Toxicity of Single-Walled Carbon Nanohorns

Jin Miyawaki,[†,⊥,]* Masako Yudasaka,[†,‡,]* Takeshi Azami,[‡] Yoshimi Kubo,[‡] and Sumio Iijima[†,‡,§]

[†]JST/SORST, c/o NEC, 34 Miyukigaoka, Tsukuba, Ibaraki 305-8501, Japan, [‡]NEC, 34 Miyukigaoka, Tsukuba, Ibaraki 305-8501, Japan, and [§]Meijo University, 1-501 Shiogamaguchi, Tenpaku, Nagoya 468-8502, Japan. [⊥]Current address (as of February 1, 2008): Institute for Materials Chemistry and Engineering, Kyushu University, 6-1 Kasugakoen, Kasuga, Fukuoka 816-8580, Japan. E-mail: miyawaki@cm.kyushu-u.ac.jp.

The expansion in the fields of nanoscience and nanotechnologies and the increased handling of manufactured nanomaterials have made the assessment of health risks and the estimation of the environmental impact of nanomaterials critical and urgent issues.[1–14] For example, the National Institute of Environmental Health Sciences in the United States has alerted "generally, the smaller the particles, the more reactive and toxic are their effects."[15] The Royal Society and Royal Academy of Engineering has presented a report on nanoscience and nanotechnologies in which it recommended treating nanomaterials as new substances because "the toxicity of chemicals in the form of free nanomaterials cannot be predicted from their known toxicity in a larger form."[16] Carbon nanotubes (CNTs)[17] are considered one of the most promising nanomaterials with ex-

ABSTRACT We extensively investigated *in vitro* and *in vivo* the toxicities of as-grown single-walled carbon nanohorns (SWNHs), a tubular nanocarbon containing no metal impurity. The SWNHs were found to be a nonirritant and a nondermal sensitizer through skin primary and conjunctival irritation tests and skin sensitization test. Negative mutagenic and clastogenic potentials suggest that SWNHs are not carcinogenic. The acute peroral toxicity of SWNHs was found to be quite low—the lethal dosage for rats was more than 2000 mg/kg of body weight. Intratracheal instillation tests revealed that SWNHs rarely damaged rat lung tissue for a 90-day test period, although black pigmentation due to accumulated nanohorns was observed. While further toxicological assessments, including chronic (repeated dose), reproductive, and developmental toxicity studies, are still needed, yet the present results strongly suggest that as-grown SWNHs have low acute toxicities.

KEYWORDS: carbon nanohorn · acute toxicity · genotoxicity · carbon nanotube · fullerene

pectations for a variety of applications due to their novel physical and chemical characteristics. Concern about occupational exposure to airborne CNTs led to assessments of pulmonary toxicities upon inhalation, but the conclusions are in disagreement.[18–24]

Furthermore, few toxicological studies of CNTs have been reported for other exposure pathways, such as percutaneous and peroral exposures. Therefore, systemic toxicological assessments of CNTs are urgently needed.

Single-walled carbon nanohorn (SWNH) aggregates,[25] composed of thousands of graphitic tubules (similar in structure to single-walled CNTs) having wide diameters of 2–5 nm, have a spherical structure with a diameter of 50−100 nm. On the basis of their morphology, they were
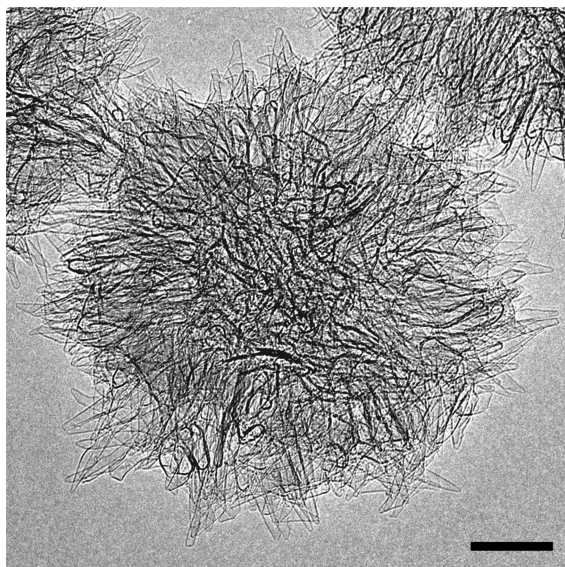

Figure 1. TEM images of dahlia-type SWNH aggregates prepared in Ar. Scale bar represents 20 nm.

TABLE 1. SWNH Toxicological Tests

test	test organism/animal	dosage	findings
reverse mutation (Ames) test	*S. typhimurium* and *E. coli* strains	78–1250 μg/plate	no positive increase in revertants; no growth inhibitory effect
chromosomal aberration test	Chinese-hamster lung fibroblast cell line	0.010–0.078 or 0.313–2.5 mg/mL	negligible positive incidences of structural chromosomal aberrations or polyploidy
skin primary irritation test	rabbits	0.015 g/site	primary irritation index (P.I.I.) = 0; no clinical signs of abnormalities; normal body weight gain
eye irritation test	rabbits	0.02 g/eye	Draize irritation score = 0; no clinical signs of abnormalities; normal body weight gain
skin sensitization (adjuvant and patch) test	guinea pigs	0.02 g/site (induction); 0.01 g/site (challenge)	mean response score = 0; no clinical signs of abnormalities; normal body weight gain
peroral administration test	rats	2000 mg/kg	no mortality; no clinical signs of abnormalities; normal body weight gain
intratracheal instillation test	rats	2.25 mg/animal (17.3 mg/kg)	no mortality; rale for all animals including control group; normal body weight gain; black lung spots and anthracosis; foamy macrophage in intra-alveolar spaces

classified into dahlia, bud, and seed types.[26] Figure 1 shows typical transmission electron microscopy (TEM) images of the dahlia-type SWNH aggregates prepared in an Ar atmosphere; their average diameter was about 100 nm. SWNHs contain no metal catalyst because they are produced by laser ablation of a pure graphite target. This means that the effects of metal impurities can be excluded when determining toxic responses, enabling us to investigate the pure toxicological effects of nanometer-sized graphitic structures. The ease of chemically modifying SWNHs facilitates investigation of the effects of different surface characteristics, such as surface charge and functional groups, on toxic responses. Moreover, the rough external surface and large pore volume inherent to internal nanospaces enable SWNHs to be used as catalyst supports,[27,28] H_2 and CH_4 storage media,[29–32] and supercapacitor electrodes.[33] Because SWNHs are also potential drug delivery carriers,[34–37] many studies of their biomedical applications have been reported.[38–43] To avoid potential health hazards caused by occupational exposure to SWNHs and to promote industrial and biomedical applications of SWNHs, the toxicity of SWNHs should be proactively investigated from various aspects.

We comprehensively investigated *in vivo* and *in vitro* the toxicities of as-grown SWNHs (Table 1) and compared our findings with the reported toxicities for CNTs and fullerenes. First, to assess the carcinogenic potential of SWNHs, we carried out two types of bacterial genotoxicity tests that are widely used as inexpensive, rapid, and simple screenings for substance carcinogenicity. Since the superficial organs, such as the skin and the eyes, have the greatest risk of exposure to SWNHs, we next investigated dermal and ocular reactions. We also performed peroral administration test for SWNHs, because oral ingestion is a likely uptake pathway upon SWNH exposure. There is a risk of breathing in fluffy SWNHs floating in air, so we investigated the effects of intratracheal instillation of SWNHs on the lungs as a sur-

rogate for inhalation exposure to SWNHs to evaluate pulmonary toxicity.

All the test results showed that as-grown SWNHs have low toxicities. This implies that spherical graphite structures with submicrometer diameter, graphitic nanotubules, and pentagons/heptagons in a hexagonal network have negligible impact on living bodies. If toxic responses are observed for other nanocarbons, it is likely that factors other than those mentioned above are the main toxic determinants.

RESULTS AND DISCUSSION

Reverse Mutation (Ames) and Chromosomal Aberration Tests. The carcinogenic potential of the as-grown SWNHs was examined using two kinds of *in vitro* genotoxicity tests, that is, bacterial reverse mutation (Ames) and chromosomal aberration tests. Although benzene is weakly mutagenic or nonmutagenic in the typical Ames test, it causes chromosomal aberrations;[44–46] therefore, test systems with different genetic end points should be used together.

The results of the Ames test for as-grown SWNHs are shown in Figure 2. Neither a positive increase in the number of revertants nor an inhibitory effect of the SWNHs on bacterial growth was observed for any of the five test strains at any of the tested dose levels in either the absence or presence of the metabolic enzyme, S9 mix. For dose levels higher than 78 μg/plate (both in the absence and presence of S9 mix), black precipitation of SWNHs was observed with the naked eye. Positive controls induced the appearance of more than twice the number of revertants for each strain (Supporting Information, Table S1), confirming the validity of the present tests.
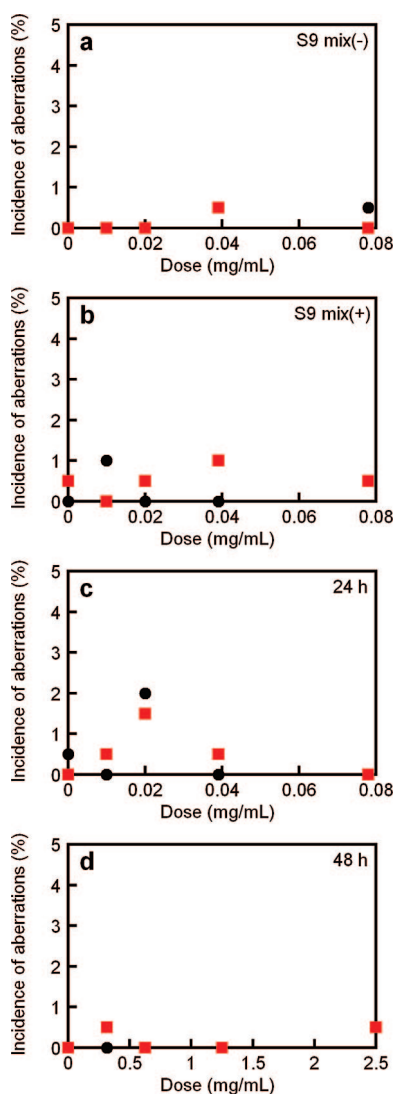
In the chromosomal aberration test, incidence of cells showing polyploidy and structural chromosomal aberrations was less than 2% at any dosage in both 6 h short-term (both in the absence and presence of S9 mix) and continuous (both 24 and 48 h treatment peri-
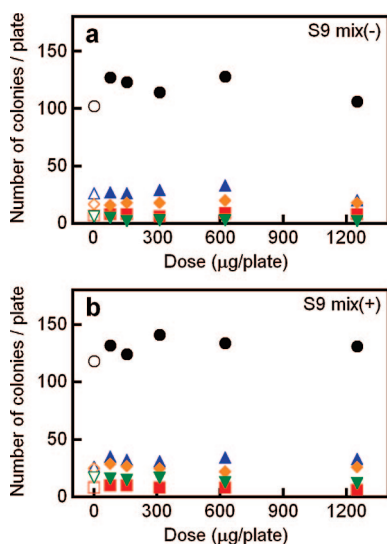
Figure 2. Dose–response curves for bacterial reverse mutation test (48 h incubation time) for as-grown SWNHs without (a) and with (b) metabolic activation by S9 mix. Bacterial strains: (black) TA100; (red) TA1535; (blue) WP2 *uvrA*; (orange) TA98; (green) TA1537. Open symbols represent solvent control.

ods) exposure experiments, as shown in Figure 3. We observed precipitation of SWNHs at concentrations higher than 0.039 mg/mL. The incidences of polyploidy cells and chromosomal aberrations observed in both untreated and vehicle controls were less than 1%, and the positive controls showed high frequencies of structural chromosomal aberrations (16.0% for the short-term S9 mix(−), 45.5% for the short-term S9 mix(+), 43.0% for the 24 h continuous, and 63.0% for the 48 h continuous exposure experiments), proving that the tests were properly conducted.

