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Dispatch

Mesothelioma: Identical Routes to Malignancy from Asbestos and Carbon Nanotubes

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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 malignant mesothelioma. Fibres of a similar nature may pose significant health risks to humans.

Malignant pleural mesothelioma (MPM) is an incurable cancer of the epithelial lining of the lungs and chest cavity [1]. Diagnosis is notoriously difficult, treatment options (inclusive of surgery) are severely limited and most patients succumb to the disease within one to two years of diagnosis [2]. Historically, this disease is causally linked to direct high-volume exposure to fibrous asbestos, particularly amongst asbestos miners, construction workers and ship-builders. Commercial use of asbestos is consequently banned in Western society although its use continues unabated in several emerging economies. There is increasing awareness of the threat posed by environmental and secondary exposures, and a legacy of asbestos-insulated buildings continues to drive new cases of MPM in countries where its active use has long ceased [3]. Moreover, there is growing concern amongst scientists that materials with physical properties similar to asbestos, such as carbon nanotubes, might pose an equivalent human health threat [4]. Given the extremely long latency between exposure and asbestos-driven MPM (typically up to 40 years), it may be decades before the actual threat posed by such materials manifests as malignancy.

Precisely how asbestos causes mesothelioma has been debated for decades. The epidemiology of MPM indicates that long fibrous types of asbestos (for example, amosite or

crocidolite) pose a much greater threat than more particulate specimen (for example, serpentine or chryostile) [5]. The long-term persistence of fibres in the pleura is thought to drive chronic inflammation, and the continued exposure of pleural epithelium to mitogens and growth-promoting cytokines likely supports ectopic cell proliferation [6]. Iron deposits in some forms of asbestos are linked to generation of reactive oxygen species that can similarly promote proliferation by altering protein function and, at high concentrations, drive mutagenesis [7]. Additionally, asbestos fibres have been proposed to physically interfere with chromosome condensation and the fidelity of chromosome segregation during mitosis [8]. A landmark study in rodents demonstrated that fibrous asbestos cannot be efficiently cleared by macrophages (engendering the term 'frustrated phagocytosis') leading to chronic inflammation, whereas the same material milled into a fine powder is efficiently cleared and the accompanying acute inflammation quickly resolved [9]. The study strongly suggests that the physical attributes of fibrous asbestos (rather than its chemical composition) are the primary determinant of MPM risk. Ominously, high aspect-ratio carbon nanotubes that physically resemble fibrous asbestos similarly resist phagocytosis, drive chronic inflammation and were previously shown to predispose rodents to peritoneal mesothelioma when injected into the abdominal cavity, suggesting that such materials may pose a significant hazard akin to asbestos [10–12].

A new study by Chernova *et al.* [13] in this issue of *Current Biology* is the first to directly compare the progressive pathological response of mice to intra-pleural injection of long-fibre asbestos (LFA) with that of long-fibre carbon nanotubes (CNT). Examination of exposed tissue at one and twelve weeks post inoculation revealed a similar degree of chronic inflammation induced by either fibre type. Transcriptomic profiling of exposed tissue showed a pattern of altered gene expression that was remarkably similar for tissue exposed to long-fibre forms of either LFA and CNT, as compared with vehicle control or indeed tissue exposed to short-fibre forms of either. Phospho-protein profiling moreover revealed that exposure to either CNT or LFA drives activation of the same signal transduction pathways that are commonly perturbed in human MPM, notably including the SRC, FAK, ERK, AKT/mTORC1 and STAT pathways [14], and that the degree of activation was strikingly similar for both fibre types. By 6 months, the inflammatory lesions showed a sustained increase in proliferation and oxidative DNA damage in both the asbestos and CNT cohorts and, by 12-20 months, 10-25% of animals exposed to CNT (3 independent cohorts) and 9% of animals exposed to asbestos developed pleural mesothelioma. Disease latency was moreover identical for both fibre types. Both LFA- and CNT-induced tumours exhibited histopathology and immuno-histochemical

markers consistent with human MPM, including mesothelial lineage markers, cytokeratins, and WT1. The study thus conclusively demonstrates that carbon nanotubes with asbestos-like dimensions drive the same disease as long-fibre asbestos and thus present a comparable risk.

A significant advantage of such longitudinal analysis in an animal model is the capacity to investigate early-stage disease and indeed progression from pre-malignant lesions to malignancy. By comparison, studies of human MPM have to date been largely restricted to late-stage disease, owing to prolonged disease latency, late presentation and the current lack of predictive clinical indicators of progression from benign asbestos-related pleural effusion to malignancy (note that approximately 15% of patients presenting with pleural effusion eventually progress to MPM). The recent comprehensive genomic characterization of human MPM has shown that the majority of end-stage disease cases contain loss-of-function mutations in one or more of three key tumour-suppressor loci, namely *CDKN2A*, *NF2* and *BAP1* [15], however, the status of these genes in pre-malignant disease is presently unknown.

