolar spaces of the lungs (13). The major deposition mechanisms are by impaction, interception, sedimentation and diffusion, and are dependent on the physical characteristics of the particles (14, 15). It results that asbestos and other elongated mineral particles have a greater inhalability than spherical particles having the same mass or volume (16). While particles and fibers are readily cleared from the tracheobronchial airways by mucociliary transport, clearance from distal airways and alveoli is slower and mediated by phagocytosis by alveolar macrophages. Fiber length impairs macrophage-mediated clearance, especially for fibers that exceed the diameter of alveolar macrophages (10–25 μm). Impaired clearance may result in penetration of fibers through the

alveolar epithelium and subsequent translocation to the pleura and distant sites (17). Fibers that enter the interstitium may cross the visceral pleural by paracellular migration or by direct penetration (18). An alternative route of translocation to the pleural space is transport via lymphatics or the bloodstream (19).

The parietal pleura lines the chest wall and the superior surface of the diaphragm, and the visceral pleura covers the lungs. The pleural space in humans is lined by a single layer of mesothelial cells approximately 1 μm thick resting on a basement membrane and underlying connective tissue and blood vessel (20). The major route of drainage of fluid, protein, particulates, and cells from the pleural space is lymphatic stomata that open between mesothelial cells on the parietal pleural lining. Lymphatic stomata are communication holes between the pleural cavity and the parietal pleura lymphatics, where the particles are not cleared and concentrate, depending on their shape and dimensions (21-23). The diameter of lymphatic stomata (~10–12 μm) limits the clearance of long fibers from the pleural space (19). The translocation of asbestos fibres in the lymph nodes and in the pleura has been reported in animal experiments, a process also found in CNT-exposed animals (8, 24-27). Asbestos persists in the lung regional lymphatics of mice one year after pharyngeal aspiration and giant cells formation is present in lymph nodes (28).

Systemic dissemination of fibers through lymphatics and the bloodstream has been described in humans following autopsy (29-31). Asbestos fibers and asbestos bodies have been noted in the liver, mesentery, spleen, and abdominal lymph nodes (32, 33). Several studies have demonstrated the presence of asbestos fibers in the human pleura (30, 31, 34). The translocation of asbestos to the pleura is also suggested by the presence of pleural plaques that develop in the parietal pleura in asbestos-exposed subjects. Parietal pleura is also the location of early MM, although MM does not seem arise from pleural plaques. However, a statistically significant association was observed between mesothelioma and pleural plaques, consistent with the role of asbestos in these pathologies (35).

Diffuse peritoneal malignant mesothelioma is also associated with exposure to asbestos fibers (36, 37). Fibers might reach the peritoneal mesothelial lining via diaphragmatic lymphatics that connect the pleura and peritoneal spaces or following systemic vascular and lymphatic dissemination. Another route of entry may be via swallowing of expectorated mucus and penetration of fibers through gastrointestinal wall. A biodisponibility of asbestos fibers may account for the occurrence, not only of MM and lung cancer, but of other types of cancers, larynx, ovary, and possibly pharynx, esophagus, stomach colon and rectum (38-42).

The Mesothelial Cell In Situ

The mesothelium consists of a monolayer of mesothelial cells lying on a basement membrane and supported by connective tissue containing fibroblasts and macrophages. It provides a protective barrier for frictionless interface for the free movement of apposing organs and tissues, and in fluid transport across the pleura (43). Mesothelial cells may have specialized functions at different anatomical sites, as demonstrated by morphological studies at the ultrastructural level (44). Mesothelial cells play a role in the resolution of inflammation and tissue repair after pleural injury (45). Fibrosis is a potential outcome of chronic inflammation. These processes are of particular interest in investigating the mechanism of action of asbestos fibers in the pleura.

So far, the mechanism of mesothelial cell regeneration remains poorly understood, mostly in the context of serosal injury following dialysis. However, some controversial hypotheses, have been formulated. Comprehensive reviews summarize our present knowledge of these potential mechanisms (46, 47). The regeneration process has been studied experimentally following mechanical, chemical, or heat injury of the peritoneal serosa. Briefly, six

mechanisms have been suggested to replace the injured mesothelial cells: (1) centripetal migration of adjacent mesothelial cells, (2) exfoliation of mature or proliferating mesothelial cells that replicate on the wound surface, (3) preexisting free-floating serosal cells having the capability to differentiate into new mesothelium, (4) macrophage transformation into mesothelial cells, (5) submesothelial mesenchymal precursors that migrate to and differentiate at the mesothelium surface, and (6) bone marrow-derived circulating precursors (47). The origin of these new mesothelial cells has not yet been confirmed, but according to Mutsaers et al. (47), mesothelial regeneration is not dependent on subserosal cells, but more likely results from implantation, proliferation, and incorporation of free-floating mesothelial cells (48). Recently, floating mesothelial cells were identified in pleural fluid after lung surgery in human, in the vein of this hypothesis (49).

Effects of Asbestos Fibers in Wild-Type Animals

The relationship between mesothelioma and exposure to asbestos, or to other fibers, erionite and fluoro-edenite, has been well demonstrated by numerous experimental studies carried out in rodents. Some samples of asbestos fiber substitutes, refractory ceramic fibers (RCF) and glass fibers, have induced MM after inhalation by rats or hamsters. These data have been described in detail in several IARC monographs (14, 39, 50). Other routes of exposure by intracavitary pleural or peritoneal injection have illustrated the carcinogenic potency of these mineral fibers. Both types of exposure have been used to assess fiber parameters modulating the oncogenic response in the pleura. It can be emphasized here that fiber-induced MM show similar morphological features in rodents as in humans (51-54).

Some studies have investigated the pleural responses to asbestos fibers following deposition in the lung. An inflammatory reaction characterized by the recruitment of inflammatory cells and the presence of growth factors in the pleural fluid was demonstrated. These growth factors were able to induce proliferation of mesothelial cells in culture (55). An inflammatory response may be triggered by fiber translocation to the pleura as demonstrated in rodents exposed to glass fibers, RCF or CNT (56-59). The pleural reactivity to asbestos was observed in mechanistic studies using CNT, and asbestos as control fibers. Shvedova et al (28) reported the occurrence of pleuritis and mesothelial hyperplasia and/or atypia one year after pharyngeal aspiration of crocidolite in mice (28).

