フェーズ2:

フェーズ1のエンドポイントへの対処で報告したように、スポンサーは適切で可能な、追加の必要な試験を決定し、フェーズ2で必要なDossierプランを開発する。留意点として、フェーズ1の全てが完了するまでフェーズ2の開始を遅らせるような必要はない。フェーズ1と2は異なる委託なので、フェーズ2のスポンサーがフェーズ1と同じかどうかは分からない。フェーズ2の領域は各担当産業用ナノ製品のハザードの可能性の把握に必要な追加エンドポイントに対処することである。フェーズ1と2で得られた試験データによってリスク評価の理論的枠組みへの応用が完全に十分ではないが可能になり、スポンサーのついた産業用ナノ製品の特定利用(十分な曝露データが利用できる場合)が検討される。フェーズ2では、産業用ナノ製品の寿命やナノマテリアル使用の副生成物の評価といった側面がフェーズ1で見込まれる以上の詳細さと特異性で考えられる可能性もある。したがって、フェーズ2試験はリスク関連決定論理によって導かれる可能性がある。

このガイダンスマニュアルはスポンサーシッププログラムのフェーズ1のみを対象とするが、フェーズ2試験で考慮される可能性のある項目について言及することもある。

スポンサーシッププログラム・フェーズ1の目的や性質は、日程/概算時間や労力に関する事項ではなく、「予備的な性質」という観点から決定されべきである。さらに、エンドポイントに対処するための試験方法の開発が研究体制として必要であると考えられたが、スポンサーシッププログラムが終わりのない研究プログラムになることをWPMNは意図しない。したがって、このような可能性を減らす実際的な対策として、フェーズ1の中間評価ステップまでに2年の期限(2011年前半の第8回WPMN会議の約2ヶ月前まで)を設定することが提案された。その時点でWPMNにプログラムの進行状況に関する報告書が提示される。中間評価はこのガイダンスマニュアルと各DDPに基づいて行われる。

この2年間の結論として、WPMNとスポンサーは以下について検討することになる。

1. 追加試験法の開発に関する状況、必要性、および調整
2. 産業用ナノ製品の特性の理解状況の評価
3. フェーズ2の試験とプログラムに向けて着手するかどうか、そして着手すべき事柄
4. フェーズ1の完了には何が必要でどのようなスケジュールか

主な用語:

スポンサーシッププログラムには3レベルでの参加が可能である。それらは、リードスポンサー、共同スポンサー、コントリビューターである。各参加レベルの責任は以下の通り。

- リードスポンサーは、リスト記載の産業用ナノ製品に関するフェーズ1試験において、エンドポイントへの対処が適切且つ可能な全試験について、実施や調整の責任がある。場合によっては、エンドポイントに対処する関与の度合いを考慮して"連合リード"スポンサーが取り決められる可能性もある。このガイダンスマニュアルではリードスポンサーや連合リードスポンサーを"スポンサー"と呼んでいる。
- 共同スポンサーは、リスト記載の産業用ナノ製品に関するフェーズ1試験において、エンドポイントへの対処が適切且つ可能ないくつかの試験を実施する。
- コントリビューターは、リードスポンサーと共同スポンサーに試験データ、参考資料、試験材料、あるいはその他の関連情報を提供する。

留意点として、リードスポンサーには試験に関する書類一式を揃える最終責任はあるが、共同スポンサーにもDDPを積極的に作成する役割がある。コントリビューターはリードスポンサーの要求によりDDPの作成に参加できる。選んだ産業用ナノ製品ごとにリードスポンサー同士で適宜情報を交換して共有することが考えられる。総合的なコミュニケーション方法をDDPの一部として開発すべきである。

- **スポンサー用ガイダンスマニュアル(version 2.1)**

スポンサー用ガイダンスマニュアルの作業部会は

ガイダンスマニュアルの開発のために2008年2月に設置され、試験プログラムでのスポンサー支援、およびWPMNプロジェクトにより達成された作業(1:代表的産業用ナノ材料の安全性試験(SG3)、2:産業用ナノ製品とテストガイダンス(SG4))の増進が目的である。

WPMNは産業用ナノ材料の試験のためのスポンサーシッププログラムに関するワークショップを開催した(2008年4月24-25日、東京)。ワークショップでは、次の点を示すことでガイダンスマニュアルがスポンサーの支援となることが認められた。

1)スポンサーの3レベル(リードスポンサー、共同スポンサー、コントリビューター)、
2)予備的フェーズ1試験とフェーズ2試験の違い、
3)エンドポイントへの対処情報の開発方法の検討、
4)第8回WPMN会議の前の2年間の審査を含むWPMNの審査・監視方法、
5)スポンサーのついた各産業用ナノ材料について試験プログラムで期待されるアウトプット、

作業部会ではガイダンスマニュアルの原案を作成し、ワークショップでの議論に基づいて第4回WPMN会議(2008年6月11-13日)でその原案を提示した。第4回WPMN会議では、さらに、第5回WPMN会議での検討を目的にDossier Development Plans(DDP)作成に役立つスポンサー用ガイダンスマニュアルの草稿を作成することとなった。スポンサー用ガイダンスマニュアルの作業部会はWPMN会議やスポンサーシッププログラムへの参加国(リードスポンサー、共同スポンサー、コントリビューター)や、関係各国への回覧や釜山で開催されたワークショップ等の議論を経て2088年末にはversion 2.1が作成された。

- **DDP作成(健康影響)**

我が国は、表1に示すようにフラーレン、SWCNT(単層カーボンナノチューブ)およびMWCNT(多層カーボンナノチューブ)のリードスポンサーとなることとなり、第5回WPMN会議にむけてDDPの作成作業が行われた。