The negative results in these two genotoxicity tests mean that as-grown SWNHs were not mutagenic or clastogenic under the test conditions, suggesting that as-grown SWNHs are not carcinogenic. Although many studies on the cytotoxicity of CNTs have been reported,[47–57] to the best of our knowledge, none has been reported on their carcinogenic risks. The genotoxicity of C_{60} fullerenes has been relatively well studied,[58–63] but the conclusions are conflicting. Using single-cell gel electrophoresis, Dhawan *et al*.[62] demonstrated dose-dependent genotoxic responses of C_{60} clusters for human lymphocytes at a concentration as low as 2.2 or 4.2 µg/L depending on the method used for preparing the aqueous suspension. On the other hand, Zakharenko *et al*.[61] reported no genotoxicity of intact C_{60} at 450 µg/L when the SOS chromotest with *Escherichia coli* (*E. coli*) was used and slight genotoxicity at 2.24 mg/L when the somatic mutation and recombination genotoxicity test with *Drosophila melanogaster* was used. Mori *et al*.[63] obtained negative results for both the Ames and chromosomal aberration tests for fullerenes (a mixture of C_{60} and C_{70}). Such inconsistencies suggest that the toxicity strongly depends on


Figure 3. Incidence of polyploidy (black) and structural chromosomal aberrations (red) in chromosomal aberration test for as-grown SWNHs: (a) short-term (6 h) exposure without S9 mix activation; (b) short-term (6 h) exposure with S9 mix activation; (c) 24 h continuous exposure; (d) 48 h continuous exposure.

the physicochemical characteristics of the material used, such as the aggregation state, size, shape, and surface properties, and on the cell type being tested. More systematic studies are thus required to conclusively determine the genotoxicies and carcinogenicities of carbon-based nanoparticles.

Skin Primary Irritation, Eye Irritation, And Skin Sensitization Tests. Dermal and ocular reactions upon as-grown SWNH exposure were investigated through widely approved skin primary irritation, eye irritation, and skin sensitization tests.

No clinical signs of abnormalities in any of the test animals (rabbits) in either a skin primary or eye irritation test were observed, and all animals showed normal body weight gain. No erythema/eschar or edema formation was observed on either the intact or abraded skin of all animals, and the primary irritation index (P.I.I.)
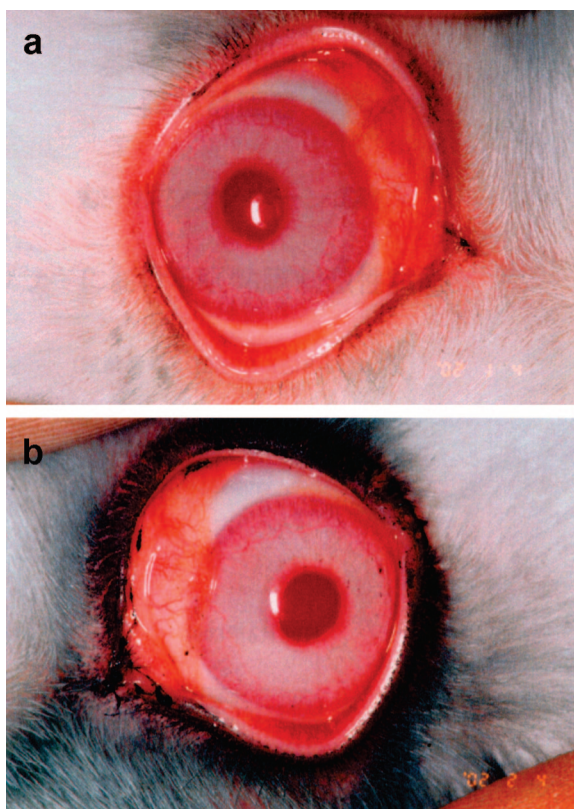
Figure 4. Photo images of rabbit eye after eye irritation test: (a) untreated; (b) 1 h after SWNH dose in conjunctival sac without eye washing.

was calculated to be 0. All animals (with or without eye washing) showed no irritation response in their conjunctivae, corneas, or irises for any observation period, and the Draize eye irritation score was 0. Typical photo images of the eye after the eye irritation test are shown in Figure 4. No corneal injury was observed in any animal.

Table 2 summarizes the dermal response scores for the test animals (guinea pigs). No erythema or edema formation was observed for any SWNH challenge in the SWNH induction group (1) and noninduction group

(2A). The positive contact allergic reactions against the 2,4-dinitrochlorobenzene (DNCB) challenge for the DNCB induction group (3A) and the negative reactions against the DNCB challenge for the noninduction group (2B) or against the ethanol challenge for the DNCB induction group (3B) indicate that the tests were properly conducted. No clinical signs of abnormalities for any animal were observed, and all animals showed normal body weight gain.

The as-grown SWNHs did not show any signs of causing skin irritation (P.I.I. = 0), eye irritation (Draize score = 0), or skin allergic (mean response score = 0) responses. Therefore, as-grown SWNHs can be considered a nonirritant for both the skin and eyes and a nondermal sensitizer under our test conditions. Nelson *et al.*[64] concluded that fullerenes (a mixture of C_{60} and C_{70}) applied in benzene at a likely industrial exposure level do not cause toxic effects on mouse skin epidermis. Huczko *et al.* reported that dermatological tests for C_{60} soot[65] and soot containing CNTs[66] (produced by arc sublimation of Co/Ni-doped homogeneous graphite anodes) indicated no signs of health problems such as skin irritation and allergic reactions. Taken together, it is unlikely that working with nanocarbons, such as fullerenes, CNTs, and SWNHs, is associated with any risk of skin irritation or allergy. In contrast, *in vitro* tests have shown that both C_{60}[67] and CNTs[47,51,54] induce cytotoxic responses on dermal cell lines.

Peroral Administration Test. The suspension of as-grown SWNHs in dimethyl sulfoxide (DMSO)/water was administered orally into the stomach at dose of 2000 mg/kg of body weight. No clinical signs of abnormalities were observed, and all animals (rats) survived the 2 week test period. The body weight changes are shown in Table 3. The vehicle control and SWNH groups showed significantly lower body weight gains than the negative control group at the next day of administration (day 1). No statistically significant differences were observed for the following days, indicating that the transient difference

TABLE 2. Dermal Response Scores in Skin Sensitization Test[a]

group	chemical induction	chemical challenge	observation time (h)	erythema score 0 1 2 3 4	edema score 0 1 2 3 4	sensitization rate	mean response
1	SWNH	SWNH	24	5 0 0 0 0	5 0 0 0 0	0/5	0
			48	5 0 0 0 0	5 0 0 0 0		0
2A	no treatment (E-FCA injection only)	SWNH	24	5 0 0 0 0	5 0 0 0 0	0/5	0
			48	5 0 0 0 0	5 0 0 0 0		0
2B		0.1% DNCB/ethanol	24	5 0 0 0 0	5 0 0 0 0	0/5	0
			48	5 0 0 0 0	5 0 0 0 0		0
3A	0.1% DNCB/ethanol	0.1% DNCB/ethanol	24	0 0 5 0 0	0 0 5 0 0	5/5	4.0
			48	0 1 4[b] 0 0	2 3 0 0 0		2.4
3B		ethanol	24	5 0 0 0 0	5 0 0 0 0	0/5	0
			48	5 0 0 0 0	5 0 0 0 0		0

[a]Abbreviations: SWNH, single-walled carbon nanohorn; E-FCA, emulsified Freund's complete adjuvant; DNCB, 2,4-dinitrochlorobenzene; sensitization rate = number of animals showing positive reaction/total number of animals tested; mean response = sum of erythema and edema scores/total number of animals tested. [b]Three of four animals showed dander.

TABLE 3. Body Weight Gains in Peroral Administration Test[a]

dose group (n = 5)	day 0	day 1	day 3	day 7	day 14
			days after administration		
negative control (untreated)	204 ± 5.8	234 ± 5.9 (30 ± 1.6)	252 ± 6.4 (18 ± 2.6)	287 ± 3.4 (35 ± 4.2)	320 ± 8.2 (33 ± 9.9)
vehicle control (DMSO/water)	203 ± 5.6	230 ± 7.8 (26 ± 2.7)[b]	245 ± 10.9 (16 ± 5.5)	279 ± 8.8 (33 ± 5.8)	313 ± 11.4 (34 ± 4.7)
SWNH	205 ± 5.1	231 ± 5.3 (27 ± 1.2)[b]	246 ± 4.5 (15 ± 3.7)	278 ± 5.9 (32 ± 6.1)	307 ± 11.5 (29 ± 6.6)

[a]Abbreviation: DMSO, dimethyl sulfoxide; upper line, mean body weight $\pm$ standard deviation; lower line, mean body weight gain from previous measurement $\pm$ standard deviation. [b]Significantly different from negative control group ($P < 0.05$).

on day 1 was not induced by the SWNHs but by the solvent, DMSO/water. Although a white substance in the bladder (one of the five animals in the negative control, two of the five in the vehicle control, and one of the five in the SWNH administered groups) was discovered upon autopsy, this is not uncommon for male rats, indicating that it did not stem from the SWNHs. These results indicate that the lowest lethal dose of as-grown SWNHs administered orally to rats is more than 2000 mg/kg of body weight.

Mori et al.[63] reported that rats administered a single oral dose of fullerenes (a mixture of C_{60} and C_{70}) at 2000 mg/kg of body weight survived the 2 week test period and showed similar body weight gain profiles as the vehicle controls. Chen et al.[68] reported that water-soluble polyalkylsulfonated C_{60} can be considered nontoxic when administered orally; no rats died after oral administration of this compound at 2500 mg/kg of body weight. The median lethal dose (LD_{50}) of carbon black was found to be 8000 mg/kg of body weight for rats,[69] though no reports on the peroral administration of CNTs have been published. These results suggest that nanometer size itself does not induce oral toxicity for pure carbons; nevertheless, further detailed studies on gastrointestinal absorption and excretion kinetics are needed to obtain a conclusive answer.

