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▶ **To cite this version:**

Agnes Kane, Didier Jean, Sakari Knuutila, Marie-Claude Jaurand. Malignant Mesothelioma: Mechanism of Carcinogenesis. Occupational Cancers, Springer London, pp.299-319, 2014, 10.1007/978-1-4471-2825-0_17 . inserm-02479963

HAL Id: inserm-02479963
https://www.hal.inserm.fr/inserm-02479963

Submitted on 14 Feb 2020

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CHAPTER 22

Mechanism of mesothelial carcinogenesis

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1. Introduction

Our present knowledge of the mechanism of mesothelial carcinogenesis results from pathophysiological and toxicological research carried out *in vivo* in rodents, in mammalian cells in culture, and from biological and molecular studies of malignant mesothelioma (MM) tissue samples and cell lines from humans and experimental animals. In this latter context, most experimental studies have been based on the cellular and/or animal responses to asbestos fibers and in genetically modified mice. 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 networks involved in transformation of the mesothelial cell. The aim of the present work is to propose a potential mechanism of mesothelial carcinogenesis by integrating data based on cellular and molecular effects of asbestos fibers on mesothelial cells, with altered physiological and molecular features of malignant mesothelioma cells.

2. Mechanism of action of asbestos fibers

a. Translocation

The initial route of entry of asbestos fibers is by inhalation and deposition in the tracheobronchial regions, distal airways, and alveolar spaces of the lungs [1]. 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 [2]. Fibers that enter the interstitium may cross the visceral pleural by paracellular migration or by direct penetration [3]. An alternative route of translocation to the pleural space is transport via lymphatics or the bloodstream [4].

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 [5]. 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 [6,7]. The diameter of lymphatic stomata (~ 10-12 μm) limits clearance of long fibers from the pleural space [4].

Systemic dissemination of fibers through lymphatics and the blood stream has been described in humans following autopsy [8-10]. Asbestos fibers and asbestos bodies have been noted in the liver, mesentery, spleen, and abdominal lymph nodes [11,12]. Diffuse peritoneal malignant mesothelioma is also associated with exposure to asbestos fibers [13,14]; fibers may reach the peritoneal mesothelial lining via diaphragmatic lymphatics that connect

the pleura and peritoneal spaces or following systemic vascular and lymphatic dissemination.

b. ***Experimental studies on biological effects of asbestos fibers***

As this volume is devoted to occupational cancer, the studies reported here will focus on asbestos as the only known etiological factor associated with MM. However, other types of 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 carbon nanotubes. Their effects will be discussed separately in subsequent paragraphs related to the fiber parameters related to carcinogenicity (see paragraphs in 22-2.c).

i. Effects of asbestos fibers in animals

Epidemiological studies have clearly linked mesothelial carcinogenesis with asbestos exposure. Nevertheless, no history of exposure can be found in about 10-20% of MM cases [15-18]. This relationship between mesothelioma and asbestos has also been well demonstrated by numerous experimental studies carried out in rodents. It must be noted that in animals, other types of fibers also induce MM. 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, and summarized in peer reviews [19]. 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 [20-23].

Some studies have investigated pleural responses to asbestos fibers after 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 [24]. These growth factors were able to induce proliferation of mesothelial cells in culture. This inflammatory response may be triggered by fiber translocation to the pleura as demonstrated in rodents exposed to glass fibers or to RCF [25,26]. Several studies have demonstrated the presence of asbestos fibers in the human pleura [9,10,27]. Hypotheses on the mechanism of asbestos translocation have been recently discussed [3,4] (see paragraphs 22.2.a).

Several fiber parameters are of importance in the mechanism of asbestos toxicity. They are discussed in paragraphs 22-2.c. In animal experiments, it was generally found that the fiber dimensions were important, with a greater carcinogenic potency of long and thin fibers in comparison with shorter fibers.

Mutations in malignant mesotheliomas have been investigated in animals, after *in vivo* exposure to asbestos fibers. Table 22-1 summarizes genomic alterations in MM identified in asbestos-exposed animals. Although few studies have been performed, these results are consistent with observations made in human MM. Chromosome rearrangements were observed in wild-type animals exposed to asbestos. Mutations and base hydroxylation have

been detected within several weeks after asbestos administration. At the gene level, no or few mutations were found in the tumor suppressor gene (TSG) Tumor protein p53 (*Trp53*), both in wild-type rats and heterozygous *NF2* mice. Interestingly, genes at the *Ink4a* locus were deleted, as found in human MM. In MM from genetically modified mice, gene inactivation occurred by loss of heterozygosity (LOH). These studies suggest that asbestos fibers are genotoxic, and can produce DNA strand breaks and chromosomal recombination.

ii. Effects of asbestos fibers on mesothelial cells in culture.

While early studies have been carried out with cells of different species and tissues, rat and human mesothelial cells have been most widely used to study the response of mesothelial cells to asbestos fibers. Detailed data can be found in a several reviews [28,29].

Various types of asbestos fibers have been found to cause cytotoxic and genotoxic defects in primary cell cultures and in animals exposed to fibers [30]. Typically, chromosomal breaks, centromeric and telomeric alterations as well as aneuploidy (an lower number of chromosomes in comparison with normal cells), polyploidy (twice or several times the normal number of chromosomes) and heteroploidy (an abnormal number of chromosomes) due to spindle defects, are seen. Because of chromosomal breaks, as well as spindle and centrosomal damage, micronucleus formation is a typical feature of asbestos-induced genotoxicity, whereby genotoxic endpoints are quantitated by scoring the number micronuclei [31].

Table 22-2 summarizes genomic alterations in mesothelial cells in culture treated with asbestos fibers. Briefly, when exposed to asbestos fibers, mesothelial cells demonstrate phagocytic properties. Within hours, responses to oxidant stress, activation of the Mitogen-Activated Protein Kinase (MAPK) pathway, and induction of transcription factors are detected. Table 22-3 summarizes activation of various signaling pathways in mesothelial cells in culture exposed to asbestos fibers. When incubated in the absence of serum or in low levels of serum concentration, cell proliferation was observed [32,33]. In proliferating mesothelial cells, asbestos provoked a p53- and p21-dependent cell cycle arrest consistent with the induction of a DNA damage-induced response [34]. P53 was also induced in serum-deprived G0 synchronized mesothelial cells exposed to asbestos, but failed to block cell cycle progression [35]. However, genotoxicity was also found suggesting that the DNA repair mechanism was incomplete, error-prone, or impaired.

Several types of genetic damage have been found in asbestos-exposed mesothelial cells (Table 22-2). Briefly, DNA damage was demonstrated directly by the occurrence of DNA breakage [36-39], and indirectly by the induction of DNA repair [40,41]. Oxidation of deoxyguanosine has been reported in several studies. Notably, recurrent chromosome abnormalities have been reported. These consist in numerical and structural changes, including aneuploidy and polyploidy, micronucleus formation, and chromosomal missegregation [42-48]. Comparison between different studies showed that significant effects were found with doses of 0.5 - 1 $\mu g/cm^2$ [29].

These studies demonstrate that asbestos fibers are genotoxic for mesothelial cells, able to produce base hydroxylation, DNA breakage, and numerical and structural chromosomal changes in 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. As discussed previously, life-or-death decisions may be at the heart of malignant transformation and defective mechanisms of arrest or apoptosis may be critical to development of malignancy [49]. Several studies with mesothelial cells in culture have emphasized the occurrence of apoptosis, which should be beneficial for the mesothelium. However, some cells can survive with gene 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 the cells could pass through cell cycle checkpoints with unrepaired DNA and chromosomal damage.

