Original Article

Asbestos and multi-walled carbon nanotubes generate distinct oxidative responses in inflammatory cells

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(Received 6 August, 2014; Accepted 26 August, 2014)

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 L-012 (8-amino-5-chloro-2,3-dihydro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-dione) to detect reactive oxygen species (ROS). Rat peripheral blood or RAW264.7 cells were used to assess the effects on neutrophils and macrophages, respectively. Crocidolite and amosite induced significant ROS generation by neutrophils with a peak at 10 min, whereas that of chrysotile was ~25% of the crocidolite/amosite response. MWCNTs with different diameters (~15, 50, 115 and 145 nm) and different carcinogenicity did not induce significant ROS in peripheral blood. However, the MWCNTs induced a comparable amount of ROS in RAW264.7 cells following asbestos treatment. The peaks for MWCNTs (0.5–1.5 h) were observed earlier than those for asbestos (1–5 h). Apocynin and superoxide dismutase significantly inhibited ROS generation for each fiber, suggesting an involvement of NADPH oxidase and superoxide. Thus, asbestos and MWCNTs induce different oxidative responses in inflammatory cells, indicating the importance of mesothelial cell evaluation for carcinogenesis.

***Key Words*:** **asbestos, multi-walled carbon nanotubes, neutrophil, macrophage, NAD(P)H oxidase**

Novel materials may generate an unexpected health risk to the human society. Asbestos, a natural fibrous mineral, is an example. Asbestos presents excellent durability, such as resistance to acid, heat and friction, versatility for mixing with other materials and huge economic merits depending on mining, and because of these beneficial properties, it was abundantly used in industries worldwide during the last century. Therefore, it was copiously released into the air surrounding mines and factories. It took a few decades of epidemiological studies to declare that asbestos is the cause of not only asbestosis but also a rare and aggressive malignant tumor, mesothelioma.[1–3] Due to mesothelioma's extremely long incubation period of 30–40 years, the peak incidence of mesothelioma in Japan is predicted to occur in 2025 with 100,000 new patients being diagnosed in the coming 40 years.[4]

Multi-walled carbon nanotubes (MWCNTs) are novel synthetic materials consisting only of carbon,[5] 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,[6,7] including mesothelial carcinogenesis in rodents.[8–11] In both cases, mesothelial cells, which are closely associated with foreign body-induced inflammation and the associated local iron overload, are the predominant carcinogenetic cells.[10,12–15] Previous studies have reported the generation of reactive oxygen species (ROS) with luminol by macrophages or isolated neutrophils exposed to asbestos,[16–22] and the toxicity of MWCNTs to macrophages.[23] 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.[10] 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 the inflammatory cells.

Materials and Methods

Materials. We obtained asbestos (crocidolite, amosite and chrysotile) from the Union for International Cancer Control (UICC; Geneva, Switzerland) and suspended it in 0.9% saline (5 mg/ml). We obtained MWCNTs and suspended them in 0.5% bovine serum albumin (BSA) (5 mg/ml in saline) as described.[10,11] The carbon nanotubes were distinguished as CNT-50, CNT-115, CNT-145 and CNT-tngl to represent the diameter of each nanotube (Table 1). Zymosan (Sigma-Aldrich, St. Louis, MO) and lipopolysaccharide (LPS) from *E. coli 011:B4* (Sigma-Aldrich) were used to initiate inflammation. Deferoxamine mesylate (DFO) from Sigma-Aldrich and nitrilotriacetate disodium salt from Nakalai Tesque (Kyoto, Japan) were used at a final concentration of 40 μM.

Antioxidants. Cu,Zn superoxide dismutase from bovine erythrocytes (SOD1; EC1.15.1.1), catalase from bovine liver (EC1.11.1.6) and apocynin (APO; a NADPH oxidase inhibitor) were purchased from Sigma-Aldrich and were used at final concentrations of 1,000 units/L, 1,000 units/L and 1 mM, respectively. NaN_3 (sodium azide; an inhibitor of catalase, peroxidase