Chromosome and DNA alterations

Chromosomal and molecular alterations have been studied in mesothelial tissue and in MM developed in rats exposed to asbestos by intraperitoneal injection. Chromosomes losses and rearrangements were observed in rats exposed to crocidolite and chrysotile (60). Significantly enhanced mutation rate of *lacI* gene from omenta in Big Blue rats (a model to detect mutation potency) was found 12 and 24 weeks post-exposure to crocidolite, and significant enhanced level of 8-Oxo-2'-deoxyguanosine (8-OHdG), a major product of DNA oxidation, in DNA from 10-20 weeks post treatment of Wistar rats (61, 62)**.** 8-OHdG in DNA was also enhanced in rats and hamters after intratracheal instillation 1 day after the exposure to crocidolite (63).

The type of mutations has been poorly investigated in animals. No mutations were found in *Trp53* (exons 5-8) or in *Kras* (exons 1, 2) (60, 64, 65). Additionally, no hot spot point mutation in *Kras* were detected, one year after pharyngeal aspiration of crocidolite in mice (28). In MM from Big blue rats, transversions G>T were predominant (29%) followed by deletion (26%), G>A (20%), G>C (12%), A>T (6%), A>G and insertion (3%), while in controls spontaneous mutations were G>T (19%), deletion (5%), G>A (57%), G>C (14%), A>T and A>G (0%) and insertion (5%) (61). Recently mutational signature was investigated in human malignant pleural mesothelioma (MPM) (66). The authors found a highest rate of mutations

C>T, which C>T mutations can be generated by spontaneous deamination of methyl-cytosine bases in 5'-CpG, and by APOBEC-catalyzed deamination of cytosine bases to uracil (67). One of the signature in human MPM may be associated to reactive oxygen species (ROS) but no significant difference in the mutational signature was found between asbestos-exposed and non exposed patients (66).

DNA mutations in asbestos-exposed cells may occur through generation of ROS by surface reactivity of particles, by asbestos uptake, or by inflammation. Oxidative DNA damage has been reported in several studies (68-71). Moller et al (72) reported a critical assessment of the association between pulmonary exposure to particles, considering carcinogens carbon-derived particles, quartz and asbestos and levels of oxidatively damaged DNA in lung tissues from animals (72). The authors mentioned that the results show that asbestos can generate genotoxicity in a dose-dependent manner and without a clear threshold, and that measurements of oxidatively damaged DNA, as marker of particle-induced genotoxicity in animal tissues, did not show evidence that inflammation is a perequisite for generating DNA oxidation (72).

Inflammation

Inflammation plays a role in cancer. Asbestos-related MPM pathogenesis is associated with fibroproliferative response (73). This process partly involves IL-1, as reported in a study comparing inflammation in wild-type (WT) and IL-□□□□/KO mice following injection of crocidolite or carcinogenic CNT fibers in the pleural cavity (74). Both types of mice developed mesothelial cell hyperplasia, leucocyte infiltration, granulomas and fibrotic responses, but fibrosis specific genes were downregulated in the IL-1/KO mice in comparison to WT mice (74).

Induction of inflammation was confirmed by transcriptomic and proteomic analyses. Inflammatory response to asbestos, crocidolite and tremolite, was studied at the acute (1 day) and subacute (7 days) phase in lung of mice exposed by oropharyngeal aspiration (75). Gene expression demonstrated inflammatory response (increased cytokine and chemokine release) and tissue damage (LDH release in the broncho-alveolar fluid) (75). Fifty six days post esposure perivascular and parenchymal inflammation, granulomas and fibrosis were moderate to severe (75). A proteomic analysis was carried out in lungs of mice exposed to crocidolite, single-walled CNT and ultrafine carbon black by pharyngeal aspiration (76). The overall pattern of protein changes was similar across treatments, and GO functional categories were related to inflammation/immune response, fibrosis and tissue remodeling (76).

Global Gene Expression

In the previously mentioned transcriptomic study (75), apart from genes of the inflammatory response, differentially expressed genes were involved in several other pathways regulating cell movement, death and survival, growth and cell proliferation, in comparison to control. In another study where amosite asbestos fibers and CNT were instillated into the pleural cavity of mice, transcitptomic microarrays analysis showed a common molecular signature of inflammatory lesions, and antibody-based array analysis showed activation of pro-oncogenic signaling pathways, including Src family kinases, Akt, mTOR, ERK1/2, and STAT3 (77). Progression of fiber-induced lesions is characterized by increased proliferation and oxidative DNA lesions (77).

Gene mutations and signal pathway dysregulation were studied in 15 MM cell lines obtained from crocidolite-induced murine MM in three different mice strains, BALB/c, CBA and C57BL/6 (78). Whole exome analysis reported homozygous deletions in in *Cdkn2a* in 14/15 cell lines, and deletion in *Trp53*, *Setd2* or *Lats2* in 1 to 3 cell lines, as well as a frequent

amplification of *Myc*(78). The genes significantly mutated belonged to pathways Wnt, Mapk and Jak/Stat, and mutations were also detected in genes form the Hedgehog and Notch pathways (78). A differential response depending on the mice strain must be noted, as the BALB/c MM cells had higher average number of mutations than MM cells from the other strains, and only mutation in one sample in the Mapk signaling pathway(78).

Exposure of laboratory mice to carbon nanotubes mimics exposure to asbestos, from initial and chronic inflammation, through loss of the same tumour-suppressor pathways and eventual sporadic development of MM. These data support that fibers of a similar nature may pose significant health risks to MM (79).

Immunological Effects

Pathogenesis of asbestos, also shared by carcinogenic CNT, may be linked to their immunosuppressive effects, as reported in different studies (80-82).

MM induction in GEM

To investigate the role of specific genes in MM development, several models of MM have been developed using genetically modified mice (GEM) unexposed or exposed to mineral fibers. A recent review analyzes the different studies (83).

GEM Unexposed to Asbestos Fbers

A few studies inverstigated the development of MM in conditional mutant mice carrying either heterozygous (Htz) or homozygous (Hom) inactivated genes in the absence of asbestos exposure (84-87)(see (83) for review). Gene inactivation was carried out by injection of Ad*Cre* (Adenovirus expressing *Cre* recombinase) in the pleural or peritoneal cavity of mice carrying floxed relevant genes. All targeted genes were tumour suppressors, *Nf2*, *Cdkn2a/Ink4a*, *Cdkn2a/Arf*, *Trp53, Rb*, *Tsc1*, *Pten* or *Bap1* alone or in combination. A high rate of thoracic MM was observed after injection of Ad*Cre* in the pleural cavity of double mutants *Nf2* and *Cdkn2a*, *Trp53* or *Rb* and in triple mutants *Nf2*, *Trp53* and *Ink4a* (almost 100%) (85). After injection of Ad*Cre* in the peritoneal cavity or in the bladder of double Hom *Trp53/Tsc1* mutants a high rate of MM developed, but none in Htz/Hom mutants showing a higher contribution of *Trp53* (84). Involvement of *Pten* was also reported in the occurrence of pleural MM, as Hom *Pten* mice developed a frequency of 7% MM, but when coupled with Hom *Trp53*, 56% of mice developed pleural MM (87). Of note, genetic alterations in *Tsc1* and *Pten* are found at very low frequency in MM. Kadariya et al (86) investigated the role ot *Bap1*, a gene predisposing to the development of MM and frequently mutated in human MM (88, 89). Interestingly, the authors generated mice with point mutations in *Bap1* identical to germline mutations found in two human families with a *BAP1* cancer syndrome, and presenting mesothelioma in several family members (86). They also studied Htz mice (knockout in exons 6 and 7). The results showed that Htz mice developed numerous types of cancers, but few or no MM (86). The tumour type with the highest incidence was ovarian sex cord stromal tumours, found in 63% *Bap1* mutant mice (86).