表1.第5回WPMN会議時点でのスポンサー登録

Manufactured Nanomaterial	Lead sponsor(s)	Co-sponsor(s)	Contributors	DDP Status
Fullerenes(C60)	Japan United States		Denmark China	1st Draft
SWCNTs	Japan United States		Canada France Germany EC China BIAC	1st Draft
MWCNTs	Japan United States	Korea BIAC	Canada France Germany EC China BIAC	1st Draft
Silver nanoparticles	Korea United States	Canada Germany Nordic Council of Ministers	Australia France EC China	1st Draft
Iron nanoparticles	BIAC China		Canada United States Nordic Council of Ministers	1st Draft
Carbon black			Denmark Germany United States	
Titanium dioxide	France Germany	Austria Canada Denmark Korea Spain United States BIAC	China	1st Draft
Aluminium oxide			Germany United States	
Cerium oxide	United States United Kingdom/BIAC	Netherlands	Australia Germany Switzerland EC	1st Draft

各物質のDDPの中には、以下の健康影響に関するエンドポイントについて、そのデータ収集の観点から、既存データの有無、試験の計画や実行状況、試験方法について、計画書として記載することとなっている。**Mammalian Toxicology**の必須項目としては、

Pharmacokinetics/Toxicokinetics (ADME)

Acute toxicity

a) Skin Corrosion
b) Skin Irritation
c) Skin sensitization
d) Acute Eye Irritation
e) Phototoxicity

Repeated dose toxicity (28 and 90 days study)

a) Oral
b) Dermal
c) Inhalation

となり、さらに、利用可能な場合は、以下の項目についても記載することになっている。

Chronic toxicity

Reproductive toxicity

Developmental toxicity

Genetic toxicity

a) In vitro Genotoxicity

b) In vivo Somatic Cell Genotoxicity

c) In vivo Germ Cell Mutagenicity

Experience with human exposure

Other relevant test data

D. 考察

本研究班のこの WPNM のスポンサーシッププログラムへの貢献としては、DDP へ記入すべき健康影響に関するエンドポイントのうち、まず、C60 の ADME に関するデータを提供できると考えられた。また、現時点では、まだ定量化までは至っていないが、MWCNT の ADME データの入手の可能性も示唆されている。さらに、C60 や MWCNT の in vivo 投与のための分散手法に関する研究を進めている観点からは、いくつかの急性毒性(Acute toxicity)試験を行う際の有用な情報を提供できることが可能であると考えられる。in vitro の試験法開発に関する研究からは、C60 の遺伝毒性(In vitro Genotoxicity)に関する成果や、その他の C60 や MWCNT の in vitro データの提供にも対応可能であると考えられた。また、厚生労働科学研究費の化学物質リスク研究事業で行っている他の(経皮暴露や吸入暴露を中心とした)研究班における研究成果の貢献も期待できるものと推定される。

E. 結論

OECD における産業用ナノマテリアルの作業グループのなかで代表的なナノマテリアルを用いた物質を選定して各国が自主的に行うおこなうスポンサシッププログラムと Dossier 作成計画書(DDP)について、その動向の情報収集を行った。本研究班の成果からは、C60 や MWCNT に関して ADME や急性毒性、遺伝毒性を含む In vitro 試験等のエンドポイントについて直接および間接的な貢献が期待できるものと考えられた。

F. 研究発表

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G. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得

(該当なし)

2. 実用新案登録

(該当なし)

3. その他

(該当なし)

Ⅲ．研究成果の刊行に関する一覧表

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Ⅳ．研究成果の刊行物・別冊

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Original Article

Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats

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ABSTRACT — The present study assessed a carcinogenic hazard of multi-wall carbon nanotube (MWCNT) in intact (not genetically modified) rodents. MWCNT (1 mg/kg body weight, 7 animals), crocidolite (2 mg/kg body weight, 10 animals) or vehicle (2% carboxymethyl cellulose, 5 animals) was administered to male Fischer 344 rats (12 weeks old) by a single intrascrotal injection. Rats were autopsied immediately after death, when becoming moribund or at the end of the maximal observation period scheduled to be 52 weeks. After 37-40 weeks, however, 6 MWCNT-treated animals died or became moribund due to intraperitoneally disseminated mesothelioma (6/7, 85.7%) with bloody ascites. Peritoneal mesothelium was generally hypertrophic, and numerous nodular or papillary lesions of mesothelioma and mesothelial hyperplasia were developed. While mesothelioid cells were predominant in relatively early stage tumors, advanced stage mesotheliomas were constituted by 2 portions occupied by mesothelioid cells on the surface and spindle-shaped sarcomatous cells in the depth. In the latter, the histological transition was apparently observed between these 2 portions. Mesotheliomas were invasive to adjacent organs and tissues, and frequently metastasized into the pleura. Only 1 rat survived for 52 weeks in the MWCNT-treated group, and similar findings except mesothelioma were observed. All 10 crocidolite-treated and 5 vehicle-treated rats survived for 52 weeks without any particular changes except deposition of asbestos in the former case. It is thus indicated that MWCNT possesses carcinogenicity causing mesothelioma at a high rate in intact male rats under the present experimental conditions. The present data identifies a carcinogenic hazard of MWCNT and will serve as one of the indispensable evidences to be used for the risk assessment crucial for not only protection and improvement of human health and welfare, but also safe and acceptable development and prevalence of this and similar upcoming materials.

Key words: Multi-wall carbon nanotube, Mesothelioma, Nanomaterial, Carcinogenicity, Hazard identification, Rat

INTRODUCTION

Hazardous and risky substances present in the human environment must be avoided or strictly controlled. For this purpose, the risk assessment process is critical and should be conducted using established protocols based on scientific evidence. In recent years, progress of research and development in the industrial field has been con-

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tinuously introducing new materials into our society to make our life more comfortable and convenient than ever. Potential human and environmental risks of these newcomers, however, should be carefully clarified at as an early stage as possible during its development and prevalence in order to protect benefits of all stakeholders, including not only health profits of general consumers and people participating in the manufacturing, but also business interests of manufacturers.

