主論文の要旨

Role of hemoglobin and transferrin in multi-wall carbon nanotube-induced mesothelial injury and carcinogenesis

（多層カーボンナノチューブに誘発した中皮細胞傷害または発がんにおけるヘモグロビン及びトランスフェリンの役割）

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[Introduction]

Carbon nanotubes (CNTs) are a promising material in nanotechnologies due to their high thermal and mechanical resistance but high electrical and thermal conductivity with flexibility and semiconductivity. Thus, CNTs are used worldwide in various industrial and mechanical applications; they are used as components in electronics, energy-storage devices, solar cells and sensors, or as fillers in polymeric composites and concrete. CNTs have also been proposed for use in medicine as nanovectors or as substrates in tissue engineering.

Asbestos is natural hydrated silicate fibers. Exposure to asbestos may induce various pathologies in humans, including pleural effusion, pleural plaques and pulmonary asbestosis. Furthermore, this material may cause malignant mesothelioma (MM) and/or lung cancer after a long incubation period. Many rodent experiments support the carcinogenicity of asbestos, especially to mesothelial cell. In 2006, at least 40 countries have banned or severely restricted asbestos use. Having a diameter less than 200 nm and a length measured in μm, CNTs have a needle-like shape with a high aspect ratio, which is similar to asbestos. Accordingly, there have been arguments about whether CNTs might have similar carcinogenicity to asbestos. Recently, three independent rodent studies revealed that multi-wall carbon nanotubes (MWCNTs) 50 nm diameter cause MM when injected intraperitoneally to $p53^{+/-}$ knockout mice or to wild-type rats or intrascrotally to Fischer-344 rats. In 2014, IARC designated MWCNTs 50 nm in diameter as a possible human carcinogen (Group 2B), based on these studies. Of note, tangled CNTs 15 nm in diameter induce no MM and CNT150 nm are less carcinogenic, which may be partially associated with their difficulty in entering mesothelial cells.

Here, we followed our previous strategy for asbestos-induced mesothelial carcinogenesis, which is similar to immunoprecipitation, to elucidate the major molecular mechanisms of MWCNT-induced mesothelial carcinogenesis. We used mass spectrometry (MS) to exhaustively identify the proteins that adsorb on the surface of four pristine MWCNTs of different diameters. Among these, we focused on hemoglobin and transferrin, both of which are associated with iron metabolism.

[Materials and Methods]

To elucidate the carcinogenic mechanisms of MWCNT in mesothelial cells, we used a variety of lysates to comprehensively identify proteins specifically adsorbed on pristine MWCNT of different diameters (50 nm, NT50; 100 nm, NT100, 150 nm, NT150 and 15 nm/tangled, NTtngl) using mass spectrometry. Among these proteins, we selected hemoglobin and transferrin for coating MWCNTs to evaluate cytotoxicity, wound healing, intracellular catalytic ferrous iron, oxidative stress and DNA damage by using a comet assay in Rat peritoneal mesothelial cells (RPMCs).

[Results]

We identified >400 proteins, which included hemoglobin (Hb), histone, transferrin (Tf) and various proteins associated with oxidative stress. Many of these proteins were identified in all four different types of MWCNTs. However, cytotoxicity to rat peritoneal mesothelial cells (RPMC) was observed with NT50 but not with NTtngl. Coating NT50 with hemoglobin or transferrin, which was confirmed with a wound-healing assay and by evaluating 4-hydroxy-2-nonenal-modified proteins, significantly aggravated cytotoxicity to RPMC. Notably, coating NT50 with hemoglobin or transferrin significantly increased cellular catalytic ferrous iron, which also revealed increased DNA damage by the comet assay. Knockdown of the transferrin receptor 1 with ferristatin II decreased not only NT50 uptake but also cellular catalytic ferrous iron.

