HARN Dimension and Pleural Retention

Craig Poland, SAFENANO Research Toxicologist

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Within the June edition of The American Journal of Pathology (vol. 178, No. 6), researchers Murphy et al. from the University of Edinburgh, U.K.[1], have published an interesting article further addressing the issue of carbon nanotubes and pathology occurring in the space between the lung surface and the chest wall known as the pleural space, a site known to be a target for harmful fibres such as asbestos.

In our second Feature Article for summer 2011, Craig Poland – Research Toxicologist for SAFENANO and co-author on the paper – provides an insightful commentary on the study and its contribution towards investigating the fibre pathogenicity paradigm, and explains the danger of blanket statements such as 'all carbon nanotubes are like asbestos'.

An Introduction to the Fibre Pathogenicity Paradigm

Carbon nanotubes are an interesting form of carbon, and have gained fame as a potential wonder material due to their exceptional structural and electrical properties which are advantageous for various industrial applications from electrical circuitry to high strength composites. Carbon nanotubes can be found in various forms, but are most concisely described as a single graphene sheet with its distinctive hexagonal structure rolled to form a cylinder or single-walled carbon nanotube. A multi-walled carbon nanotube can be described as several graphene cylinders stacked concentrically one inside the other - these were the variety investigated by Murphy et al.
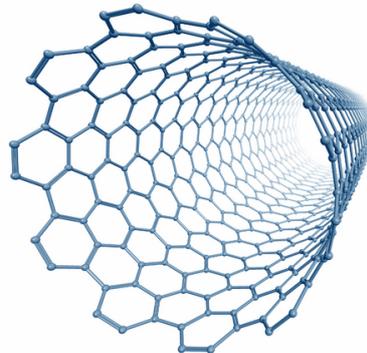

Figure 1 - Single Walled Carbon Nanotube (SWCNT)

Carbon nanotubes are often described as being a high aspect ratio nanoparticle (HARN; a high length-to-width ratio) but can actually vary greatly in length, diameter, structural properties, and contaminants such as metals left over from the production process. They can be found as highly curled, semi-spherical agglomerates such as those described recently by Pauluhn[2] or straight/semi-straight high aspect ratio fibres as described by Poland et al.[3] meaning that, despite having the same name, two samples of carbon nanotube can vary substantially.

The fibrous shape of carbon nanotubes has raised suspicions of a possible similarity between their toxicological profile and that of pathogenic fibres such as asbestos[4]. Such concerns often surround new industrially relevant fibres to which there is the potential for human exposure. However, as discussed recently within a review article by several of the authors of the Murphy

article, not all fibres are actually dangerous to health and many are benign[5]. Over the last 30 years or so, the investigation of what exactly causes a fibre to be dangerous led to the development of a set of criteria often referred to as the fibre pathogenicity paradigm, which describes the attributes required of a fibre if it is to exert fibre-toxicity. It is worth noting that fibre toxicity represents a degree of added toxicity, thus if a particle is not classified as a fibre, it does not mean that it is harmless, it simply means it won't exert fibre-toxicity. The paradigm brings together several key attributes which can be summarised as Dose, Dimension, and Durability or the "3 D's".

The fibre paradigm

Dose

When thinking about the fibre paradigm, the issue of dose (quantity of material to which the subject is exposed) is of course the most basic of criteria for all toxic substances; an absence of dose means an absence of effect, since a dose is required to drive an effect.

Dimension

The importance of dimension firstly relates to the diameter of a fibre, which must be below 3µm in order for it to be able to negotiate the progressively narrowing airways of the lung and deposit in the deep respiratory zone. Fibres which are far thicker than this are simply not respirable and so can never deposit in the deep lung (hence no dose). However, there is an additional issue of length in the fibre paradigm, which has its origin in the hindrance of clearance mechanisms that enable the body to deal with unwanted foreign material. Typically, material depositing in the lung is broadly dealt with in one of two ways depending on where it lands in the lung. In the upper airways, specialist cells with small hair-like projections called cilia, continually waft mucus and trapped foreign material such as bacteria and particles upwards towards the throat where they are swallowed or spat out. A slower method of clearance happens in gas exchange or respiratory zone of the lung. Here the removal of unwanted material is performed by specialist immune cells called macrophages which are mobile and patrol this region, ingesting anything they detect as foreign to the body and clearing it – a process called phagocytosis. The progressively narrowing airways of the lung means that only very small particles (less than ~3µm) can find their way into this region and so these are easily dealt with by macrophages. However fibres, by a quirk of aerodynamics, can align themselves in the airflow and as such present a small diameter but have a length far greater than the 3µm cut-off.

