Asbestos exposure is considered a social burden by causing mesothelioma. Despite the use of synthetic materials, multi-walled carbon nanotubes (MWCNTs) are similar in dimension to asbestos and produce mesothelioma in animals. The role of inflammatory cells in mesothelial carcinogenesis remains unclear. Here, we evaluated the differences in inflammatory cell responses following exposure to these fibrous materials using a luminometer and luminol and LPS.

Materials and Methods

Asbestos and multi-walled carbon nanotubes (MWCNTs) are novel synthetic materials consisting only of carbon, and due to its superior properties, are already used in industries for production of semiconductors, fuel cells and structural materials. However, the physical dimensions and the biopersistence of MWCNTs were found to be similar to asbestos, and they indeed have revealed asbestos-like pathogenicity, including mesothelial carcinogenesis in rodents. In both cases, mesothelial cells, which are closely associated with foreign body-induced inflammation and the associated local iron overload, are the predominant carcinogenic cells. Previous studies have reported the generation of reactive oxygen species (ROS) with luminol by macrophages or isolated neutrophils exposed to asbestos and the toxicity of MWCNTs to macrophages. However, there are limited data available on the oxidative responses of inflammatory cells to MWCNTs.

Our recent studies revealed that MWCNT diameter and rigidity are critical factors in mesothelial injury and carcinogenesis. Here, we compared neutrophil and macrophage responses to asbestos and MWCNTs of various defined diameters by measuring ROS generation, and thus studied the contribution of inflammation in fiber-induced mesothelial carcinogenesis. There has been a recent progress in the luminometer and its probe as well as protocols, thus allowing peripheral blood to be used as the source of neutrophils without performing separation procedures. In the present study, we found that asbestos and MWCNTs generate distinct responses in inflammatory cells.
and cytochrome oxide) was from Wako and was used at a final concentration of 1 mM.

**Peripheral blood and macrophage cell line.** Male Sprague-Dawley rats 15 weeks old (Shizuoka Laboratory Center, Hamamatsu, Japan) were used (n = 3 for each group). The animals were anesthetized with pentobarbital, and the blood was collected from the inferior vena cava with heparinization immediately before use. The animal experiment committee of Nagoya University Graduate School of Medicine approved this experiment. We used the murine macrophage cell line RAW264.7 (DS Pharma Biomedical, Osaka, Japan).

**Determination of ROS generated from inflammatory cells.** We measured ROS with a luminometer (AB-2280; Atto Corporation, Tokyo, Japan; detection range, 350–900 nm) using L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt; Wako Pure Chem. Co., Ltd., Osaka, Japan) as a chemiluminescent probe. L-012 develops strong chemiluminescence with a $\lambda_{\text{max}}$ of 458 nm when it reacts with ROS, including superoxide ($O_2^-$), hypochlorite (HClO) and hydroxyl radical (OH), among which OH 'causes the highest chemiluminescence.'

In the peripheral blood experiments, blood (20 μl) and glucose (5 μl; final concentration 10 mM) were incubated at 37°C for 3 min. L-012 (20 μl; final concentration 2 μM), a material sample (final concentration 1.0, 2.0 or 4.0 mg/ml) and an antioxidant (10 μl) were combined and adjusted to a total volume of 250 μl with 10 mM phosphate-buffered saline (PBS; pH 7.4). After ample pipetting and vortexing, we started each measurement. Measurements were performed on the luminometer for 10 s and were repeated every 30 s for a period of ~50 min. Zymosan (1 mg/ml) was used as a positive control, and 0.9% NaCl and 0.5% BSA in saline were used as a negative control for asbestos and MWCNTs, respectively.

For the macrophage experiments, RAW264.7 cells (1 × 10⁴) were incubated in a 6-well plate in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and antibiotic/antimycotic (Life Technologies, Carlsbad, CA) at 37°C in 5% CO₂ for 24 h. Asbestos (5 μg/cm²) or MWCNTs (5 μg/cm²) were then added to the culture, and the cells were further incubated for up to 7 h. The cells were removed with a scraper and recovered by centrifugation at 720 × g. New medium with FBS (230 μl) was then added, followed by incubation at 37°C for 3 min. Then, L-012 (20 μl; final concentration 2 μM) was added for a total volume of 250 μl, and the measurements were performed as described above. LPS (1.2 μg/ml) was used as a positive control for macrophage stimulation, and 0.9% NaCl and 0.5% BSA in saline were used as negative controls for asbestos and MWCNTs, respectively.