GEM Exposed to Asbestos

Several studies investigated the development of MM in mice carrying Htz mutation in genes homologous to the most frequently inactivated in human MM, *NF2*, *CDKN2A/INK4A*, *CDKN2A/ARF*, *BAP1* and *TP53* (83). Studies were carried out on mice Htz for one tumor suppressor *Trp53*, *Nf2, Cdkn2a/Ink4a*, *Cdkn2a/Arf* or *Bap1*. A higher level of MM was found in asbestos-exposed Htz mice (crocidolite) in comparison with asbestos-exposed WT mice. No MM was observed in untreated mice (54, 86, 90-93).

Interestingly MM cells obtained from ascites in *Trp53*$^{+/-}$mice exhibited *Trp53* LOH and polyploidy (94). A loss of heterozygozity (LOH) of the *Nf2* gene was found in *Nf2*$^{+/-}$mice, suggesting a common mechanism for loss of the WT allele (54, 91). Moreover, in *NF2*$^{+/-}$mice, two other TSG, *Cdkn2a/Ink4a* and *Cdkn2a/Ink4b*, were deleted at a high rate, while *Trp53* was mutated at a much lower rate similar to human MM, (91, 92). A loss of the WT allele was also observed in MM Htz *Bap1* mice exposed to asbestos (86).

Gene alteration and expression were studied in mesothelioma cells from asbestos-treated MexTAg transgenic mice carrying SV40 large T Antigen (SV40Tag), in comparison with WT mice (95). Analysis of the *Cdkn2* locus revealed deletion in WT animals, but not in MexTAg mice (95). As SV40Tag protein targets and impairs the p53 protein, this is consistent with different pathways of mesothelial cell transformation involving *Cdkn2a/b* and *Trp53*. Differentially expressed genes were involved in cell cycle regulation and DNA replication (95).

Murine MM closely mimics the human disease characterized by peritoneal ascites, a long latency between fiber injection and MM appearance, and histological subtypes, epithelioid, sarcomatoid, and biphasic, similar to human MM. The results obtained with GEM show that in most cases, MM progression could follow several routes involving different TSG with *Cdkn2a* and *Trp53* as independent key players. This is consistent with the specific clinical features and molecular alterations in human MM.

Collectively, results obtained in the different GEM experiments with or without asbestos exposure show that the most frequently altered murine genes homologous to the human genes, *NF2*, *CDKN2A* and *TP53* are important in the neoplastic transformation of mesothelial cells, consistent with findings in human MM. The potential of other genes, *Rb* and *Pten*, is dependent on the inactivation of other key MM genes. *Bap1* plays a role as cancer predisposing gene and is not specifically linked to the development of MM. The data obtained with asbestos-exposed mice are consistent with this observation.

Effects of Asbestos Fibers on Mesothelial Cells in Culture

While early studies have been carried out with cells of different species and tissues, human and rodent normal mesothelial cells have been most widely used to study the response of mesothelial cells to asbestos fibers (96).

Genotoxicity

In cultures of normal rat pleural mesothelial cells, asbestos induces chromosome alterations and abnormal mitoses (97-102). DNA breaks, base oxidation and stimulation of DNA repair were also evidenced (68, 103-108). Furthermore, DNA breakage and cell cyle arrest were detected in rabbit pleural mesothelial cells exposed to crocidolite (68). Interestingly DNA breakage was related to the phagocytosis of fiber by mesothelial cells as reduction of phagocytosis reduced the level of DNA breakage (68). When incubated in the absence of serum or in low levels of serum concentration, cell proliferation was observed (109, 110). However, in proliferating mesothelial cells, asbestos provoked a p53- and p21-dependent cell cycle arrest consistent with the induction of a DNA damage-induced response (102). P53 was also induced in serum-deprived G0-synchronized mesothelial cells exposed to asbestos, but failed to block cell cycle progression (111). Comparison between different studies showed that significant effects were found with doses of 0.5–1 µg/cm2 (71).

To summarize, studies on genotoxicity of asbestos fibers demonstrate that asbestos fibers are genotoxic for mesothelial cells. DNA repair processes are stimulated in asbestos-treated mesothelial cells. The consequences of DNA damage will be dependent on the efficiency and

fidelity of repair. When genomic damage is extensive, an apoptotic program should be induced. Life-or-death decisions may be at the heart of malignant transformation, and defective mechanisms of arrest or apoptosis may be critical to the development of malignancy (112). Several studies with mesothelial cells in culture have emphasized the occurrence of apoptosis (68, 102, 113). However, some cells can survive with genetic alterations that can be inherited in daughter cells. In that context, it is remarkable that mesothelial cells show both cell cycle arrest and mitotic abnormalities, suggesting that some cells could pass through cell cycle checkpoints with unrepaired DNA and chromosomal damage. Recent findings suggest that BAP1 could play a role as recucing apoptosis (114).

Inflammation

The ability of mesothelial cells to interact and internalize asbestos fibers is an important feature that is linked to the deleterious effects of asbestos, especially production of inflammatory factors by these cells, and interaction with the dynamic of mitosis. Activation of the Nalp3 inflammasome that triggers inflammation, is observed in mesothelial cells exposed to asbestos (73, 115).

Epigenetic Changes

Recently, some data on epigenetic changes in asbestos-exposed cells in culture were reported. DNA methylation profiling and gene expression were studied in Met5A cell line exposed to asbestos (chrysotile and crocidolite) (116). Only 26 CpG sites were differentially methylated after treatment by both asbestos types and methylation changes were the same for 15 of them (116). Results did not show correlation between methylation and gene expression, except for *DKK1,* an inhibitor of Wnt signal pathway, whose expression is upregulated by chrysotile treatment. With chrysotile, differential methylation occurred in genes involved in cell response to stimuli, cell adhesion and cellular matrix (116). With crocidolite, several genes from the DNA damage response were downregulated and upregulated genes were involved in metabolic process (116).