The problem comes when the length of the fibre exceeds the maximum size a macrophage can realistically deal with (~10–20µm), meaning that the lungs own defence mechanism cannot clear the particle. Macrophages are nothing if not determined and, irrespective of length, will try to clear the particle. This can result in a situation where the macrophage is struggling to clear a fibre far longer than itself and is unable to fully enclose it leading to a stressful situation for the cell called 'frustrated phagocytosis'. When thinking of this, I'm often reminded of the iconic image of Laurel and Hardy trying to handle a long plank of wood in the film 'Finishing Touch' and creating havoc with their lack of finesse - a macrophage handling a long fibre is oddly similar.

**Figure 2 - 'The Finishing Touch' © Hal Roach/MGM**

Durability

When considering a long fibre in the deep lung unable to be cleared by normal mechanisms, there is only one route of removal – namely the fibre either dissolving or breaking into smaller pieces which can be cleared by macrophages. Therefore, it is easy to see how a fibre that is not durable and rapidly dissolves or breaks (such as certain forms of glass fibre) will not lead to the build-up of a large dose so long as exposure/deposition does not exceed the rate of dissolution. However a very durable fibre such as crocidolite asbestos will not easily dissolve in the lung and, as such, every exposure can lead to a further accumulation of fibres until a critical threshold dose is reached and harmful effects begin to manifest.

The importance of each of these attributes to the overall pathogenicity of a fibre can be seen most simply in an adapted version of the classic Fire or Combustion Triangle (Fig. 3). Rather than Heat, Oxygen and Fuel we have Dose, Dimension and Durability, where removal of any of these 'ingredients' results in a loss of pathogenicity, in the same way that removal of heat, oxygen or fuel would extinguish a fire.

Fibre **dimensions** are important in establishing whether a fibrous particle is able to reach sites (such as the distal lung) where cellular interactions may take place, including hindrance of clearance mechanisms

Fibre **durability** is important in its relation to how long a fibrous particle can persist at its site of deposition as well as how the surface of a particle may be altered
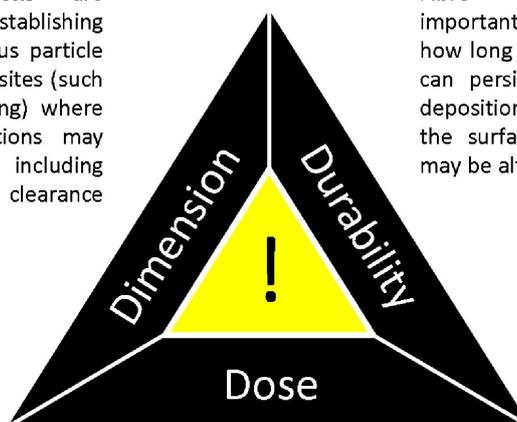
The build up of **dose** relates to exposure to a particle (e.g. in the occupational environment), its dimension in relation to entering the body and hindering removal, and resistance to dissolution or fracture in the biological milieu (biopersistence).

Figure 3 - The Fibre Pathogenicity Triangle

An Overview of the Study

The interest of Murphy et al. was in the fibre paradigm, how it relates to high aspect ratio nanoparticles (HARN) and the comparison that has been made in the literature between HARN and asbestos. The authors focused on a region of the body which is almost uniquely susceptible to asbestos-related disease including the cancer, mesothelioma, which is almost exclusively associated with asbestos exposure. Mesothelioma is a cancer of the lining of the chest cavity (the pleura) and is uniformly fatal. The authors focused upon diseases of the pleura as several are considered a response almost unique to fibrous particles[6] and as such is an ideal candidate for examining if HARN such as carbon nanotubes follow this paradigm. The study itself did not set out to demonstrate or detect mesothelioma, as this requires whole of life exposure studies, but rather looked for other responses such as inflammation and scar formation in the pleural space that can indicate a harmful effect. However it must be taken into account that demonstration of such effects do not necessarily mean the development of cancer or disease.