According to our knowledge, no data on epigenetic changes in asbestos-exposed cells in culture, or in animals have been reported. Further investigations would be of great interest for our understanding of the mechanism of action of asbestos fibers in carcinogenesis.

iii. MM in genetically modified mice

Several models of MM have been developed using genetically modified mice exposed to mineral fibers. One study was based on mice carrying a heterozygous mutation in the TSG *Trp53* (*Trp53*$^{+/-}$), and others on mice heterozygous for a mutation on the neurofibromin 2 gene (*NF2*), a TSG

known to be inactivated in human MM (*Nf2*$^{+/-}$ mice). Interestingly MM cells obtained from *Trp53*$^{+/-}$ mice exhibited *Trp53* LOH and polyploidy [50]. LOH of the *Nf2* gene was found in *Nf2*$^{+/-}$ mice suggesting a common mechanism for loss of the wild type (WT) allele [23,51]. Moreover, in *NF2*$^{+/-}$ mice, two other TSG, cyclin-dependent kinase inhibitor 2a (*p16/cdkn2a*) and cyclin-dependent kinase inhibitor 2b (*p15/cdkn2b*) were deleted at a high rate, similar to human MM, while *Trp53* was mutated at a much lower rate [51,52]. In studies carried out by one of us (MCJ), *Nf2* and *Trp53* were exclusively inactivated. Spontaneous MM in the absence of asbestos exposure have been generated in double mutants *Nf2*$^{+/-}$;*Trp53*$^{-/-}$ and *Nf2*$^{+/-}$;*Ink4a*/*Arf*$^{-/-}$ mice. MM developed rapidly and at a high incidence [53]. These results suggest that MM development can be associated with inactivation of TSG involving several pathways including *Trp53* or *Nf2* and genes at the *Ink4a* locus, the two latter genes being more specific targets of asbestos effects. Murine MM closely mimicked the human disease characterized by peritoneal ascites, a long latency between fiber injection and MM development, and histological subtypes, epithelioid, sarcomatoid and biphasic, similar to human MM. The results obtained with genetically modified mice show that MM progression could follow several routes involving different TSG, and are in good agreement with (i) specific clinical features and molecular alterations in human MM, and (ii) the role of tobacco smoke in cancer development. It is generally accepted that MM is not related to smoking, and that p53 mutation is a signature of tobacco smoke, consistent with no signature of tobacco smoke in MM development.

Nevertheless, this strongly suggests that other carcinogens targeting p53 that could reach the pleura would be able to induce MM.

c. ***Fiber properties in relation to the biological effects and carcinogenic potency***

This chapter will discuss the biological mechanisms leading to development of diffuse malignant mesothelioma focusing on the physiochemical properties of asbestos fibers, carbon nanotubes, and other engineered high aspect ratio nanomaterials relevant for the pathogenesis of this cancer. The reader is referred to the comprehensive reviews cited above for a detailed summary of the toxicological studies related to biological activity of carbon nanotubes.

i. Mineral fibers

Asbestos and erionite are naturally-occurring fibrous minerals that have been associated with the development of diffuse malignant mesothelioma in epidemiological studies [54,55]. 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 [56,57]. Erionite fibers are a form of the mineral zeolite characterized by a high internal surface area [58]. These naturally-occurring fibrous minerals are variable with respect to chemical composition, associated minerals, and trace contaminants depending on their geographic origin [59]. Asbestos fibers may contaminate other mineral deposits, for example, talc [54,60] and vermiculite from Libby, Montana [60,61] and exposure to these mixed materials have also been linked with diffuse

malignant mesothelioma [58,62]. The physiochemical properties of mineral fibers associated with biological activity include shape and dimensions, surface chemistry and reactivity, and biopersistence [19].

ii. Shape and dimensions

Elongated fibers with a high aspect ratio, defined as a length: diameter ratio of 3:1 or greater, are characteristic of the crystalline structure of the mineral. Asbestos fibers occur as bundles of individual crystals or fibrils that split longitudinally at the silicate layers. Fiber length and diameter determine respirability and site of deposition in the lungs and fiber length is related to efficiency of phagocytosis by alveolar macrophages and rate of clearance from the lungs [19].

Titanium dioxide nanorods have been shown to induce frustrated phagocytosis and activation of the Nalp3 inflammasome [63] similar to asbestos fibers [64]. Carbon nanotubes have also been shown to induce frustrated phagocytosis by macrophages in vitro [65]. In rodents, long rigid carbon nanotubes have been shown to translocate to the subpleural regions of the lungs [66-69] and to induce inflammation, frustrated phagocytosis, and granulomas similar to asbestos fibers following intraperitoneal injection [65]. Direct intraperitoneal [70] or intrascrotal injection [71] of some commercial carbon nanotubes induced diffuse malignant mesothelioma in heterozygous p53-deficient mice and wild type rats, respectively. However, short multiwalled carbon nanotubes (< 1 μm long) did not induce mesotheliomas in rodents following intraperitoneal injection [72].

iii. Surface chemistry and reactivity

Serpentine or chrysotile asbestos is a magnesium silicate ($Mg_3 Si_2O_5(OH)_4$); Al^{3+} or Fe^{2+} may substitute for Si^{4+} or Mg^{2+}. Amphibole asbestos fibers are double-chain silicates containing a variety of cations including Fe^{2+}, Fe^{3+}, Mg^{2+}, Al^{3+}, Ca^{2+}, and Na^{+}. Surface chemistry determines interactions between the fiber, physiological fluids, and cells with possible proton transfer, oxidation-reduction reactions, and adsorption of biological macromolecules [58]. Broken chemical bonds at the fiber surface are highly reactive with molecular oxygen and can generate free radicals in aqueous fluid [73]. Surface Fe^{2+} and Fe^{3+} ions on amphibole asbestos fibers are bioavailable and catalyze formation of reactive oxygen species (ROS) [74]. Erionite fibers can acquire Fe^{2+} and Fe^{3+} ions and become redox active in the presence of intracellular chelators or reductants such as citrate or ascorbate [75]. Iron-catalyzed redox activity has been associated with biological effects of mineral fibers including lipid peroxidation, oxidative DNA damage, and activation of intracellular signaling pathways [76,77].

Genotoxicity of natural and man-made fibers has been linked with surface reactivity, especially redox activity, as detected using acellular assays for free radical generation [78], induction of micronuclei [28], and mutagenicity using a hamster-human hybrid cell line [79]. Amphibole and chrysotile asbestos fibers show strong activity using these assays, while silicon carbide fibers show no free radical activity [78]. Refractory ceramic fibers contain bioavailable iron and are active in the salicylate assay to detect release of hydroxyl radicals [78]. Chrysotile asbestos fibers, tremolite (an amphibole fiber that contaminates chrysotile deposits), and erionite are mutagenic in the

hamster-human hybrid cell line, while refractory ceramic fibers are non-mutagenic [79].

