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Role of ferroptosis in nanofiber-induced carcinogenesis

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Abstract

Biopersistent nanofibers with specified physical dimension are unexpected human carcinogens whether they are natural or synthetic. Asbestos, a natural fibrous mineral, is classified as a definite human carcinogen (IARC Group 1) to cause malignant mesothelioma (MM) and lung cancer. Multi-walled carbon nanotube of 50 nm-diameter was defined in 2014 as a possible carcinogen (IARC Group 2B) toward MM, fortunately with no authorized patients thus far. Carcinogenic mechanism of asbestos has been a mystery for a long time. It is now recognized that asbestos goes through lung parenchyma by collecting hemoglobin-derived iron to reach pleural cavity, which takes several decades. Iron-loaded asbestos can induce oxidative damage directly to mesothelial cells, carcinogenesis-target cells lining somatic cavities. Recently, it was clarified that surrounding stromal environment are as important for mesothelial carcinogenesis. The novel concept here is ceaseless ferroptosis of macrophages, which forms a Fe(II)-dependent stromal mutagenic milieu indirectly for mesothelial cells and indeed is a revised understanding of frustrated phagocytosis. Deposition of foreign materials eventually causes iron accumulation *in situ* due to the innate characteristic of preserving iron inside cells. Nanofiber-induced carcinogenesis may be involved in other human carcinogenesis, including ovarian cancer. Alternatively, iron excess can be an optimal target of cancer prevention and cancer treatment.

Key words: asbestos, mesothelioma, ferroptosis, macrophage, iron, carbon nanotube

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Abbreviations:

DMT1	divalent metal transporter 1
EV(s)	extracellular vesicle(s)
IARC	International Agency for Research on Cancer
IRE	iron-responsive element
IRP(s)	iron-regulatory protein(s)
MM	malignant mesothelioma
MWCNT	multi-walled carbon nanotube
NCOA4	nuclear receptor coactivator 4
PCBP1/2	poly-repeated cytidine-binding protein 1/2
UTR	untranslated region

Introduction

Cancer is one of the leading causes of human mortality all over the world (https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates). Whereas molecular carcinogenic mechanism of each human cancer is diversely different and unidentified in most of the cases [1], some of the environmental carcinogenesis reveals unequivocally significant epidemiological association, including ionizing radiation with leukemia [2] and asbestos exposure with malignant mesothelioma (MM) [3].

Asbestos is a natural fibrous mineral, which has been used in the human history since 2,500 B.C. due to its resistance to heat, acid and friction, such as in pottery and sacred cremation garments for the Egyptian pharaohs [4]. After the industrial revolution period, wide use of asbestos started worldwide because of the economical merits of mining [5], which continued till epidemiologist recognized the association between asbestos exposure and MM or lung cancer [6]. MM has been and is a rare tumor [7] in that the tumor retains the characteristics of thin and flat mesothelial cells which line somatic cavities and decrease the friction-derived heat by producing hyaluronic acid [8]. We have ~2,000 new patients yearly in Japan (https://www.mhlw.go.jp/toukei/saikin/hw/jinkou/tokusyu/chuuhisyu17/dl/chuuhisyu.pdf) in comparison to ~120,000 new lung cancer patients. Historically, prolonged average human life was essential for the recognition of this tumor due to an extremely long incubation period of 30~40 years after asbestos exposure, which has been a long-time mystery [9]. After the recognition of all the asbestos as a definite human carcinogen (Group 1) by the International Agency for Research on Cancer (IARC) in 1987 [5], scientists have lost interest in the mechanistic elucidation for a while. Of note, asbestos is negative for Ames test [10].

In Japan, asbestos issue was reminded in 2005 when Kubota shock occurred by newspaper report [11], when inhabitants near the asbestos factory obtained a high incidence of MM. Even now the prognosis of MM is quite poor because of the difficulty in diagnosing the early stage of MM [12]. It is established that carcinogenicity of asbestos fibers depends on its high affinity for histones and hemoglobin-derived iron. Indeed, asbestos (ferruginous) body found in the lung parenchyma of those people exposed to high amounts of asbestos supports this mechanism, thus generating physical scissors for cutting genomic DNA via the Fenton reaction [3, 9] (**Figure 1**). Size (3 nm ~ 5 μm in diameter) [13] and length as an aspect ratio (fiber length/diameter) of > 3 were important to reach alveolar space and this needle-like structure with biopersistence was essential to reach pleural and sometimes peritoneal cavities eventually.