Loss of *CDKN2A* occurs in approximately 50% of human MPM [16]. In the present study, Chernova and colleagues examined expression of the protein products of *Cdkn2a* during progression to malignancy [13]. This gene encodes two important tumour-suppressor proteins that function in distinct pathways: p16^{INK4a} blocks progress through the cell cycle by inhibiting CDK4/Cyclin D, whereas p14^{ARF} (p19^{Arf} in mouse) antagonizes MDM2-dependent p53 degradation. *In vivo* mouse models have shown that both p16^{Ink4a} and p19^{Arf} proteins suppress asbestos-induced tumourigenesis and, although p19^{Arf} must be lost for mesothelioma pathogenesis to occur, inactivation of both *Cdkn2a* gene products cooperate to accelerate asbestos-induced tumourigenesis [17]. Consistent with this, Chernova *et al.* [13] detected reduced mRNA and protein expression of both p19^{Arf} and p16^{Ink4a} in tumours induced by either LFA or CNT, along with allelic loss of p19^{Arf} in tumours induced by CNT. Somewhat surprisingly, allelic loss of p19^{Arf}-encoding sequences occurred in the absence of deletion of p16^{Ink4a}-encoding sequence, suggesting that the two pathways are subject to distinct selective pressures. Importantly, examination of pre-malignant inflammatory lesions also revealed sporadic loss of p19^{Arf} and p16^{Ink4a} protein and mRNA expression, this time in the absence of allelic loss, suggesting an epigenetic mechanism of *Cdkn2a* suppression in pre-malignant lesions. The *CDKN2A* locus is silenced through CpG-island hypermethylation in multiple human cancers and, accordingly, bisulphite sequencing of the locus in pre-malignant chronic inflammatory lesions showed hypermethylation of CpG islands within and adjacent to both p19^{Arf} and p16^{Ink4a}-coding sequences in mice exposed to either LFA or CNT. These data

would suggest that epigenetic silencing of the *Cdkn2a* locus occurs prior to progression to malignancy and that progression is associated with further selective pressure to delete the locus, in particular the p19^{Arf}-encoding regions.

As noted above, *Cdkn2A* is one of three loci that show a high frequency of loss-of-function mutation in human MPM, the others being *BAP1* and *NF2*. Surprisingly, tumours induced by either LFA or CNT showed no loss of NF2 protein expression whereas immunohistochemistry for BAP1 suggested preferential cytosolic localisation occurring in both instances. Although again demonstrating clear molecular similarities between progressive disease induced by LFA and that induced by CNT, these data also point to the need for further in vivo investigation of the molecular, cellular, and intercellular processes that drive disease evolution from the pre-malignant state to clinical MPM.

Several outstanding questions remain: Do reactive oxygen species play a direct role in driving the epigenetic changes detected in pre-malignant lesions? Why is hypermethylation-induced silencing of *Cdkn2a* not sufficient for progression to malignancy and what is the selective pressure for locus deletion to occur subsequent to gene silencing? A possible answer may lie in the fact that *CDKN2B*, syntenic with *CDKN2A* on chromosome 9p21.3, is co-deleted in the vast majority of MPM cases with loss of *CDKN2A* [18]. What is unquestionably clear from this study is that carbon nanotubes (and potentially other high-aspect-ratio fibres) present the same carcinogenic hazard as does long-fibre asbestos. Carbon nanotubes have a broad spectrum of commercial uses, ranging from use in automotive parts, sports equipment and boat hulls to more direct contact via use in water filtration and drug formulation [19], presenting substantial opportunity for exposure during manufacturing or later, due to attrition of materials containing CNT. The study by Chernova and colleagues [13] presents a clear opportunity to mitigate against future MPM epidemics through legislation and tighter regulation of CNT production, use and disposal.

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Figure 1. Identical disease progression after injection of long-fibre asbestos or carbon nanotubes

Intra-pleural injection of either long-fibre asbestos (LFA) or carbon nanotubes (CNT) drives equivalent acute inflammation, followed by frustrated phagocytosis, leading to chronic inflammation. Sustained growth-factor signalling and continued exposure to reactive oxygen species eventually leads to transformation of pleural epithelial cells. Epigenetic silencing of *Cdkn2a* is subsequently found in pre-malignant inflammatory lesions, with sporadic deletion of p19^{Arf} and/or p16^{Ink4a}-encoding sequences appearing later, coincident with progression to malignant pleural mesothelioma. Progressive disease appears to be agnostic to fibre composition and rather is dependent upon fibre dimensions and bio-persistence.