To investigate the role of length in the toxicity of HARN, Murphy and colleagues put together a panel of particles consisting of both long (>10µm) and short (<2µm) carbon nanotubes as well as long (~24µm) and short (~5µm) nickel nanowires, another form of HARN. In addition, to show the importance of confirmation (straight vs. curled), they also incorporated two highly curled carbon nanotube samples which were described by the manufacturer as being 1-5µm and 5-20µm long, but rather than possessing this as straight length, instead formed far smaller compact agglomerates. These particles were compared with a range of control particles including amosite asbestos (fibrous) and carbon black (non-fibrous), selected to either meet or not meet the length criteria of the paradigm.

The clearance mechanisms within the fluid-filled pleural space centre around either the outflow of liquid through pores in the outer (chest) wall of the pleura (the parietal pleura), or uptake by resident cells (macrophages) which specialise in engulfing material such as particles, bacteria and dead or dying cells. These pores, or stomata, within the pleural wall feed into the underlying lymphatic channels which lead to outlying lymph nodes. The mouths of these stomata are relatively conserved in size with an opening of only 2-10µm. Based in this, it was therefore hypothesised by Murphy et al. that small particles (<10µm) could negotiate these stomata and leave the pleural space (hence a removal of dose) whilst long fibres (>10µm) would be caught and deposit at the pleural surface in a manner reminiscent of spaghetti caught in a plug hole.

Mesothelioma arises at the parietal pleura rather than at the surface of the lung (the visceral pleura) which is reflected in the various staging criteria of the cancer (e.g. the tumour-node-metastasis (TNM) staging system[7]) where if the tumour is associated only with the parietal pleura then it is classed as very early stage, indicating its genesis. Taking the simple idea of cause and effect, one would suggest that dose must be present at the parietal pleura in order to trigger a negative response such as mesothelioma.

Murphy and colleagues placed their particles into suspension and injected directly into the pleural space of mice. After a period of 24hrs, the mice were humanely culled and the pleural space washed with saline to remove inflammatory cells and other markers of adverse effects to measure the response to the injected particles. The authors found that only those particles which were long (specifically three forms of HARN and long fibre asbestos) could induce a response in the pleural space and small particles caused no significant effects. To emphasise the role of size, the authors took the interesting 'proof-of-concept' approach of injecting in polystyrene beads which were identical except in size. Based on the size of the stomata, and if the size selective retention theory was correct, the small (3µm) bead should be able to pass freely out of the pleural space or be taken up easily by resident cells, whilst the large (10µm) beads should not. This proved correct with only the 10 µm polystyrene bead causing significant inflammation. The importance of this size-dependent retention was further demonstrated by injecting quartz or coal dust particles, which are known to cause effects in the lung, into the pleural space. These particles were small enough to negotiate the stomata and as such passed out of the pleural space without causing inflammation.

To confirm that the particles were indeed leaving the pleural cavity and track their route, Murphy and colleagues injected radioactively-labelled short carbon nanotubes into the pleural space of mice, which they hypothesised would not be retained. The animals were then placed in a single-photon emission computed tomography scanner and observed over time. They noted that, at first, the labelled carbon nanotubes remained in the pleural space showing up as a diffuse glow across the chest region which rapidly became more focal in a region corresponding to lymph nodes located in the neck of the animals. By 24hrs after injection, the signal from the labelled carbon nanotubes was almost exclusively found in these lymph nodes. The authors also removed and analysed these lymph nodes from animals injected with short and long nickel nanowires and found a far higher fibre number in the lymph nodes after injection of short fibres than for long fibres demonstrating the ease with which short fibres/particles can exit the pleural space and end up in the lymph nodes.

Looking at later time points stretching up to 24 weeks after injection, Murphy et al. monitored the effects long and short carbon nanotubes in the pleural space. At 1 day, they noted that injection of short curled carbon nanotubes into the pleural space led to small (non-significant) numbers of inflammatory cells infiltrating the pleural space and aggregating on the parietal pleura but this rapidly cleared over the course of the first week. This was in stark contrast to the

long carbon nanotubes, which maintained high numbers of inflammatory cells in the pleural space over the course of a week with evidence of increasing cell accumulation and long carbon nanotubes visible at the surface of the pleura. Over the course of the next 24 weeks, the small cellular accumulations on the surface of the parietal pleura after injection with short carbon nanotubes rapidly resolved whilst the lesions (areas of abnormal tissue) caused by long carbon nanotubes continued to grow and become more organised as scar formation (fibrosis) occurred and blood vessels began infiltrating the forming lesion. Over time, this lesion retracted in size from covering much of the pleura, to occupying mostly those regions between the ribs. In addition, the authors noted a change in the surface from an inflamed surface composed of numerous inflammatory cells to the formation of a scar and finally signs of overgrowth of the lesion by the cells which line the pleura.