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doi: 10.3164/jcbn.14-92

Table 1. Characteristics of asbestos and MWCNTs

	Fibers	Structural formula	Diameter (nm)	Length (μm)	*Mesothelial carcinogenicity (50% incidence, days)
Asbestos	Crocidolite	$Na_2(Fe^{2+})_3(Fe^{3+})_2Si_8O_{22}(OH)_2$	40–150	4.54	~600
	Amosite	$(Fe\text{-}Mg)_7Si_8O_{22}(OH)_2$	60–350	5.45	~600
	Chrysotile	$Mg_3(Si_2O_5)(OH)_4$	20–80	3.87	~400
MWCNTs	CNT-50	C_n	**52.40 ± 0.72	**4.60 ± 0.10	~280
	CNT-115	C_n	116.25 ± 1.58	4.88 ± 0.10	Not determined
	CNT-145	C_n	143.5 ± 1.56	4.34 ± 0.08	~320***
	CNT-tngl	C_n	~15	Not applicable	No carcinogenicity[36]

*Data are based on 10 mg intrapenitoneal injection to F1 rats between Fischer-344 and Brown-Norway. **Means ± SEM.[10,14] ***Almost no carcinogenicity at the dose of 1 mg intraperitoneal injection at day 350.[10] MWCNTs, multi-walled carbon nanotubes.

and cytochrome oxidase) 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 λ_{max} of 458 nm when it reacts with ROS, including superoxide (O_2^-), hypochlorite ($HClO^-$) and hydroxyl radical ($^{\bullet}OH$), among which $^{\bullet}OH$ causes the highest chemiluminescence.[24]

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 99 times 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 controls for asbestos and MWCNTs, respectively.

For the macrophage experiments, RAW264.7 cells (1×10^6) 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% CO_2 for 24 h. Asbestos (5 μg/cm^2) or MWCNTs (5 μg/cm^2) 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 centrifuge 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:[25]

$$HP\ (\%) = (D_t - D_{nc}) / (D_{pc} - D_{nc}) \times 100$$

D_t is the absorbance of the test samples; D_{pc} 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 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 = 40.6×10^3). 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^-, H_2O_2, cytochrome oxidase and NADPH oxidase. DFO also inhibited ROS generation, whereas nitrilotriacetate 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