The ability of carbon nanotubes to generate free radicals is controversial. Some commercial carbon nanotube samples have not been shown to generate carbon or oxygen-centered free radicals using spin-trapping and electron spin resonance [68,80]. In fact, carbon nanotubes can scavenge hydroxyl and superoxide radicals which has been attributed to defects in the graphene sidewalls creating gaps in the carbon lattice and dangling bonds [81]. Multiwalled carbon nanotubes are not directly mutagenic in bacterial reverse mutation assays [82]. Agglomerated multiwalled carbon nanotubes are also negative in this assay and do not induce chromosome aberrations in the V79 cell assay [83]. Long multiwalled carbon nanotubes, but not short multiwalled carbon nanotubes or long single-walled carbon nanotubes, induced DNA strand breaks in human lung epithelial cells [84]. Multiwalled carbon nanotubes also induced micronuclei in rat lung epithelial cells in culture and in animals [85]. Single-walled carbon nanotubes, carbon nanofibers, and graphite nanofibers induced micronuclei in V79 cells [86] and human bronchial epithelial cells [87]. Both single-walled and multiwalled carbon nanotubes have been shown to induce oxidative stress, DNA damage, and activation of intracellular signaling pathways in cultures of human mesothelial cells [88,89].

Direct generation of ROS at the surface of asbestos or erionite fibers may be amplified by secondary generation of reactive oxygen and nitrogen species by target cells, including inflammatory cells and mesothelial cells in the

pleural lining [76,90]. Target cells generate endogenous ROS and reactive nitrogen species during the process of phagocytosis [91], disruption of mitochondrial electron transport [77], and activation of inducible nitric oxide synthase generating nitrogen-derived radicals [92]. These exogenous and endogenous reactive oxygen and nitrogen species have multiple effects on target cells in the pleura that amplify the inflammatory response, activate inflammatory cells to release chemokines, cytokines, and other mediators, stimulate cell proliferation, and induce cell injury and apoptosis [64,76].

Fiber length has also been associated with induction of aneuploidy and chromosomal damage due to direct physical interference with the mitotic apparatus [28,93] or by binding to cell cycle regulatory proteins [94]. Induction of chromosomal breaks and aneuploidy has been shown for single-walled carbon nanotubes and carbon nanofibers in V79 cells [86] and for single-walled and multiwalled carbon nanotubes in rat [85] and human [87] lung epithelial cells. These direct physical effects of long, thin fibers on target cells in the lungs and pleura raise concern about potential carcinogenicity of man-made mineral fibers that have been developed as asbestos substitutes [19] or engineered fibrous nanomaterials including carbon nanotubes [4,95] and metal and metal oxide nanorods or nanowires [63]. Although these man-made fibers and engineered nanomaterials may not have intrinsic redox activity, other surface properties (e.g., structural defects or carbonaceous residues on the surface of carbon nanotubes) may generate oxygen-derived radicals.

iv. Biopersistence

A major determinant of fiber pathogenicity is biopersistence in the lungs [19]. If long fibers are not efficiently cleared or destroyed by physical breakage, splitting or chemical dissolution in the lungs, they are called biopersistent [19]. Differences in biopersistence of asbestos fibers have been linked with carcinogenic potency, as biopersistent fibers could sustain a local inflammatory response [96]. Amphibole asbestos fibers are more potent than chrysotile asbestos fibers due to their increased biopersistence in the lungs. However, 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 [97,98].

Biopersistence of natural and man-made fibers in the lungs [99] or peritoneal cavity [100] is an important characteristic of fibrous materials that induce lung cancer and diffuse malignant mesothelioma in rodents following inhalation [19]. Man-made mineral fibers developed as asbestos fiber substitutes, especially refractory ceramic fibers [26] and silicon carbide whiskers, have been shown to be biopersistent [101] following inhalation by rodents. Following inhalation by hamsters, refractory ceramic fibers translocated to the pleura and induced mesothelial cell proliferation and fibrosis [26]. Refractory ceramic fibers also induced pleural malignant mesotheliomas after chronic inhalation by rats and hamsters [19]. Intrapleural [102] or intraperitoneal injection of silicon carbide whiskers [103] also induced diffuse malignant mesothelioma in rats. Although no malignant mesotheliomas have been reported in worker cohorts involved in

manufacturing and application of refractory ceramic fibers, the rodent carcinogenicity assays raise concern that long thin biopersistent mineral fibers may be carcinogenic [104]. Erionite fibers are very potent in induction of malignant mesotheliomas following intrapleural injection [105] or inhalation [106].

Natural and man-made fibers are not unique in induction of rodent malignant mesotheliomas following intraperitoneal or intrapleural injection. A variety of chemicals, radionuclides, SV40 virus, and metallic nickel particles are also carcinogenic in this rodent bioassay [107]. From a mechanistic viewpoint, ferric saccharate, nitrilotriacetic acid, nickel particles, and alpha- or beta- emitting radionuclides are notable in their abilities to generate reactive oxygen species [108].

Unfunctionalized carbon nanotubes are bioperistent when assessed in acellular assays [109]; however, carboxylated single-walled carbon nanotubes are susceptible to enzymatic [110] or oxidative degradation [111]. In principle, carbon nanotubes could be engineered to alter their physiochemical properties in order to decrease their biological reactivity and potential carcinogenicity.

High aspect ratio and biopersistence [4,112] have been hypothesized to be important properties of engineered nanomaterials that raises concern about their potential to be translocated to and retained in the pleural following inhalation. So far, this hypothesis has not been tested in any long-term inhalation studies of high aspect ratio engineered nanomaterials in rodents.

v. Unique characteristics of nanomaterials

Additional features of engineered carbon nanomaterials that may alter their biological activity include their purity, rigidity, hydrophobicity, and agglomeration state. Carbon nanotubes are frequently produced commercially in the presence of metal catalysts including nickel, iron, cobalt, and yttrium [113]. Other potential contaminants include combustion-derived products such as polycyclic aromatic hydrocarbons [113]. Amorphous carbon residues at the graphenic surface of carbon nanotubes may also contribute to surface reactivity [85]. Bioavailability of metal catalyst residues is variable depending on the purity of carbon nanotubes; redox active metal catalyst residues can generate reactive oxygen species leading to cell toxicity, inflammation, activation of intracellular signaling pathways involving the MAPK and the nuclear factors NF-KB and AP-1 [76], and genotoxicity [95]. Carbon nanotubes can be highly variable in length ranging from 1nm to 1mm. Although short nanotubes and nanofibers less than 5 μm in length should be more easily phagocytized and cleared following inhalation [4], they may behave as needles and penetrate into cells and the nucleus where they could directly damage chromosomes and DNA [95]. Unfunctionalized carbon nanomaterials are very hydrophobic and tend to form agglomerates or bundles called nanoropes, although individual carbon nanotubes have been detected in aerosols [114]. Hydrophobic nanomaterials may interact differently with biological macromolecules in comparison with hydrophilic crystalline mineral fibers [115]. Very thin, hydrophobic carbon nanotubes may bend and agglomerate to form spherical

aggregates that are more readily phagocytized than long, rigid multiwalled carbon nanotubes that have been shown to induce frustrated phagocytosis resulting in impaired clearance and translocation to the pleura [4,116]. The extent of agglomeration has also been shown to influence cell toxicity: rope-like agglomerates of carbon nanotubes were shown to be more toxic than crocidolite asbestos fibers using a mesothelioma cell line [115]. Finally, structural defects at carbon nanotube surfaces attributed to imperfections in the graphene lattice or defects leading to surface oxidation and increased hydrophilicity have been shown to contribute to acute toxicity and genotoxicity of even short multiwalled carbon nanotubes [85].

