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Evaluation of the genotoxic potential of single-wall carbon nanotubes by using a battery of *in vitro* and *in vivo* genotoxicity assays

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ABSTRACT

The genotoxic potential of a high purity sample of single-wall carbon nanotubes (SWCNTs) was evaluated using a battery of *in vitro* and *in vivo* genotoxicity assays. These comprised a bacterial reverse mutation test (Ames test), an *in vitro* chromosomal aberration test, and an *in vivo* mouse bone marrow micronucleus test. The SWCNTs exerted no genotoxicity in *Salmonella typhimurium* TA97, TA98, TA100, and TA1535, or in *Escherichia coli* WP2 *uvr*A/pKM101, whether in the absence or presence of metabolic activation and at concentrations of 12.5–500 μg/plate. In the chromosomal aberration test, at 300–1000 μg/mL, the SWCNTs did not increase the number of structural or numerical chromosomal aberrations, whether the test was conducted with or without metabolic activation. In the *in vivo* bone marrow micronucleus test, doses of 60 mg/kg and 200 mg/kg SWCNTs did not affect the proportions of immature and total erythrocytes, nor did it increase the number of micronuclei in the immature erythrocytes of mice. The results of these studies show that the high purity and well-dispersed sample of SWCNTs are not genotoxic under the conditions of the *in vitro* bacterial reverse mutation assay, chromosomal aberration assay, or *in vivo* bone marrow micronucleus test, and thus appear not to pose a genotoxic risk to human health *in vivo*.

1. Introduction

Carbon nanotubes (CNTs) are one of attractive nanomaterials because of its unique physical and chemical characteristics. Manufactured CNTs exist in many forms, including single-wall carbon nanotubes (SWCNTs), double-wall carbon nanotubes (DWCNTs), and multi-wall carbon nanotubes (MWCNTs). Biological and toxicological responses to CNTs may vary, depending on their form, manufacturing processes, route of exposure, and dosage. There is little information about the possible human health and environmental impacts of manufactured CNTs. Early studies suggested that SWCNTs did not induce mutagenic effects in *Salmonella typhimurium* YG1024 and YG1029 strains or increase micronuclei in V79 lung fibroblast cells (Kisin et al., 2007), and MWCNTs did not induce mutagenic effects in the bacterial reverse mutation assay (Di Sotto et al., 2009; Wirnitzer et al., 2009). In an *in vitro* comet assay (single cell gel electrophoresis), a mixture of SWCNTs and other CNTs increased the extent of DNA damage in human bronchial epithelial cells in a dose dependent manner (Lindberg et al., 2009). In an *in vivo* micronucleus assay, MWCNTs induced an increase in micro-nucleated pneumocytes (MNPNCEs) in rats (Muller et al., 2008). However, the existing information on the genotoxicity of CNTs is limited and remains inconclusive (Landsiedel et al., 2009; Singh et al., 2009). A battery of genotoxicity assays serves as a reference point for the type of screening information on carcinogenicity that should be addressed as a new product is being developed. MWCNTs induced mesothelioma in $p53^{+/-}$ mouse (Takagi et al., 2008) or asbestos-like pathological changes in wild-type mice (Poland et al., 2008). However, there was little information on carcinogenicity of SWCNTs.

It is recommended that a battery of standard regulatory approved methods, which are validated, for which guidelines exist, and cover a wide range of different endpoints, should be used for assessing the genotoxicity of nanomaterials. This would facilitate comparison of the obtained results and provide information on mechanisms of action (Landsiedel et al., 2009; Singh et al., 2009).

The genotoxicity test battery comprised a bacterial reverse mutation assay (Ames test), an *in vitro* mammalian chromosomal aberration test, and an *in vivo* mouse bone marrow micronucleus test has been established specifically for hazard identification, the first step in risk assessment (Elespuru et al., 2009). Genotoxicity

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assessment of new substances using this test battery is essential in the field of regulatory toxicology. To our knowledge, however, there are no studies in which genotoxicity of SWCNTs have been assessed using this test battery. In the present study, we therefore conducted an Ames test with *S. typhimurium* and *Escherichia coli*, a chromosomal aberration test in cultured Chinese hamster cells, and an *in vivo* mouse bone marrow micronucleus test to assess the genotoxic potential of the high purity and well-dispersed sample of SWCNTs.