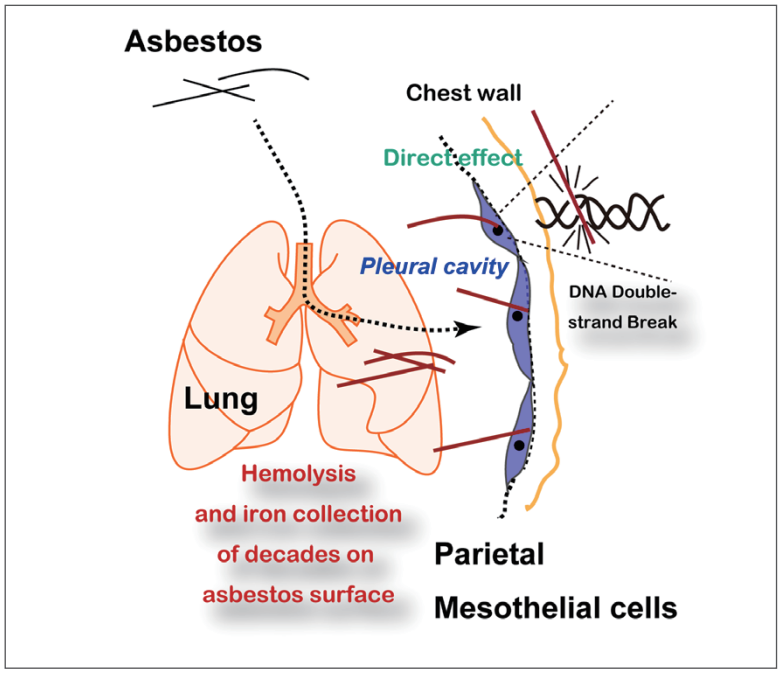

Figure 1. **Current understanding of the mechanism of asbestos-induced mesothelial carcinogenesis in humans.**
Note that a few decades are required for asbestos fibers to go through lung parenchyma to the pleural cavity. Asbestos fibers have hemolytic activity and high affinity for hemoglobin, thus accumulating massive amounts of iron on its surface (red fibers in the figure) to cause DNA double-strand breaks via Fenton reaction in parietal mesothelial cells, targets for carcinogenesis (direct effect). Refer to text for details.

Iron metabolism revised

No life on the earth can live without iron from bacteria to humans [14, 15]. There are recent advancements in the understanding of iron metabolism in higher animals, which started from transferrin/ferritin system, iron transporters (DMT1, ferroportin, etc.) [16], posttranscriptional regulation (IRE-IRPs system) and IRP2/FBXL5-ubiquitin-proteasome system [17, 18].

The recent noteworthy new concepts in iron metabolism would be ferritinophagy [19, 20], cytosolic iron chaperones [21] and ferritin secretion via IRE-IRP/CD63-regualted extracellular vesicles [22]. All of these suggest that intracellular iron levels are strictly regulated not to abandon but efficiently reuse iron and to maintain the iron in a safe non-catalytic fashion. Ferritinophagy

is ferritin-specific autophagic process directed by nuclear receptor coactivator 4 (NCOA4) to release iron from ferritin cores into lysosomes [23]. Poly(rC)-binding protein 1 and 2 (PCBP1/2) has been first reported as intranuclear RNA-binding proteins but are now recognized as mutually exclusive cytosolic iron chaperones [24-26]. Only PCBP1 can load Fe(II) to ferritin cores whereas PCBP2 play a role in other intracellular Fe(II) deliveries [27, 28]. Theoretically, PCBP1/2 carries 3 molecule of Fe(II) in non-catalytic manner [21]. In general, PCBP1 works as tumor suppressor gene [29] and PCBP2 as oncogene [30]. The last one is quite new reported in 2021. We discovered a canonical IRE in the 5'-untranslated region (UTR) of CD63 mRNA responsible for regulating its expression in response to increased iron. We showed that under iron-loading, intracellular ferritin is transferred via NCOA4 to CD63(+) extracellular vesicles (EVs) that are then secreted. Such iron-dependent secretion of the major iron storage protein ferritin is performed through CD63(+) EVs [22].