doi: 10.3164/jcbn.14-92

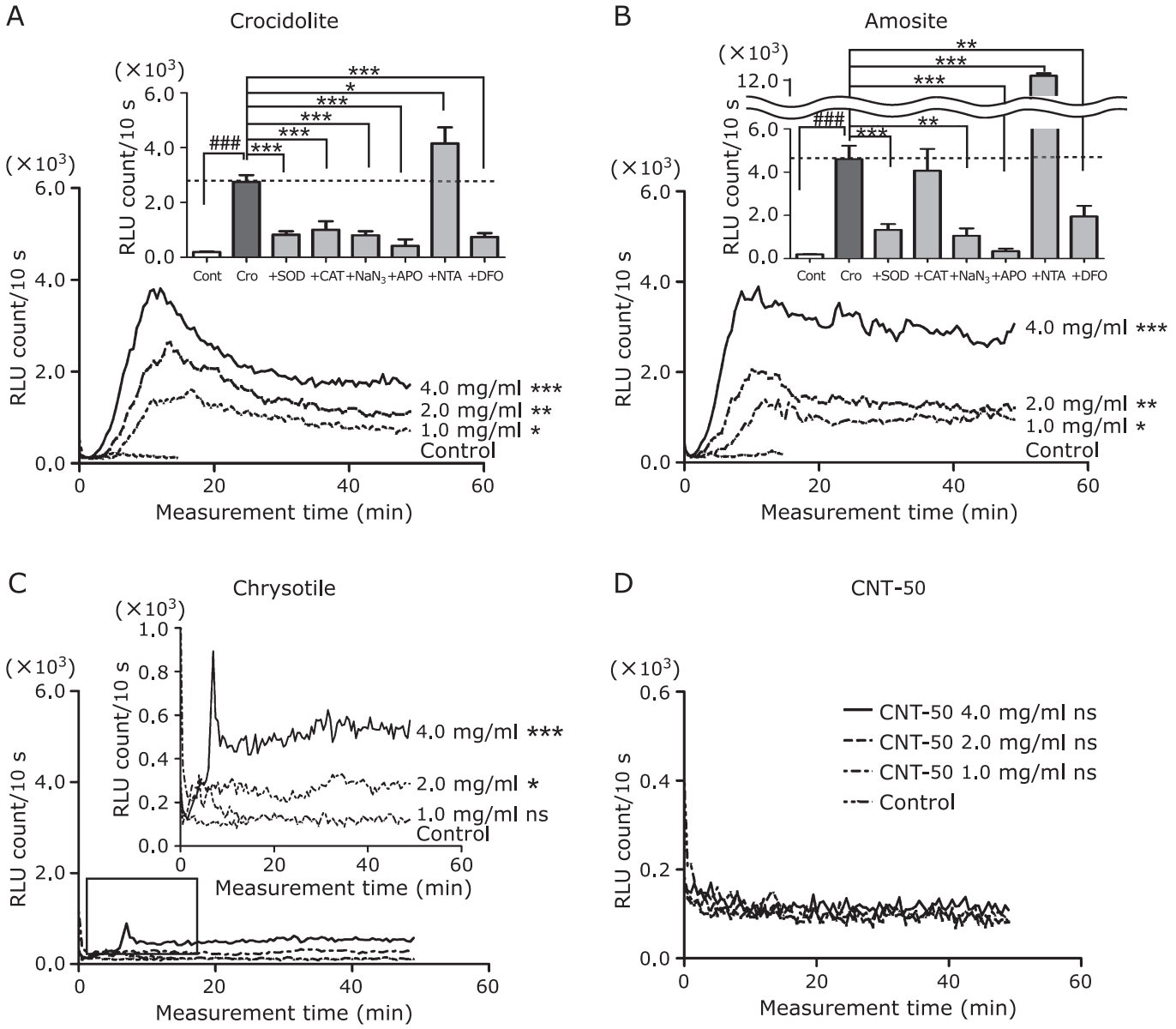

Fig. 1. ROS generation by neutrophils was different following asbestos and MWCNT exposure. A luminometer and L-012 were used to measure ROS generation from neutrophils (rat whole blood) after stimulation by each type of fiber. (A) crocidilite; (B) amosite; (C) chrysotile; (D) CNT-50 (CNT-115, CNT-145 and CNT-tngl showed similar results). The inset in A and B shows the results of the inhibition experiments using antioxidants and iron modulators. Refer to the text for further details. *P* values were determined by one-way ANOVA with Dennett's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001, ns, not significant vs each fiber; $^{\#\#}p$<0.01; $^{\#\#\#}p$<0.001 vs control; means ± SEM from at least three independent samples). APO, apocynin; CAT, catalase; CNT, carbon nanotube; Cont, control; DFO, deferoxamine mesylate; MWCNT, multi-walled carbon nanotube; NTA, nitrilotriacetate; RLU, relative luminescence unit; ROS, reactive oxygen species; SOD, superoxide dismutase.

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).

SOD and apocynin consistently inhibited ROS generation, but catalase did not work in all experiments. NaN_3 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-