Effects on Signaling Pathways

Two studies investigated the response of human mesothelial cells to crocidolite asbestos by transcriptomic analyses (117, 118). Gene expression was investigated in normal pleural human mesothelial cells and in LP9, an h-TERT immortalized human mesothelial cell line exposed to crocidolite by transcriptomic analysis (118). Several genes were upregulated (*ATF3*, *PTGS2*, *FOSB*, *IL8*, *NR4A2*, and *TFPI2*). Among them the transcription factor *ATF3* regulated levels of asbestos-induced inflammatory cytokines, IL-1b, IL-13, G-CSF, and the growth factor, PGDF-BB, in LP9/TERT-1 cells (118). *ATF3* silencing by specific siRNA reduced cytokines and PGDF-BB expression levels (118).

The response of Met-5A cells to crocidolite was investigated using a Protein Pathway Array, which assesses proteins and phosphoproteins functionally linked to proliferation, apoptosis, cell cycle regulation, DNA repair, signaling, and transcription activity (119). Three pathways were only affected by crocidolite, ILK signaling, PPARa/RXRa and G1/S phase checkpoint regulation (119). Interaction between pathways, investigated by Ingenuity Pathway Analysis identified several proteins regulating the networks, P53, CCND1, RB1 and CTNNB1 in asbestos-treated Met-5A cells in comparison with untreated cells (119). These results confirm the effects of asbestos on cell cycle progression. Concerning the role of P53, it must be noted that Met-5A are SV40-transformed cells, which show a basal accumulation of nuclear P53(120).

An upregulation of genes involved in invasion, including *MMP2*, was reported in a transcriptome microarray analysis of Met-5A mesothelial cells exposed to CNT and crocidolite at subcytotoxic concentrations (121). Gene signaling network analysis found other genes

involved in the asbestos- or CNT-induced invasion network as potential regulators of *MMP2* (121).

Fiber Properties in Relation to the Biological Effects and Carcinogenic Potency

These paragraphs summarize the biological mechanisms leading to the development of diffuse malignant mesothelioma, focusing on the physiochemical properties of asbestos fibers, and other carcinogenic natural mineral fibers known to induce MM in human. Several recent mechanistic studies have been carried out with CNT providing new perspectives to account for the mechanism of action of elongated particles. The reader is referred to comprehensive reviews for a details on the fiber properties in relation to the biological effects and carcinogenic potency (8, 10, 11, 19, 122). Several fiber parameters are of importance in the mechanism of asbestos toxicity.

Physico-chemical Properties of Asbestos Fibers and Elongated Particles.

Asbestos fibers are fibrous silicates and are classified into two groups based on their crystal structure and chemical composition: serpentine asbestos which is called chrysotile and amphibole asbestos which includes crocidolite, amosite, tremolite, actinolite, and anthophyllite (123, 124). Fluoro-edenite is a fibrous amphibole not used in the industry, but naturally occurring present in quarry stones (125). Erionite fibers are a form of the mineral zeolite characterized by a high internal surface area. They are associated with the development of diffuse malignant mesothelioma in epidemiological studies (126-128). These naturally occurring fibrous minerals are variable with respect to chemical composition, associated minerals, and trace contaminants depending on their geographic origin (129). Asbestos fibers may contaminate other mineral deposits, for example, talc (126, 130) and vermiculite from Libby, Montana (130, 131), and exposure to these mixed materials has also been linked with diffuse malignant mesothelioma (128, 132). The physiochemical properties of mineral fibers associated with biological activity include shape and dimensions, surface chemistry and reactivity, and biopersistence (8).

Shape and dimensions

Shape and diumensions are fiber parameter modulating the biological effects of asbestos and elongated fibres. Fiber length and diameter determine the respirability and site of deposition in the lungs, and clearance mechanisms. Short fibers are taken more easily by macrophages than long fibers and can be eliminated by the clearance mechanims. In experimental studies, it was generally found that the fiber dimensions are important, with long and thin fibers more active that shorter fibers on cultured cell and with a greater carcinogenic potency in animals.

Phagocytosis is an important function of macrophages and other cells as it determines the intracellular availability of the fibers and possible interactions with cell components (73, 115). A recent study investigated phagocytosis of CNT according to their geometry and demonstrated that geometry and volume influence the efficiency of phagocytosis (133).

Fiber length has been associated with the induction of aneuploidy and chromosomal damage due to direct physical interference with the mitotic apparatus or by binding to cell cycle regulatory proteins (134, 135) (136). Chromosome damage and mitosis impairement is also a feature of CNT as observed in several types of rodent and human cells (137-139).

Surface chemistry

Surface chemistry determines interactions between the fiber and the molecules present in the fiber vicinity. Fibers may interact with macromoleucules in the biological fluids (proteins,

phospohlipids…) (128). Surface iron, especially on amphiboles surface may be released, which could catalyze the formation of reactive oxygen species (ROS), and may be associated with biological effects of mineral fibers including lipid peroxidation, oxidative DNA damage, and activation of intracellular signaling pathways (140-143).

Biopersistence

Biopersistence is considered as a major determinant of fiber pathogenicity in the lungs (144). It is dependent on fiber clearance and on the ability of fibers to be broken, splitted, or attacked by the biological medium in the lungs (144). Differences in biopersistence of asbestos fibers have been linked with carcinogenic potency, as biopersistent fibers could sustain a local inflammatory response (145). Amphibole asbestos fibers are more potent than chrysotile asbestos fibers due to their increased biopersistence in the lungs (8). However, chryotile fibers are detected in autopsic lungs several years past-exposure to asbestos, and their biopersistence and effects could be linked to the surface modification of the fibers (146-148). Additionally, thes fibers should be stable regarding the lun pH (149). Fiber biopersistence in the pleura is not documented; in particular, there are no data on the relationship between biopersistence in the lung and translocation of fibers from the lung to the pleura, nor on the pleural clearance of fibers following inhalation (150, 151).

High aspect ratio and biopersistence have been hypothesized to be important properties of engineered nanomaterials that raise concern about their potential to be translocated to and retained in the pleura following inhalation (19, 152). A long-term study, after intratracheal instillation of CNT in rats, reported that pulmonary lung burden did not decrease significantly over time up to more that one year after instillation (153).

Summary Hypotheses on the Mechanism of Action of Asbestos Fibers to Generate MM

The development of diffuse malignant mesothelioma is a complex, multistage process that is governed by the physicochemical properties of crystalline mineral fibers and their propensity to migrate to the pleural and peritoneal linings. The most important properties of asbestos fibers related to carcinogenicity are fibrous shape and dimensions, surface chemistry and reactivity, and biopersistence (39).