Intratracheal installation test. Pulmonary toxicity upon inhalation of fluffy SWNHs was evaluated using intratracheal instillation of as-grown SWNHs in surfactant Tween 80/saline. Although the intratracheal instillation route is a less realistic pathway for airborne materials than inhalation, it has several advantages in terms of testing:[70] (1) intratracheal instillation is better suited for administering a scheduled dosage to the lungs; (2) experimental setup is easy, simple, and cost-effective; (3)

exposure risk to the tester is minimal; (4) materials that are respirable by people but not by many laboratory animals can be tested; and (5) plenty of test data are available, which facilitate assessment of the toxicity level. While the validity of intratracheal instillation as an alternative exposure pathway has been debated,[20,23,71] and although there are distinct differences between these two exposure routes, the intratracheal instillation test is still useful as long as the limitations are fully understood.

The animal groupings for the intratracheal instillation test are shown in Table 4. All animals (rats) survived the test period (7 or 90 days). Rale was observed for all animals including those in the vehicle control group immediately after instillation (day 0), and most animals had recovered by the next day (day 1). A few animals in each group showed rale continuously or sporadically over the following days. It was generally observed when foreign material was aspirated into the lungs, indicating that it was a nonspecific response induced by the instillation of suspensions into the lungs. Seven days after the instillation (day 7), symptoms were observed in only two animals in the finely ground quartz particles (Qz) dose group: one (no. 32) showed rale on day 24, and the other (no. 33) showed it on day 36. This lack of continuity indicates that the rale found occasionally after day 7 was accidental. Besides the rale, bradypnea and deep respiration were observed for one animal in the single-walled CNT (SWCNT) dose group (no. 19) and one in the Qz group (no. 28) on day 0, and another animal in the SWCNT group (no. 27) showed deep respiration on day 1. Although the dosing of the SWCNT or Qz may have induced these symptoms, the number of animals showing these clinical

**TABLE 4. Groupings for Intratracheal Installation Test[a]**

sample	sample dose (mg/animal)	dose liquid volume (mL/animal)	7 day group (autopsied on day 7)		90 day group (autopsied on day 90)	
			no. of animals	animal no.	no. of animals	animal no.
vehicle control (Tween 80/saline)	0	0.3	4	1–4	5	5–9
SWNH	2.25	0.3	4	10–13	5	14–18
SWCNT	2.25	0.3	4	19–22	5	23–27
Qz	2.25	0.3	4	28–31	5	32–36

[a]Abbreviations: SWCNT, single-walled carbon nanotubes; Qz, finely ground quartz particle.

signs is too small to judge toxicity strength of SWCNT and Qz.

The macroscopic findings of the pathological autopsies are as follows. For animals autopsied on day 7 (7 day group), no abnormalities were found except for one of the four animals (no. 21) in the SWCNT group (black spots were observed on lungs). For animals autopsied on day 90 (90 day group), such spots were found for three of the five animals in the SWNH group (no. 16–18) and one of the five in the SWCNT group (no. 27), and black spots on the liver were observed for one of the five in the vehicle control group (no. 9). All animals in the 90 day Qz group showed swelling of the thoracic lymph node and white spots on the lungs.

As shown in Figure 5, there was no statistically significant difference in body weight gain between the vehicle control group and the SWNH or SWCNT group. There was a tendency toward less body weight gain in the Qz group compared with the vehicle control group after day 28. It is attributed to the deterioration in
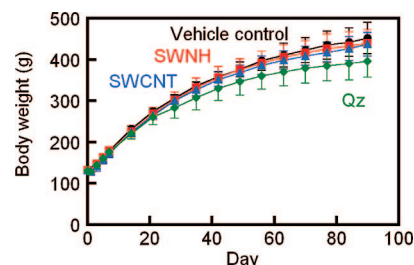
Figure 5. Time profiles of body weight change of rats after single-dose intratracheal instillation: (black) vehicle control group; (red) SWNH group; (blue) SWCNT group; (green) Qz group ($n = 5$ for each group). Weights are mean ± standard deviation.

health due to silicosis caused by the lengthy presence of Qz in the intra-alveolar spaces.

For animals in the 7 day group, higher actual and relative weights of the lungs compared with the control group were observed for one of the four animals in the SWCNT group (no. 21) and two of the four in the Qz group (no. 28 and 29). For animals in the 90 day Qz group, there was a tendency toward higher actual lung

**TABLE 5. Histopathological Findings for Intratracheal Instillation Test**

| organ	finding	degree	Tween 80[a]/saline day 7 autopsy, 4 animals	Tween 80[a]/saline day 90 autopsy, 5 animals	SWNH day 7 autopsy, 4 animals	SWNH day 90 autopsy, 5 animals	SWCNT day 7 autopsy, 4 animals	SWCNT day 90 autopsy, 5 animals	Qz day 7 autopsy, 4 animals	Qz day 90 autopsy, 5 animals
lungs	foreign body granuloma	+	0	0	0	0	0	1	0	0
		++	0	0	0	0	1	0	0	0
	inflammatory cell infiltration	+	0	0	0	0	1	0	0	0
	foamy macrophage in intra-alveolar spaces	±	0	0	1	1	0	0	0	0
		+	0	0	0	0	1	0	0	1
		++	0	0	0	0	0	0	2	4
	granuloma	++	0	0	0	0	0	0	2	0
	sarcoido-like granulomatous inflammation	++	0	0	0	0	0	0	0	2
		+++	0	0	0	0	0	0	0	3
	anthracosis	+	0	0	0	3	0	0	0	0
	fibrin deposition in intra-alveolar spaces	+	0	0	0	0	0	0	0	2
		++	0	0	0	0	0	0	0	2
		+++	0	0	0	0	0	0	0	1
	fibrosis	+	0	0	0	0	0	0	0	3
heart	focal infiltration of mononuclear cells	±	0	3	0	0	0	2	0	0
kidneys	basophilic tubules	±	0	1	0	0	0	0	0	0
liver	microgranuloma	±	0	1	0	0	0	0	0	0
spleen	abnormality		0	0	0	0	0	0	0	0
brain	abnormality		NA	0	NA	0	NA	0	NA	0
thoracic lymph node	sarcoido-like granulomatous inflammation	+	0	0	0	0	0	0	0	2
		++	0	0	0	0	0	0	0	2
		+++	0	0	0	0	0	0	0	1

[a]Vehicle control. NA: not applicable.

weight (Qz, 3.80 ± 0.67 g; vehicle control, 1.72 ± 0.08 g), and a significant increase ($P < 0.05$) in the relative lung weight (Qz, 0.97 ± 0.19 g/100 g of body weight; vehicle control, 0.38 ± 0.02 g/100 g of body weight) was found as compared with the control group. These weight increases were observed for the same animals showing histopathological lesions described below, meaning that the weight increases apparently reflect pulmonary abnormalities. In comparison with the vehicle control group, moreover, significant decreases in the actual liver weight for the 90 day Qz group (Qz, 12.57 ± 1.43 g; vehicle control, 15.62 ± 2.29 g) and the relative weight for the 90 day SWCNT group (SWCNT, 3.05 ± 0.21 g/100 g of body weight; vehicle control, 3.44 ± 0.28 g/100 g of body weight) were observed. However, we consider these differences to be toxicologically insignificant because they were found in either the actual or relative weight, and we observed no histological changes in the liver. No abnormal organ weight gain was observed for other animals.

The histopathological findings for animals autopsied 7 or 90 days after intratracheal instillation are summarized in Table 5. The vehicle control group showed no pathosis in the lungs autopsied either on day 7 or day 90 (Figure 6a and Figure 7a). For the SWNH groups, one of four animals in the 7 day group (no. 10, Figure 6b) and one of five animals and in the 90 day group (no. 14) showed formation of foamy macrophage in the intra-alveolar spaces, and anthracosis was observed for three of the five animals in the 90 day group (no. 16–18, Figure 7b). Because the anthracosis was not accompanied by any inflammatory responses, the number of animals showing the foamy macrophage was small, and the degree of the lesions was low, it is likely that as-grown SWNHs rarely damage lung tissues under the conditions of this test. For the SWCNT groups, foreign body granuloma together with inflammatory cell infiltration and foamy macrophage in the intra-alveolar spaces were observed for one of the four animals in the 7 day group (no. 21, Figure 6c), and one of the five animals in the 90 day group showed formation of foreign body granuloma in which many black particles were completely surrounded by the granuloma (no. 27, Figure 7c). Although there was not much difference in the degree of the lesions of the foreign body granuloma observed for both periods, it is difficult to predict the long-term outcome of the lesions at the moment. For the Qz groups, various pathoses were observed in the lungs: two of the four animals in the 7 day group showed foamy macrophage in the intra-alveolar spaces and granuloma formation (no. 28 and 29, Figure 6d), and in the 90 day group, sarcoido-like granulomatous inflammation, foamy macrophage, and fibrin deposition in the intra-alveolar spaces were observed for all animals, and three of them also showed fibrosis (no. 33, 34, and 36, Figure 7d). These findings suggest that the administered Qz stayed in the intra-alveolar spaces for a
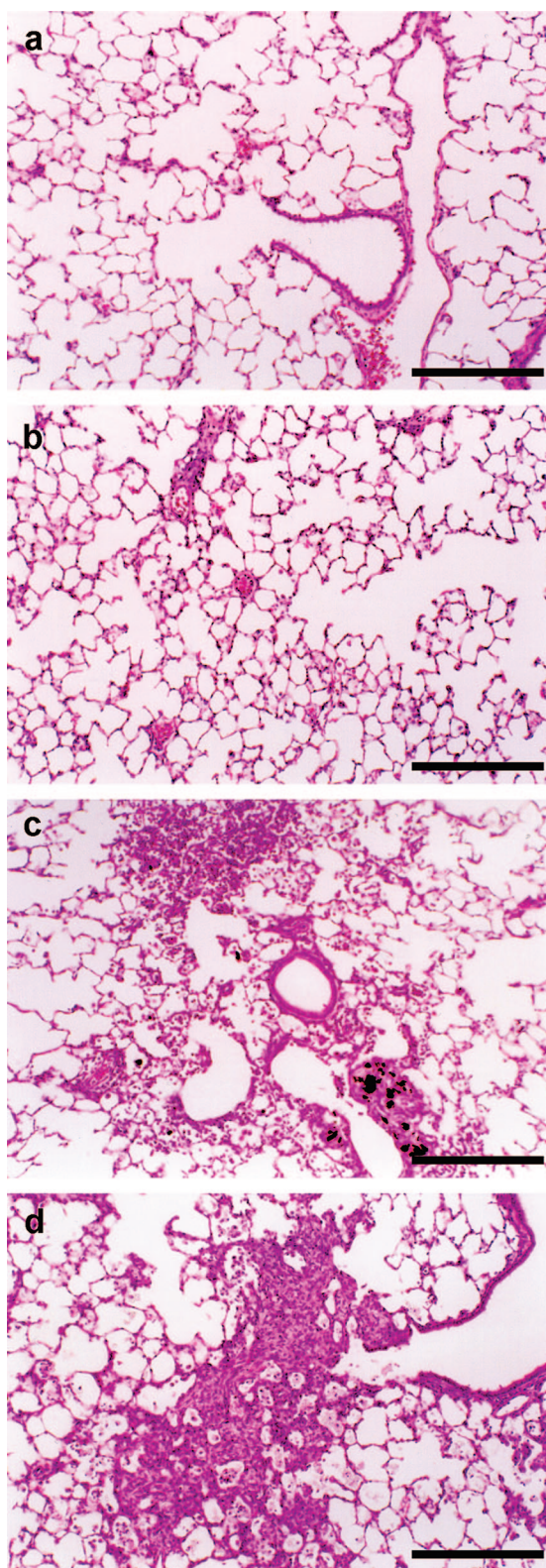
Figure 6. Optical microscope images of lung tissues of rats autopsied 7 days after intratracheal instillation: (a) animal no. 1 in vehicle control group showing no pathosis; (b) animal no. 10 in SWNH group showing foamy macrophage; (c) animal no. 21 in SWCNT group showing foreign body granuloma, inflammatory cell infiltration, and foamy macrophage; (d) animal no. 28 in Qz group showing granuloma and foamy macrophage. The tissues were hematoxylin and eosin stained. Scale bars represent 200 μm.
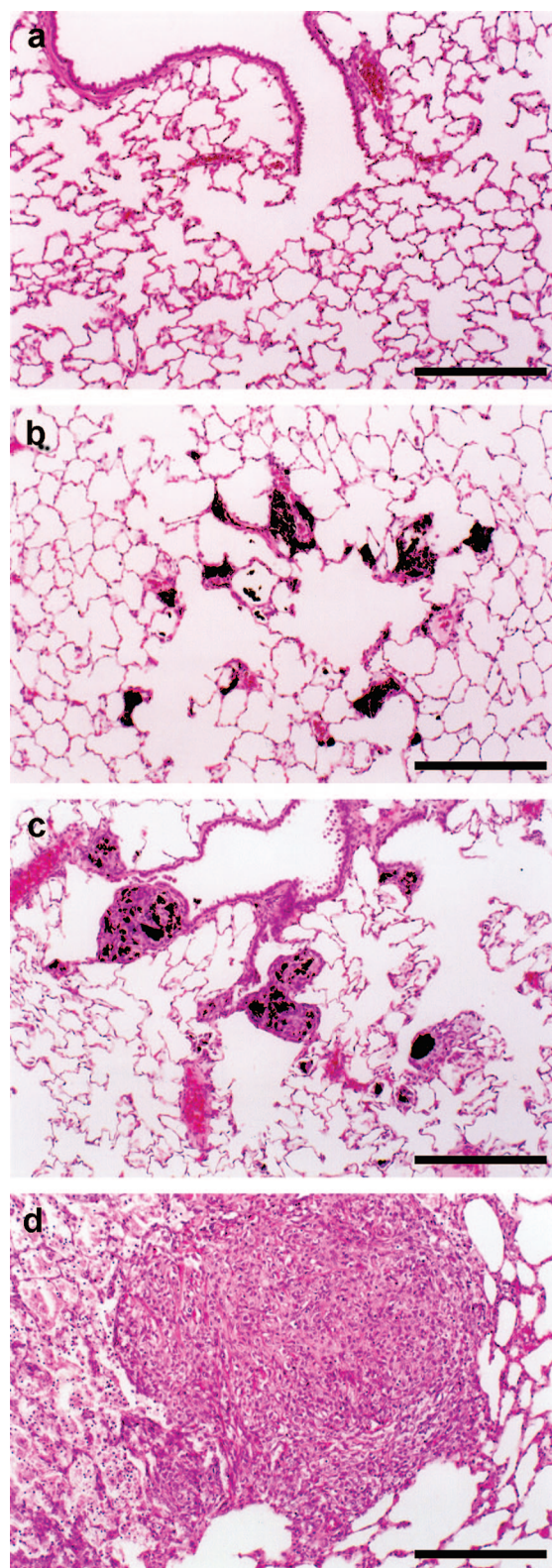
Figure 7. Optical microscope images of lung tissues of rats autopsied 90 days after intratracheal instillation: (a) animal no. 6 in vehicle control group showing no pathosis; (b) animal no. 16 in SWNH group showing anthracosis; (c) animal no. 27 in SWCNT group showing foreign body granuloma; (d) animal no. 33 in Qz group showing sarcoido-like granulomatous inflammation, foamy macrophage, fibrosis, and fibrin deposition. The tissues were hematoxylin and eosin stained. Scale bars represent 200 μm.