Nanomaterials, provisionally defined as substances of which sizes are smaller than 100 nm at least in 1 dimension, have attracted much attention and being enthusiastically developed and supplied because of its promising future with a wide variety of potential application. It should be noted, however, that their outstanding reactivity due to the enormous surface area achieved by the infinitesimal size serves as their best merit at one side but also their worst demerit at the other side, especially when they come into the human and other living creatures' bodies (Medina *et al.*, 2007). Possible human and environmental risks of nanomaterials should thus be urgently but carefully elucidated, and efforts have globally started to be made on this issue (Donaldson *et al.*, 2006; Lam *et al.*, 2006; Singh and Nalwa, 2007).

Carbon nanotube, a new form of technological crystalline carbon, is one of the most anticipated nanomaterials because of its unique properties that are suitable for a variety of industrial products such as high-strength materials, electronics and biomedical apparatuses (Martin and Kohli, 2003; Scott, 2005). Regarding potential biological risks of carbon nanotube, only limited information is currently available, especially for its chronic effects. Nevertheless, cohesive and thus easily agglomerating nature and estimated long stability in the body are considered as possible factors for toxic influence (Lam *et al.*, 2006; Luo *et al.*, 2004; Takagi *et al.*, 2008a). There is another concern that carbon nanotube might cause a tragedy similar to that caused by asbestos, because of their similarities such as a fibrous/rod-shaped structure with a superbly high aspect ratio and a contamination of iron, and of the fact that carbon nanotube may be used as an asbestos substitute (Donaldson *et al.*, 2006; Maynard *et al.*, 2004; Lam *et al.*, 2006; Singh and Nalwa, 2007).

In 2008, our colleagues of the National Institute of Health Sciences of Japan have reported that multi-wall carbon nanotube (MWCNT) induces peritoneal mesotheliomas at an incidence (14/16, 87.5%) similar to the case of crocidolite (14/18, 77.8%), within 25 weeks after a single intraperitoneal administration to male mice heterozygously deficient in the *p53* gene, in which some of us participated (Takagi *et al.*, 2008a). While the study clearly indicates a potential carcinogenic hazard of MWCNT, it is then absolutely necessary to perform the hazard identification using intact (not genetically modified) animals before forwarding steps/stages of the risk assessment process. In this context, we planned and conducted the present small-sized study simply aiming at an identification of a carcinogenic hazard of MWCNT in intact rodents, before starting a more detailed dose-dependent study that is now being executed in our laboratories.

MATERIALS AND METHODS

Ethical consideration of the experiments

An experimental protocol was approved by the Experiments Regulation Committee and the Animal Experiment Committee of the Tokyo Metropolitan Institute of Public Health prior to its execution and monitored at every step during the experimentation for its scientific and ethical appropriateness, including concern for animal welfare, with strict obedience to the National Institutes of Health Guideline for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals and other similar laws, guidelines, rules and *etc.* provided domestically and internationally.

Animals

A total of 22 male Fischer 344 DuCrlCrlj rats were purchased at their age of 4 weeks old from Charles River Inc. (Kanagawa, Japan). Rats were housed individually in stainless-steel cages (220 x 200 x 160, in millimeter) with wire-netting fronts and floors. The cages were suspended from belt-type racks with an automatic water-supply system providing tap water. The animal room was air-conditioned as 24-25°C, 50-60% relative humidity, and 10 times ventilation per hour using air drawn into the animal room by passing through a filter at the efficiency of 99.9% (HEPA filter). Fluorescent lighting was controlled to give a 12-hr light (6:00-18:00)/dark cycle. After an 8-week acclimation, rats were used for experimentation at their age of 12 weeks old, when the average body weight was 235 g. Animals were given tap water and a CE-2 pellet diet (Clea Japan Inc., Tokyo, Japan) *ad libitum*, critically monitored to detect any clinical signs and deaths, and weighed weekly throughout the acclimation and experimental periods.

Test chemicals

The presently utilized test chemicals, MWCNT (MITSUI MWCNT-7; lot number, 060125-01k) and UICC-

grade crocidolite (stocked at the Tokyo Metropolitan Institute of Public Health) were exactly identical to those used in the *p53* gene-deficient mice study of Takagi *et al.* (2008a). To examine the property of MWCNT, therefore, the same methods were utilized as described by Takagi *et al.* (2008a) such that particle number per unit weight as well as width and length distribution were measured scanning electron microscopically using a 5% Triton X-100 (Qbiogene, CA, USA) suspension (1.03 mg/5 ml), while contents of elements such as iron, sulfur, chlorine, fluorine and bromine were determined by a collision type inductively coupled plasma mass spectrometer (ICP-MS 7500ce, Agilent Technologies, Inc., Santa Clara, CA, USA) and a combustion ion chromatography (AQF-100, DX-120, Dia Instrument Co., Ltd, Kanagawa, Japan).

MWCNT and crocidolite were administered to rats as suspensions in 2% carboxymethyl cellulose (CMC) (Kanto Chemical Co., Inc., Tokyo, Japan) solution at concentrations of 0.5 and 1.0 mg/ml for MWCNT and crocidolite, respectively. These suspensions as well as a vehicle 2% CMC solution were sterilized by an autoclave at 120°C for 20 min and vigorously mixed by hand-shaking immediately prior to the administration. States of MWCNT and crocidolite in the administering suspensions in 2% CMC were assessed light microscopically, and in addition a state of MWCNT in a water suspension was separately assessed using a transmission electron microscope (TEM), because CMC cannot be used as a medium for the ultrastructural assessment.

Animal treatments

Rats at an average body weight of 235 g were administered vehicle (5 animals), crocidolite (10 animals, 2.0 mg/kg body weight corresponding to 0.47 mg/rat) or MWCNT (7 animals, 1.0 mg/kg body weight corresponding to 0.24 mg/rat) by a single intrascrotal injection for which the anterior skin of the scrotum was surgically incised 2-3 mm in length under the anesthesia with pentobarbital (Nembutal; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), and suspensions of test chemicals or a vehicle solution were administered into the scrotal cavity at a volume of 2 ml/kg body weight. The doses of test chemicals were decided as described below. Rats were then maintained under critical monitroing for the maximal observation period scheduled to be 52 weeks. When rats died or became moribund in the middle of such a period, they were immediately autopsied. At the end of week 52, all surviving animals were sacrificed under light ether anesthesia by exanguination.