Interactions between mesothelial cells and fibers can cause genetic and chromosomal changes. There is a great body of evidence (1) that asbestos fibers can directly interfere with chromosomes and the mitotic spindle and (2) that they induce the formation of reactive ROS resulting in DNA breaks, oxidation, and mutations (154-157). Further, (3) the physical interaction of fibers with target cells causes persistent inflammation and, consequently, modulation of inflammatory and immune responses. ROS have been clearly indicated to cause genetic damage including chromosomal breaks and mutations and they are well known to initiate signal transduction pathways that are, in turn, linked to inflammation, proliferation, and apoptosis (157, 158). Free radical scavengers decrease genotoxic endpoints such as micronucleus formation induced by fibers and antioxidant enzymes can protect cells against genotoxicity induced by chrysotile fibers (159, 160).

Prolonged interaction between pleural inflammatory cells and adjacent mesothelial cells causes persistent release of chemokines and cytokines, inflammatory mediators, ROS and reactive nitrogen species, and growth factors, that trigger repeated episodes of inflammation resulting in mesothelial cell injury, death and/or proliferation (161). This may be also linked to altered gene methylation patterns and to epigenetic gene silencing identified in human MM (162-164). Genomic instability and acquired gene and chromosomal alterations in mesothelial cells may lead to altered cell cycle and growth regulation, resistance to apoptosis, impaired repair of DNA and chromosomal damage, activation of oncogenes and inactivation

of tumor suppressor genes (134, 135, 163, 165). This persistent inflammatory microenvironment in combination with oxidative stress and cell division impairement generates a strong selective force for mesothelial cells that have acquired genetic and epigenetic changes that promote their survival, proliferation, and tumor progression (164).

Molecular Alterations in Human MM

Carcinogens provoke several types of somatic gene mutations, consisting of DNA and chromosome alterations. Some mutations are the signature of past exposure to given carcinogens. Somatic mutations in tumors are of interest both to determine the mechanism of action of carcinogens and to elucidate their adverse consequences on cellular homeostasis.

Chromosomal imbalance

Structural and numerical chromosomal abnormalities are numerous and complex in MM. A detailed review can be seen in Chapter 19 (166). It can be summarized here that one of the most frequent alterations are losses in the 3p21 region including the frequently inactivated gene *BAP1*, and other less frequenty altered gene *SETD2* (167). Frequent losses also occur in 9p21, which encloses the *CDKN2A*(*INK4A/ARF*) locus, encoding both the $P16^{INK4A}$ and the $P14^{ARF}$ proteins, and the *CDKN2B* locus, encoding P15 protein, and in 22q12 which encloses the *NF2* locus, which encodes the protein merlin.

Gene mutations

In MPM, there are a limited number of genes known to be recurrently mutated in a high percentage of MM.

Inactivation of *CDKN2A* and *CDKN2B* TSG are mostly due to large deletions (168-170). *CDKN2A* deletions have been considered as a marker of asbestos exposure in a study of non-small cell lung carcinomas (171). In MM, DNA methylation of *CDKN2A* and *CDKN2B* have been reported at a frequency of 13 % (nine patients) and 4 % (three patients) respectively, and positively correlated with asbestos body counts in the lung (172, 173). The average methylation frequency of these genes in the literature is about 10 % (92, 172, 174-178). It was also suggested that mesotheliomas express microRNA (miRNA) that could inhibit *P16*/*CDKN2A* expression, based on an in silico analysis for miRNA target gene prediction (179). Interstingly, a recent experimental study of instillation of either long asbestos fibers (amosite) or long CNT showed hypermethylation of *Cdkn2a*(*Ink4a/Arf*) in early lesions that precedes mesothelioma (77). Both $P16^{INK4A}$ and $P15^{INK4B}$ are inhibitors of the kinase function of cyclin/cdk complexes involved in cell cycle progression. The protein $P14^{ARF}$ has an indirect function on cell cycle regulation, by positively regulating the level of P53 through interaction with P53 inhibitors. Consequently, cells with damaged DNA can proliferate and survive in the absence of $P14^{ARF}$. In murine models of asbestos-induced mesothelioma, the orthologous genes, *Cdkn2a*/*Ink4a* and *Cdkn2b*, are also inactivated by deletion (91, 92, 180)((83).

TP53 mutations occur at a lower rate in comparison with other human cancers, they are mainly due to non- or missense substitutions (66, 168, 170, 181, 182). Different frequencies of 7.4% and 16.3% are reported in 2 studies respectively(66, 170). None *TP53* mutation was reported in the epithelioid molecular MM subtype, in a whole exome analysis of 202 MPM (66), but TP53 mutations were found in MM of epithelioid histologic type in other studies (170, 183). The protein P53 is activated in response to DNA damage and is a regulator of senescence, apoptosis and autophagy. In animal models of MM (see above), the mutated status of *Trp53* was investigated in mice exposed to mineral fibers by intraperitoneal inoculation. In C57Bl/6 $p53^{+/-}$mice, a strain having one allele mutated in the gene *Trp53*, loss of the WT allele was found at a high rate in MM induced by asbestos fibers (90).

In large-scale analyses alterations of *NF2* TSG are frequently found, in about 20 % of MPM (66, 170). Higher percentages were previously reported in smaller series (184-187). *NF2* has pleiotropic functions, being involved in regulation of cell proliferation, apoptosis, and endocytic trafficking and acting upstream of several signaling pathways including the Hippo signaling pathway (188). Mutations in *NF2* consist of both point mutations and deletions (189). In $Nf2^{WT}$ and $Nf2^{+/-}$ FVB mice, *Trp53* alterations were infrequent. *Nf2* mutations were detected in mice exposed to asbestos and exposed to ceramic fibers (92, 180). Alteration in the chromosomal region of the *Trp53* locus was infrequent (190). These results suggest that deletions would be more likely a consequence of the mechanism of action of asbestos, while p53 point mutations could be related to "spontaneous" gene alterations in this model.

Alteration of *NF2* is also consistent with a physical mechanism of action of asbestos fibers with mesothelial cells. The encoded protein, merlin is a regulator involved in signaling pathways that control, among other parameters, cell shape, proliferation (involving the hyaluronic acid receptor, CD44, which is important for proliferation of MM cells), survival, and motility (188). Merlin is a component of the adherens junctions and other types of cell-to-cell contacts (191, 192). As cell division is mechanically impaired by the presence of asbestos fibers, mutation of *NF2* could be responsible for enhanced proliferation as well as impaired mitotic control.