long time, which would induce formation of granuloma with high fibrogenic properties, resulting in irreversible fibrosis. In addition, sarcoido-like granulomatous inflammation was found in the thoracic lymph node for all animals in the 90 day Qz group. We considered that lung macrophages phagocytizing Qz were trapped in the lymph node, and an accumulation of Qz induced the sarcoido-like granulomatous inflammation. Focal cell infiltration of mononuclear cells in the heart was observed for three of the five animals in the 90 day vehicle control group (no. 5, 8, and 9) and for two of the five in the 90 day SWCNT group (no. 24 and 26), and basophilic tubules in the kidneys and microgranuloma in the liver were observed for one of the five animals in the 90 day vehicle control group (no. 5). Since these symptoms are spontaneous changes often observed in normal rats, they have no toxicological relevance to the test materials.

Although the pulmonary toxicity of CNTs has been studied comparatively well, the conclusions are inconsistent.[23,24] Huczko et al.[18] reported that a single intratracheal instillation of CNT soot containing Co and Ni catalysts (produced by the arc discharge method) at 100 mg/kg of body weight in guinea pigs did not affect the pulmonary functions and did not induce any measurable inflammation in the bronchoalveolar spaces at 4 weeks after instillation. Warheit et al.[19] investigated lung toxicity in rats upon a single intratracheal instillation at a maximum dose of 5 mg/kg of body weight of SWCNT soot containing 30–40% amorphous carbon and 5% each of Co and Ni (produced by the laser ablation process). The exposure of SWCNT soot resulted in transient inflammatory and cell injury effects as well as the formation of multifocal granulomas surrounding the SWCNT aggregates. The authors questioned the toxicological relevance because of the lack of a dose–response relationship and the nonuniform distribution of lesions. The formation of granulomas was suspected to be the consequence of instilling a bolus of agglomerated nanotubes. Lam et al.[20] assessed the pulmonary toxicity in mice for three kinds of SWCNTs containing different metal species and contents at two dose levels (3.3 or 16.7 mg/kg of body weight). They found that, even for purified HiPco SWCNTs containing only 2 wt % residual Fe, a single intratracheal instillation of SWCNTs induced a dose-dependent formation of epithelioid granulomas and, in some cases, interstitial inflammation. These lesions persisted and were more pronounced in a prolonged exposure period, 90 days; the authors concluded that these SWCNTs could be more toxic than quartz if they reach the lungs. Similar data were reported by Shvedova et al.:[21] a pharyngeal aspiration of purified HiPco SWCNTs (containing 0.23 wt % Fe) at a maximum dose of 2 mg/kg of body weight induced a robust acute inflammatory reaction with very early onset of a fibrogenic response and formation of granuloma in mice. Muller et al.[22] concluded that CNTs,

even in the absence of agglomerates, are potentially toxic to the lungs. They administered intact or ground multiwalled CNTs (MWCNTs) intratracheally to rats at a maximum dose of about 22 mg/kg of body weight. They found that when the MWCNTs reached the lungs, they were biopersistent, and the intact (long) MWCNTs were apparently retained longer than the ground (short) ones. The retained MWCNTs induced dose-dependent lung inflammation and fibrosis and caused the formation of collagen-rich granulomas.

In our tests, the quartz (Qz) induced the most serious toxic responses upon single-dose intratracheal instillation; the SWCNTs and SWNHs showed much weaker toxicities. However, Lam et al.[20] and Shvedova et al.[21] found that SWCNTs are more toxic than Qz even though they used the same Qz sample (Min-U-Sil-5) that we did. We attribute this contradiction to differences in the animal species used and/or in the SWCNT samples. Their observed lesions upon Qz exposure for mice were much weaker than ours for rats, suggesting that different species have significantly different susceptibilities to Qz. Indeed, Warheit et al.[19] reported that, for rats, exposure to the same Qz induced more intense and progressive toxic responses than those to SWCNTs. Under our test conditions, the SWCNTs induced worse tissue damage than the SWNHs. However, a conclusive determination of their relative toxicities requires further dose-dependence studies with a larger number of test animals because the number of animals showing lesions in the present study was at an incidental level. At the same time, assessment of the biomarkers of inflammatory, fibrogenic, and oxidative-stress responses in bronchoalveolar lavage fluid after exposure to SWNHs should give useful information about the mechanisms of toxic pulmonary responses. Furthermore, quantitative study of biopersistence (clearance kinetics) is also a key issue because it often affects toxicity.[72,73]

Summary. We performed extensive in vivo and in vitro toxicological assessments of as-grown SWNHs for various exposure pathways, showing that as-grown SWNHs have low toxicities. Because SWNHs contain no metal catalyst, we can exclude the toxicological effects of metal impurities and consider only those of the nanometer-sized graphitic structure. That is, the present results show that spherical graphite structures with submicrometer diameter, graphitic nanotubules, and pentagons/heptagons in a hexagonal network have negligible impact on a living body. We believe that these findings should help deepen our understanding of the toxicological factors of carbonaceous nanomaterials and thus facilitate the fabrication of carbonaceous nanomaterials designed for industrial and biomedical applications with low toxicity.

MATERIALS AND METHODS

SWNH Preparation. The SWNHs were prepared by CO_2 laser ablation of a pure graphite target containing no metal catalyst in an Ar atmosphere (760 Torr) at room temperature.[25]

Reverse Mutation (Ames) Test. The mutagenic activity of the as-grown SWNHs was examined by means of the well-known Ames test.[74] Histidine-auxotrophic bacterial strains will starve to death if grown in a histidine-free medium. In the presence of a mutagenic chemical, however, the defective histidine gene may mutate back, permitting the bacterium to grow in the histidine-free medium. Although many chemicals are not mutagenic in their native form, they can be converted into mutagenic substances by metabolism in the liver. Because bacteria lack the same metabolic capabilities as mammals, activation enzymes are used to promote the metabolic activation of chemicals.

In our test, four Salmonella typhimurium (S. typhimurium) strains (TA100, TA1535, TA98, and TA1537) and an E. coli strain (WP2 uvrA) were used. All bacteria were obtained from Japan Bioassay Research Center. The type of mutation for TA100, TA 1535, and WP2 uvrA was base-pair substitution and that for TA98 and TA1537 was frameshift. The activation enzyme was S9 mix (S9 supplemented with essential cofactors), where S9, prepared from Sprague–Dawley male rat liver, was purchased from Oriental Yeast Co., Ltd. A 5% w/v as-grown SWNHs suspension in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd., 99.9%) was diluted with 0.1 M sodium phosphate buffer (pH 7.4) (or cofactors for the metabolic activation test) to the desired concentration (78, 156, 313, 625, or 1250 μg/plate). A 0.4 mL portion of this SWNH suspension mixed with 0.2 mL of the buffer (or 0.2 mL of S9 mix for the metabolic activation test) and 0.1 mL of each bacterium was preincubated with shaking at 37 °C for 20 min and then mixed with 2 mL of molten agar (top agar) and poured onto the surface of a minimal-agar plate containing glucose (bottom agar). The top agar contained 1:10 (v/v) of 0.5 mM histidine−0.5 mM biotin solution for the S. typhimurium or 0.5 mM tryptophan solution for E. coli strains. The plates were incubated at 37 °C for 48 h, and then the number of bacterial colonies was counted using a stereoscopic microscope. As positive controls, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2, 99.0%), sodium azide (NaN_3, 99.4%), and 6-chloro-9-[3-(2-chloroethylamino)-propylamino]-propylamino]-2-methoxacridine dihydrochloride (ICR-191) were used in experiments without S9 mix, and 2-aminoanthracene (2AA, 93.3%) was used with S9 mix. The chemicals were purchased from Wako Pure Chemical Industries, Ltd. (AF-2, NaN_3, and 2AA) and Polyscience, Inc. (ICR-191). Two plates were incubated for each concentration, except for the solvent control (DMSO) where three plates were prepared. We adopted assay evolution criteria for a positive response as follows: mean number of revertants should be greater than twice the corresponding solvent control values and a reproducible dose-dependent increase in mutant colonies should be evident.