Pathological assessment

At autopsy, rats were macroscopically examined throughout the body including celomic cavities. All major organs and tissues, especially tumor tissues of the inner-surface of such cavities and organs suspected to be involved by tumor, were taken, fixed in 10% neutrally-buffered formalin, embedded in paraffin and processed by routine hematoxylin and eosin staining for the histological examination.

Statistical analysis

Statistical significance of intergroup difference for the tumor incidence was assessed using Fisher's exact test, and *p*-values less than 0.05 were considered significant.

RESULTS

Property of test chemicals, their state in suspensions and decision of their administrating doses

As aforementioned, the test chemicals used in the present study were exactly identical to those used in the *p53* gene deficient mice study of Takagi *et al.* (2008a), and these 2 studies utilized the same methods to determine their property. As expected, therefore, property of test chemicals of the present study was virtually the same as that described by Takagi *et al.* (2008a). Number of particles per unit weight of MWCNT was 3.55×10^8 particles/mg, while that of crocidolite was 2.93×10^9 particles/mg (Moalli *et al.*, 1987). Width of MWCNT particles formed Gaussian distribution with a peak at 90 nm, and 82% of particles belonged in a range of 70-110 nm (Fig. 1a). In the case of crocidolite, a peak of width distribution located between 110-200 nm, and 81.3% of particles belonged in a range of 30-400 nm (Moalli *et al.*, 1987). Length of MWCNT particles also formed Gaussian distribution with a peak at 2 μm, and 72.5% of particles belonged in a range of 1-4 μm (Fig. 1b). In the case of crocidolite, a peak of length distribution located between 1.1-2.0 μm, and 91.5% of particles belonged in a range of 0.1-5 μm (Moalli *et al.*, 1987). Within a certain weight of MWCNT, therefore, substantially fewer and thinner particles were present when comparing with crocidolite, while length distribution was similar for both test chemicals.

The contents of iron, sulfur and chlorine of MWCNT were 3,500, 470 and 20 ppm, respectively, whereas fluorine and bromine (the respective detection limits being 5 and 40 ppm) could not be detected. The iron content of MWCNT was thus only 1.2-1.3 hundredths of that of crocidolite, estimated to be approximately 26-29% (Roller *et al.*, 1996) or 27% (Matsuoka *et al.*, 2003; Poser *et al.*,

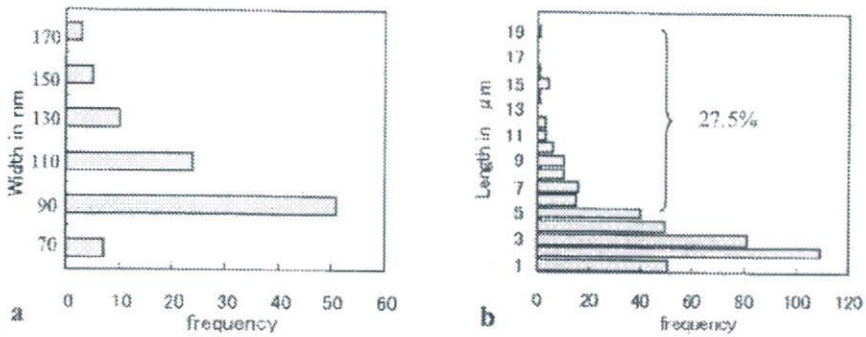

Fig. 1. Size distribution, (a) width and (b) length, of MWCNT used in the present study.

2003).

In a water suspension, the state of MWCNT was demonstrated by TEM as fine fibrous particles in occasional association with agglomerates (Fig. 2a). At higher magnifications, MWCNT particles appeared as multi-layered hollow fibers with round ends and highly electron-dense areas scattering outside of the fiber walls (Figs. 2b-d). In a CMC suspension, the state of MWCNT was light microscopically demonstrated as the coexistence of agglomerates and dispersed as multi-sized rod-shaped or fibrous particles (Fig. 2e), well in accordance with the above TEM image and suggesting the coexistence of minute fibrous particles that could not be seen under a light microscope. In contrast, crocidolite was dispersed in CMC as rod or needle-shaped particles (Fig. 2f).

Taking above-mentioned property and state in suspensions into consideration, the dose of MWCNT was decided to be 0.24 mg/animal (1 mg/kg body weight), corresponding to 0.85 x 10^5 particles/animal (3.62 x 10^8 particles/kg body weight) to be approximately 1/10 of a moderate value of the reported ranges (Roller *et al.*, 1997) corresponding to the maximum value recommended by the draft guideline for man-made mineral fibers (Bernstein and Riego Sintes, 1999). The dose of crocidolite was then decided to be 0.47 mg/animal (2 mg/kg body weight), corresponding to 13.77 x 10^5 particles/animal (58.60 x 10^8 particles/kg body weight), because we planned to make the doses of test chemicals in an equivalent range at least as a weight basis under consideration of the fact that the dose of crocidolite needs to be high enough to cause carcinogenicity (Adachi *et al.*, 2001; Cullen *et al.*, 2002; Davis, 1976; Mackay *et al.*, 1987; Vasilieva *et al.*, 1998; Wagner *et al.*, 1984; Whitaker *et al.*, 1984).