2. Materials and methods

The experiments were performed at the Institute of Environmental Toxicology (Ibaraki, Japan) in compliance with the Guideline for Animal Experimentation (1987), the Law Concerning the Protection and Control of Animals (1973), and the Standards Relating to the Care and Management of Experimental Animals (1980). The study was approved by the Institutional Animal Care and Use Committee of the Testing Facility and performed in accordance with the ethics criteria contained in the bylaws of the Committee of National Institute of Advanced Industrial Science and Technology.

2.1. Test material

SWCNTs were synthesized using a water-assisted chemical vapor deposition method (denoted as "super-growth CVD" method) with iron as the catalyst at the National Institute of Advanced Industrial Science and Technology, Japan. The super-growth CVD efficiently produces SWCNTs when the activity and life of the catalysts are enhanced by addition of water vapor (Hata et al., 2004). The bulk SWCNTs were characterized according to their chemical composition (ash impurities), residual catalyst, length, and diameter by thermogravimetric analysis, inductively coupled plasma mass spectrometry (ICP–MS), and transmission electron microscopy (TEM).

2.2. Sample preparation

Based on the result of a preliminary homogeneity examination, sodium carboxymethyl cellulose (CMC-Na, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Tween 80 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were selected as a dispersant for SWCNTs. In the bacterial reverse mutation test and *in vitro* chromosomal aberration test, SWCNTs were dispersed in 0.1% and 1% CMC-Na aqueous solution respectively using an ultrasonic bath for 90 min at 135 W and a frequency of 42 kHz. In the *in vivo* mouse bone marrow micronucleus test, SWCNTs were also dispersed in phosphate-buffered saline with 1% Tween 80 using the ultrasonic bath in the same manner as describe above.

2.3. Bacterial reverse mutation test (Ames test)

The experiments were conducted in compliance with the Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances (Kashin-hou in Japanese), which is equivalent to the OECD guideline for the testing of chemicals No. 471. The histidine-requiring *S. typhimurium* strains TA97, TA98, TA100, and TA1535 and the tryptophan-requiring *E. coli* mutant WP2 *uvr*A/pKM101 were cultured in a nutrient broth at 37 °C with shaking. SWCNTs were insoluble in the preparation vehicle for the bacterial reverse mutation test; therefore, 0.1% sodium carboxymethyl cellulose (CMC-Na) solution was applied for suspension in this test system. The maximum concentration of SWCNTs in 0.1% CMC-Na was 1 mg/mL without agglomeration. In a preliminary cytotoxicity study, all strains were tested using 2 plates per dose (ranging from 12.5 to 500 μg/plate) of SWCNTs to determine the appropriate concentrations range for the mutagenicity study. The preliminary study was conducted with and without metabolic activation, and no inhibition of growth of the colony was observed at any of the tested concentrations. Based on the results of the preliminary study, concentrations of 12.5, 25, 50, 100, 200 and 500 μg/plate were selected for the main mutagenicity study. The preliminary study and main study were conducted using duplicate plates for each dose.

The Ames test (Ames et al., 1975; Gatehouse et al., 1994) was performed with a pre-incubation method in the presence and absence of metabolic activation, via S9 mix. S9 fraction, which was prepared from male Sprague–Dawley rats pretreated with the enzyme-inducing agents phenobarbital and 5,6-benzoflabone, was purchased from Kikkoman Corporation (Chiba, Japan) mixed with co-factor (Oriental Yeast Co., Ltd., Tokyo, Japan), give a final concentration of 10% (v/v) S9. Distilled water and 0.1% CMC-Na (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used for the negative control and vehicle control, respectively. The positive controls used were 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2; Wako Pure Chemical Industries, Ltd., Osaka, Japan), sodium azide (NaN_3; Wako Pure Chemical Industries, Ltd., Osaka, Japan), 9-aminoacridine hydrochloride (9AA; Aldrich Chemical Co. Inc., Wisconsin, USA), and 2-aminoanthoracene (2AA; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