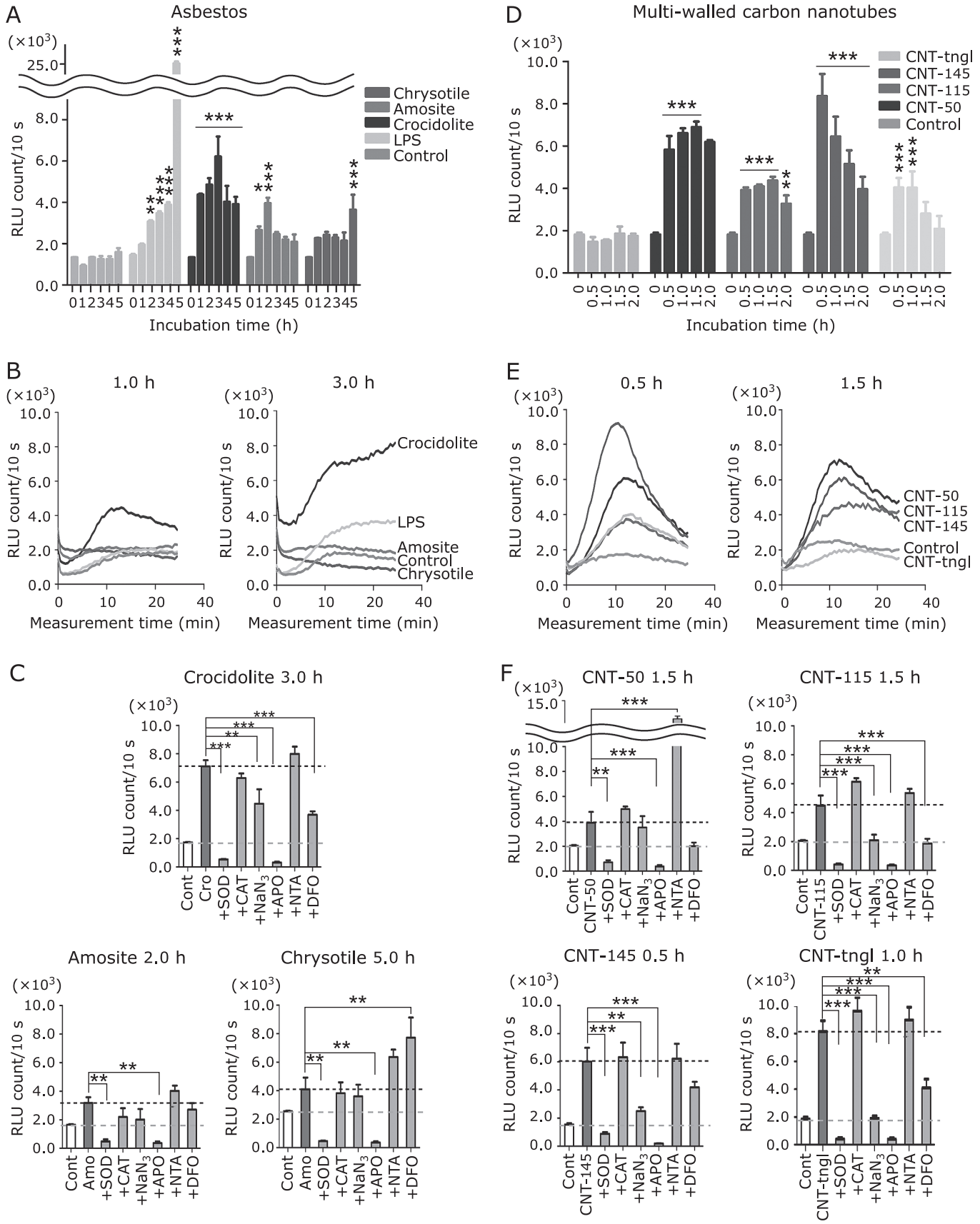

Fig. 2. ROS generation by macrophages after exposure to asbestos and MWCNTs. A luminometer and L-012 were used to measure ROS generation from macrophages (RAW264.7) after incubation with each fiber. Refer to the text for further details. (A–C) asbestos; (D–F) MWCNTs. LPS, lipopolysaccharide. Refer to the legend of Fig. 1 for abbreviations.