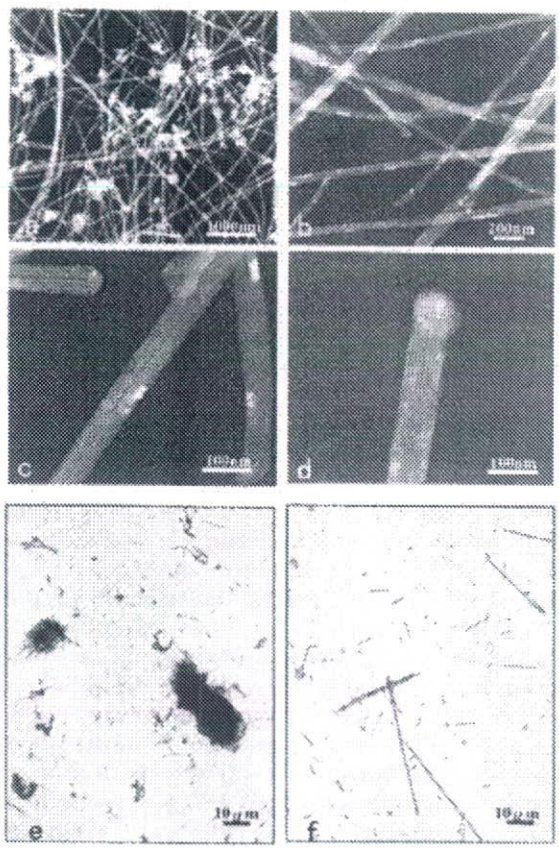

Fig. 2. Representative TEM image of a water suspension of MWCNT (a, x 10000; b, x 30000; c, x 50000; d, x 100000), and light microscopic appearance of 2% CMC suspensions of (e) MWCNT and (f) crocidolite used for the administration.

Animal experiment

General findings

The timings of autopsy are summarized in Table 1. All vehicle- and crocidolite-treated rats healthily survived throughout the 52-week maximal observation period. In contrast, 4 out of 7 MWCNT-treated rats died during weeks 37-40, 2 other rats became moribund at the ends of week 40 and week 50, and only 1 rat healthily survived until the end of week 52. In dead and moribund rats treated with MWCNT, severe anemia and enlargement of abdomen due to accumulation of ascites were commonly observed, and their body weights were decreased, or in some cases conversely increased due to marked ascites, for several weeks before autopsied. It is thus meaningless to compare group values of body weight between MWCNT-treated rats and other rats, whereas body weight values or a growth trend of crocidolite-treated animals were not different from those of vehicle-treated, control animals.

Macroscopic findings

The macroscopic findings observed in rats are summarized as an individual animal basis in Table 2. There were

Table 1. Summary of timing of autopsy

Treatment	Total number of rats	Timing of autopsy (weeks after commencement)							
		26	27	30	37	39	40	50	52
Vehicle	5								5 (S)*
Crocidolite	10								10 (S)
MWCNT	7				1 (D)	1 (D)	2 (D), 1 (M)	1 (M)	1 (S)

*Number of rats autopsied with the autopsy status specified in the parenthesis: S, scheduled sacrifice; D, sacrifice after death; and M, moribund sacrifice.

Table 2. Macroscopic findings as an individual animal basis

Treatment	Animal number	Autopsy status*	Timing of autopsy (weeks after commencement)	Findings**			
				Hemorrhagic ascites	Adhesion	Tumor nodule	
						Peritoneal cavity	Thoracic cavity
Vehicle	V1-V5	all S	all 52	-	-	-	-
Crocidolite	C1-C10	all S	all 52	-	-	-	-
MWCNT	M1	M	50	++	+++	+++	-
	M2	D	37	++	+++	+++	+
	M3	D	40	++	++	++	+
	M4	D	39	+++	+++	+++	+
	M5	S	52	-	++	+	-
	M6	M	40	+	++	++	-
	M7	D	40	+++	+++	+++	+

*S, scheduled sacrifice; D, sacrifice after death; and M, moribund sacrifice.

**Presented as grade detetmined under the following criteria.

Hemorrhagic ascite (volume): +, < 20 ml; ++, 20-50 ml; +++, > 50 ml.

Adhesion (affected area): +, < 5%; ++, 25-75%; +++, > 75%.

Tumor nodules: +, < 2 mm in diameter, focal; ++, 2-10 mm in diameter, diffuse; +++, 2-10 mm in diameter, diffuse and the presence of large tumor mass involving in the diaphragm, liver, stomach, pancreas and spleen.

no apparent macroscopic findings observed in vehicle-treated, control rats. In crocidolite-treated rats, scattered bluish-green spots were observed on the serosal surface.

Representative macroscopic appearances of MWCNT-treated rats upon autopsy are demonstrated in Fig. 3. Hemorrhagic ascites at an amount of 5-75 ml was present in the abdominal cavity of 4 dead and 2 moribund rats in association with severe fibrous adhesion of organs/tissues and the peritoneum, especially among the diaphragm, liver, stomach, pancreas, spleen and omentum. The liver was strongly deformed, resulting in difficulty to identify lobular segmentation. In such animals, whitish nodules with varied sizes and polypoid or papillary shapes were disseminated throughout the peritoneal wall including that of the scrotal cavity and occupied visceral peritoneum of organs/tissues. While the majority of such nodules were small (up to 2 mm in diameter), large tumors (5-20 mm in diameter) were occasionally observed, mostly around the diaphragm and involving the liver, stomach, pancreas, spleen and their surrounding stroma. Abdominal adipose tissues were largely replaced by tumor nodules. In the thoracic cavity, tumor nodules were observed only on the surface of the diaphragm with the exceptions of metastatic lesions detected on the peri- and epicardium detected of 4 dead animals. In the MWCNT-treated rat surviving at the end of week 52, moderate fibrous adhesions and small tumor nodules were observed on the parietal and visceral peritoneum including the wall of the scrotal cavity, but ascites were not present. In addition, small black-colored spots scattered on the peritoneum of all MWCNT-treated rats.