Ferroptosis

Ferroptosis is a recently defined regulated necrosis. The characteristic in this cell death mode is the dependence on catalytic Fe(II) leading to lipid peroxidation [18, 31]. This was first reported on *H-ras* mutated fibrosarcoma cells with the use of erastin, an inhibitor for cystine/glutamate antiporter (SLC7A11), resulting in decrease in reduced GSH as an antioxidant [32]. The current revised concept of ferroptosis is the imbalance between Fe and S (-SH; sulfhydryls) in favor of iron, causing Fenton-reaction [18]. Iron is one of the most basic elements of the cell, working as cofactors in enzymes, such as ribonucleotide reductase (DNA synthesis), cytochrome oxidase (energy production) and catalase (antioxidant), and hemoglobin in higher species. Thus, every cell tries to maintain the amounts of iron, and bacterial and fungal infections might be a fight to obtain iron for the continued growth of those invaders [15].

It is worth mentioning here that there is no mechanisms to abandon iron from an individual in higher species except for bleeding though Fe(II) can be secreted extracellularly via ferroportin [25, 33]. Accordingly, accumulated iron or decreased antioxidant systems results in ferroptosis. Cancer cells collects iron for persistent proliferation [34-36]. Thus, it is not hard to imagine that cancer cells specifically fall into ferroptosis when the overused antioxidant pathway is squeezed with certain chemicals [1]. Furthermore, autophagic process promotes ferroptosis via ferritin degradation [37] whereas ferritinophagy inhibition via NCOA4 deficiency in the heart mitigates the development of pressure overload-induced dilated cardiomyopathy [38].

Novel mechanism in asbestos-induced mesothelial carcinogenesis

Mesothelial cells are the major target cells in asbestos-induced carcinogenesis. Thus, the research has been performed to clarify how asbestos causes genotoxicity on mesothelial cells directly. However, we recently recognized that the surrounding microenvironment is as important for mesothelial carcinogenesis, based on animal model experiments [39]. In this peritoneal injection model in rats, we noticed after 1 month of injection that virtually all the asbestos fibers are inside the macrophages, which formed granuloma, a foreign body reaction.

Granuloma formation is a specific inflammation mainly of macrophages to confine uncontrollable materials/agents within those barriers, whether they are independent species or man-made synthetic materials (**Figure 2**). Macrophages are phagocytic and antigen-presenting cells. Furthermore, they play a central role in iron metabolism, especially regarding iron recovery from dead or dying cells. We found that asbestos due to its specified physical dimension kills macrophages consistently at first via lysosomal-dependent cell death and finally ferroptosis, which generates Fe(II)-dependent mutagenic stromal milieu, causing β-catenin induction in mesothelial cells [39]. This is an indirect effect to mesothelial cells and may be a revised understanding of frustrated phagocytosis [40].

Carbon nanotubes

Carbon nanotube was discovered with electron microscopic observation in 1991 [41]. This is a synthetic material, consisting exclusively of carbon and with a tubular structure of a few nanometer (single-walled) to several hundred nanometers (multi-walled) in diameter. This material has been and is used for numerous industrial purposes, such as in lithium battery and liquid crystal film, based on its physical nature of rigidity, electrophilicity and heat conductivity [42, 43].

However, its similarity to asbestos fibers in physical dimension was questioned in the early 2,000's, and thus we worked on this issue. We found that diameter of carbon nanotube is the most critical risk factor, where multi-walled carbon nanotube (MWCNT)