The potential of engineered carbon nanotubes to induce pathological reactions (lung inflammation, fibrosis, and diffuse malignant mesothelioma) similar to asbestos fibers has generated significant controversy and concern for occupational safety and health [112,117]. Occupational exposures via inhalation, skin contact, and ingestion are possible during the synthesis, handling, and fabrication steps of engineered carbon nanotubes; airborne mass concentrations in the range of 0.7 – 430 $\mu g/m^3$ have been detected at eight worksites and research laboratories [118]. Several recent reviews have summarized the numerous in vitro cellular and rodent toxicology studies investigating biological activity and potential toxicity of carbon nanotubes [90,114,116,118].

d. Summary hypotheses on the mechanism of action of asbestos fibers to generate mesothelioma

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 as summarized in Figure 22-1. The most important properties of asbestos fibers related to carcinogenicity are fibrous shape and dimensions, surface chemistry and reactivity, and biopersistence [19]. Long, rigid biopersistent fibers that are translocated to the pleura are trapped on the parietal pleura lining at the sites of lymphatic openings [27] and incite a persistent inflammatory response [4]. The pleura is covered by a thin, single layer of mesothelial cells that have lower antioxidant defenses than lung epithelial cells [119].

Interactions between mesothelial cells and fibers can cause genetic and chromosomal changes. There is a great body of evidence that 1) asbestos fibers can directly interfere with chromosomes and the mitotic spindle [120-122], and 2) that they induce formation of reactive oxygen species (ROS) resulting in DNA breaks, oxidation, and mutations [123]. 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 [123]; and they are well shown to initiate signal transduction pathways that are, in turn, linked to inflammation, proliferation, and apoptosis [124]. Free radical scavengers have reported to decrease genotoxic endpoints such as micronucleus formation induced by

fibers [125]. Further, there is clear-cut evidence that antioxidant enzymes can protect cells against genotoxicity induced by chrysotile fibers [126].

Prolonged interaction between pleural inflammatory cells and adjacent mesothelial cells causes persistent release of chemokines and cytokines, inflammatory mediators, reactive oxygen and nitrogen species, and growth factors that trigger repeated episodes of inflammation resulting in mesothelial cell injury, death, and proliferation [127]. In this chronic inflammatory microenvironment, genomic instability and acquired genetic and chromosomal alterations in mesothelial cells may lead to altered cell cycle and growth regulation, resistance to apoptosis [128], impaired repair of DNA and chromosomal damage induced directly or indirectly by asbestos fibers [28,93], and activation of oncogenes and inactivation of tumor suppressor genes [129]. Persistent inflammation has also been linked with altered gene methylation patterns identified in diffuse pleural malignant mesotheliomas in humans [130]. DNA methylation leads to epigenetic gene silencing and has been linked to inflammation-mediated damage to cytosine [131] or endogenous generation of methyl radicals [132]. This persistent inflammatory microenvironment in combination with oxidative stress generates a strong selective force for mesothelial cells that have acquired genetic and epigenetic changes that promote their survival, proliferation, and tumor progression [133].

3. Mesothelial cells and malignant mesothelioma

a. ***The mesothelial cell in situ. Normal cells.***

The mesothelium consists of a monolayer of mesothelial cells lying on a basement membrane and supported by connective tissue stroma containing fibroblasts. Mesothelial cells provide a protective barrier for frictionless interface for the free movement of apposing organs and tissues, and in fluid transport across the pleura [134]. Mesothelial cells may have specialized functions at different anatomical sites, as demonstrated by morphological studies at the ultrastructural level [135]. Mesothelial cells play a role in the resolution of inflammation and tissue repair after pleural injury. 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 is poorly understood, mostly in the context of serosal injury following dialysis; however some controversial hypotheses have been formulated. Recent comprehensive reviews summarize our present knowledge of these potential mechanisms [136,137]. 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 : (i) centripetal migration of adjacent mesothelial cells, (ii) exfoliation of mature or proliferating mesothelial cells that replicate on the wound surface, (iii) pre-existing free-floating serosal cells having the capability to differentiate into new mesothelium, (iv) macrophage transformation into mesothelial cells, (v) submesothelial mesenchymal precursors that migrate to and differentiate at the mesothelium surface, and (vi) bone marrow-derived circulating precursors [137].

The origin of these new mesothelial cells has not yet been confirmed, but according to Mutsaers *et al*. [136], mesothelial regeneration is not dependent on subserosal cells, but

more likely results from implantation, proliferation, and incorporation of free-floating mesothelial cells [138].

b. ***The malignant mesothelioma cell***

i. Role of gene mutations in the neoplastic transformation of mesothelial cells

Carcinogens provoke several types of somatic gene mutations, consisting of DNA and chromosome alterations. Some mutations are the signature of past exposure to one or several 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.

In malignant pleural mesothelioma (MPM), there is a limited number of genes known to be recurrently mutated. Mutations in TSG cyclin-dependent kinase inhibitor 2A (*P16/CDKN2A*), an alternative open reading frame of *CDKN2A* generating a distinct protein (*P14/ARF*), cyclin-dependent kinase inhibitor 2B (*P15/CDKN2B*) and *NF2* have been reported in a high percentage of MM, and *TP53* (tumor protein p53) has been found mutated at a lower rate in comparison with other human cancers [139-141]. These genes play a role in cell cycle regulation at different levels. The *CDKN2A* locus encodes two different proteins, $p16^{INK4A}$ and $p14^{ARF}$ while *P15/CDKN2B* encodes one protein $p15^{INK4B}$. Both $p16^{INK4A}$ and $p15^{INK4B}$ are inhibitors of the kinase function of cyclin/cdk complexes involved in cell cycle progression. *TP53* encodes a protein, p53, which is activated in response to DNA damage and is a regulator of apoptosis. The protein $p14^{ARF}$ has 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 $p14^{ARF.}$ Interestingly, all of these genes carry different types of mutations. The most frequent alteration at the *P16/CDKN2A* and *P15/CDKN2B* loci is homozygous deletion in about 70% of MM cases [141]. In murine models of asbestos-induced mesothelioma, the orthologous genes, *p16/Cdkn2a* and *p15/Cdkn2b*, were also inactivated by deletion [51,52,142]. It can be also noted that *P16/CDKN2A* deletions have been considered as a marker of asbestos exposure in a study of non small cell carcinomas [143]. However, in MM, DNA methylation of *P16/CDKN2A* and *P15/CDKN2B* has been reported at a frequency of 13% (9 patients) and 4% (3 patients) respectively, and positively correlated with asbestos body counts in the lung [130,144]. The average methylation frequency of these gene in the literature is about 10% [52,144-149]. It was also suggested that mesotheliomas express microRNA (miRNA) that could inhibit *P16/CDKN2A* expression, based on *in silico* analysis for miRNA target gene prediction [150].

Point mutations are the main types of alterations of *TP53* in MM. Six point mutations are indicated in the IARC p53 database, five missense mutations and one stop mutation [151]. So far, no specific type of mutation in *TP53* has been related to asbestos exposure. In lung cancer, G:C-to-T:A transversions are generally interpreted as mutagenic fingerprints of tobacco smoke [152]. This base substitution can be due to the formation of 8-OH-deoxyguanosine generated by oxidative damage, which in turn causes primarily G-to-T transversions. A few studies have reported *TP53* mutations

in relationship to asbestos exposure. In lung cancer, the frequency of mutations was diminished in lung adenocarcinomas of asbestos-exposed subjects in comparison with unexposed patients but the difference was not significant [153]. G-to-T transversions in asbestos-exposed lung cancers have been reported, but not in all studies, and G:C-to-A:T transitions are rare [153].