[Discussion]

Risk assessment of CNTs is important because CNTs are already in the market due to their superb utility as an industrial material. We previously observed that carcinogenic NT50 was likely to enter mesothelial cells, probably via penetration. Based on our previous asbestos studies, we used lysates from various rat organs including lung, which is a putative major target for exposure in humans. Of note, asbestos did not adsorb Tf in our previous experiments. Many identified proteins were associated with oxidative stress in the current experiments on MWCNT, which included Keap1, cytochrome P450, aldehyde dehydrogenase, thioredoxin, glutathione S-transferase, heat shock protein, peroxiredoxin and proteasome.

Among those proteins, we decided to focus on Hb and Tf, considering not only the result that only CNTs, especially NT50, adsorbed Tf but also a close association between excess iron and carcinogenesis. Approximately 60% of the iron is present in the heme of Hb in erythrocytes. Due to its richness in capillaries, lung tissue contains a large amount of Hb.

Coating NT50 with Hb or Tf significantly increased mesothelial damage and significantly delayed wound healing with Hb or lung lysate; a similar effect was not observed with NTtngl, likely because NTtngl does not enter mesothelial cells. We evaluated the effects of NT50 coated with Hb and Tf from the viewpoint of catalytic Fe(II) and lipid peroxidation. Catalytic Fe(II) can initiate Fenton reaction that generates hydroxyl radicals to start lipid peroxidation. Hb and Tf coating significantly increased the catalytic Fe(II) in RPMCs detected with RhoNox-1 and HNE-modified proteins simultaneously, suggesting that NT50 exposure induces high levels of oxidative stress in mesothelial cells. This was also supported by an observation of increased intracellular Tf itself with Western blot analysis.

Then, we evaluated whether oxidative stress can cause DNA damage with comet assay and found that only Hb- or Tf-coated NT50 induced DNA strand breaks in mesothelial

cells, whereas pristine NT50 did not. Mesothelial damage with less cellular death in the case of Hb or Tf coating might contribute to more mutations in mesothelial cells through NT50. We interpret here that Hb- or Tf-coated NT50 can induce various kinds of DNA damage including DNA double-strand breaks. Thus, further studies are necessary to identify and quantify precise DNA lesions.

In the previous carcinogenesis experiments, we observed iron accumulation in areas near CNT deposits. Excess iron has been associated with DNA strand breaks, which may lead to homozygous deletion of Cdkn2A/2B as observed in Fenton reaction-induced renal carcinogenesis in rats. Reportedly, iron overload is a major pathogenesis in asbestos-induced mesothelial carcinogenesis, including the case of chrysotile containing no iron per se, where hemolysis followed by surface Hb adsorption, induces similar pathology of iron overload. Together with our previous finding of a high incidence of homozygous deletion of Cdkn2A/2B in CNT-induced mesothelial carcinogenesis, these new results strongly support the hypothesis that excess iron possibly derived from Hb and Tf plays a role in the molecular mechanism of NT50-induced mesothelial carcinogenesis.

Finally, we evaluated the role of Tf receptor 1, based on the result that coating NT50 with lung lysate or Tf significantly increased the uptake of NT50 by RPMCs. Decreasing Tf receptor 1 with ferristatin II significantly decreased NT50 uptake and cytoplasmic catalytic Fe(II). These findings demonstrate, for the first time, the involvement of Tf and its receptor in the NT50 uptake by mesothelial cells, in addition to simple penetration, which provided a new molecular mechanism of MWCNTs in mesothelial cell damage. Surprisingly, 18% decrease in the uptake of NT50 dramatically changed intracellular catalytic Fe(II). This may be associated with iron metabolism in mesothelial cells, especially storage and export, which needs further investigation.

[Conclusion]

Our results suggest that adsorptive activity of NT50 for proteins, especially hemoglobin and transferrin, is a major mechanism in mesothelial damage followed by carcinogenesis. It works for the efficient NT50 uptake by mesothelial cells and also for the increased catalytic Fe(II), leading to DNA damage. Therefore, chemical modification of CNT to avoid Hb and Tf adsorption might decrease the human risk to CNT-induced mesothelial carcinogenesis. Many more adsorptive proteins on MWCNTs await evaluation.