In the direct method (no metabolic activation), 0.5 mL of 100 mM sodium-phosphate buffer (pH 7.4) and 0.1 mL of each bacterial suspension were added to sterile tubes with distilled water (100 μL), 0.1% CMC-Na (100 and 500 μL), SWCNTs (100, 200, and 500 μL), or positive control (100 μL each). In the metabolic activation method, 0.5 mL of S9 mix and 0.1 mL of each bacterial suspension were added to sterile tubes with distilled water (100 μL), 0.1% CMC-Na (100 and 500 μL), SWCNTs (100, 200, and 500 μL), or positive controls (100 μL each). The mixtures were incubated at 37 °C with shaking (at a rate of 85 times per minute) for 20 min. Two milliliters of top agar, maintained at 45 °C, was then added. The mixtures were poured into plates and incubated at 37 °C for 48 h. The number of revertant colonies was counted using a colony counter. The test substance was considered to be mutagenic if either (a) a twofold or greater increase in the mean number of revertants per plate was observed compared with the mean number of revertants per plate in the appropriate negative control, in at least 1 of the tested strains, in the absence of cytotoxicity or (b) a dose-related increase in the mean number of revertants per plate, compared with that of the appropriate negative control, was observed at least 2–3 concentrations of the test substance and in at least 1 bacterial strain, in the absence of cytotoxicity (Ames et al., 1975; US FDA, 2000).

2.4. In vitro chromosomal aberration test

The experiments were conducted according to the OECD guideline for the testing of chemicals No. 473, and the Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances (Kashin-hou in Japanese). The Chinese hamster lung fibroblast cell line CHL/IU, which was obtained from National Institute of Health Science, Tokyo, Japan, was cultured in minimum essential medium (GIBCO®, Invitrogen Corp., California, USA) and 10% heat-inactivated newborn calf serum (HyClone Laboratories, Inc., Utah, USA). Cells were incubated at 37 °C and 5% CO_2. A preliminary test of SWCNTs was performed for cell growth inhibition at concentrations of 300 and 1000 μg/mL. The maximum concentration of SWCNTs in 1% CMC-Na was 3 mg/mL without agglomeration and 10 mg/mL with minimum agglomeration. These suspensions of SWCNTs in 1% CMC-Na were added to the culture medium to give a 10% (v/v) concentration, resulting in final concentrations of SWCNTs in the culture medium of 300 and 1000 μg/mL. Duplicate

plates were examined at each dose. According to the results of the preliminary test, the concentrations of SWCNTs selected for the main study were 300, 500, and 1000 μg/mL.

The main study consisted of a short-term exposure (6 h, with and without metabolic activation) and a continuous exposure (24 h, without metabolic activation) according to the method of Ishidate et al. (1984). Furthermore, 1% CMC-Na (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a negative control. The positive control substances used were mitomycin C (MMC, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) and benzo(a)pyrene (BaP, Wako Pure Chemical Industries, Ltd., Osaka, Japan). In both the short-term and continuous exposure methods, 1.5×10^5 cells were seeded on each tissue culture plastic plate (diameter, 60 mm) and incubated at 37 °C in a volume of 5 mL culture medium for 2 days. In the short-term exposure method, 2.7 mL of culture medium with or without S9 mix was changed after 2 days, and 300 μL of SWCNT suspension was added. The S9 mix components were the same as in the bacterial reverse mutation test, except that the final concentration of S9 in the treatment medium was 5% (v/v). After 6-h exposure, the medium was discarded and the cells were washed with phosphate buffer. Fresh medium was then added and the cells were cultured for an additional 18 h. In the continuous exposure method, 4.5 mL of culture medium without S9 mix was changed after 2 days, and 500 μL of SWCNT suspension was added, and the cells were cultured for 24 h. In both the short-term and continuous exposure methods, colcemid (Wako Pure Chemical Industries, Ltd., Osaka, Japan; 0.2 μg/mL final concentration) was added 2 h before cell harvesting. The cells were then harvested, treated with 0.075 M KCl solution, fixed in fixative (methanol:acetic acid = 3:1), placed onto clean glass slides, and stained with 2% Giemsa solution (Merck Ltd., Germany) for 15 min at room temperature. One hundred metaphases per slide (200 metaphases per dose) were examined blindly for structural aberrations and polyploidy. Pearson's chi-square test was used to determine significant differences between the negative control and the other groups. A cell growth inhibition test was also concomitantly conducted under the same conditions. In the cell growth inhibition test, 1.5×10^5 cells were seeded on a plastic plate (diameter, 60 mm), cultured as describe above, fixed in 10% (v/v) neutral buffered formalin, and stained with 0.1% crystal violet. The growth rates of the treated cells, relative to those of the negative control cells, were evaluated using a monolayer cell densitometer (Monocellater®, Olympus Optical Co., Ltd).