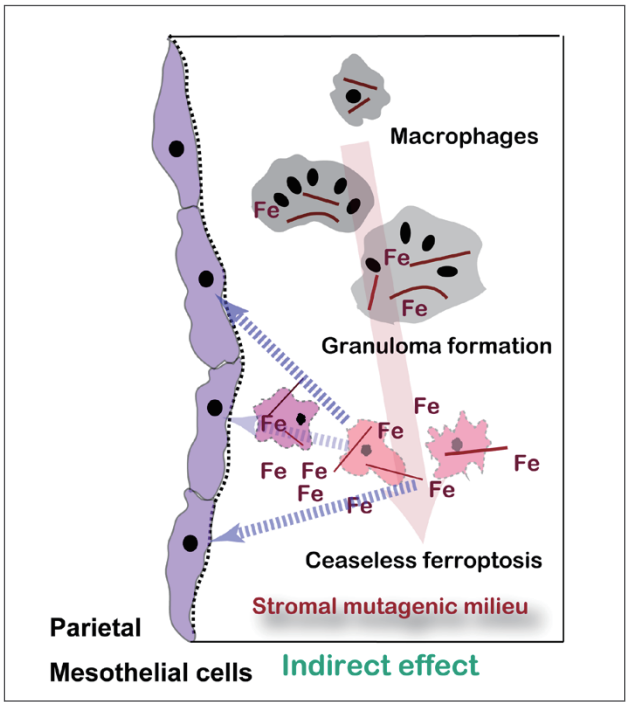

Figure 2. Stromal mutagenic milieu generated by ceaseless macrophage ferroptosis.
Mesothelial cells have phagocytic activity and asbestos fibers in the somatic cavity are eventually transferred to stromal tissue supporting the somatic walls, where macrophages take up the fibers coated with hemoglobin-derived iron. Macrophages try to accommodate all the asbestos fibers by making granuloma, a collection of macrophages with multinucleated giant cells. However, many of the macrophages die through ferroptosis because they cannot cope with the disposal of these thin and long fibers. Finally, abundant iron is released to the stroma of somatic wall, which constitutes the mutagenic milieu for the surface-lining mesothelial cells (indirect effect).

of ~50 nm-diameter was most carcinogenic to mesothelial cells in rat intraperitoneal injection studies [44] and that MWCNT of ~15 nm-diameter was not carcinogenic to mesothelial cells even after > 3 years of observation [45]. The interesting point was that carbon nanotubes have a high affinity not only for histone and hemoglobin but also for transferrin, an iron transporting protein in the serum, and that only MWCNT of -50 nm could go into mesothelial cells [46]. There observations suggest that excess iron play a role in MWCNT-associated mesothelial carcinogenesis. This is strongly confirmed with the similar genetic alterations observed in the rat and human MMs between asbestos origin [47-49] and MWCNT of 50 nm-diameter origin [44], where homozygous deletion of *p16*^{Ink4a} tumor suppressor gene was the most prominent . Indeed, we believe that deletion of *p16*^{Ink4a} tumor suppressor gene is a marker of Fenton reaction-induced carcinogenesis [35, 50], based on the studies of ferric nitrilotriacetate (Fe-NTA)-induced renal carcinogenesis model (**Table 1**). Regarding Fe-NTA-induced renal carcinogenesis,

Table 1. Similarities and differences among three *wild-type* animal models causing cancer through excess iron.
Fe-NTA, ferric nitrilotriacetate; *ip*, intraperitoneal; MWCNT, multi-walled carbon nanotube. Refer to text for details.

Models	Fe-NTA	Asbestos (chrysotile, crocidolite and amosite)	MWCNT of 50-nm diameter
Species	Rat, mouse	Rat, mouse	Rat, mouse
Injection	*ip*, 3~5 times a week/10-12 weeks	*ip*, 1~3 times	*ip*, 1~3 times
Major pathology	Repeated Fenton reaction in the renal proximal tubules	Direct action to mesothelial cells with prolonged foreign body reaction (ceaseless ferroptosis of macrophages)	Direct action to mesothelial cells with prolonged foreign body reaction
Origin of excess iron	Fe-NTA itself	Asbestos itself (crodidolite, amosite), hemolysis (chrysotile) and affinity to hemoglobin	Affinity to hemoglobin and transferrin
Induced cancer	Renal cell carcinoma; 50% of pulmonary metastasis in rats	Malignant mesothelioma	Malignant mesothelioma
Major target gene	Homozygous deletion of *16*^{ink4a} tumor suppressor gene	Homozygous deletion of *16*^{ink4a} tumor suppressor gene	Homozygous deletion of *16*^{ink4a} tumor suppressor gene
References	[15, 50, 71, 72]	[49, 73]	[44, 46]

refer to other published reviews [15, 51].