TP53 mutations reported in MM consisted of different types of base substitution and base deletion but G:C-to-A:T transitions seems to be the most frequent [151] (unpublished data from MCJ). In animal models of MM, 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 wild type allele was found at a high rate in MM induced by asbestos fibers [154]. In $Nf2^{WT}$ and $Nf2^{+/-}$ FVB mice, *Trp53* alterations were infrequent. Two point mutations A:T-to-C:G were detected in mice exposed to asbestos, and two point mutations, A:T-to-G:C and A:T-to-T:A, and a duplication of 12 bases in MM were found in mice exposed to ceramic fibers [52,142]. Frequency of alteration in the chromosomal region of the *Trp53* locus was infrequent [155]. 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.

Alterations of *NF2* TSG are frequently found in about 50% of MPM [156,157]. This gene encodes merlin, a protein found in cell-cell junctions and microvilli and regulating contact-dependent cell proliferation [158,159].

NF2 has pleiotropic functions, being involved in regulation in cell proliferation, apoptosis and endocytic trafficking, and acting upstream of the Hippo signaling pathway [160]. Mutations in *NF2* consist of both point mutations and deletions [129]. So far, there is no explanation for the high level of alterations in *NF2* in MPM. However, some hypotheses can be formulated and will be discussed below (see 4. Concluding remarks).

ii. Role of genomic alterations in the neoplastic transformation of mesothelial cells

Chromosome banding, fluorescence in situ hybridization, flow cytometry, Southern blotting and chromosome and array comparative genomic hybridization, single nucleotide polymorphism (SNP) array and representational oligonucleotide microarray analysis (ROMA) as well as second generation sequencing analyses all indicate complex genomic alterations in MM [161-171]. Typically, chromosomal abnormalities are very complex, even chaotic, that is involving alterations both in chromosome structure and number [161,163-165,172-174]. It is characteristic for this disease that the chromosome number is mostly hypodiploid (less than 46 chromosomes, the normal number of chromosomes in human), but it varies greatly within a specimen, as a given tumor can exhibit a variety of hypodiploid metaphases [161]. Similarly, polyploid forms (with a number of chromosomes twice or more the number of chromosomes present in the parental cell) of the hypodiploid clone are commonly encountered. Other cytogenetic alterations may be observed such as diplochromosomes of endoreduplication which are

a signature of alteration of the mitotic process. The polyploidization and non-disjunction type of aneuploidy are due to fiber-induced damages to the structures involved in cell division, i.e. the middle spindle, centrosome, centriole, cleavage furrow, and cell membrane.

Similar to numerical chromosomal aberrations, structural aberrations in MM are highly variable. Typically, translocations, deletions, insertions and inversions are seen. Occasionally, double minutes and a homogenously staining region, representing cytological manifestations of gene amplification are also observed. So far, translocations are mainly unbalanced and no recurrent chromosomal translocations with known fusion genes have been reported. Due to chaotic nature of these aberrations and methodological difficulties, the detection of specific chromosomal aberrations has been very difficult in karyotypic analysis. Novel next generation sequencing methods, such as exome sequencing, has facilitated overcoming the above-mentioned problems and, for the first time, fusion genes have been described in MM [169].

These described structural changes are mainly due to DNA breaks. The mechanism known as breakage-fusion-bridge phenomenon nicely explains severe chromosomal imbalances and intratumoral heterogeneity in MM [175]. As already mentioned, asbestos fibers are capable of causing DNA breaks either directly or indirectly through ROS generation. Whether there are hot spots in the genome for DNA breaks caused by fibers is still largely an open question. However, experiments with cells in culture have indicated that chromosome aberrations induced by fibers may be recurrent. Certain

numerical chromosomal abnormalities have been reported to be over-represented in human pleural cell cultures exposed to asbestos [176]. Even though no distinct hot spots were seen in this study, chromosome 1 seemed to be involved more often than other chromosomes. Interestingly, we have previously reported that structural aberrations in the short arm of chromosome 1 and loss of material in the short arm chromosomes 1 and 4 were associated with high asbestos fiber burden in MM [177,178]. More recently, one of us (DJ) identified a recurrent region of chromosome loss, 14q11.2-q21, in MM from asbestos-exposed patients that was not found in unexposed patients. The syntenic region that also lost in fiber-induced MM in mice, suggesting that this region might be a target of action of mineral fibers [155].

Very recent experiments from one of us (SK), carried out with cell lines and with lung tumor tissues (not mesotheliomas) of patients who had been either exposed or unexposed to asbestos fibers, indicated a couple of asbestos associated chromosomal areas. These findings are described in detail in Chapter 24. Even though chromosomal aberrations in MM are complex, they are not random and they are clonal in nature originating from one cell. Chapter 24 describes, in more details, these recurrent aberrations and their clinical significance.

The chromosomal alterations characteristic of MM, such as hypodiploid chromosomes number as well as deletions and losses in chromosome 14 and 10, are not seen in lung adenocarcinoma, which helps in differential diagnosis of these malignancies [179,180]. Interestingly, chromosomal

aberrations in gastrointestinal stromal tumors (GIST) resemble those seen in MM [181]. To our knowledge GIST is not, however, an asbestos related tumor, but the similarities of chromosomal alterations may, instead, be related to similarities in mesenchymal stem cells from which the tumors originate.

To conclude, asbestos fibers cause a wide variety of chromosomal imbalances. Even though some of these changes may be recurrent, most of them are random. Various genetic changes caused by asbestos fibers offer a versatile genomic aberration reservoir, from which the aberrations promoting uncontrolled growth and malignant transformation are selected during the long initiation and progression (latency) period before tumor diagnosis. Variable chromosomal aberrations together with multifocal clonal evolution are consistent with this mechanism.

iii. Role of epigenetic alterations in the neoplastic transformation of mesothelial cells

Altered gene expression in MPM could also be due to epigenetic mechanisms. MPM show specific patterns of gene methylation as compared to normal pleura or other tumors [130,145,148,182,183]. Data on methylation profiles of MPM will be described in detail chapter 24. Several studies suggested that DNA methylation at specific gene loci could be correlated with asbestos exposure. Significant associations between asbestos exposure and DNA methylation were first described in genes encoding heavy metal binding proteins, *MT1A* and *MTA2*, with a positive association for *MT1A*, but not for *MT2A*. Asbestos exposure does not seem to be an

independent variable in this study [184]. A trend towards an increasing number of methylated cell cycle control genes (*APC*, *CCND2*, *CDKN2A*, *CDKN2B*, *HPPBP1* and *RASSF1*) and increasing asbestos body counts was observed [144]. These findings were confirmed in a more recent, high-throughput methylation analysis underlining distinct methylation profiles between MPM from asbestos-exposed and unexposed patients, and a significant positive association between asbestos fiber burden and methylation status of *CDKN2A*, *CDKN2B*, *RASSF1* and *MT1A* and about one hundred other loci [130].

MiRNAs are small (around 22 base pairs in size) RNAs that have a crucial role in posttranscriptional gene regulation. Their biosynthesis and functions have been described in more detail in Chapter 24. It has been demonstrated that MPM has a characteristic miRNA profile and that different MPM histopathological subtypes can be discriminated according to their profiles (see Chapter 24). Even though significantly differentially expressed miRNAs discriminated MPM patients according to smoking habit, this did not significantly discriminate asbestos-exposed patients versus unexposed [150]. The reason for this may be the low number of non-smoking patients. On the other hand, it is possible that patients classified into the unexposed category were actually exposed to asbestos fibers. Recent results provide evidence that a group of miRNAs differentiates asbestos associated lung adenocarcinomas from the non-associated tumors [185]. The results of these lung carcinoma studies are presented in detail in Chapter 24. As the mechanisms of the miRNA regulation are yet poorly understood, it is

premature to speculate how asbestos fibers cause miRNA dysregulation seen in MM and in lung carcinomas. Nevertheless, some of them could be lost, as their loci are located in chromosomal regions frequently altered in MPM and possibly linked to asbestos exposure, as was demonstrated for miR31 which is close to the *CDKN2A* locus [186]. So far, no experiments using cell cultures or experimental animals have been published that investigate miRNA profiles in asbestos-exposed cells or animals. Further investigations are needed to elucidate the mechanisms responsible for miRNA dysregulation and function in MM. In two MPM cell lines lacking either miR31 or miR29C, overexpression by transfection of these miRNAs decreased proliferation, migration, invasion, and colony formation [186,187].