2.5. In vivo mouse bone marrow micronucleus test

The experiments were conducted in compliance with the OECD guideline for the testing of chemicals No. 474, and the Law Concerning the Examination and Regulation of Manufacture etc. of Chemical Substances (Kashin-hou in Japanese). Six-week-old Crlj: CD1 (ICR) mice were purchased from Charles River Laboratories, Japan, Inc. (Yokohama, Japan). The mice were kept in an animal facility housed in positive-pressure air-conditioned units (21–23 °C, 45–64% relative humidity) on a 12:12-h light/dark cycle. After 7-day acclimation, the mice were assigned to the study. The experimental protocol was approved by the internal ethical committee for the use of animals in research.

SWCNTs were insoluble in the preparation vehicle; therefore, 1% Tween 80 in phosphate-buffered saline (PBS) was applied to achieve particle suspension. The maximum concentration of SWCNTs in 1% Tween $80^{-/-}$ PBS was 3 mg/mL without agglomeration and 10 mg/mL with minimum agglomeration. The volume of administration was 20 mL/kg body weight; hence, 3 and 10 mg/mL corresponded to 60 and 200 mg/kg, respectively. A dose-finding test was carried out for maximal tolerance in 6 male and 6 female mice. SWCNTs were administered orally by gavage once daily at doses 60 and 200 mg/kg/day for 2 days. From the results of the dose-finding test, no difference in SWCNT toxicity between male and female mice, and SWCNT dosages of 60 and 200 mg/kg/day were used for the main study. SWCNTs were administered to each 5 male mice per dose via oral gavage twice at an interval of 24 h. For the negative control, a 20 mL/kg of 1% Tween $80^{-/-}$ PBS was administered orally to 5 male mice for 2 days in a similar manner. Mitomycin C was used for the positive control and administered orally to 5 male mice at a dose of 10 mg/kg (20 mL/kg) for 1 day. After dosing, the mice were examined regularly for mortality and clinical signs of toxicity until they were euthanized. The femurs of mice in all groups were removed 24 h after the final administration, and the bone marrow cells were flushed into tubes with fetal bovine serum to prepare bone marrow smear specimens. The specimens were air-dried, fixed with ethanol and stained with 3% Giemsa solution for 30 min at room temperature.

The frequency of micronucleated polychromatic erythrocytes (MNPCEs) was calculated on the basis of observations of 2000 polychromatic erythrocytes (PCEs) per animal. The proportion of PCEs among total erythrocytes was determined by counting 1000 erythrocytes for each mouse. Significant differences in the frequency of MNPCEs between the negative control and SWCNT groups were determined using Kastenbaum–Bowman's test (Kastenbaum and Bowman, 1970). Pearson's chi-square test was applied for comparison of the frequency of MNPCEs between the negative control and positive control. The proportion of PCEs to total erythrocytes was analyzed by Wilcoxon's Rank Sum test.