Somatic *BAP1* mutations are frequent in MM. A frequency of about 20% was reported in several studies, although higher rates, up to 60% are reported (88). Bueno et al (66) reported a frequency of mutations in 23% in MPM, that was the highest rate of mutations in comparison with the other predominantly mutated genes *NF2*, *TP53* and *SETD2* (66). In another series including MPM tumors and cultured MPM cells, a higher percentage of *BAP1* mutations was found in the subgroup of epithelioid MPM (subgroup C1) in comparison with subgroup including both epithelioid and sarcomatoid MPM (subgroup C2) (169). *BAP1* germline mutations were found in a few cases of sporadic mesotheliomas (193). However, no germline mutations in *BAP1* was found in a cohort of patients in Australia (194). So far, the weight of germline *BAP1* mutations in asbestos-induced MM is not clear.

Up to recently, *BAP1* was the only gene reported as possibly confering an increased susceptibility to MPM. A recent paper reported a gene sequencing analysis of 85 cancer susceptibility genes on germline DNA of patients with pleural, peritoneal, and tunica vaginalis MM (195). Twelve percent of patients with MM carried mutations in genes such as *BRCA2*, *CHEK2*, *CDKN2A* and *ATM*, especially those with peritoneal MM, minimal asbestos exposure, young age, and a second cancer diagnosis(195).

So far, no recurrent mutations have been reported in oncogenes. However, a 'hot spot' of mutations in the *TERT* gene core promoter has been reported in 15% of MPM MPM (196). *TERT* promoter mutations were significantly more frequent in MPM with sarcomatoid histologic subtype (196).

Regulatory Pathways in MM Cells

Constitutive activation of several signaling pathways has been demonstrated in MPM by the occurrence of mutations and/or deregulated expression of specific regulators in comparison with normal mesothelial cells. These studies have been carried out in primary tumor samples but also in malignant mesothelial cell cultures developed from tissue samples. Pathway deregulation in MM has been shown by gene sequencing and gene expression profiling (197, 198). Alterations were recurrently reported in several pathways: hippo, MAPK, PI3K/AKT/mTOR hedgehog, Wnt signaling pathways, cell cycle, P53/DNA repair and apoptosis and ubiquitin/proteasome system due to the frequent alteration of the deubiquitinase, BAP1.

The Hippo Pathway

The Hippo pathway is of special interest regarding the high frequency of mutations detected in merlin encoded by the *NF2* gene. As mentioned above, merlin negatively regulates cell proliferation and other cell functions (199, 200). Its activity is affected by interaction between extracellular signals and membrane proteins, and activated merlin transduces signals suppressing the transcriptional activity of YAP coactivator for TEAD and other transcription factors (168, 201). YAP and LATS1/2, regulator kinases of the hippo pathway, may mediate proper organization of cytokinesis machinery and mitosis progression (199). *NF2* co-inactivation with *LATS2* led to loss of cell contact inhibition in human MM cells (202). *LATS2* gene was found to be deleted in three out of six MM cell lines and in one out of 25 tumors by DNA sequencing analyses (203). A more recent study reported *LATS2* mutations in 11% (7/61) MPM cells (202). Merlin exists in two forms, active unphosphorylated or inactive phosphorylated. This later form is found in MPM cells possibly accounting for another mechanism for the deregulation of the hippo pathway in these cells (204).

In an integated analysis of gemomics data hippo pathway was identified as altered in all histological type of MPM due to gene alterations in several members of the pathway (66).

Cell Cycle

The alteration of CDK inhibitor genes located at the *CDKN2* (*CDKN2A* and *CDKN2B*) locus, as mentioned above, contributes to uncontrolled cell proliferation. Cell cycle control can be affected in MM cells not only by the loss of other negative regulators but also by the overexpression of cyclin-dependent kinases (CDKs), cyclins (CCNs), and regulators of the mitotic checkpoints. These alterations have been shown by gene profiling analyses using microarrays (205-207). Overexpressed genes were involved in the regulation of all phases of the cell cycle and cell replication and control of cell cycle progression (205).

Several genes involved in the control of entry in mitosis and mitosis progression were also detected. Overexpression of aurora kinases (AURK) has been reported in several studies (206, 208). In a recent study, higher expression of aurora kinase A (AURKA) mRNA expression was reported in a subset of MM with poor prognosis (170). Stathmin, a gene involved in the regulation of the microtubule dynamics by inhibiting the formation of microtubules and/or promoting their depolymerization, was strongly overexpressed in MPM, resulting in protein overexpression, possibly by an epigenetic regulation (209-211).

These results can account for the complex, even chaotic, chromosomal alterations mentioned above, as the result of a defective control of cell cycle progression through different phases of the cell cycle, including dysregulation of mitosis.

P53/DNA repair and apoptosis

Mutations in *TP53* and *BAP1* play a role in MM pathogenesis. *TP53* has multifunctional tumor-suppressor response, including the DNA damage response (DDR) function and regulation of senescence and apoptosis (212). Additionally, *BAP1* encodes a multifunctional ubiquitin C-terminal hydrolase, which is also involved in DNA repair and stress response (213, 214). Epigenetic mechanism was identified as a mechanism involved in gene silencing in DDR responses (215). A nanostring analysis reported that mRNA expression of 12 target genes involved in different DDR pathways was significantly associated with expression levels of miRNAs in a series of 24 epithelioid MPM (216).

Otherwise, specific regulators can contribute to MM resistance to apoptosis, such as low expression of proapoptotic proteins (Bax, Bak, Bad, Bid, or Bim) and high levels or activity of antiapoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1) regulating mitochondrial function (217-220). Approaches to control MM proliferation have focused on the resistance of MM cells to

apoptosis (221, 222). Integrated analysis of the genomics data identified alteration of P53 signaling pathways (66).

From several studies, P53 appears to be stabilized in MM, suggesting basal overexpression and/or another type of dysregulation. The P53 protein is constitutively expressed, not only in MM cells in culture, but also in immunohistological sections of primary tumors (223-226). Candidates for P53 activation could be up-regulation of IGF-1/AKT/mTOR pathway and altered energy metabolism, which have been identified as additional functions of P53, as recently reviewed (227). Energy metabolism of MM cells is characterized as aerobic glycolysis (the Warburg effect), and the P53 protein could be induced to shut down this pathway (227, 228).

MAPK and PI3K/AKT/mTOR Signaling Pathway

The MAPK and PI3K/AKT/mTOR signaling pathway control various cellular processes, cell proliferation and differentiation, cell migration, survival, apoptosis, response to stress and mitogens, and is deregulated in solid cancers (229). In normal cells, these pathways are triggered by the activating phosphorylation of tyrosine kinase receptors (RTKs), followed by a protein kinase cascade. Downstream networks from RTKs can be activated by RTK mutation or sustained signaling through autocrine or paracrine mechanisms.