doi: 10.3164/jcbn.14-92

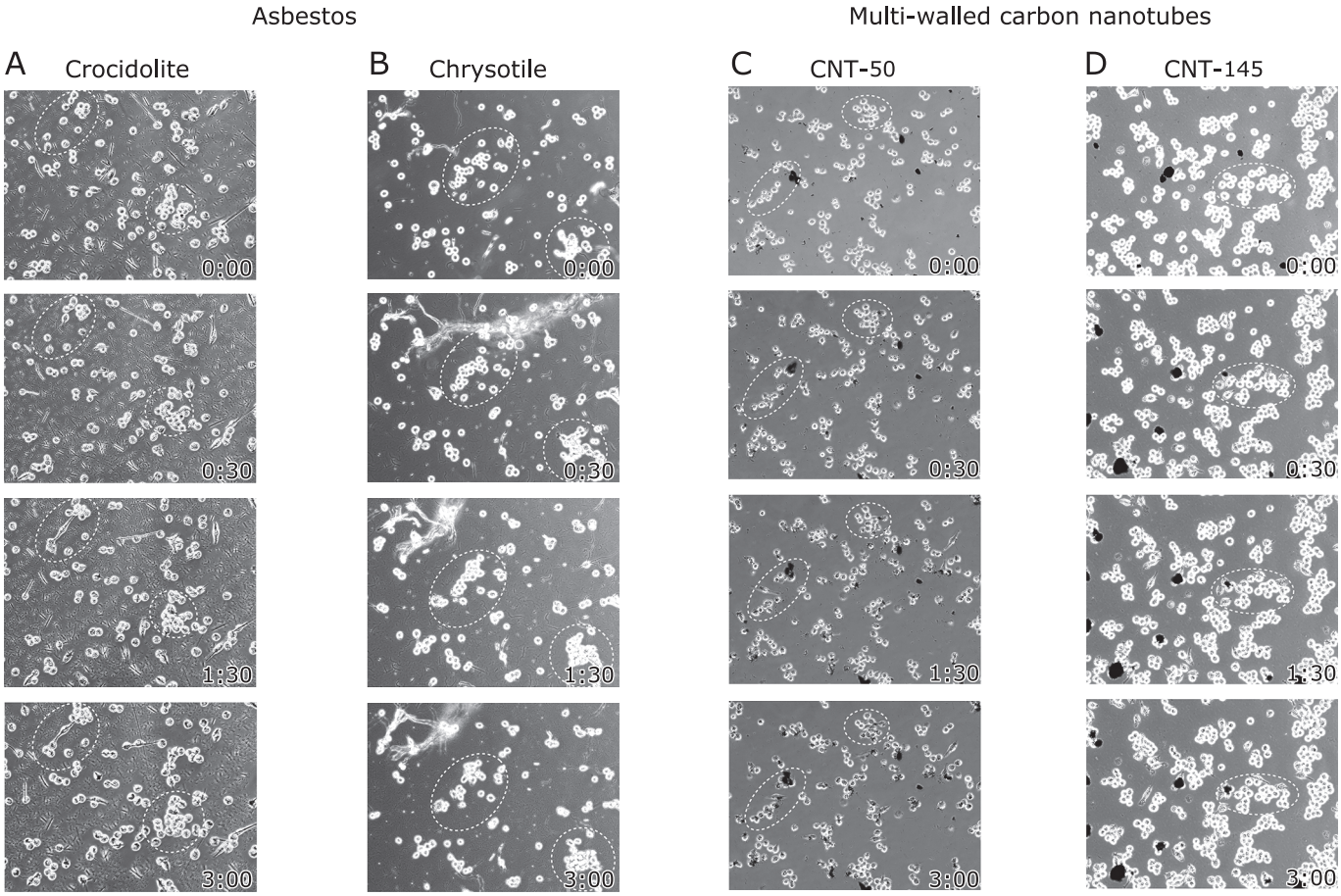

Fig. 3. Time-lapse microscopic analysis of cellular movements by macrophages after exposure to each fiber. Cellular movements were distinct between asbestos and MWCNT exposures. (A) crocidolite; (B) chrysotile; (C) CNT-50; (D) CNT-145. The number indicates h and min. Differences between asbestos and MWCNTs are indicated by the areas with disrupted circles (cellular aggregation). Refer to the text for further details (bar = 80 μm).

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 O_2^-, H_2O_2, and $^{\bullet}OH$, 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

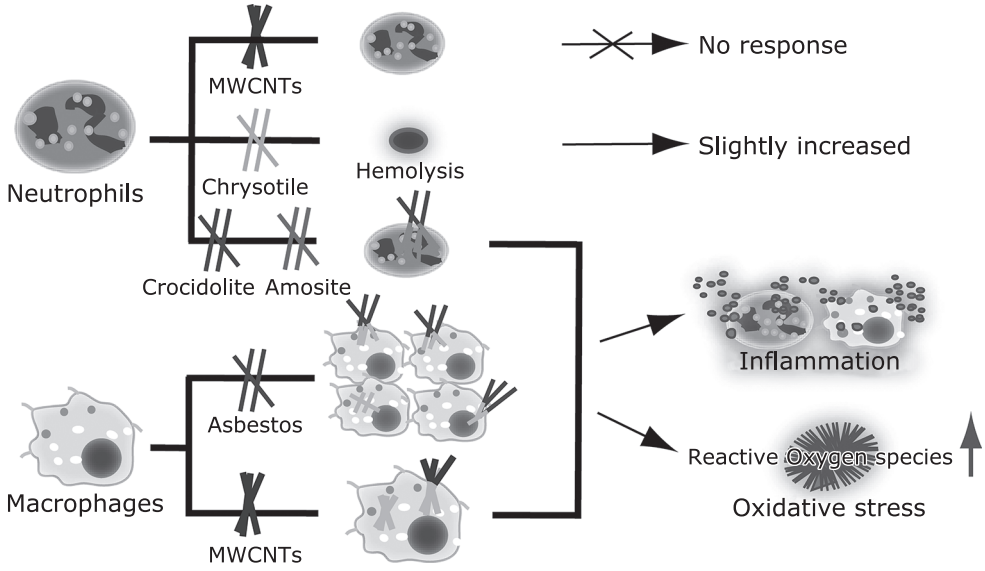

Fig. 4. Summary of the differences in oxidative response of inflammatory cells to each fiber.

inflammasome activation by nanomaterials.[34,35] Catalase did not work for any of the fibers, suggesting that H_2O_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.[10] 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 inflammatogenicity.[10] 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,[10] CNT-145 is less carcinogenic and CNT-tngl was not carcinogenic (Table 1).[36] 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 sodium salt
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.

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