3. Results

3.1. Characterization of SWCNT

The main characteristics of the super-growth SWCNT bulk material and the dispersed SWCNTs in the testing solution (1% Tween 80 in PBS) are presented in Figs. 1 and 2 and Table 1. The diameter and length of the SWCNT bulk material was measured to be 3.0 ± 1.1 nm (mean ± SD) and 1200 nm (maximum length), respectively. The surface area of the SWCNT bulk material was determined to be 1064 ± 37 m^2/g (mean ± SD). TEM and chemical analysis of the bulk material showed a high purity for the SWCNTs with little indication of impurities due to metal catalyst, oxide, or ash. In the testing solutions, minimal agglomeration was observed (Fig. 2), and the diameter and length of the SWCNT bundles were measured to be 12.0 ± 6.5 nm (mean ± SD) and 0.51 ± 1.6 μm (mean ± SD), respectively.

3.2. Bacterial reverse mutation test (Ames test)

In the preliminary cytotoxicity test, there was no growth inhibition at any concentration of SWCNTs, with or without metabolic activation. In the mutagenicity test (Table 2), SWCNTs did not increase the mean number of revertants of any *S. typhimurium* strain or the *E. coli* strain per plate at any concentration, with or without metabolic activation, in comparison to the spontaneous reversion rate in the negative control. Precipitation of the SWCNTs was observed at concentrations between 50 and 500 μg/plate. There was no evidence of cytotoxicity under any of the test conditions. Positive controls induced increases in the number of revertant colonies, confirming the sensitivity of the test system. SWCNTs were not mutagenic in the bacterial system.

3.3. In vitro chromosomal aberration test

In the preliminary cytotoxicity test (Table 3), precipitation of SWCNTs was observed at concentrations 300 and 1000 μg/mL,

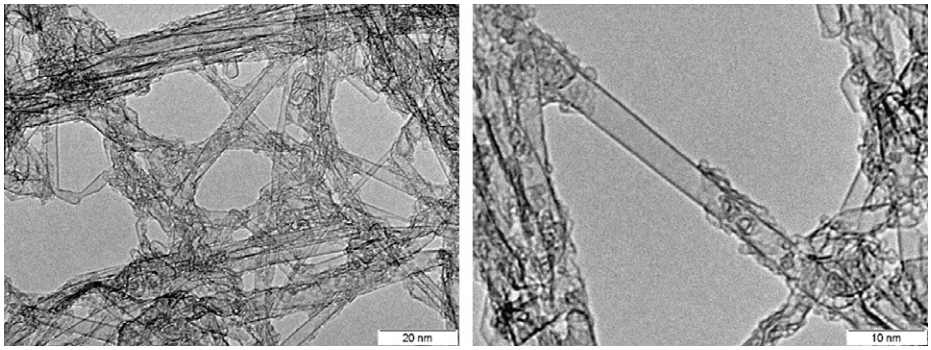

Fig. 1. Transmission electron microscopy (TEM) images of bulk SWCNTs.

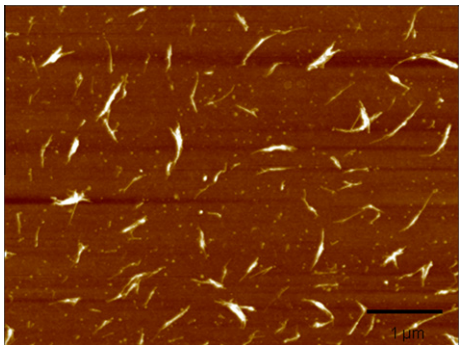

Fig. 2. AFM image of SWCNTs in testing solution.

but, there was no growth inhibition at any concentration of SWCNTs with or without metabolic activation. The results of the main chromosomal aberration test are shown in Table 4. SWCNTs did not increase the number of structural (excluding gaps) or numerical chromosomal aberrations at any of the tested concentrations with or without metabolic activation. The positive controls MMC and BaP produced marked increases in structural aberrations, confirming the sensitivity of the test system. SWCNTs did not show genotoxicity in the *in vitro* chromosomal aberration test.

3.4. In vivo mouse bone marrow micronucleus test

In the dose finding test, SWCNTs were tested up to their maximum preparatory concentration (10 mg/mL) in 1% Tween $80^{-/-}$ PBS, by administering 20 mL/kg orally by gavage once a day for 2 days to the male and female mice. No mortality or clinical signs were observed in mice receiving 60 and 200 mg/kg of SWCNTs. The dose of 200 mg/kg was confirmed to be a practical upper limitation to mouse micronucleus test in the amount that could be administered orally by gavage via gastric tube. The results of the examinations of micronuclei in bone marrow are shown in Table 5. The SWCNTs did not induce increases in the MCPCE frequency at any dose. The positive control MMC increased the frequency of MCPCEs but decreased the frequency of PCEs, which might result in suppression of the bone marrow. SWCNTs did not show any potential for genotoxic activity in the *in vivo* micronucleus assay.