MPM cells express both vascular endothelial growth factor (VEGF) and the VEGF receptors (fms-related tyrosine kinases, FLT1 and FLT4, and fetal liver kinase, KDR/FLK1) (230-233). VEGF expression was enhanced in a large proportion of MPM in comparison with nonneoplastic specimens (234). An autocrine role for VEGF in cell proliferation has been suggested (232, 235).

MM cell growth may also be linked to autocrine or paracrine stimulation growth factors such as PDGF (236-242)

Epidermal growth factor receptor (EGFR) is overexpressed in 44–97 % of MM as found by immunohistochemical studies, but no mutation was detected in contrast with others types of cancer (243)

Human MM cells express insulin growth factor (IGF) and insulin growth factor receptors (IGFR), and the activation of IGFR activates downstream signaling (244, 245). IGF-I appears to function as an autocrine growth factor in human mesothelial cells (246). IGFBPs also regulate IGF-dependent growth (245, 247, 248).

Hepatocyte growth factor receptor (MET) is a proto-oncogene and the receptor for the ligand hepatocyte growth factor/scattering factor (HGF/SF). Both MET and HGF/SF proteins are expressed in some MPM suggesting the establisment of an autocrine loop of the (249). *In vitro* HGF/SF increases spreading, motility and/or invasiveness of mesothelial cell lines and inhibition of MET reduced cell proliferation (250-252). The activation status of MET and other RTKs, EGFR family, PDGF-A and PDGFR-B has been investigated in 20 MPM cell lines and 23 primary specimens of MPM, and the effect of MET-specific inhibitors was investigated on cell lines (253). The results showed that inhibition of a single RTK was not sufficient to obtain a tumor suppressor effect but that inhibition of multiple RTK was required (253).

The MAPK signaling pathway is constitutively activated in MM as demonstrated by the phosphorylation and activation of downstream proteins of the MAPK cascade, ERKs, Jun amino-terminal kinases/stress-activated kinases (JNKs/SAPKs) and p38 MAPK, and inhibition of cell proliferation and induction of apoptosis by inhibitors of the pathway (254-256). RTK activation can be achieved by a variety of growth factors, such as EGF family, PDGF, FGF, and HGF/SF, and cytokines such as TGF-ß, TNF, and IL1. The relative levels of tyrosine phosphorylation of 42 distinct RTKs were determined in MM cell lines established from surgical specimens. A coordinated activation of several RTKs **–** EGFR, ERBB3, AXL, and MET –

was reported (257). No recurrent mutations were identitified in members of MAPK signaling pathway in MM.

Activation of RTKs also induces activation of other downstream signaling cascades including PI3K-AK) pathway. PI3K/AKT/mTor is activated in MM (258). Phosphorylation of AKT protein, the active form of the protein, and activation of the Akt pathway have been demonstrated in MM cells (198, 219, 259). In MM cells, PTEN, a TSG and negative regulator of the PI3K-AKT pathway, homozygous deletion has been reported in a very small subset of MPM cell lines (260, 261). Integrated analysis of the genomics data identified mTOR pathway as deregulated in MPM (66). Uppregulation of PI3K and mTOR signaling pathways were associated to poor prognosis (170).

Other signaling pathways

Other signal transduction pathways Wnt, Hedgehog and Notch are activated in MM cells. These pathways are important in embryonic development, and also as regulators of cancer stem cells (CSC), a side population which is resistant to chemotherapy and radiotherapy (262, 263).

The Wnt signaling pathway regulates cell proliferation and cell polarity, its activation prevents beta-catenin inactivation, a coactivator of transcription, allowing the expression of a variety of genes exerting pleiotropic effects (264). However, cell growth inhibition and apoptosis of MPM cells was observed according to a beta-catenin-independent inhibition of Wnt signaling (265, 266). In MPM, the Wnt pathway could be altered as a result of promoter hypermethylation of regulatory genes (265, 267, 268). Gene expression profiling of MM cell lines, primary MPM tumors and normal pleural tissue demonstrated that numerous Wnt and Wnt-related genes were upregulated and that some Wnt antagonists were downregulated (269). These results suggest that deregulation of the Wnt signaling pathway is involved in mesothelial carcinogenesis. Hedgehog signaling pathway is inactive in normal mesothelium, it can be reactivated in some MM and targeted to reduce the stemness-related cell population (270-272). Mutations in genes of these pathway have been suggested in MM (273). The deregulation of Notch signaling pathways has been reported in MM, with expression levels of Notch1 and Notch2 being elevated and reduced, respectively in human MM cell lines (274). These proteins act as positive and negative modulators, respectively, of PI3K/Akt/mTOR signaling pathway.

Epigenetic pathways

More recently, alterations in epigenetic pathways, DNA methylation, histone modification, nucleosome remodeling and RNA-mediated targeting (noncoding RNAs) have been reported in MPM. These pathways are important as they are connected to cancer (275). Modifications of DNA methyl transferases, chromatin remodelers and differential expression of noncoding RNAs in comparison with normal mesothelium are found in MM. DNA methylation was associated to silencing TSGs (276). Mutations in genes *SMARCA4*, *ARID1A* and *ARID2* involved in the chromatin remodeling SWI/SNF complexes have been found in a low percentage of MM, and in the histone methyl transferases *SETD2* and *KMT2D* (66, 276). Promoter methylation was associated with alterations of gene expression and an upregulation of several DNA methyltransferases in MM (276, 277). High-throughput integrated analysis of the genomics data identified histone methylation, RNA helicases pathways are altered in MPM (66).

Immune checkpoints

Immune checkpoints are modified in cancer cells. In normal tissue, they permit the maintenance of a self-tolerance function. In cancer cells, the expression of immune-checkpoint proteins is modified, allowing tumor evasion, and blockade of the immune

checkpoints is a developing field in anticancer immunotherapy (278). Among them inhibitory T cell receptors, CTL4 and PD1, or ligand PDL1 are presentely targeted using specific antibodies to enhance immune recognition (279). Studies have investigated the level of expression of checkpoints proteins in MM, and an heterogeneity between tumors of their expressions and also of the immune cell content has been reported (66, 170, 280). Further studies should improve the knowledge of immune microenvironment of tumor cell and improve targeted immunotherapy (281).

Human MM Molecular Heterogeneity

MM heterogeneity appears to be one cause of the limited efficiency of treatments (282). Histological diversity reflects the various morphological patterns of MM defined through detailed classifications of the tumors (283). Immunohistochemical markers are useful for differential diagnosis of MPM and molecular markers, such as BAP1 protein expression and deletion of *CDKN2A* locus are currently used (284). CGH arrays and gene mutations analyses of MM have added a level of complexity in MM heterogeneity. DNA sequencing have revealed numerous copy number alteration and gene mutations (166). Moreover, within tumors, mutations are not detected in every mesothelioma cell in the tumor, possibly linked to polyclonal evolution (282, 285).