Digest

The approach of injecting particles directly into the pleural space may seem, on the face of it, a very non-physiological route of exposure. Indeed one of the only situations where particles are injected into the pleural space of humans is the injection of talc slurry as a therapeutic approach to pleural effusion (build up of liquid in the pleural space) and as such, this is obviously not as a result of environmental exposure. However as discussed in the recent reviews the same authors[5,8], we know from post-mortem studies of people exposed to high dusts concentrations (such as city dwellers) that there is evidence of particle transit from the lungs into the pleural space resulting in characteristic 'black spots' on the parietal pleura[9].

Indeed two studies have now been published which add further weight to the contention that inhaled carbon nanotubes may pass through into the pleural space from the lungs. The first, published in the journal Nature Nanotechnology by Ryman-Rasmussen et al., showed evidence of deposition of carbon nanotubes after inhalation in the areas directly adjacent to the pleura covering the lung surface and integration of these particles into the lung tissue[10]. In a later study, Mercer and colleagues instilled carbon nanotubes into the lungs of mice and observed carbon nanotubes protruding out of the lung into the pleural space and transiting into the pleural space[11]. These two studies, together with the human evidence of particle transfer from the lung to the pleura, add teeth to the argument that the important question is not 'do carbon nanotubes reach the pleura?' but instead 'are they retained in the pleural space and what do they do when they are there?'.

The article by Murphy goes some way to answering the questions of what attributes cause nanoparticles to be retained in the pleural space and what this may mean in terms of inflammation and fibrosis. As a concept, the issue of retention and length is not new, but the evidence that it is also relevant to nanoparticles is. Indeed this work builds upon theory put forward by Stanton in the early eighties. Within their seminal paper, Stanton and colleagues over the course of 72 experiments implanted a range of mineral fibres into the pleural space of rats and monitored them over a period of a year or more for signs of tumour formation. The resultant tumours were recorded and the data analysed for correlation between the particle dimensions and the ensuing tumours. Stanton and colleagues concluded that the probability of developing such pleural pathology correlated best with fibres that were 8µm in length and 0.25µm in diameter[12]. The particular strength of this study was the large array of particles tested, which allowed them to conclude that the carcinogenicity of fibres depended on its dimensions and durability. The study of Murphy et al. advances the theory of Stanton and shows its relevance to HARN whilst providing evidence for a plausible mechanism for the presentation and retention of dose to the parietal pleura as advanced by Kane and colleagues[13,14].

As discussed at the beginning of this commentary, another important attribute of the fibre paradigm is the issue of biopersistence. While this wasn't directly dealt with by Murphy and colleagues in this article, they have, in collaboration with the Australian Commonwealth Scientific and Industrial Research Organisation (CSIRO) and the Institute of Occupational Medicine (IOM), recently published an article in the journal Particle and Fibre Toxicology (Osmond-McLeod et al. 2011) looking at this issue with several of the particles used within the Murphy study. They found that of the carbon nanotubes evaluated, most were highly durable but one sample did lose both mass and length indicating that it may not be as durable as the others[15].

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So, what does all of this mean?

Well, based on both the historical evidence concerning fibres and particles, it seems likely that a small proportion of particles depositing in the lung will pass through the pleural space. The evidence, advanced by Murphy et al., suggests that long, durable fibres that find their way into the pleural space are likely to be retained and cause adverse effects, whilst short, or non-durable fibres are not and this is relevant to both conventional fibres and HARN.

It is important, however, to realise that just as with conventional fibres, not all HARN will meet the criteria of the fibre paradigm - indeed there exists enormous variability within even a single class of HARN. For example, this means that whilst they share the same name, there may be considerable variability between types of carbon nanotube, which in turn may impact significantly on their potential to cause harm. This emphasises the need for careful and reasoned evaluation of potential risks and shows the inappropriateness of blanket statements such as 'all carbon nanotubes are like asbestos'. Thus when considering the potential risks (particle or fibre) it is important to firstly know what you are working with, perform an adequate risk assessment that identifies, if appropriate, the potential hazards of long fibres if they meet all the criteria of the fibre paradigm.

However it should also be stressed that much work still needs to be done to confirm the generation of disease such as mesothelioma after inhalation exposure of HARN. Further research should be undertaken, taking heed of the fibre paradigm, to establish this.

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