Recently, Tim4 was identified as a receptor for MWCNT in macrophages leading to granuloma formation [52, 53]. It was recently reported that Tim4+ macrophages sequester and impair proliferation of CD8+ T cells [54] whereas TIM4 expression by dendritic cells mediates uptake of tumor-associated antigens and anti-tumor responses [55]. Whether persistent ferroptosis of macrophages is at work for mesothelial carcinogenesis requires further investigation. We have to stress here that no definite MM case has been reported thus far in terms of carbon nanotubes. We believe that this depends on early recognition (IARC Group 2B) [56] and the efforts on the industry side to avoid the use of high-risk carbon nanotubes and to develop the large-scale automated systems to minimize human exposure. The continued use of MWCNT in industry and in material science, even for the robotic systems [57], is very different from the asbestos case and we can declare that this is one of the successful examples of experimental pathology using animal models and regulatory science.

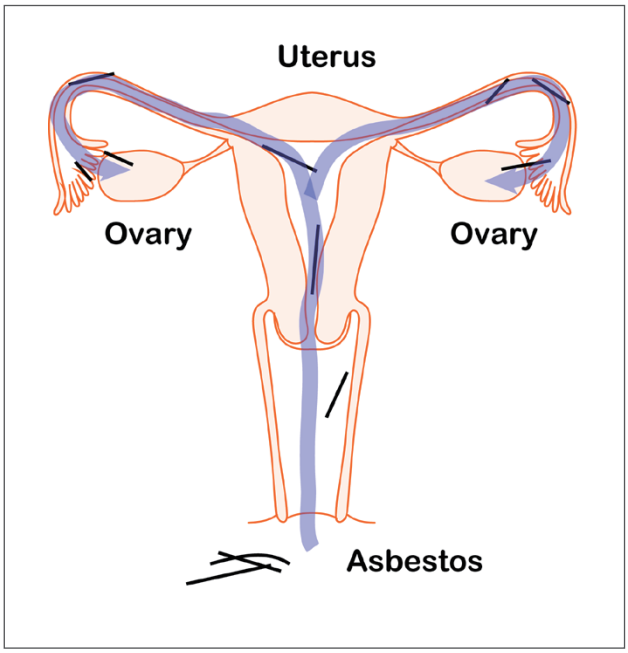

Figure 3. Possible pathway of asbestos fibers reaching ovaries in humans.
Asbestos and other nanofibers may exist in the vulva of reproductive-age women as contaminants of family laundry or cosmetic applications. Liquid flow from vulva, vagina, uterine cavity, oviduct to ovary may carry these fibers to ovarian epithelial cells. Refer to text for details.

Ovarian carcinogenesis

Another example we suspect for nanofiber-induced carcinogenesis accompanied by excess iron is ovarian carcinogenesis. Endometriosis is a female disease of reproductive age where endometrium exists outside of uterine cavity, leading to iron excess due to monthly bleeding. In the case of ovarian endometriosis, this iron excess is established as a risk for adenocarcinoma, especially clear cell carcinoma and endometrioid adenocarcinoma, in addition to menstrual pain and infertility [58, 59]. In addition to this, fibrous materials, including asbestos, are suspected to be carcinogenic to ovarian epithelial cells. There are two points that are true but not well recognized: 1) there is a pathway in women through vagina, endometrium, oviduct to ovary [60-62], which happens at fertilization; 2) frequent use of baby power, including talc and some amounts of asbestos, may be epidemiologically associated with ovarian cancer [63, 64] though it is still controversial [65, 66] (**Figure 3**). We believe that this is an important question to be explored to prevent ovarian carcinogenesis.

Conclusion

Biopersistent fibrous materials may provide humans with unexpected risk of cancer, depending on its physical parameters and exposure route. This is closely associated with the general response of our cells against foreign materials to collect/recover iron as much as possible and to deplete iron in the extracellular space. Alternatively, if the macrophages cannot accommodate or scavenge these fibrous materials and die through ferroptosis, mutagenic stromal milieu is generated [39]. Finally, it is important to recognize that iron excess can be a target for cancer prevention [67, 68] and also for cancer therapy [18, 69, 70], including non-thermal plasma.

Declaration of competing interest

The authors declare no conflict of interest to present.

Acknowledgments

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