**Hemolysis.** Heparinized blood (100 μl) and a fibrous material (5 mg/ml; in PBS or 0.5% BSA in PBS) were mixed and incubated at 37°C for 4 h. Thereafter, samples were centrifuged at 1,500 × g for 5 min, and the collected supernatant was measured for absorbance at 540 nm (hemoglobin) using a spectrophotometer (ND-2000, Thermo, Japan). The hemolysis percentage (HP) was calculated using the following equation as described previously:

$$\text{HP} (\%) = \left( \frac{D - D_c}{D_{nc} - D_c} \right) \times 100$$

where $D$ is the absorbance of the test samples; $D_c$ and $D_{nc}$ are the absorbances of the positive and negative control, respectively. The results are shown as the average of three independent measurements.

**Time-lapse microscopic observation.** BZ-9000 (Keyence, Osaka, Japan) was used for time-lapse video microscopy of RAW264.7 cells up to 5 h.

**Statistics.** The peak values of chemiluminescence during the observation period were analyzed by one-way ANOVA with Dennett’s multiple comparison test through Prism5 (GraphPad Software Inc., San Diego, CA). Means ± SEM are shown.

**Results**

ROS from neutrophils increased with asbestos treatment but not with MWCNT treatment. Using zymosan as a positive control, we confirmed that the whole system works well (average RLU ≈ 40.6 × 10⁴). The peak time (~20 min) after addition (data not shown) also confirmed that we observe the function of neutrophils in the peripheral blood. All asbestos treatments significantly increased ROS generation in a dose-dependent manner with a peak increase at ~10 min (Fig. 1A–C). ROS generation by crocidolite and amosite were significantly higher than that of chrysotile (~25% of crocidolite/amosite; RLU < 1,000). Amosite induced the highest ROS generation, followed by crocidolite and chrysotile (amosite > crocidolite >> chrysotile). In contrast, MWCNTs of all diameters (Table 1) did not induce significant ROS generation under the same experimental conditions (Fig. 1D and data not shown).

SOD1, catalase (crocidolite only), sodium azide and apocynin significantly inhibited the ROS generation induced by crocidolite and amosite, indicating the involvement of $O_2^-$, H₂O₂, cytochrome oxidase and NADPH oxidase. DFO also inhibited ROS generation, whereas nitritotriacetae promoted it. Inhibitory experiments were not performed for chrysotile due to its relatively low ROS generation.

ROS from macrophages increased with both asbestos and MWCNT treatment. LPS-mediated ROS increased in a concentration-dependent manner (0.12 < 1.2 < 12 μg/ml; data not shown), revealing that the system worked. Stimulation with 1.2 μg/ml LPS caused a continual ROS generation for more than 24 h in RAW264.7 cells (Fig. 2A and data not shown). Furthermore, the peak time was different for each type of asbestos: crocidolite was 3 h, amosite was 2 h and chrysotile was >5 h (Fig. 2A–C).

Similar to asbestos, MWCNTs of various diameters consistently induced ROS generation in the macrophage cells (Fig. 2D–F). The peak time for MWCNTs occurred much earlier than that

---

Table 1. Characteristics of asbestos and MWCNTs

| Fibers    | Structural formula | Diameter (nm) | Length (μm) | *Mesothelial carcinogenicity (50% incidence, days) |
|-----------|--------------------|---------------|-------------|-----------------------------------------------|
| Asbestos  |                    |               |             |                                               |
| Crocidolite | Na₂(Fe³⁺)(Fe²⁺)₂Si₃O₇(OH)₄ | 40–150       | 4.54        | 600                                           |
| Amosite   | (Fe-Mg)₂Si₃O₇(OH)₄ | 60–350        | 5.45        | 600                                           |
| Chrysotile | Mg₄(Si₄O₁₁)OH₈ | 20–80         | 3.87        | 400                                           |
| MWCNTs    |                    |               |             |                                               |
| CNT-50    | Cₙ                | **52.40 ± 0.72** | **4.60 ± 0.10** | 280                                           |
| CNT-115   | Cₙ                | 116.25 ± 1.58  | 4.88 ± 0.10  | Not determined                                |
| CNT-145   | Cₙ                | 143.5 ± 1.56   | 4.34 ± 0.08  | 320***                                        |
| CNT-tngl  | Cₙ                | −15           | Not applicable | No carcinogenicity**                          |