Chromosomal Aberration Test.[75–77] The clastogenic potentials of the as-grown SWNHs were examined using mammalian cultured cells. A clonal subline of a Chinese hamster lung fibroblast cell line (CHL/IU) was obtained from the National Institute of Health Sciences, Japan. The model chromosome number of this cell line is 25, and the doubling time is about 15.8 h. The cells were maintained in Eagle's minimum essential medium (GIBCO, Invitrogen Co.) supplemented with 10% heat-inactivated calf serum (HyClone) at 37 °C in a 5% CO_2 humidified incubator and subcultured every 3–5 days. The passage numbers of cells were 10 (growth inhibition tests), 14 (short-term chromosomal aberration tests), and 20 (continuous chromosomal aberration tests). The activation enzyme, S9 mix, was prepared immediately before use by mixing 3 mL of S9 and 7 mL of the cofactors (40 μmol of HEPES buffer, 50 μmol of $MgCl_2$, 330 μmol of KCl, 50 μmol of G-g-P, and 40 μmol of NADP). The as-grown SWNHs were suspended in a 1% carboxymethyl cellulose sodium salt (CMC-Na, Wako Pure Chemical Industries, Ltd.) aqueous solution.

Growth inhibition tests were carried out prior to the chromosome aberration tests to estimate the 50% growth inhibition dose. The cells were seeded in 60 mm dishes (2×10^4 cells/5 mL of medium/dish) and incubated for 3 days. For the tests with metabolic activation, 0.83 mL of the medium was removed and then 0.83 mL of S9 mix was added (final S9 concentration was 5 vol %). A 0.5 mL portion of the SWNH suspension was added at different dose levels (0.005, 0.010, 0.020, 0.039, 0.078, 0.156, 0.313, 0.625, 1.250, or 2.500 mg/mL). We prepared one dish for each dose level. For the short-term exposure experiments, the cells with the SWNHs were incubated for 6 h, rinsed with saline, supplemented with 5 mL of fresh medium, and then incubated for another 18 h. For the continuous exposure experiments, the cells were continuously incubated for 24 or 48 h after the addition of the SWNH suspension. After the incubation, the cells were washed with saline, fixed with 10% formalin solution, and then stained with 0.1% crystal violet solution. The cell productivity was estimated from the color absorption value of the stained cells using a densitometer (Monocellater, Olympus Co.). That of the vehicle control (1% CMC-Na aqueous solution) group was set to 100%.

The growth inhibition tests revealed that the 50% growth inhibition concentrations of the SWNHs were more than 2.500 mg/mL for both the short-term and continuous exposure experiments. We observed precipitation and coloring of the SWNHs in the medium at concentrations higher than 0.039 mg/mL. Although no cytotoxicity of the SWNHs was observed in the short term and 24 h continuous exposure experiments, the cell productivity was 64% at the 2.500 mg/mL dose level in the 48 h continuous exposure experiments, indicating weak SWNH cytotoxicity. Therefore, we set the dose levels of the SWNHs in the chromosomal aberration tests to 0.010, 0.020, 0.039, and 0.078 mg/mL (the latter two dose levels showed the precipitations) for the short-term exposure experiments with and without S9 mix metabolic activation (denoted S9 mix(+) and S9 mix(−), respectively) and the 24 h continuous exposure experiments; they were set to 0.313, 0.625, 1.250, and 2.500 mg/mL for the 48 h continuous exposure experiments.

In the chromosomal aberration tests, four 60 mm dishes were prepared for each dosage. The cells were seeded in the dishes (2×10^4 cells/5 mL of medium/dish) and incubated for 3 days. The mitomycin C (MMC, 0.05 μg/mL) treated group was used as the positive control in the S9 mix(−), 24 h and 48 h continuous exposure experiments; the N-nitrodimethylamine (DMN, 0.4 mg/mL) treated group was used as the positive control in the S9 mix(+) experiments. The MMC and DMN were purchased from Kyowa Hakko Kogyo Co., Ltd., and Wako Pure Chemical Industries, Ltd., respectively. Untreated and vehicle groups were prepared as the negative controls. After treatment with the test materials, the cell productivity was estimated for two of the four dishes in the same manner as for the growth inhibition tests. The chromosome preparations were made for the other two dishes as follows. Cells were treated with 0.2 μg/mL of colcemid (GIBCO, Invitrogen Co.) to accumulate metaphase cells 2 h before the end of the incubation, supplemented with 2 mL of 0.25% trypsin solution, and centrifuged (1000 rpm, 5 min). After removal of the supernatant, 5 mL of 0.075 M KCl hypotonic solution was added, and then the cells were incubated at 37 °C for 15 min. The cells were then fixed with ice-cold fixative (methanol−acetic acid mixture, 3:1 v/v), which was changed three times. The cell suspension was dropped on a slide glass, dried in air, and then stained with 1.7% Giemsa's solution for 15 min.

The number of cells with chromosomal aberrations was counted on 200 (100 cells/dish) well-spread metaphases using a microscope. The types of aberrations were classified into six groups: chromatid breaks (ctb), chromatid exchanges such as four radial exchanges (cte), chromosome breaks (csb), chromosome exchanges such as dicentric and ring formations (cse), gaps (g), and others such as acentric fragmentation (frg). In our criteria, distinct unstained parts wider than the chromatid on the vertical axis of the chromatid or fragments slipped from the chromosome/chromatid axis were classified as breaks. Gaps were defined as distinct unstained parts narrower than the chromatid on the vertical axis of the chromatid. Exchanges were defined

as mutual exchanges of more than two breaks in a chromosome or chromatid. The incidence of polyploid cells was also counted; cells that had more than 37 chromosomes including triploidy ones were recorded as polyploidy. For objectivity, all observations were done by blind trials.

The clastogenic potential was judged in accordance with Sofuni's criteria:[77] negative (−) if the incidence of cells showing any aberrations except for gaps was less than 5%; suspicious (±) if 5−10%; and positive (+) if more than 10%. When dose-independent suspicious/positive results were obtained, additional experiments were carried out to confirm their reproducibility.

Irritation Tests (Draize Test).[78] Specific-pathogen-free (SPF) female rabbits (9 weeks old upon arrival) of the Japan White variety (Kbl:JW) obtained from KITAYAMA LABES Co., Ltd. were used for skin primary and eye irritation tests of as-grown SWNHs. The animals were housed individually in stainless steel suspended cages and allowed free access to standard pellets (RC-4, Oriental Yeast Co., Ltd.) and water under controlled laboratory conditions (20−26 °C, 40−70% relative humidity, 12 h/12 h light/dark cycle). The period of acclimation to this environment was 1 week. The animals were treated in accordance with the Institutional Animal Care and Use Guidelines. Because as-grown SWNHs have low bulk density, the SWNHs were compressed by centrifugation at 3500 rpm for 20 min.

Skin Primary Irritation Test. The back of each animal ($n = 6$, 10 weeks old, 2.06 ± 0.09 kg body weight) was clipped free of fur with electric clippers 1 day before testing. A 0.015 g portion of the compressed as-grown SWNHs was spread on a cotton lint (2.5×2.5 cm), and then lint was applied to two sites (one site intact, and the other abraded with a sterilized syringe needle). Although the dose (0.015 g) used did not correspond to the specified dosage (0.5 g or 0.5 mL), this was the maximum quantity not to overflow when patched and the sample fully contacted the skin. Therefore, we considered this dose to be adequate for examining the skin irritation effects of SWNHs. The patches were backed with a moisture-proof rubber sheet, secured with a bandage, and covered with a protective cloth. The patches were removed after 24 h, and the test sites were gently sponged to remove any sample residue. The test sites were examined for dermal reactions in accordance with the Draize criteria[78] at 1, 24, 48, and 72 h after patch removal. The P.I.I., the sum of the scored reactions (both for erythema/eschar and edema formations) for all six animals at 1 and 48 h divided by 24, was calculated following test completion. Body weight was measured on the day before administration and at test termination for all animals.

Eye Irritation Test. Within 24 h before the Draize eye irritation tests, the anterior eye parts were confirmed to have no abnormalities for all animals (10 weeks old, 1.95 ± 0.15 kg body weight). A 0.02 g portion of the compressed as-grown SWNHs was administered into the left conjunctival sac; the right eye was used as an untreated control (no administration). We used a 0.02-g dose because it was the maximum quantity not to overflow when administered into the conjunctival sac although it does not meet the specifications (0.1 g) for the Draize method.[78] For the eye-washing group ($n = 3$), the eyes were forcibly closed for about 1 s after the administration and then, 30 s after, gently washed with warm normal saline (Otsuka Pharmaceutical Factory, Inc.) for about 30 s. For the non-eye-washing group ($n = 3$), the eyes were forcibly closed for about 1 s after the administration and there was no subsequent eye washing. Both eyes for all six animals were examined and scored for irritation responses in accordance with the Draize criteria[78] before and 1, 24, 48, and 72 h after the administration. On the basis of the scores, the eye irritation score was determined in accordance with Kay's criteria.[78] After the observation at 24 h postadministration, both eyes of all animals were examined for corneal injury using a slit lamp (SL-14, Kowa Co.) equipped with a blue filter after corneal staining with fluorescein sodium dye (Showa Yakuhin Kako Co.). Body weight was measured on the day before administration and at test termination for all animals.

Skin Sensitization (Adjuvant and Patch) Test.[80−83] Male guinea pigs (Crj:Hartley, SPF, 5 weeks old upon arrival) from Charles River Laboratories Japan, Inc., were used. The animals were housed in

stainless steel suspended cages (two animals/cage during 1-week acclimation, one or two animals/cage during induction, and one animal/cage from the day before challenge) under controlled laboratory conditions (20–26 °C, 40–70% relative humidity, 12 h/12 h light/dark cycle). They had free access to standard pellets (RC-4, Oriental Yeast Co., Ltd.) and water. On the day before the first stage of induction, the animals were 6 weeks old and weighed 356–391 g. They were treated in accordance with the Institutional Animal Care and Use Guidelines.

The chemicals were purchased from Difco Laboratories (Freund's complete adjuvant: FCA), Otsuka Pharmaceutical Factory, Inc. (injection solvent), Wako Pure Chemical Industries, Ltd. (2,4-dinitrochlorobenzene: DNCB, ethanol, and sodium lauryl sulfate: SLS), and Nikko Rica Co. (petrolatum, Sun White P-1). The SWNHs were compressed by centrifugation at 3500 rpm for 20 min.