The molecular mechanisms responsible for epigenetic changes in MPM are poorly understood and it is not known whether they are directly induced by asbestos or they are indirect effects. Nevertheless, as with chromosomal imbalances, they most likely play a role in mesothelial carcinogenesis.

iv. Pathways involved in the neoplastic transformation of mesothelial 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 activation in MM has been shown by gene expression profiling. So far, the relationship between pathway activation and asbestos exposure has not been specifically

investigated in MM. The effects of asbestos on mesothelial cells are discussed in paragraph 22-2.b.ii.

The Hippo pathway is of special interest regarding the high frequency of mutations detected in merlin encoded by the *NF2* gene. Merlin negatively regulates cell proliferation. Its activity is affected by interaction between extracellular signals and membrane proteins, and activated merlin transduces signals suppressing the transcriptional activity of YAP coactivator [141]. In a recent study, another negative regulator of the hippo pathway, *LATS2*, was found to be deleted in 3 out of 6 MM cell lines and in 1 out of 25 tumors by DNA sequencing analyses [188]. Merlin exists in two forms, active unphosphorylated or inactive phosphorylated. This later form is found in MPM cells possibly accounting for another mechanism for deregulation of the hippo pathway in these cells [189].

Cell cycle. Alteration of CDK inhibitor genes located at the *INK4* (*CDKN2A* and *CDKN2B)* locus, as mentioned above, contributes to uncontrolled cell proliferation. However, 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 [190-192]. Overexpressed genes were involved in the regulation of all phases of the cell cycle, cell replication and control of cell cycle progression: cyclin-dependent kinase 1 (*CDK1/CDC2*); cell division cycle 6 (*CDC6*), a regulator of replication; cyclin-dependent kinase inhibitor 2C, p18, (*CDKN2C*); cyclin H (*CCNH*); cyclin B1 (*CCNB1*),

controlling the cell cycle at the G2/M transition; forkhead box M1 transcription factor (*FOXM1*), a regulator of gene expression in the G2 phase. Others are more specific of a response to DNA damage such as checkpoint kinase 1 (*CHEK1*). The protein encoded by this gene, Chk1, is required for checkpoint-mediated cell cycle arrest in response to DNA damage. Underexpression of cyclin D2 (*CCND2*), a regulator of Cdk4 and Cdk6, which controls the cell cycle at the G1/S transition), was also detected [190].

Several genes involved in the control of entry in mitosis and mitosis progression were also detected. Overexpression of aurora kinases has been reported in several studies [191,193]. Stathmin, a gene involved in the regulation of the microtubule dynamics, by inhibiting the formation of microtubules and/or promoting their depolymerisation, was strongly overexpressed in MPM, resulting in protein overexpression [194,195].

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

Signaling pathways. The MAPK signaling pathway controls cell proliferation and differentiation, survival, apoptosis and Wnt signaling [196]. In normal cells, the MAPK pathway is 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.

The MAPK signaling pathway is constitutively activated in MM as demonstrated by the phosphorylation and activation of downstream proteins of the MAPK cascade, extracellular-regulated kinases (ERKs), Jun amino-terminal kinases/stress-activated kinases (JNKs/SAPKs), and p38 MAPK [197,198] and inhibition of cell proliferation and induction of apoptosis by inhibitors of the pathway [199]. RTKs activation can be achieved by a variety of growth factors, EGF family, PDGF, FGF, HGF/SF and cytokines such as TGF-ß, TNF and IL1. In a recent study, the relative levels of tyrosine phosphorylation of 42 distinct RTKs was determined in MM cell lines established from surgical specimens. Coordinated activation of several RTKs**:** EGFR, ERBB3, AXL and MET was found [200].

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*) [201-204]. VEGF expression was enhanced in a large proportion of MPM in comparison with nonneoplastic specimens [205]. An autocrine role for VEGF in cell proliferation has been suggested [203,206].

MM cell growth may also be linked to autocrine or paracrine stimulation by platelet-derived growth factor (PDGF), and the regulation by PDGF appears to be complex in MM cells. PDGF has been suggested as a regulatory factor for proliferation of MM cells, either directly or indirectly via the hyaluronan/CD44 pathway [207,208]. Human MM cells express high levels of PDGF-A, and PDGF-B and PDGFR-B while normal human mesothelial cells express low levels of PDGF-A mRNA chain and PDGFR-A [209,210].

PDGF-A could contribute to tumor formation via a paracrine mechanism [211,212].

Epidermal growth factor receptor (EGFR) is over-expressed in 44–97% of MM as found by immunohistochemical studies, but no mutation was detected in contrast with others types of cancer [213].

Human MM cells express insulin growth factor (IGF) and insulin growth factor receptors (IGFR), and the activation of IGFR activates downstream signaling [214,215]. IGF-I appears to function as an autocrine growth factor in human mesothelial cells [216]. IGFBPs also regulate IGF-dependent growth [215,217,218].

Hepatocyte growth factor receptor (MET) is a proto-oncogene. It is the receptor for the ligand hepatocyte growth factor/scattering factor (HGF/SF). Mutation in the *MET* gene has been detected in a few MM cell lines [219,220]. Both MET and HGF/SF proteins are expressed in some MPM [221,222]. *In vitro* HGF/SF increases spreading, motility and/or invasiveness of mesothelial cell lines and inhibition of MET reduced cell proliferation [219,223,224]. The activation status of MET and other RTKs, EGFR family (Erb1, Erb2, Erb3), 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 (MET-shRNA interference vector and RTK inhibitors) was investigated on cell lines [220]. 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 [220].

Activation of RTKs also induces activation of other downstream signaling cascades including phosphatidylinositol-3-kinase (PI3K-AKT) pathway, regulating cell survival and proliferation, cell migration and apoptosis. Phosphorylation of AKT protein, the active form of the protein, and activation of the Akt pathway have been demonstrated in MM cells [225,226]. In *PTEN*, a TSG and negative regulator of the PI3K-AKT pathway, homozygous deletion has been reported in a small subset of MPM cell lines [227,228].

The Wnt signaling pathway regulates developmental processes, cell proliferation, and cell polarity and its activation prevents beta-catenin inactivation, a coactivator of transcription, allowing the expression of a variety of genes exerting pleiotropic effects [229]. However, cell growth inhibition and apoptosis of MPM cells was observed according to a beta-catenin-independent inhibition of Wnt signaling [230,231]. In MPM, the Wnt pathway could be altered as a result of promoter hypermethylation of regulatory genes [230,232,233]. 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 [234]. These results suggest that deregulation of the Wnt signaling pathway is involved in mesothelial carcinogenesis.

Apoptosis. Deregulation of signaling pathways likely plays an important role in dysfunction of apoptosis in MPM. Moreover specific regulators can contribute to MM resistance to apoptosis. In MM cells, apoptosis alteration

can be due to overexpression of the caspase-8 inhibitor, *FLIP*/*CFLAR*, to the methylation of cell death agonist TRAIL receptors and/or by the 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 [226,235-238].