*Data are based on 10 mg intraperitoneal injection to F1 rats between Fischer-344 and Brown-Norway. **Means ± SEM.***Low carcinogenicity (17%) at the dose of 1 mg intraperitoneal injection at day 350. MWCNTs, multi-walled carbon nanotubes.
observed for asbestos: CNT-50 and CT-115 occurred at 1.5 h, CNT-145 at 0.5 h and CNT-tngl at 1 h (Fig. 2D–F).

SOD1 and apocynin consistently inhibited ROS generation, but catalase did not work in all experiments. NaN₃ inhibited the ROS generated by CNT-115, CNT-145 and CNT-tngl. NTA significantly promoted ROS generation only with CNT-50 treatment, whereas DFO inhibited it for crocidolite, CNT-115 and CNT-tngl. Of note, DFO promoted ROS generation only with chrysotile (Fig. 2C and F).

Hemolysis was induced by chrysotile. Among the asbestos and MWCNTs used, only chrysotile caused massive hemolysis, which was 75% after a 4 h incubation.

Distinct motion of macrophages after exposure to asbestos and MWCNTs. We observed RAW264.7 cells using time-lapse microscopy analysis after exposure to either asbestos or MWCNTs. In the case of asbestos, we observed cell movements toward the fibers, leading to the isolation of fibers from media by groups of cells (Fig. 3A and B). In contrast, the cells remained dispersed with any MWCNTs (Fig. 3C and D).

Discussion

The biological assessment of novel synthetic materials is important to evaluate human health risk. We compared the inflammatory response in neutrophils and macrophages following expo-
Fig. 2. ROS generation by macrophages after exposure to asbestos and MWCNTs. A luminometer and L-012 were used to measure ROS generation from macrophages (RAW264.7) after incubation with each fiber. Refer to the text for further details. (A–C) asbestos; (D–F) MWCNTs. LPS, lipopolysaccharide. Refer to the legend of Fig. 1 for abbreviations.
sure to asbestos and MWCNTs with various diameters ex vivo. There was no difference observed in the chemiluminescence emitted by whole blood and with neutrophil isolation with L-012 probe.\(^ {24}\) Furthermore, the present ex vivo system worked well as demonstrated by the use of positive controls (zymosan and LPS). We found, for the first time to our knowledge, that ROS generation in neutrophils was completely different between asbestos and MWCNTs exposure (Fig. 1). Of note, we did not observe neutrophil stimulation by any of the MWCNTs used, indicating that the response was independent of the diameter (Fig. 1D). We believe that this effect is associated with the formulation of MWCNTs. MWCNTs consist only of carbon,\(^ {5,15}\) an element in the backbones of most biomolecules, whereas asbestos is a fibrous crystal made of silicon, oxygen and minerals.\(^ {1}\) The results indicate that the acute neutrophilic inflammation following exposure to MWCNTs may be minimal compared to other similar fibrous materials, which may call for medical attention.

Indeed, neutrophils reacted to all the types of asbestos tested within 10 min. The ROS generation was much higher with amosite and crocidolite treatment than with chrysotile. This finding is consistent with the direct catalytic activity of each type of asbestos for Fenton reactions observed by electron spin resonance analysis.\(^ {26}\) There are two indications on the results: amosite and crocidolite were found to contain large amounts of iron (27.3% and 28.5%, respectively), and chrysotile caused massive hemolysis. The presence of surface iron may facilitate ROS generation, and conversely, hemoglobin and heme in the reaction mixture may delay or inhibit ROS generation by their toxicity.\(^ {27–29}\) The ROS generated were $\cdot O_2$, $H_2O_2$, and $'OH$, and based on the inhibition experiments, their generation was associated with cytochrome oxidase and NADPH oxidase. It is known that DFO blocks catalytic iron and nitrilotriacetate promotes it.\(^ {30–32}\) The results indicate that catalytic iron is also involved in the ROS generation from neutrophils.