4. Discussion

Manufactured CNTs exist in many forms, such as SWCNTs, DWCNTs, and MWCNTs, and are exploited for their unique physical and chemical characteristics. There is little information about the possible human health impacts of manufactured CNTs. A key area governing the risk assessment of new substances for human health is genotoxicology (the study of genetic damage after exposure) due to the fact that classic genotoxic substances lead to carcinogenesis (Singh et al., 2009). Genotoxicity testing, and thus the evaluation of the carcinogenicity and mutagenicity of new substances, is the most important part of the safety testing of chemical compounds. In the present study, well-dispersed and well-characterized SWNCTs were assessed for their genotoxic potential by means of a battery of genotoxicity assays, comprising a bacterial reverse mutation assay (the Ames test), a Chinese hamster lung cell line chromosomal aberration assay, and an *in vivo* mouse bone marrow micronucleus test. This is the first report showing that SWNCTs are not genotoxic when tested using the genotoxicity test battery.

The bacterial gene mutation and chromosomal aberration assays clearly showed that the SWCNTs had no genotoxic effects,

Table 1
The main characteristics of bulk SWCNT.

	Characteristic	Value	Measuring method
Bulk material	Tube diameter[a]	3.0 ± 1.1 nm	Transmission electron microscopy
	Maximum length	1200 nm	Transmission electron microscopy
	BET Surface area[a]	1064 ± 37 m^2/g	N_2 adsorption (BET method)
	D/G ratio	0.14	Raman spectroscopy
	Carbon impurity [a] (Amorphous carbon)	<2.3 ± 0.56%	Thermogravimetric analysis
	Total metal impurity[a]	0.051 ± 0.155%	
	Metal impurities		Inductively coupled plasma – mass spectrometry
	Iron	145 ppm	
	Nickel	103 ppm	
	Chromium	34 ppm	
	Manganese	2 ppm	
	Aluminum	12 ppm	
In testing solution	Tube diameter[a]	12.0 ± 6.5 nm	Atomic force microscope
	Length[a]	0.51 ± 1.6 μm	

[a] Values are expressed as mean ± standard deviation.

Table 2
Results of bacterial reverse mutation test on SWCNT.

Dose (μg/plate)	S9 mix	No. of revertant colonies/plate (Mean)				
		Base substitution type			Flameshift type	
		TA100	TA1535	WP2 *uvrA*/pKM101	TA98	TA97
Distilled water	–	140	12	89	15	168
0.1% CMC-Na		123	8	95	19	155
0.1% CMC-Na*		154	9	105	13	156
12.5		130	7	97	20	156
25		134	9	103	21	167
50[a]		132	12	74	20	159
100[a]		123	9	88	13	150
200[a]		124	9	93	14	147
500[a]		114	6	92	17	168
AF-2 0.005				1131		
AF-2 0.01		612				
AF-2 0.1					402	
NaN_3 0.5			378			
9-AA 80						1527
Distilled water	+	125	7	145	18	197
0.1% CMC-Na		129	6	149	31	203
0.1% CMC-Na*		125	7	160	24	216
12.5		109	9	176	27	214
25		130	8	174	21	211
50[a]		115	9	134	23	180
100[a]		133	9	143	15	202
200[a]		121	7	139	22	189
500[a]		106	6	132	16	143
2AA 0.5					297	
2AA 1		717				
2AA 2			162	611		688

AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide.
NaN_3: sodium azide.
9AA: 9-aminoacridine hydrochloride.
2AA: 2-aminoanthoracene.
* 500 μL of 0.1% sodium carboxymethyl cellulose (CMC-Na) were added.
[a] Precipitation.