A day before the first stage of induction (day −1), the animals were separated into three groups and ($n = 5$ for each): (1) treated with the compressed as-grown SWNHs (SWNH induction group), (2) not treated (noninduction group), and (3) treated with DNCB (DNCB induction group). Their nuchal area was clipped with electric clippers and shaved with an electric razor. The first stage of the induction was done as follows. (1) On day 0, the first day of the first stage of induction, 0.1 mL of emulsified FCA (E-FCA), prepared from equal volumes of FCA and injection solvent, was injected intradermally at the four corners of the clipped and shaved 2 × 4 cm induction area. (2) Abrasions forming a criss-cross lattice were made using a syringe needle in this area. (3) A 0.02 g portion of SWNHs or 0.1 mL of 0.1% w/v DNCB/ethanol solution was spread on a cotton lint (2 × 4 cm), which was then applied occlusively to an injection area. Each pad was covered with a moisture-proof rubber sheet and secured with a bandage. (4) After 24 h, these patches were removed, and the test sites were gently sponged to remove any remaining sample residue. (5) Steps 2–4 were repeated once a day on the following two days (days 1 and 2). Although the dose (0.02 g) was less than the prescribed dosage (0.1 g (mL)/site or 0.2 g (mL)/site for the first or second induction phase), this was the maximum quantity not to overflow when patched and the sample fully contacted the skin. On day 6, the day before the second stage of induction, the first induction area and the surroundings were clipped and shaved. In the second stage of induction, day 7, 0.1 mL of 10% SLS in petrolatum was applied to this area. Twenty-four hours later, the SLS was sponged, and then 0.02 g of SWNHs or 0.2 mL of 0.1% w/v DNCB/ethanol solution was again similarly patched for 48 h. The animals in the noninduction group received only the E-FCA injection.

On day 22, the animals, whose hair on the back had been clipped and shaved on day 21, were exposed to a challenge dose. A 0.01 g portion of SWNHs, or 0.01 mL of 0.1% w/v DNCB/ethanol solution or ethanol, was applied occlusively to a challenge area (2 × 2 cm) and patched in the similar way as the inductions. Two challenge areas were defined for each animal in the noninduction and DNCB induction groups, and different materials were applied to each area. After 24 h, the patches were removed, and the test sites were gently sponged to remove any remaining sample residue. The dermal reactions were evaluated and scored in accordance with the Draize criteria[78] at 24 and 48 h after patch removal (days 24 and 25, respectively). Body weight was measured on days −1 and 25 for all animals.

Peroral Administration Test. The single-dose peroral toxicity of the SWNHs was examined for a 2 week test period using SPF male rats (Crj:Wistar, 6 weeks old upon arrival) purchased from Charles River Laboratories Japan, Inc. The animals were housed in aluminum cages (two or three animals/cage) during 7 day acclimation under controlled laboratory conditions (22–26 °C, 40–70% relative humidity, 12 h/12 h light/dark cycle). They had free access to standard pellets (CRF-1, Oriental Yeast Co., Ltd.) and water. At the time of administration, the animals were 7 weeks old and weighed 197–213 g. They were treated in accordance with the Institutional Animal Care and Use Guidelines.

A 2.5 g portion of SWNHs compressed by repeated centrifugation at 3000 rpm for 10 min was first wetted with 7.5 mL of DMSO (Wako Pure Chemical Industries, Ltd., 99.9%) and then suspended in 17.5 mL of distilled water (Wako Pure Chemical Industries, Ltd.). The SWNH suspension was administered at a dose of 20 mL/kg of body weight (2000 mg of SWNH/kg of body weight) into the stomach using a plastic syringe barrel attached to a gastric sonde. This administration level was the maximum dose for toxicity studies of drugs given in the applicable test guideline.[84] We also prepared two control groups: a negative control group without any treatment and a vehicle control group to which only a DMSO/water (3:7 v/v) solution was administered orally at 20 mL/kg of body weight. Each group contained five animals. Their body weight was measured immediately before the administration (day 0) and on days 1, 3, 7, and 14. After the evaluation of distribution uniformity by Bartlett's test,[85] the difference in body weight between the animals in the administered groups and the negative control group was assayed by Dunnett's test[86] (significance levels: ±0.05, ±0.01). After the 2 week test period, each animal was euthanized, and all organs and tissues were observed with the naked eye.

Intratracheal Instillation Test. SPF male rats (4 weeks old upon arrival) of the Wistar Hannover GALAS variety (BrlHan: WIST@Jcl(GALAS)) were obtained from CLEA Japan, Inc. The animals were housed in stainless steel suspended cages (two or three animals/cage) during 8 day acclimation under controlled laboratory conditions (20–26 °C, 40–70% relative humidity, 12 h/12 h light/dark cycle). They had free access to standard pellets (FR-2, Funabashi Farms Co., Ltd.) and water. At the time of instillation, the animals were 5 weeks old and weighed 124–140 g. They were treated in accordance with the Institutional Animal Care and Use Guidelines.

As-grown SWNHs, single-walled carbon nanotubes (SWCNTs, HiPco, Carbon Nanotechnologies Inc.) and finely ground quartz particles (Qz, Min-U-Sil 5, U.S. Silica Company) were used. The nominal size of the SWCNTs was 1.0 ± 0.2 nm in diameter and several hundred nanometers to several micrometers long. However, the SWCNTs were rarely individual units; instead, they were mostly aggregated into bundles. The purity of the SWCNTs was >90%; metal catalyst (Fe) residue accounted for about 5–10 wt %. According to the distributor's product data, 97% or more of the Qz was finer than 5 μm. All samples were used without further purification.

A suspension of test materials was prepared by mixing 0.15 g of the test materials with a small amount of 0.025% w/v Tween 80 (Wako Pure Chemical Industries, Ltd.)/saline (Otsuka Pharmaceutical Factory, Inc.) solution using a mortar and pestle and then adding the 0.025% w/v Tween 80/saline solution to bring the total volume up to 20 mL (final concentration: 7.5 mg/mL). Surfactant Tween 80 was used to obtain a good dispersion of the test materials in the solution, enabling homogeneous instillation into all pulmonary lobes.[87] A 0.3 mL/animal (≈ 2.3 mL/kg of body weight) portion of the suspension was intratracheally instilled into unanesthetized but immobilized rats using a 1 mL disposable syringe barrel with a common metal oral sonde. The dosage was calculated to be 2.25 mg/animal (≈17.3 mg/kg of body weight), which is comparable to a dose of 0.5 mg/animal (≈17 mg/kg for mice of about 30 g body weight) used in a study of pulmonary toxicity after a single intratracheal instillation of SWCNT suspension.[20] Rats into which only Tween 80/saline was instilled served as a vehicle control. Seven or 90 days after instillation, each animal was euthanized by exsanguination under pentobarbital anesthesia. Their organs and tissues were autopsied, and the heart, lungs, kidneys, liver, and spleen were weighed. These organs, the brain (only for the 90 day group), and other organs/tissues showing macroscopic abnormalities were fixed in 10% buffered formalin, embedded in paraffin, thin sectioned, stained with hematoxylin-eosin, and observed using an optical microscope. Body weight was measured on the day of instillation (day 0), at 1, 3, 5, 7 days after instillation, and thereafter once a week. Distribution uniformity was evaluated by Bartlett's test[85] for body weight and for individual organ weight (actual weight and weight relative to body weight). When the dispersion was uniform (not uniform), Dunnett's test[86] (nonparametric Dunnett's test) was used to compare the weight to the corresponding control value. Significance was judged at the 0.05 and 0.01 probability levels.