Macrophages are the second cells following neutrophils to arrive at the site of inflammation and play a major role in chronic inflammation when the inflammatory stimulus is not quickly eliminated.\(^ {33}\) Both asbestos and MWCNTs induced ROS generation in RAW264.7 cells. Following asbestos exposure, the peak time observed in macrophages was later than that observed for neutrophils. Furthermore, the peak time occurred earlier following MWCNTs exposure (0.5–1.5 h) compared to asbestos (2–5 h) (Fig. 2A, B, D and E). This result suggests that different mechanisms exist for sensing the presence of different fibrous materials that have similar dimensions. Using video microscopy, we observed that the isolation activity for fibrous materials by macrophages, indicated by cell gathering, is stronger for asbestos than for MWCNTs (Fig. 3).

At the same time, the results observed for macrophages treated with antioxidants and iron chelators were much different from the results observed for neutrophils and also for each fiber. NADPH oxidase and $O_2$ were consistently involved as indicated by the effects observed with apocynin and SOD treatment. This is consistent with recent reports on the involvement of NLRP3

---

**Fig. 3.** Time-lapse microscopic analysis of cellular movements by macrophages after exposure to each fiber. Cellular movements were distinct between asbestos and MWCNT exposures. (A) crocidolite; (B) chrysotile; (C) CNT-50; (D) CNT-145. The number indicates h and min. Differences between asbestos and MWCNTs are indicated by the areas with disrupted circles (cellular aggregation). Refer to the text for further details (bar = 80 µm).
inflammasome activation by nanomaterials. Catalase did not work for any of the fibers, suggesting that H$_2$O$_2$ is not involved or was present in an unapproachable fashion. DFO worked for crocidolite and CNT-115, and NTA promoted ROS generation only in CNT-50 (Fig. 2C and F). We recently reported different mechanisms for the uptake of asbestos and MWCNTs by mesothelial cells. The former was phagocytosis, whereas the latter was penetration/piercing, which was most prominent for CNT-50. The effect of NTA may be associated with the intracellular localization of fibers in macrophages, which may induce catalytic iron in the cell. The enhancement in ROS generation in chrysotile-exposed macrophages with DFO was unexpected (Fig. 2C). Among the types of asbestos used in the study, chrysotile displays the highest toxicity and inflammogenicity. Thus, iron removal by DFO may have stimulated the macrophages. The time required for macrophage activation was different between asbestos and MWCNTs (Fig. 2A and D). The identification of molecules on the outer membrane and inside of neutrophils and macrophages that are associated with the present results is an interesting issue to pursue in the near future.

In our previous experiments of mesothelial carcinogenesis in rats, the MWCNTs used in the present study revealed that CNT-50 is potently carcinogenic, CNT-145 is less carcinogenic and CNT-tngl was not carcinogenic (Table 1). However, in the studies presented here, neutrophil responses were subtle and macrophage responses were basically the same among the MWCNTs used (Fig. 4). Therefore, we believe that mesothelial cell injury is a more important indicator than the responses induced by neutrophils and macrophages in fiber-induced mesothelial carcinogenesis. On the other hand, neutrophils and macrophages may play a role in the inflammatory conditions often observed after airway exposure, such as bronchitis, pneumonitis and pulmonary fibrosis. Whether MWCNTs cause different types of pulmonary disease from those caused by asbestos should be carefully monitored in workers involved in MWCNT production.

Acknowledgments

We would like to thank Nobuyuki Misawa for excellent technical assistance. This work was supported in part by a grant-in-aid for research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (24390094; 221S0001-04; 24108001).