Recently comprehensive genomic analyses allowed classifying MM in different subtypes through transcriptome analysis alone or coupled to other analysis such as sequencing (66, 169, 170, 202, 208, 286). One transcriptomic study reported a clusterisation of MPM in 2 subtypes loosely correlated with tumor histology, consistent with a molecular diversity partly related to morphological pattern (286). In another study, gene expression profiles of epithelioid versus sarcomatoid MM were analyzed, and led to the identification of genes related to lower survival expressed in sarcomatoid MM, such as aurora kinases A and B and functionally related genes involved in mitosis and cell cycle control (208). The authors developed a prognostic classifier based on their microarray data, but found a limited predictive value (208). Howerver, the identification of diagnostic markers is of potential interest for better patients management (208). Another transcriptomic analysis defined two robust molecular MPM subtypes, C1 and C2, only partly related to histologic types but closely related to prognosis (169). Interestingly, epithelioid MPM were found in both groups, with a worse survival prognosis in the C2 subtype. These MM groups also exhibited differential rate of mutations, with more frequent *BAP1* alterations in C1 subtype. Pathways analysis revealed that EMT was differentially regulated between MPM subtypes, C2 subtype being characterized by a mesenchymal phenotype (169). A subtype of C2, the $C2^{LN}$ subtype, characterized by the double inactivation of *NF2* and *LATS2* TSG, was identified by coupling transcriptomic and genetic analyses (202). Another publication has identified four distinct molecular subtypes: sarcomatoid, epithelioid, biphasic-epithelioid (biphasic-E) and biphasic-sarcomatoid (biphasic-S) using RNA-seq data (66). Exome analysis in the same tumor samples confirmed already identified and less commomly known mutated genes, *BAP1*, *NF2*, *TP53*, *SETD2*, *DDX3X*, *ULK2*, *RYR2*, *CFAP45*, *SETDB1* and *DDX51*, and alterations in Hippo, mTOR, histone methylation, RNA helicase and P53 signaling pathways without establishing a link with the 4 molecular subtypes (66). Investigation of the immune microenvromment found highest rates of T cells and M2 macrophages in the sarcomatoid group (66). Finally, one publication reported a comprehensive integrated genomic study providing histology-independent determinants of poor prognosis (170). Four clusters, namely iCluster 1 to 4, were characterized. The authors also defined a genomic subtype with *TP53* and *SETDB1* mutations and extensive loss of heterozygosity, and a strong expression of the immune-checkpoint gene *VISTA* in icluster 1 related to epithelioid MPM (170). Gene methylation seems associated to

prognosis as the methylation level is different between clusters, and higher in better prognosis clusters (170).

The mechanisms of mesothelioma heterogeneity have been recently discussed emphasizing the different levels of MM heterogeneity (287). The recent publications on molecular characterization of MPM and the definition of distinct groups with specific molecular biomarkers linked to prognosis is of paramount interest to refine the diagnosis, to guide the therapeutic option and to develop targeted therapies. In the future, it may be expected that integration of metabolic, epigenetic and genomic data will succeed in proposing therapy adaped to the patients tumor.

Conclusions

Recent studies brought some light on the mechanism of MM carcinogenesis, and some questions remain to be addressed. Carcinogenesis progresses through multi-dependent steps, from fibers inhalation to neoplastic transformation of mesothelial and tumor growth. Asbestos remains the major risk factor for MM and past exposure can explain most of the MM, demonstrating a strong link between asbestos activity and mesothelial cell responsiveness. Lung, larynx, ovary, possibly stomach colon and rectum cancers are other cancers linked to asbestos exposure, but asbestos is not the unique cause for these cancers. Fibers can reach these organs via clearance, translocation and ingestion mechanisms, after inhalation. The relationship with past-asbestos exposure addresses the question of the specific sensitivity of mesothelial cells. The recent investigations carried out with CNT demonstrated a pleural translocation. Further studies would account for a more precise mechanism of particle translocation.

The *BAP1* gene was discovered and suggested as predisposing to MM in a context of asbestos exposure. In human, this gene is mutated in *BAP1* tumor predisposition syndrome (BAP1-TPDS), which increases the risk of a variety of malignant and benign tumors. In MM, *BAP1* mutation may be not a predisposing factor, as other cancers are associated in BAP1-TPDS families, but *BAP1* mutation is more likely a sensitivity factor in subjets exposed to asbestos, asking the question of the role of *BAP1* in mesothelial cell physiology. The results obtained with GEM are consistent with this hypothesis as no MM is found in unexposed *Bap1*$^{+/-}$ mice, in contrast with asbestos-exposed *Bap1*$^{+/-}$ mice. A recent study suggest the association of other germinal gene mutations associated to MM formation (195).

Carcinogenesis is defined by several capabilities that cells acquire during the neoplastic process (288, 289). Asbestos can induce genotoxicity, an early step in mesothelial cell transformation, due to DNA oxidation generated by oxidative stress and inflammation, and chromosome aberrations generated by mitosis impairement. It seems that there is no evidence that inflammation is a perequisite for generating DNA oxidation. Chromosome alterations are also reported in human cells exposed to CNT. Further studies carried out with HARNs should improve our knowledge of the mechanism of fiber-induced genotoxicity.

Studies of human MM cells and tissue samples have identified cellular and molecular changes in comparison with normal cells. MM is characterized by numerous copy number alterations including frequent deletions, gene fusions and point mutations in a limited number of genes, most being TSG. In MM, genes are inactivated by mutation or by methylation. Apart from activating mutations in *TERT* promoter, no other recurrent oncogenic activation has been reported. Inactivated genes in MM are involved in the regulation of several pathways, cell cycle, hippo, P53/DNA repair and MAPK and PI3K/AKT/mTOR regulatory pathways. Moreover, epigenetic changes in DNA methylation, histone modification, nucleosome remodeling and miRNA-mediated targeting were more recently reported to occur in

mesothelioma cells. Ongoing researches will improve our knowledge on the molecular ways followed by mesothelial cells during neoplastic transformation.

Several recent clinico-biological studies have performed a molecular classification of MM, based on transcriptomic and multi-omic studies. The results have highlighted the molecular heterogeneity of MM, where tumors can be classified in different subtypes with different gene mutations, level of epithelial-mesenchymal transition, deregulated pathways, immunological microenvironment, and linked to survival outcome. These studies demonstrate that MM are heterogenous tumors, not only clinically and morphologically but also on a molecular basis. The results are encouraging to go forward and define biomarkers to develop efficient precision medicine.

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