REFERENCES AND NOTES

1. Service, R. F. Nanotubes: the Next Asbestos. *Science* **1998**, *281*, 941.
2. Service, R. F. Nanomaterials Show Signs of Toxicity. *Science* **2003**, *300*, 243.
3. Colvin, V. L. The Potential Environmental Impact of Engineered Nanomaterials. *Nat. Biotechnol.* **2003**, *21*, 1166–1170.
4. Brumfiel, G. A Little Knowledge. . .. *Nature* **2003**, *424*, 246.
5. Hoet, P. H. M.; Nemmar, A.; Nemery, B. Health Impact of Nanomaterials. *Nat. Biotechnol.* **2004**, *22*, 19.
6. Proffitt, F. Yellow Light for Nanotech. *Science* **2004**, *305*, 762.
7. Hoet, P. H. M.; Brüske-Hohlfeld, I.; Salata, O. V. Nanoparticles - Known and Unknown Health Risks. *J. Nanobiotechnol.* **2004**, *2*, 12.
8. Kipen, H. M.; Laskin, D. L. Smaller Is Not Always Better: Nanotechnology Yields Nanotoxicology. *Am. J. Physiol.: Lung Cell. Mol. Physiol.* **2005**, *289*, L696–L697.
9. Robichaud, C. O.; Tanzil, D.; Weilenmann, U.; Wiesner, M. R. Relative Risk Analysis of Several Manufactured Nanomaterials: An Insurance Industry Context. *Environ. Sci. Technol.* **2005**, *39*, 8985–8994.
10. Nel, A.; Xia, T.; Mädler, L.; Li, N. Toxic Potential of Materials at the Nanolevel. *Science* **2006**, *311*, 622–627.
11. Balbus, J. M.; Florini, K.; Denison, R. A.; Walsh, S. A. Getting It Right the First Time. *Ann. N.Y. Acad. Sci.* **2006**, *1076*, 331–342.
12. Panessa-Warren, B. J.; Warren, J. B.; Wong, S. S.; Misewich, J. A. Biological Cellular Response to Carbon Nanoparticle Toxicity. *J. Phys.: Condens. Matter* **2006**, *18*, S2185–S2201.
13. Hurt, R. H.; Monthioux, M.; Kane, A. Toxicology of Carbon Nanomaterials: Status, Trends, and Perspectives on the Special Issue. *Carbon* **2006**, *44*, 1028–1033.
14. Helland, A.; Wick, P.; Koehler, A.; Schmid, K.; Som, C. Reviewing the Environmental and Human Health Knowledge Base of Carbon Nanotubes. *Environ. Health Perspect.* **2007**, *115*, 1125–1131.
15. National Institute of Environmental Health Sciences (NIEHS) OPPE Factsheet #03 July 2003 Nanotechnology Safety Assessment. NIEHS: Washington, DC, 2003.
16. Nanoscience and Nanotechnologies: Opportunities and Uncertainties. The Royal Society and Royal Academy of Engineering: London, 2004 (http://www.nanotec.org.uk/finalReport.htm).
17. Iijima, S. Helical Microtubles of Graphitic Carbon. *Nature* **1991**, *354*, 56–58.
18. Huczko, A.; Lange, H.; Całko, E.; Grubek-Jaworska, H.; Droszcz, P. Physiological Testing of Carbon Nanotubes: Are They Asbestos-like. *Fullerene Sci. Technol.* **2001**, *9*, 251–254.
19. Warheit, D. B.; Laurence, B. R.; Reed, K. L.; Roach, D. H.; Reynolds, G. A. M.; Webb, T. R. Comparative Pulmonary Toxicity Assessment of Single-Wall Carbon Nanotubes in Rats. *Toxicol. Sci.* **2004**, *77*, 117–125.
20. Lam, C.-W.; James, J. T.; McCluskey, R.; Hunter, R. L. Pulmonary Toxicity of Single-Wall Carbon Nanotubes in Mice 7 and 90 Days after Intratracheal Instillation. *Toxicol. Sci.* **2004**, *77*, 126–134.
21. Shvedova, A. A.; Kisin, E. R.; Mercer, R.; Murray, A. R.; Johnson, V. J.; Potapovich, A. I.; Tyurina, Y. Y.; Gorelik, O.; Arepalli, S.; Schwegler-Berry, D.; *et al.* Unusual Inflammatory and Fibrogenic Pulmonary Responses to Single-Walled Carbon Nanotubes in Mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2005**, *289*, L698–L708.
22. Muller, J.; Huaux, F.; Moreau, N.; Misson, P.; Heilier, J.-F.; Delos, M.; Arras, M.; Fonseca, A.; Nagy, J. B.; Lison, D. Respiratory Toxicity of Multi-Wall Carbon Nanotubes. *Toxicol. Appl. Pharmacol.* **2005**, *207*, 221–231.
23. Muller, J.; Huaux, F.; Lison, D. Respiratory Toxicity of Carbon Nanotubes: How Worried Should We Be. *Carbon* **2006**, *44*, 1048–1056.
24. Donaldson, K.; Aitken, R.; Tran, L.; Stone, V.; Duffin, R.; Forrest, G.; Alexander, A. Carbon Nanotubes: A Review of Their Properties in Relation to Pulmonary Toxicology and Workplace Safety. *Toxicol. Sci.* **2006**, *92*, 5–22.
25. Iijima, S.; Yudasaka, M.; Yamada, R.; Bandow, S.; Suenaga, K.; Kokai, F.; Takahashi, K. Nano-aggregates of Single-Walled Graphitic Carbon Nano-horns. *Chem. Phys. Lett.* **1999**, *309*, 165–170.
26. Kasuya, D.; Yudasaka, M.; Takahashi, K.; Kokai, F.; Iijima, S. Selective Production of Single-Wall Carbon Nanohorn Aggregates and Their Formation Mechanism. *J. Phys. Chem. B* **2002**, *106*, 4947–4951.
27. Yoshitake, T.; Shimakawa, Y.; Kuroshima, S.; Kimura, H.; Ichihashi, T.; Kubo, Y.; Kasuya, D.; Takahashi, K.; Kokai, F.; Yudasaka, M.; *et al.* Preparation of Fine Platinum Catalyst Supported on Single-Wall Carbon Nanohorns for Fuel Cell Application. *Physica B* **2002**, *323*, 124–126.
28. Yuge, R.; Ichihashi, T.; Shimakawa, Y.; Kubo, Y.; Yudasaka, M.; Iijima, S. Preferential Deposition of Pt Nanoparticles Inside Single-Walled Carbon Nanohorns. *Adv. Mater.* **2004**, *16*, 1420–1423.
29. Murata, K.; Miyawaki, J.; Yudasaka, M.; Iijima, S.; Kaneko, K. High-Density of Methane Confined in Internal Nanospace of Single-Wall Carbon Nanohorns. *Carbon* **2005**, *43*, 2826–2830.
30. Murata, K.; Kaneko, K.; Kanoh, H.; Kasuya, D.; Takahashi, K.; Kokai, F.; Yudasaka, M.; Iijima, S. Adsorption Mechanism of Supercritical Hydrogen in Internal and Interstitial Nanospaces of Single-Wall Carbon Nanohorn Assembly. *J. Phys. Chem. B* **2002**, *106*, 11132–11138.
31. Bekyarova, E.; Murata, K.; Yudasaka, M.; Kasuya, D.; Iijima, S.; Tanaka, H.; Kanoh, H.; Kaneko, K. Single-Wall Nanostructured Carbon for Methane Storage. *J. Phys. Chem. B* **2003**, *107*, 4681–4684.
32. Murata, K.; Hashimoto, A.; Yudasaka, M.; Kasuya, D.; Kaneko, K.; Iijima, S. The Use of Charge Transfer to Enhance the Methane-Storage Capacity of Single-Walled, Nanostructured Carbon. *Adv. Mater.* **2004**, *16*, 1520–1522.
33. Yang, C.-M.; Kim, Y.-J.; Endo, M.; Kanoh, H.; Yudasaka, M.; Iijima, S.; Kaneko, K. Nanowindow-Regulated Specific Capacitance of Supercapacitor Electrodes of Single-Wall Carbon Nanohorns. *J. Am. Chem. Soc.* **2007**, *129*, 20–21.
34. Murakami, T.; Ajima, K.; Miyawaki, J.; Yudasaka, M.; Iijima, S.; Shiba, K. Drug-Loaded Carbon Nanohorns: Adsorption and Release of Dexamethasone in vitro. *Mol. Pharm.* **2004**, *1*, 399–405.
35. Ajima, K.; Yudasaka, M.; Murakami, T.; Maigné, A.; Shiba, K.; Iijima, S. Carbon Nanohorns as Anticancer Drug Carriers. *Mol. Pharm.* **2005**, *2*, 475–48.
36. Murakami, T.; Fan, J.; Yudasaka, M.; Iijima, S.; Shiba, K. Solubilization of Single-Wall Carbon Nanohorns Using a Peg-Doxorubicin Conjugate. *Mol. Pharm.* **2006**, *3*, 407–414.
37. Matsumura, S.; Ajima, K.; Yudasaka, M.; Iijima, S.; Shiba, K. Dispersion of Cisplatin-Loaded Carbon Nanohorns with a Conjugate Comprised of an Artificial Peptide Aptamer and Polyethylene Glycol. *Mol. Pharm.* **2007**, *4*, 723–729.
38. Kase, D.; Kulp III, J. L.; Yudasaka, M.; Evans, J. S.; Iijima, S.; Shiba, K. Affinity Selection of Peptide Phage Libraries against Single-Wall Carbon Nanohorns Identifies a Peptide Aptamer with Conformational Variability. *Langmuir* **2004**, *20*, 8939–8941.
39. Sano, K.-I.; Ajima, K.; Iwahori, K.; Yudasaka, M.; Iijima, S.; Yamashita, I.; Shiba, K. Endowing a Ferritin-Like Cage Protein with High Affinity and Selectivity for Certain Inorganic Materials. *Small* **2005**, *1*, 826–832.

40. Miyawaki, J.; Yudasaka, M.; Imai, H.; Yorimitsu, H.; Isobe, H.; Nakamura, E.; Iijima, S. In vivo Magnetic Resonance Imaging of Single-Walled Carbon Nanohorns by Labeling with Magnetite Nanoparticles. *Adv. Mater.* **2006**, *18*, 1010–1014.

41. Isobe, H.; Tanaka, T.; Maeda, R.; Noiri, E.; Solin, N.; Yudasaka, M.; Iijima, S.; Nakamura, E. Preparation, Purification, Characterization, and Cytotoxicity Assessment of Water-Soluble, Transition-Metal-Free Carbon Nanotube Aggregates. *Angew. Chem., Int. Ed.* **2006**, *45*, 6676–6680.

42. Ajima, K.; Yudasaka, M.; Maigné, A.; Miyawaki, J.; Iijima, S. Effect of Functional Groups at Hole Edges on Cisplatin Release from Inside Single-Wall Carbon Nanohorns. *J. Phys. Chem. B* **2006**, *110*, 5773–5778.

43. Ajima, K.; Maigné, A.; Yudasaka, M.; Iijima, S. Optimum Hole-Opening Condition for Cisplatin Incorporation in Single-Wall Carbon Nanohorns and Its Release. *J. Phys. Chem. B* **2006**, *110*, 19097–19099.

44. Dean, B. J. Recent Findings on the Genetic Toxicology of Benzene, Toluene, Xylenes and Phenols. *Mutat. Res.* **1985**, *154*, 153–181.

45. McMichael, A. J. Carcinogenicity of Benzene, Toluene and Xylene: Epidemiological and Experimental Evidence. *IARC Sci. Publ.* **1988**, *85*, 3–18.

46. Waters, M. D.; Stack, H. F.; Brady, A. L.; Lohman, P. H. M.; Haroun, L.; Vainio, H. Use of Computerized Data Listings and Activity Profiles of Genetic and Related Effects in the Review of 195 Compounds. *Mutat. Res.* **1988**, *205*, 295–312.

47. Shvedova, A. A.; Castranova, V.; Kisin, E. R.; Schwegler-Berry, D.; Murray, A. R.; Gandelsman, V. Z.; Maynard, A.; Baron, P. Exposure to Carbon Nanotube Material: Assessment of Nanotube Cytotoxicity Using Human Keratinocyte Cells. *J. Toxicol. Environ. Health, Part A* **2003**, *66*, 1909–1926.

48. Sato, Y.; Shibata, K.; Kataoka, H.; Ogino, S.; Bunshi, F.; Yokoyama, A.; Tamura, K.; Akasaka, T.; Uo, M.; Motomiya, K.; *et al.* Strict Preparation and Evaluation of Water-Soluble Hat-Stacked Carbon Nanofibers for Biomedical Application and Their High Biocompatibility: Influence of Nanofiber-Surface Functional Groups on Cytotoxicity. *Mol. BioSyst.* **2005**, *1*, 142–145.

49. Sato, Y.; Yokoyama, A.; Shibata, K.; Akimoto, Y.; Ogino, S.; Nodasaka, Y.; Kohgo, T.; Tamura, K.; Akasaka, T.; Uo, M.; *et al.* Influence of Length on Cytotoxicity of Multi-Walled Carbon Nanotubes against Human Acute Monocytic Leukemia Cell Line THP-1 in vitro and Subcutaneous Tissue of Rats in vivo. *Mol. BioSyst.* **2005**, *1*, 176–182.

50. Jia, G.; Wang, H.; Yan, L.; Wang, X.; Pei, R.; Yan, T.; Zhao, Y.; Guo, Z. Cytotoxicity of Carbon Nanomaterials: Single-Wall Nanotube, Multi-Wall Nanotube, and Fullerene. *Environ. Sci. Technol.* **2005**, *39*, 1378–1383.

51. Manna, S. K.; Sarkar, S.; Barr, J.; Wise, K.; Barrera, E. V.; Jejelowo, O.; Rice-Ficht, A. C.; Ramesh, G. T. Single-Walled Carbon Nanotube Induces Oxidative Stress and Activates Nuclear Transcription Factor-κb in Human Keratinocytes. *Nano Lett.* **2005**, *5*, 1676–1684.

52. Ding, L.; Stilwell, J.; Zhang, T.; Elboudwarej, O.; Jiang, H.; Selegue, J. P.; Cooke, P. A.; Gray, J. W.; Chen, F. F. Molecular Characterization of the Cytotoxic Mechanism of Multiwall Carbon Nanotubes and Nano-Onions on Human Skin Fibroblast. *Nano Lett.* **2005**, *5*, 2448–2464.