Table 3
Results of preliminary cytotoxicity test for chromosomal aberration test on SWCNT.

Dose (μg/mL)	% Relative cell growth		
	Short-term exposure, 6–18 h		Continuous exposure
	−S9 mix	+S9 mix	−S9 mix, 24 h
1% CMC-Na	100	100	100
300 [a]	101	93	114
1000 [a]	94	93	108

CMC-Na: 1% sodium carboxymethyl cellulose.
[a] Precipitation.

Table 4
Results of chromosomal aberration test on SWCNT in Chinese hamster lung cell line.

Dose (μg/mL)	S9 mix	No. of cells	% Structure aberrations		% Polyploid cells	% Relative cell growth
			+gap	−gap		
0[a]	−6 to 18[c]	200	0	0	0.5	100
300[b]		200	0.5	0.5	0	99
500[b]		200	2.0	0.5	0.5	96
1000[b]		200	1.5	1.0	0	94
MMC 0.1	200	21.0	19.0**	0	99	
0[a]	+6 to 18[c]	200	0.5	0	1.0	100
300[b]		200	4.0	2.5	0	100
500[b]		200	2.0	0.5	0	97
1000[b]		200	1.5	0	0	97
BaP 40	200	46.5	43.5**	0	82	
0[a]	−24 to 0[c]	200	1.5	0.5	0	100
300[b]		200	1.5	1.0	0	112
500[b]		200	2.0	0.5	0.5	109
1000[b]		200	4.0	2.0	0	93
MMC 0.1		200	38.0	37.0**	0	118

MMC: Mitomycin C.
BaP: Benzo (a) pyrene.
[a] 1% sodium carboxymethyl cellulose (CMC-Na).
[b] Precipitation.
[c] Exposure time – recovery time (h).
** Significantly different from negative control at $p < 0.01$ (Pearson's chi-squared test).

Table 5
Results of bone marrow micronucleus test on SWCNT in mice.

Dose (mg/kg)	No. of dosing	No. of mice	Mean ± S.D.	
			% MNPCE	% PCE
0[a]	2	5	0.28 ± 0.10	54.7 ± 2.2
60	2	5	0.28 ± 0.17	57.4 ± 4.9
200	2	5	0.27 ± 0.14	50.1 ± 8.4
MMC 10	1	5	4.95 ± 1.68**,b	40.5 ± 10.0*,c

MNPCE: Micronucleated polychromatic erythrocytes.
PCE: Polychromatic erythrocytes.
MMC: Mitomycin C.
[a] 1% Tween 80 in phosphate-buffered saline.
[b] Statistical analysis using Pearson's chi-squared test.
[c] Statistical analysis using Wilcoxon's Rank Sum test.
* Significantly different from negative control at $p < 0.05$, respectively.
** Significantly different from negative control at $p < 0.01$, respectively.

with or without a metabolic activation system, when tested at or above their limit of solubility. Furthermore, there was no evidence of cytotoxicity or inhibition of cell proliferation, neither in bacteria nor in the Chinese hamster cell line. When tested using an *in vivo* genotoxicity test, the SWCNTs had no genotoxic activity, as evidenced by the lack of induction of micronuclei or chromosome damage in mouse bone marrow erythrocytes at the maximum dosing limit.

Several genotoxicity studies of SWCNTs have been previously performed. Positive responses were observed in an *in vitro* comet assay (Kisin et al., 2007; Pacurari et al., 2008) and in an *in vivo* assay for mitochondrial DNA damage (Li et al., 2007), oxidatively damaged DNA (Folkmann et al., 2009), and mutation of the K-*ras* gene locus (Shevedova et al., 2008). Savolainen et al. (2010) noted that the doses used in the *in vivo* studies have, in most cases, been relatively high, thus rendering the assessment of their relevance for risk assessment problematic. Studies should be performed using doses closely reflecting expected levels of human exposure. Negative outcomes were obtained in the *Salmonella* gene mutation assay with strains YG1024/YG1029 (Kisin et al., 2007) and in the *in vitro* micronucleus assay (Kisin et al., 2007) and in the *in vitro* and *in vivo* comet assays (Jacobsen et al., 2008; Zeni et al., 2008). The inconsistencies in the results of these studies might be attributable to the differences in the test conditions, such as cell types, exposure time, concentrations, the specific endpoint measured, the dispersal of the materials, the impurities of metals, and the SWCNT physico-chemical characteristics. In our present *in vitro* cytogenetics study, CMC-Na was used as a dispersant to prepare testing solutions, and we made SWCNTs disperse uniformly in aqueous solution without any modification of their structure. A bovine serum albumin (BSA) has been reported to be a good dispersant for CNTs (Elgrabli et al., 2007). BSA solution alone induced no modification of the biological responses, but altered cell viability with CNTs in human A549 and U937 cells. Although it is unknown if CMC will have any biological interaction with SWCNTs or affect the efficiency of cellular uptake of SWCNTs, it is not considered that CMC may be involved in genotoxicity of SWCNTs after uptake into cells.