Abbreviations

Amo amosite (brown asbestos)  
ANOVA analysis of variance  
APO apocynin  
BSA bovine serum albumin  
CAT catalase  
Chry chrysotile (white asbestos)  
CNT carbon nanotube  
Cro crocidolite (blue asbestos)  
DFO deferoxamine mesylate  
FBS fetal bovine serum  
L-012 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione  
LPS lipopolysaccharide  
MWCNT(s) multi-walled carbon nanotube(s)  
PBS phosphate-buffered saline  
RLU relative luminescence unit  
ROS reactive oxygen species  
SEM standard error of means  
SOD superoxide dismutase

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Roggli VL, Oury TD, Sporn TA. Pathology of Asbestos-Associated Diseases. 2nd ed. New York: Springer Verlag, 2004.
2 IARC, WHO. Asbestos (chrysotile, amosite, crocidolite, tremolite, actinolite, and anthophyllite). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. A Review of Human Carcinogens; Part C: Arsenic, Metals, Fibres, and Dusts. France: Lyon, 2012; 219–309.
3 Aierken D, Okazaki Y, Chew SH, et al. Rat model demonstrates a high risk of tremolite but a low risk of anthophyllite for mesothelial carcinogenesis.
Nagoya J Med Sci 2014; 76: 149–160.

4. Robinson BW, Luke RA. Advances in malignant mesothelioma. N Engl J Med 2005; 353: 1591–1603.

5. Iijima S. Helical Microtubules of Graphitic Carbon. Nature 1991; 354: 56–58.

6. Poland CA, Duffin R, Kinloch I, et al. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat Nanotechnol 2008; 3: 423–428.

7. Murphy FA, Schinwald A, Poland CA, Donaldson K. The mechanism of pleural inflammation by long carbon nanotubes: interaction of long fibres with macrophages stimulates them to amplify pro-inflammatory responses in mesothelial cells. Part Fibre Toxicol 2012; 9: 8.

8. Takagi A, Hirose A, Nishimura T, et al. Induction of mesothelioma in p53+/− mouse by intraperitoneal application of multi-wall carbon nanotube. J Toxicol Sci 2008; 33: 105–116.

9. Sakamoto Y, Nakae D, Fukumori N, et al. Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats. J Toxicol Sci 2009; 34: 65–76.

10. Nagai H, Okazaki Y, Chew S, et al. Diameter of multi-walled carbon nanotubes is a critical factor in mesothelial injury and subsequent carcinogenesis. Proc Natl Acad Sci U S A 2011; 108: E1330–E1338.

11. Toyokuni S. Genotoxicity and carcinogenicity risk of carbon nanotubes. Adv Drug Deliv Rev 2013; 65: 2098–2110.

12. Toyokuni S. Mechanisms of asbestos-induced carcinogenesis. Nagoya J Med Sci 2009; 71: 1–10.

13. Nagai H, Toyokuni S. Biopersistent fiber-induced inflammation and carcinogenesis: lessons learned from asbestos toward safety of fibrous nanomaterials. Arch Biochem Biophys 2010; 502: 1–7.

14. Jiang L, Akatsuka S, Nagai H, et al. Iron overload signature in chrysotile-asbestos-induced oxidative DNA damage. Cancer Sci 2008; 99: 2142–2151.

15. Nagai H, Ishihara T, Lee WH, et al. Asbestos surface provides a niche for oxidative modification. Cancer Sci 2011; 102: 2118–2125.

16. Kubo Y, Takenaka H, Nagai H, Toyokuni S. Distinct affinity of nuclear proteins to the surface of chrysotile and crocidolite. J Clin Biochem Nutr 2012; 51: 221–226.

17. Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. Toxicol Lett 2005; 157: 175–188.

18. Toyokuni S, Sagripanti JL. DNA single- and double-strand breaks produced by engineered nanomaterials. Small 2013; 9: 1595–1607.

19. Toyokuni S. Role of iron in carcinogenesis: cancer as a ferrotoxic disease. Cancer Sci 2009; 100: 9–16.

20. Kumar V, Abbas AK, Aster JC. Robbins Basic Pathology. 9th ed. Philadelphia, PA: Elsevier Saunders, 2013.

21. Martinon F. Signaling by ROS drives inflammasome activation. Eur J Immunol 2010; 40: 616–619.

22. Sun B, Wang X, Li Z, Li R, Xia T. NLRP3 inflammasome activation induced by engineered nanomaterials. Small 2013; 9: 1595–1607.

23. Nagai H, Okazaki Y, Chew SH, et al. Intraperitoneal administration of tangled multiwalled carbon nanotubes of 15 nm in diameter does not induce mesothelial carcinogenesis in rats. Pathol Int 2013; 63: 457–462.