53. Cui, D.; Tian, F.; Ozkan, C. S.; Wang, M.; Gao, H. Effect of Single Wall Carbon Nanotubes on Human HEK293 Cells. *Toxicol. Lett.* **2005**, *155*, 73–85.

54. Monteiro-Riviere, N. A.; Nemanich, R. J.; Inman, A. O.; Wang, Y. Y.; Riviere, J. E. Multi-Walled Carbon Nanotube Interactions with Human Epidermal Keratinocytes. *Toxicol. Lett.* **2005**, *155*, 377–384.

55. Magrez, A.; Kasas, S.; Salicio, V.; Pasquier, N.; Seo, J. W.; Celio, M.; Catsicas, S.; Schwaller, B.; Forró, L. Cellular Toxicity of Carbon-Based Nanomaterials. *Nano Lett.* **2006**, *6*, 1121–1125.

56. Dumortier, H.; Lacotte, S.; Pastorin, G.; Marega, R.; Wu, W.; Bonifazi, D.; Briand, J.-P.; Prato, M.; Muller, S.; Bianco, A. Functionalized Carbon Nanotubes are Non-cytotoxic and Preserve the Functionality of Primary Immune Cells. *Nano Lett.* **2006**, *6*, 1522–1528.

57. Becker, M. L.; Fagan, J. A.; Gallant, N. D.; Bauer, B. J.; Bajpai, V.; Hobbie, E. K.; Lacerda, S. H.; Migler, K. B.; Jakupciak, J. P. Length-Dependent Uptake of DNA-Wrapped Single-Walled Carbon Nanotubes. *Adv. Mater.* **2007**, *19*, 939–945.

58. Tokuyama, H.; Yamago, S.; Nakamura, E.; Shiraki, T.; Sugiura, Y. Photoinduced Biochemical Activity of Fullerene Carboxylic Acid. *J. Am. Chem. Soc.* **1993**, *115*, 7918–7919.

59. Boutorine, A. S.; Tokuyama, H.; Takasugi, M.; Isobe, H.; Nakamura, E.; Hélène, C. Fullerene-Oligonucleotide Conjugates: Photoinduced Sequence-Specific DNA Cleavage. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2462–2465.

60. An, Y.-Z.; Chen, C.-H. B.; Anderson, J. L.; Sigman, D. S.; Foote, C. S.; Rubin, Y. Sequence-Specific Modification of Guanosine in DNA by a C60-linked Deoxyoligonucleotide: Evidence for a Non-singlet Oxygen Mechanism. *Tetrahedron* **1996**, *52*, 5179–5189.

61. Zakharenko, L. P.; Zakharov, I. K.; Vasyunina, E. A.; Karamysheva, T. V.; Danilenko, A. M.; Nikiforov, A. A. Determination of Genotoxicity of Fullerene C60 and Fullerol by the Somatic Mutation and Recombination Test in Drosophila Melanogaster and SOS Chromotest. *Genetika* **1997**, *33*, 405–409.

62. Dhawan, A.; Taurozzi, J. S.; Pandey, A. K.; Shan, W.; Miller, S. M.; Hashsham, S. A.; Tarabara, V. V. Stable Colloidal Dispersions of C60 Fullerenes in Water: Evidence for Genotoxicity. *Environ. Sci. Technol.* **2006**, *40*, 7394–7401.

63. Mori, T.; Takada, H.; Ito, S.; Matsubayashi, K.; Miwa, N.; Sawaguchi, T. Preclinical Studies on Safety of Fullerene upon Acute Oral Administration and Evaluation for No Mutagenesis. *Toxicology* **2006**, *225*, 48–54.

64. Nelson, M. A.; Domann, F. E.; Bowden, G. T.; Hooser, S. B.; Fernando, Q.; Carter, D. E. Effects of Acute and Subchronic Exposure of Topically Applied Fullerene Extracts on the Mouse Skin. *Toxicol. Ind. Health* **1993**, *9*, 623–630.

65. Huczko, A.; Lange, H.; Calko, E. Fullerenes: Experimental Evidence for a Null Risk of Skin Irritation and Allergy. *Fullerene Sci. Technol.* **1999**, *7*, 935–939.

66. Huczko, A.; Lange, H. Carbon Nanotubes: Experimental Evidence for a Null Risk of Skin Irritation and Allergy. *Fullerene Sci. Technol.* **2001**, *9*, 247–250.

67. Sayes, C. M.; Fortner, J. D.; Guo, W.; Lyon, D.; Boyd, A. M.; Ausman, K. D.; Tao, Y. J.; Sitharaman, B.; Wilson, L. J.; Hughes, J. B.; *et al.* The Differential Cytotoxicity of Water-soluble Fullerenes. *Nano Lett.* **2004**, *4*, 1881–1887.

68. Chen, H. H. C.; Yu, C.; Ueng, T. H.; Chen, S.; Chen, B. J.; Huang, K. J.; Chiang, L. Y. Acute and Subacute Toxicity Study of Water-Soluble Polyalkylsulfonated C60 in Rats. *Toxicol. Pathol.* **1998**, *26*, 143–151.

69. *Carbon Black User's Guide: Safety, Health & Environmental Information*; International Carbon Black Association, 1999 (http://www.continentalcarbon.com/safety/Default.html).

70. Brain, J. D.; Knudson, D. E.; Sorokin, S. P.; Davis, M. A. Pulmonary Distribution of Particles Given by Intratracheal Instillation or by Aerosol Inhalation. *Environ. Res.* **1976**, *11*, 13–33.

71. Driscoll, K. E.; Costa, D. L.; Hatch, G.; Henderson, R.; Oberdörster, G.; Salem, H.; Schlesinger, R. B. Intratracheal Instillation as an Exposure Technique for the Evaluation of Respiratory Tract Toxicity: Uses and Limitations. *Toxicol. Sci.* **2000**, *55*, 24–25.

72. Hesterberg, T. W.; Chase, G.; Axten, C.; Miller, W. C.; Musselman, R. P.; Kamstrup, O.; Hadley, J.; Morscheidt, C.; Bernstein, D. M.; Thevenaz, P. Biopersistence of Synthetic Vitreous Fibers and Amosite Asbestos in the Rat Lung Following Inhalation. *Toxicol. Appl. Pharmacol.* **1998**, *151*, 262–275.

73. Oberdörster, G. Determinants of the Pathogenicity of Manmade Vitreous Fibers (MMVF). *Int. Arch. Occup. Environ. Health* **2000**, *73*, S60–S68.

74. Ames, B. N.; McCann, J.; Yamasaki, E. Methods for Detecting Carcinogens and Mutagens with the

Salmonella/Mammalian-Microsome Mutagenicity Test. *Mutat. Res.* **1975**, *31*, 347–364.

75. Ishidate, M., Jr; Odashima, S. Chromosome Tests with 134 Compounds on Chinese Hamster Cells in vitro - A Screening for Chemical Carcinogens. *Mutat. Res.* **1977**, *48*, 337–353.

76. Matsuoka, A.; Hayashi, M., Jr. Chromosomal Aberration Tests on 29 Chemicals Combined with S9 Mix in vitro. *Mutat. Res.* **1979**, *66*, 277–290.

77. Sofuni, T. *Data book of chromosomal aberration test in vitro, Revised edition 1998*; Sofuni, T. Ed.; Life-Science Information Center: Tokyo, 1999.

78. Draize, J. H.; Woodard, G.; Calvery, H. O. Methods for the Study of Irritation and Toxicity of Substances Applied Topically to the Skin and Mucous Membranes. *J. Pharmacol. Exp. Ther.* **1944**, *82*, 377–390.

79. Kay, J. H.; Calandra, J. C. Interpretation of Eye Irritation Test. *J. Soc. Cosmet. Chem.* **1962**, *13*, 281–289.

80. Draize, J. H. Dermal Toxicity. In *Appriaisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics*; The Association of Food and Drug Officials of the United States, Texas State Department of Health: Austin, TX, 1959; p *46.*

81. Magnusson, B.; Kligman, A. M. The Identification of Contact Allergens by Animal Assay. The Guinea Pig Maximization Test. *J. Invest. Dermatol.* **1969**, *52*, 268–276.

82. Sato, Y.; Katsumura, Y.; Ichikawa, H.; Kobayashi, T.; Kozuka, T.; Morikawa, F.; Ohta, S. A Modified Technique of Guinea Pig Testing to Identify Delayed Hypersensitivity Allergens. *Contact Dermatitis* **1981**, *7*, 225–237.

83. Nakamura, A.; Momma, J.; Sekiguchi, H.; Noda, T.; Yamano, T.; Kaniwa, M.; Kojima, S.; Tsuda, M.; Kurokawa, Y. A New Protocol and Criteria for Quantitative Determination of Sensitization Potencies of Chemicals by Guinea Pig Maximization Test. *Contact Dermatitis* **1994**, *31*, 72–85.

84. Notification No. 24 of the First Evaluation and Registration DivisionPAB (Pharmaceutical Affairs Bureau, Ministry of Health and Welfare): Tokyo, Japan, September 11, 1989.

85. Bartlett, M. S. Properties of Sufficiency and Statistical Tests. *Proc. R. Stat. Soc. A* **1937**, *160*, 268–282.

86. Dunnett, C. W. A Multiple Comparison Procedure for Comparing Several Treatments with a Control. *J. Am. Stat. Assoc.* **1955**, *50*, 1096–1211.

87. We compared two solutions as agents suitable for dispersing SWNHs in the intratracheal instillation test: 0.25% w/v methyl cellulose (MC, Shin-Etsu Chemical Co., Ltd.)/saline solution and 0.025% w/v Tween 80/saline solution. The latter had less effect on animal health and was thus used in our test. We instilled 0.2 or 0.4 mL/animal of the 0.25% w/v MC/saline or the 0.025% w/v Tween 80/saline solution intratracheally into female rats (BrlHan:WIST@Jcl(GALAS), 7 weeks old, 130–143 g, five/group). The conditions for animal handling and intratracheal instillation were the same as those for the SWNH examination. All animals survived the 2 week test period. Crepitant whistle was observed for all animals in the MC/saline and Tween 80/saline groups. Most of the animals in the MC/saline group recovered within 1 h of the instillation, though two in the high dose group (0.4 mL/animal) suffered a relapse and did not fully recover until 2−5 days after the instillation. Moreover, one of the five animals in the high dose group showed bradypnea at 3 and 4 h after the instillation and a decrease in locomotor activity at 4 h after the instillation; both symptoms were no longer evident the next day. Most of the animals in the Tween 80/saline group recovered within 30 min or 1 h for the low dose group (0.2 mL/animal) or within 1 h for the high dose group. The two animals in the high dose group suffered a relapse, but no clinical signs of abnormalities were observed after the second day. There were no statistical differences in body weight gains or organ (lung and bronchus) weight between the control group (syringed only air at 0.4 mL/animal) and the MC/saline or Tween 80/saline group, and no macroscopic pathosis was found in anatomicopathlogical observations of the animals following euthanasia after the 2 week test period. Histopathological examinations showed an increase in foamy macrophages in the intra-alveolar spaces for two and four animals (degree: ± or +) in the low and high MC/saline dose groups and for one animal each (degree: ±) at both dose levels in the Tween 80/saline groups.