4. Concluding remarks

Several hallmarks of cancer have been considered to contribute to neoplastic transformation [239]. These include direct molecular damage induced by carcinogens that alter the genome and induce dysregulated cellular functions and resistance to apoptosis. Neoplastic progression is associated with genetic and chromosomal instability. Genetic instability reflects unrepaired DNA damage which may arise either from increased rates of damage or defective mechanisms responsible for genetic integrity. Chromosomal instability arises from dysregulation of mitotic checkpoints. As a consequence, cancer cells fail to control the cell cycle and to correct error-free DNA and to repair chromosome damage. Investigation of the mechanism of asbestos carcinogenicity has focused on interactions between asbestos and target cells, especially mesothelial cells, and early responses of lung and pleural cells to asbestos exposure. Studies of human MM cells provide the opportunity to identify the cellular and molecular changes that have accumulated over the latent period of thirty to forty years since the beginning of asbestos exposure. However, the body of data obtained by these mechanistic studies using cells and experimental animals reveal that all types

of asbestos fibers induce early genetic changes directly and also indirectly due to the early recruitment of macrophages and inflammatory cells. These early genetic changes cause molecular alterations that perturb cell cycle control giving rise to sustained cell proliferation, and additional genetic and chromosomal instability. Early activation of proliferation and survival pathways has been shown in asbestos-exposed mesothelial cells in culture in short-term experiments. The relationship between these early effects and the characteristics of MM cells studied 30 to 40 years after the beginning of exposure remains to be explored.

When the molecular status of human MM is placed in the context of results from studies with cells in culture and in animals, consistent mechanisms emerge. Among genes inactivated in MM, those at the *INK4* locus control the cell cycle, and loss of their function results in failure of cell cycle control. The functional consequences of *P14/ARF* loss are more complex. This does not seem to be associated with p53 degradation, as expected from the known negative regulation of p53 stability by $p14^{ARF}$ loss. In contrast, 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 [240-243]. 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 [244]. The AKT/mTOR cell survival and growth pathway is activated in MM and linked to apoptosis resistance. It is remarkable that

current approaches to control MM proliferation have focused on the resistance of MM cells to apoptosis [245,246]. 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 [244,247]. The low rate of p53 mutations found in asbestos-induced MM in both humans and mice and the functional response of p53 in asbestos-exposed cells are consistent with these observations.

Transcriptional analyses suggest that cell cycle checkpoints are compromised in MM. Differential expression of genes encoding proteins involved in the control of mitosis, *AURKA*, *AURKB* and *CHEK1* has been reported in comparison with normal mesothelium or normal mesothelial cells. Aurora B (encoded by *AURKB*) is localized in the internal part of kinetochore, and is the enzymatic component of the "chromosome passenger complex", which also includes the internal protein of the centromere, and is involved in mitotic spindle organization, chromosome segregation, and cytokinesis [248]. Those events are compromised in cells that have internalized asbestos fibers as demonstrated using different target cells, including mesothelial cells [249-251] (see in paragraph 22-2.b.ii). In their review, Lampson and Cheeseman [248] suggest Aurora B activity to be modulated by tension forces. Chromosome segregation is controlled at several levels and chromosome movement is driven by motors that are linked to kinetochore associated microtubules and the centrosome. Tensile strengths are developed during this process. So far, mechanical properties of carcinogenic fibers have not been taken into consideration, but it would be of

interest to consider this parameter in the context of fiber interactions with the mitotic apparatus during cell division. Tensile strengths induce tissue and cell deformation. In a recent study carried out with nanoparticles, Mijailovich *et al*. [252] investigated the mechanisms by which deposited particles exert mechanical forces and provoke the particle indentation into alveolar tissue. They found that these mechanisms are centred on a mechanical balance between surface tension forces and tissue elastic forces. These concepts should be considered to account for the effects of fibers on cells and tissues, especially during cytosqueleton remodeling and mitosis progression.

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 [160]. Merlin is a component of the adherens junctions and other types of cell-to-cell contacts [158,159]. 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.

The overall consequences of these effects would be genetic and chromosomal instability and possibly, evasion from apoptosis. It would be important to investigate the repair processes induced by exposure to asbestos, and whether these processes are impaired, leading to additional damage such as gene deletions. So far, we do not know which gene(s) are

initiator(s) of the asbestos-induced neoplastic transformation of mesothelial cells. An activated oncogene has not clearly been identified yet. From studies carried out in genetically modified mice, it seems that NF2 could facilitate tumor progression, but Nf2 deficiency does not act as an initiator, as the latent period for development of MM is similar in WT and heterozygous $Nf2^{+/-}$ crocidolite-exposed mice [23]. In "spontaneous" MM that develops in double mutants $Nf2^{-/-}$;$p14/ARF^{-/-}$, the first MM develops at three months, confirming the role of null status of both genes in mesothelial cell transformation [53]. Further studies will improve our knowledge of the nature and relative role of gene alterations in MM.

In human epidemiologic studies, pleural fibrosis and pleural plaques are a marker of past exposure. The consequences of the interaction between a mesothelial cell and asbestos fibers towards a fibrotic or a neoplastic pathway are dependent on several parameters as discussed above. Other important variables could include the anatomical location of the mesothelial cell injured by asbestos, the severity of injury and the dose of fibers. Knowledge of these variables is important in understanding the mechanisms of asbestos carcinogenesis and in assessing the carcinogenic potential of other particles or chemicals that may to reach the pleura.

5 References

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Legend to figure

Figure 15-1. Multistage Development of Asbestos-Induced Mesothelioma

Adapted from Shukla *et al*., 2003 [76]; Nymark *et al*., 2008 [253]; Pacurari *et al*., 2010 [114]; Broaddus *et al*., 2011 [254].

Tables

Table 22-1. Molecular alterations in mesothelial tissue and malignant mesothelioma developed in asbestos-exposed animals

Reference	Animal, type of experiment	Fiber type	Molecular alteration
	Rat		
Libbus *et al*., 1988 [255]	Rat, i.p. administration Chromosome analysis in MM.	Crocidolite Chrysotile	Loss of chromosomes X, 8, 16, 18 and 20. Translocations involving 5, 10 and 13, repeated points.
Ni *et al*., 2000 [256]	Rat, i.p. administration. Investigation of p53 (exons 5-8), and *K-ras* (exons 1, 2) mutations in MM.	Crocidolite	No mutation detected.
Unfried *et al*., 1997 [257]	Rat, i.p. administration. Investigation of p53 mutations in MM.	Crocidolite	No mutation detected in p53 while numerous base substitution were found in B[a]P-treated animals.
Unfried *et al*., 2004 [258]	Big Blue rat, i.p. administration.	Crocidolite	Significantly enhanced mutation rate of *lacI* gene from omenta 12 and 24 weeks post-exposure*.
Schürkes *et al*., 2004 [259]	Rat, i.p. administration.	Crocidolite	Significantly enhanced level of 8-OHdG in DNA from *omenta* 10-20 weeks post treatment.