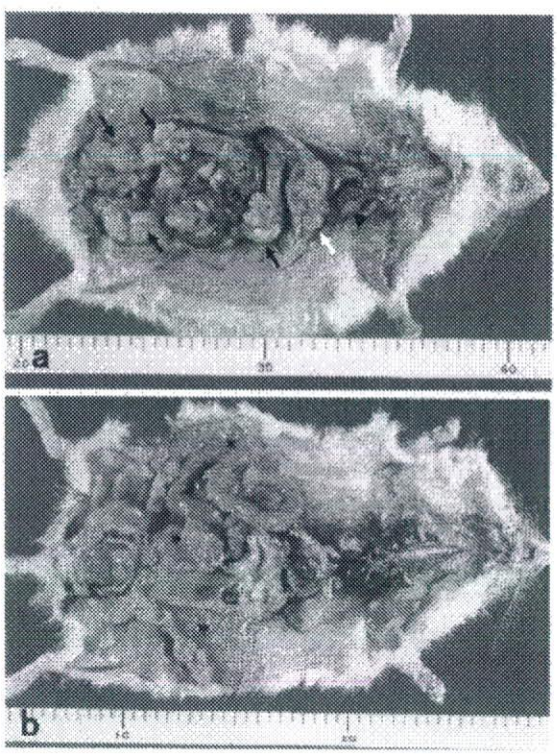

Fig. 3. Representative macroscopic appearances of rats treated with MWCNT. (a) Multiple tumor nodules were observed on the peritoneum (black arrows) and the epicardium (arrowhead), and a large tumor mass involved in diaphragm and liver (white arrow). Visceral organs were severely adhered. (b) Multiple small nodules spread on visceral and parietal peritoneum (asterisk). Black deposits of MWCNT were observed on various sites of the peritoneum.

Histological findings regarding mesothelial proliferating lesions

Histological findings for mesothelial proliferative lesions observed in rats are summarized as an individual animal basis in Table 3. While no mesothelial abnormality was found in vehicle- or crocidolilte-treated rats, mesothelial hyperplasias and mesotheliomas were observed in 7 and 6 out of 7 MWCNT-treated rats, respectively. The overall incidence of mesothelioma in MWCNT-treated rats was thus calculated to be 86%, and this value was significantly higher than those of vehicle- or crocidolite-treated rats (both 0%) ($p < 0.05$).

In the peritoneum of MWCNT-treated rats, methotelial cells were generally hypertrophic, and mesothelial hyperplasias and mesotheliomas were frequently observed. Small-sized polypoid or papillary mesotheliomas were early-stage tumors (representative histology demonstrating in Fig. 4) that grew up toward the celomic cavity and consisted of enlarged, pleomorphic mesothelioid tumor cells having nuclei with prominent nuclear membrane and nucleoli, and basophilic cytoplasm. In the central region of such tumors, necrotic tissue and/or a fibrous matrix were often present. In contrast, massive mesotheliomas were advanced-stage tumors (representative histology demonstrating in Fig. 5) that invaded into adjacent organs/tissues and destructed their architecture. These tumors were composed of 2 morphologically different portions; *id est*, the superficial layer consisting of mesothelioid tumor cells and the deep layer consisting of spindle-shaped sarcomatoid cells; and the histological transition between these portions was apparently observed. Furthermore in these large tumors, osteoid and carcifying osteoid changes were sometimes observed as a secondary, reactive phenomenon. These histological characteristics of mesotheli-

Table 3. Histological findings for mesothelial proliferating lesions as an individual animal basis

Treatment	Animal number	Autopsy status*	Timing of autopsy (week after commencement)	Mesothelial hyperplasia	Mesothelioma**		
					Development	Invasion	Osteoid change
Vehicle	V1-V5	all S	all 52	-	-	-	-
Crocidolite	C1-C10	all S	all 52	-	-	-	-
MWCNT	M1	M	50	+	+	+	+
	M2	D	37	+	+	+	-
	M3	D	40	+	+	+	+
	M4	D	39	+	+	+	+
	M5	S	52	+	-	-	-
	M6	M	40	+	+	+	-
	M7	D	40	+	+	+	+

*S, scheduled sacrifice; D, sacrifice after death; and M, moribund sacrifice.

**The overall incidence of mesothelioma in rats treated with MWCNT was 6 out of 7 (86%), significantly higher than those in rats treated with vehicle (0 out of 5, 0%) or crocidolite (0 out of 10, 0%) ($p < 0.05$).

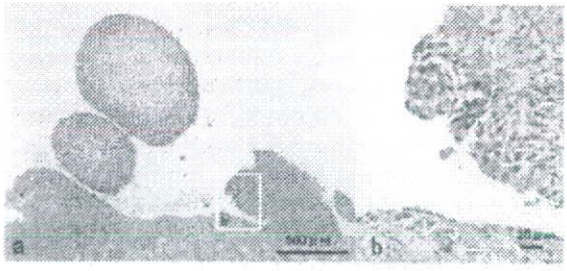

Fig. 4. Representative histology of relatively early-stage mesotheliomas observed in rats treated with MWCNT. (a) Mesotheliomas arose from the peritoneal surface with necrotic tissue and/or a fibrous matrix in its central region of nodules. (b) Under high magnification, round and basophilic mesothelioid tumor cells proliferated in the peripheral region of polypoid or papillary mesothelioma nodules.

oma were identically observed in the thoracic metastatic lesions without any continuity from the mesothelial neoplastic or non-neoplastic changes seen in the peritoneal cavity and the thoracic-side of the diaphragm.

Histological findings regarding granulomatous lesions in the celom

Granulomas were found in the celom of both crocidolite- and MWCNT-treated, but not vehicle-treated, animals (Fig. 6).

In the MWCNT-treated rats, granulomas scattered in the submesothelial layer of the fibrously thickened parietal and visceral peritoneum and were relatively large in the scrotal cavity. Such granulomas were with high cellularity including macrophages and multinucleated giant cell, and contained MWCNT agglomerates and non-agglomerated particles (Fig. 6a), indicating to be active. The distribution patterns of these granulomas and mesotheliomas were totally independent.