There is no positive report of SWCNTs on the bacterial gene mutation assays. It is said that the negative response in bacterial

gene mutation assays might be caused by the difficulty of penetration of nanomaterials through the bacterial cell wall (Landsiedel et al., 2009; Singh et al., 2009), even though the Ames *Salmonella* strains have a *rfa* mutation in their cell walls to facilitate the incorporation of large molecular sized materials into the inside of cells.

A battery of genotoxicity assays, comprising a bacterial gene mutation assay, an *in vitro* chromosomal aberration assay, and an *in vivo* micronucleus test, serves as a reference point for the type of screening information that should be addressed for new chemicals including pharmaceutical drugs (OECD, 2003; ICH guideline, 1998). The bacterial gene mutation assay and *in vitro* chromosomal aberration assay are components of the minimum base set of genotoxicity screening studies which provide a fundamental characterization of the potential hazards of nanomaterials (Warheit et al., 2007). Although we, therefore, performed the bacterial gene mutation assay of SWCNTs and obtained negative results, the possibility of mutational potential of SWCNTs may not be completely ruled out in mammalian cells in some condition since the inhalation exposure of SWCNTs was found to be induced K-*ras* gene mutation in the lung of C57BL/6 mice (Shevedova et al., 2008).

The main molecular mechanism of toxicity of nanomaterials is the induction of oxidative stress by free radical formation (Lanone and Boczkowski, 2006; Singh et al., 2009). SWCTNs have been shown to generate reactive oxygen species (ROS), induce oxidative stress, and exert toxic effects (Shevedova et al., 2007, 2008; Folkmann et al., 2009; Jacobsen et al., 2008; Pacurari et al., 2008).

The generation of ROS is considered to be a major mechanism in the induction of genotoxicity by nanomaterials, and ROS are able to cause the oxidation of DNA, DNA stand breaks, or lipid peroxidation-mediated DNA adducts (Møller et al., 2010). Determination of the ability of SWCNTs to produce ROS is necessary to elucidate the mechanism for genotoxicity of the SWCNTs. Other mechanisms may also be relevant to interference with DNA, chromatin structures, or the mitotic apparatus, especially with fiber-shaped materials. In view of its relevance for risk assessment purposes, an important issue in terms of the genotoxicity of nanomaterials is to distinguish between primary and secondary pathways. Primary genotoxicity is defined as genetic damage elicited by particles in the absence of inflammation. In contrast, secondary genotoxicity is defined as genetic damage resulting from ROS and reactive nitrogen species (RNS), which are known to be generated during inflammation, because secondary genotoxicity is considered to involve a threshold (Schins and Knaapen, 2007).

The respiratory tract is one of the target organs of nanomaterials when exposure occurs via inhalation. In a series of our research, comet assays are now planning to be conducted using the lung epithelial cells of rats given intratracheal instillation of SWCNTs, at concentrations that do and do not elicit significant pulmonary inflammation, to determine the presence or absence of genotoxicity and primary or secondary genotoxicity of SWCNTs.

Conflict of interest statement

The authors declare that there are no conflicts of interest. The views expressed in this article are those of the authors and do not necessarily reflect the views and policies of the National Institute of Advanced Industrial Science and Technology (AIST).

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