	Mice		
Vaslet *et al*., 2002 [154]	Mice, *Trp53* heterozygous, i.p. administration. Gene analysis.	Crocidolite	LOH at the *Trp53* locus.
Fleury-Feith *et al*., 2003 [23]	Mice, *Nf2* heterozygous, i.p. administration. Gene analysis.	Crocidolite	LOH at the *Nf2* locus.
Altomare *et al*., 2005 [51]	Mice, *Nf2* heterozygous, i.p. administration. Gene analysis.	Crocidolite	LOH at the *Nf2* locus. Deletion *INK4* locus.
Lecomte *et al*., 2005 [52]	Mice, *Nf2* heterozygous, i.p. administration. Gene analysis.	Crocidolite	LOH at the *Nf2* locus. Deletion *INK4* locus.
Altomare *et al*., 2009 [260]	Mice, *Arf* heterozygous, i.p. administration. Gene analysis.	Crocidolite	LOH at the *Arf* locus. Hemizygous loss of *Faf1* (Fas-associated factor 1).

i.p. : intraperitoneal
LOH : Loss Of Heterozygozity
* G to T predominant (29%) followed by deletion (26%), G to A (20%), G to C (12%), A to T (6%), A to G and insertion (3%), while controls spontaneous mutations were G to T 19%, deletion 5%, G to A 57%, G to C 14%, A to T and A to G 0% and insertion 5%

Table 22-2. Molecular alterations in mesothelial cells in culture treated with asbestos fibers

Reference	Cells. Type of experiment	Fiber type	Molecular alteration in comparison with untreated or sham cells
	Human		
Lechner *et al*., 1985 [120]	Normal cells. Karyotype analysis of cells after several passages.	Amosite	Numerical and structural chromosomal abnormalities from passage 5.
Olofsson *et al*., 1989 [176]	Normal cells. Karyotype analysis (G banding).	Crocidolite Chrysotile Amosite	Non random aneuploidy, deletion, translocations, inversions (but not breaks, dicentrics, fragments, polyploidization).
Pelin *et al*., 1995 [46]	Normal cells from different donors***. Chromosomal aberrations in metaphases in six donors.	Amosite	Increased chromosome breakage in four cases. Independent of *GSTM1* status.
Burmeister *et al*., 2004 [261]	Normal cells and human Met-5A. DNA breakage (comet assay, quantification of DNA-strand breaks and Fpg-sensitive sites by alkaline unwinding*).	Crocidolite Chrysotile	DNA breakage in both assays, but no increase in Fpg-sensitive sites. No effect on MeT-5A cells.
Poser *et al*., 2004 [48]	Normal cells. Micronucleus assay and kinetochore analysis.	Crocidolite Chrysotile	Micronucleus formation, chromosome breakage. Role of ROS** and metals.

Chen *et al*., 1996 [262]	MeT-5A. Formation of 8-oxo-2'-deoxyguanosine released in the culture medium (HPLC).	Crocidolite	Increased level of of 8-oxo-2'-deoxyguanosine.
Fung *et al*., 1997 [263]	MeT-5A. Formation of 8-OH-dG in DNA (HPLC).	Crocidolite	Decreased level of 8-OH-dG.
Jensen and Watson, 1999 [250]	MeT-5A. High-resolution time-lapse microscopy.	Crocidolite Chrysotile	Delayed cytokinesis. Formation of bi- multinucleated cells.
Nygren *et al*., 2004 [264]	MeT-5A. DNA breakage (comet assay).	Crocidolite	Increased DNA breakage, more pronounced in cells associated with fibers than in cells without fibers.
	Rat		
Jaurand *et al*., 1986 [265]	Pleural mesothelial cells. Morphological study of metaphases.	Chrysotile	Increased chromosome breakage.
Achard *et al*., 1987 [42]	Pleural mesothelial cells. Sister chromatid exchanges.	Crocidolite	Increased sister chromatid exchanges.
Wang *et al*., 1987 [43]	Pleural mesothelial cells. Ultrastructural study of metaphases.	Crocidolite Chrysotile	Polyploidization, chromosome deformities (vacuolization).
Renier *et al*., 1990 [266]	Pleural mesothelial cells. DNA repair (unscheluled DNA synthesis).	Chrysotile	Increased DNA repair.

Yegles *et al*., 1993 [44]	Pleural mesothelial cells. Morphological study of mitotic cells.	Crocidolite Chrysotile	Increased aneuploidy and few structural chromosomal abnormalities. Increased anaphase/telophase abnormalities.
Dong *et al*., 1994 [40]	Pleural mesothelial cells. DNA repair (unscheluled DNA synthesis).	Crocidolite Chrysotile	Increased DNA repair. Partial involvement of ROS.
Dong *et al*., 1995 [41]	Pleural mesothelial cells. DNA repair (poly(ADP-ribose) synthesis).	Crocidolite Chrysotile	Increased DNA repair. Partial involvement of ROS.
Yegles *et al*., 1995 [47]	Pleural mesothelial cells. Morphological study of mitotic cells.	Crocidolite Chrysotile Amosite	Induction of abnormal anaphases and telophases.
Fung *et al*., 1997 [263]	Pleural mesothelial cells. Formation of 8-OH-dG in DNA (HPLC)	Crocidolite	Increased level of 8-OH-dG.
Levresse *et al*., 1997 [34]	Pleural mesothelial cells. Cell cycle analysis.	Crocidolite Chrysotile	G2/M accumulation. G0/G1 accumulation and time-dependent p53 and p21 expression (chrysotile). Delay in the G1/S transition paralleling a low rate of p53 expression (crocidolite).
Fung *et al*., 1998 [267]	Pleural mesothelial cells, induction de l'enzyme apurinic/apyrimidinic endonuclease.	Crocidolite	Increased level (mRNA and protein).

	Rabbit		
Liu *et al*., 2000 [37]	Pleural mesothelial cells. DNA breakage (alkaline unwinding ethidium bromide fluorometric assay).	Crocidolite	DNA breakage. Cell cyle arrest in G2/M. Phagocytosis reduction by cytochalasin reduces DNA breakage.

Met-5A : an SV40-transformed human mesothelial cell line.

ROS : Reactive Oxygen Species

* Fpg protein, which recognizes oxidized bases such as 8-oxo-guanine, is used as indicative of oxidative DNA-base modifications.

** Reduction of micronucleus formation by antioxidants (metal chelators and ROS scavengers). ROS produced by fibers (crocidolite) and phagocytosis.

*** The glutathione S-transferase M1 (GSTM1) genotypes of the patients were determined.

Table 22-3. Activation of signaling pathways in mesothelial cells in culture exposed to asbestos fibers

Reference	**Cells/Experiment**	**Fiber type**	**Signaling response in comparison with untreated cells**
Janssen *et al*., 1994 [268]	Pleural mesothelial cells from rat.	Crocidolite Chrysotile	Increased mRNA expression of c-*fos* and c-*jun*.
Timblin *et al*., 1998 [269]	Pleural mesothelial cells from rat.	Crocidolite	Increased mRNA and protein expression of c-*fos* and c-*jun*.
Zanella *et al*., 1999 [270]	Pleural mesothelial cells from rat.	Crocidolite	Increased expression of mRNA c-*fos* via enhancement of EGFR level.
Berken *et al*., 2003 [271]	Pleural mesothelial cell line non tumorigenic (4/4) from rat.	Crocidolite	Activation of Erk1/2 and Akt in a β-integrin dependent manner.
Altomare *et al*., 2009 [260]	Culture of mesothelioma cells from mesothelioma form heterozygous *Arf* $^{+/-}$ mice i.p. administration.	Crocidolite	Regulation of NF-κB dependent on *Faf1* expression in response to TNF-α. Upregulated in cell showing loss of *Faf1* (see Table 22-1).

Met-5A : a SV40-transformed human mesothelial cell line.