In the crocidolite-treated rats, granulomas were distributed similarly to the MWCNT-treated rats. The cellularity was, however, much lesser, and crocidolite was found as fine fiber-shaped particles within a rich collagenous matrix (Fig. 6b), indicating them to be almost inactive.

Histological findings regarding intraorgan distribution of test chemicals

Fibrously shaped MWCNT particles were found also within organs, for instance in the cytoplasms of portal macrophages (Fig. 7a) and Kupffer cells (Fig. 7b) of the liver, as well as macrophages and multinuclear giant cells of the mesenteric lymph nodes (Fig. 7c). Similar phenomena were found in crocidolite-treated rats infrequently and with lesser amount of particles (data not shown).

Other histological findings

Several other lesions were histologically found in rats, but their incidences, multiplicities and severity were in a

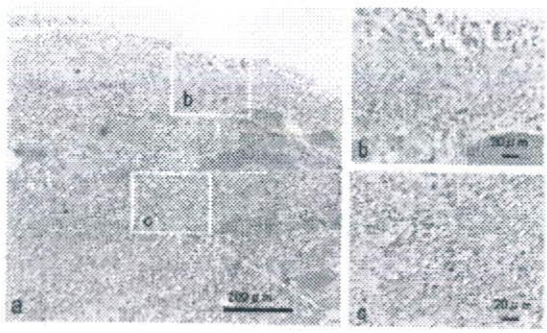

Fig. 5. Representative histology of an advanced stage mesotheliomas observed in rats treated with MWCNT. (a) Mesothelioma cells invaded and destructed the smooth muscle layer of adjacent organs/tissues (in this case, the diaphragm). Under high magnification, the tumor consisted of (b) mesothelioid cells in the surface and (c) spindle-shaped sarcomatous cells in depth.

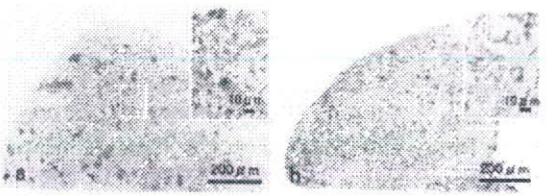

Fig. 6. Representative histology of granulomas observed in rats treated with (a) MWCNT and (b) crocidolite. Granulomas of MWCNT-treated rats were with a high cellularity and contained agglomerates and non-agglomerated particles of MWCNT (a, inset). On the other hand, granulomas of crocidolite-treated rats were with the lesser cellularity and contained fine fiber-shaped particles of asbestos within a rich collagenous matrix (b, inset).

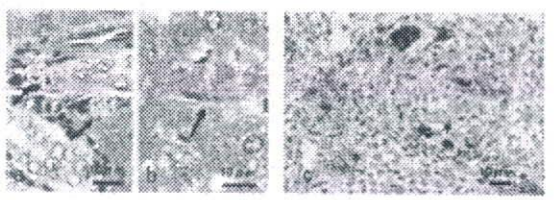

Fig. 7. Intraorgan distribution of the particles in rats treated with MWCNT. In the liver, fibrous MWCNT particles were observed in the cytoplasm of (a) macrophages present in the portal area (arrows) and of (b) a Kupffer cell present in the sinusoid (arrow). (c) MWCNT particle were detected also in the cytoplasm of multinuclear giant cells and macrophages in the mesenteric lymph node (asterisk).

similar range among 3 groups. These were thus considered as spontaneous lesions occuring without any relationship to the administration of test chemicals (data not shown).

DISCUSSION

The above data clearly indicates that MWCNT possesses carcinogenicity to cause mesothelioma at a considerably high rate in intact male rats under the present experimental conditions. Data regarding potential *in vivo* toxicity of carbon nanotubes have mostly been obtained by short-term studies featuring intracelomic, intratracheal or inhaled administration of test chemicals, simply indicating that carbon nanotubes are capable of producing inflammatory changes (Lam *et al.*, 2006; Poland *et al.*, 2008). The only long-term toxicity report available in the literature at this moment is the *p53* gene deficient mice study for MWCNT of Takagi *et al.* (2008a), and long-term toxicity of carbon nanotubes in intact animals is absolutely obscure. In this context, the present results are important and useful for the future risk assessment on MWCNT or related substances, even though this was a small-sized 1-dose study, the obtained data thus being still somewhat immature. More detailed and larger-sized studies are apparently demanded to elucidate long-term toxicity and carcinogenicity of MWCNT in intact animals, by aiming to elucidate dose-dependency and underlying mechanisms. In addition, it is especially necessary to assess whether and how MWCNT causes toxicity/carcinogenicity in intact animals when administered via human-relevant routes, and how differently MWCNT behaves according to its property and state upon exposure. These studies are now underway in our laboratories.

Histological characteristics of mesotheliomas induced by MWCNT in the present study were in good accordance with those previously published in the literature for animals and humans exposed to asbestos and other man-made mineral fibers (Adachi *et al.*, 2001; Blobel *et al.*, 1985; Davis *et al.*, 1976; Mackay *et al.*, 1987). Although the sarcomatoid portion of the advanced-stage tumors might need to be differentiated from fibrosarcoma, the differential diagnosis is not difficult, because the histological transition from the mesothelioid portion was apparently observed, and mitoses were much less than ordinary fibrosarcoma. Pleural mesothelioma lesions are conceived to be distant metastatic lesions, because peritoneal lesions/changes (inflammatory changes, fibrous thickening, granuloma, effusion, mesothelial hypertrophy and mesothelial hyperplasia) were absent in the pleura, except at the diaphragm, and pleural tumors were sufficiently distant from

the diaphragm lacking macroscopic or histological continuity from the peritoneal cavity and the diaphragm.

The present study, as well as the *p53* gene-deficient mice study (Takagi *et al.*, 2008a), was conducted to identify a potential hazard of MWCNT, and mechanistic assessments were not performed. Mechanisms underlying carcinogenicity of MWCNT are thus still obscure. Significant relation has been indicated between the size of substances and their tumorigenicity in the case of asbestos and other man-made mineral fibers, and number of such fibers must reach a sufficient level to cause chronic activation of inflammatory cell, genotoxicity, fibrosis and cancer in the target tissue (Davis, 1986, 1988 and 1989; Kamp, 1992; Miller *et al.*, 1999; Mossman and Churg, 1998; Kane, 1996; Pott *et al.*, 1987; Stanton *et al.*, 1981). In the case of MWCNT, the thinner and longer fibers are, the stronger the magnitude of asbestos-like inflammatory response is, when intraperitoneally administered (Poland *et al.*, 2008). In the present study, MWCNT had an average width of about 100 nm, and its length ranged between 100-20,000 nm, among which considerably long fibers with the 5,000 - 20,000 nm length occupied 27.5%. As aforementioned and also described by Takagi *et al.* (2008c), it is likely that dispersed and free fibrous particles are present and can also continuously come off agglomerates. Furthermore, large masses of MWCNT agglomerate present in the administering suspension are supposed to be trapped within the scrotal cavity before entering the peritoneal cavity at least partly and anyway in both cavities segregated from mesothelia by the granuloma formation. The supportive data obtained in the present study includes the presence of inflammatory changes throughout the peritoneal cavity, the lack of direct relationship between the granuloma formation and the mesothelioma development, and the detection of fibrous particles and small agglomerates of MWCNT in peripheral and resident macrophages or macrophage-oriented multi-nuclear giant cells in the liver and lymph nodes. The last data also suggest the circulatory spread of MWCNT, another important issue to be carefully assessed. Peritoneal mesothelia may thus be exposed to a sufficient amount of thin and long fibrous MWCNT particles that affect the peritoneum as a whole to make diffuse mesothelial hypertrophy and may introduce the environment sensitive for further carcinogenic stimuli in the region. It is conceivable that mesothelial hyperplasias are induced from some of such generally affected mesothelial cells receiving promoting stimuli by chance, and mesotheliomas are then developed from some of such preneoplastic lesions receiving progressive stimuli by chance.

It has been proposed that the exposure to MWCNT causes chronic inflammation in which frustrated macrophages, mediators derived from such macrophages or other sources and oxidative stress are involved, and that these play major roles in the toxicity/carcinogenicity of MWCNT, similar to the case of asbestos and other man-made mineral fibers (Poland *et al.*, 2008; Shukla *et al.*, 2003; Takagi *et al.*, 2008a, 2008b and 2008c). Active granulomas possibly containing frustrated macrophages observed in MWCNT-treated rats in the present study may be participated in such chronic inflammation and then secondary involved in the carcinogenicity as a source of inflammatory mediators including reactive oxygen or nitrogen oxide species and cytokines. Peripheral and resident macrophages as well as multi-nuclear giant cells in organs/tissues containing MWCNT may also serve as frustrated macrophages. Iron has been believed to play a crucial role in the pathogenesis of asbestos-induced diseases, by acting as a major catalyst in oxidative stress reactions (Shukla *et al.*, 2003). In the present study, however, the iron content of MWCNT was only 1.2-1.3 hundredths of that of crocidolite (Matsuoka *et al.*, 2003; Poser *et al.*, 2003; Roller *et al.*, 1996). While Lam *et al.* (2006) described that single-wall carbon nanotube containing 2,300 ppm, a little less than the iron content of the presently utilized MWCNT, induces oxidative stress and inflammatory reactions in the lung when intratrachally administered, roles of iron in carcinogenicity of MWCNT should be clarified in the future.

Comparing the present study with the *p53* gene deficient mice study of Takagi *et al.* (2008a), there are 3 clear differences regarding the route to administer test chemicals, the detection of crocidolite's carcinogenicity and the dose of test chemicals. We administered test chemicals by an intrascrotal injection, not by an ordinary intraperitoneal injection used by Takagi *et al.* (2008a), in order to increase sensitivity. The background is based on the fact that in male Fischer 344 rats mesotheliomas are spontaneously developed from the tunica vaginalis adherent to the epididymis or the tunica albuginia of the testis, and chemically induced also specifically in the scrotum (Johnson *et al.*, 1986). Furthermore, we expect that the bursal and small space of the scrotal cavity disturbs the diffusion of test chemicals, then retains them at a relatively high level for a considerable period, and thereby causes efficient exposure in the region. The scrotal cavity of rats is, however, freely connected with the peritoneal cavity, and mesotheliomas were developed throughout the peritoneal cavity. It is thus possible that there are no essential differences in reality between intrascrotal and ordinary intraperitoneal administrations.

Crocidolite did not cause carcinogenicity in the present

study. This may be simply a matter of the dose of crocidolite. Takagi *et al.* (2008a) used 3 mg/mouse corresponding to 80.79 x 10^8 particles/mouse (Moalli *et al.*, 1987), 120 mg/kg body weight (estimating an average body weight to be 25 g) and 3516 x 10^8 particles/kg body weight, whereas we used 0.47 mg/rat corresponding to 13.77 x 10^8 particles/rat, 2 mg/kg body weight and 58.60 x 10^8 particles/kg body weight. Previous studies to show the induction of mesotheliomas by asbestos administered intraperitoneally (Adachi *et al.*, 2001; Cullen *et al.*, 2002; Davis, 1976; Mackay *et al.*, 1987) or intrathoracically (Vasilieva *et al.*, 1998; Wagner *et al.*, 1984; Whitaker *et al.*, 1984) were generally performed with higher doses and/or longer periods than those in the present study. The dose of crocidolite in the present study may thus be too low to induce mesothelioma, which is also supported by the observation of inactive granuloma in crocidolite-treated rats. The reason why we set the dose of crocidolite as was used, was in order to make it in an equivalent range with that of MWCNT at least as a weight basis. It should be noted, however, that the present results cannot be used for the comparison of the strength of carcinogenicity between crocidolite and MWCNT, because even though their weight-based doses were in a similar range, their particle number-based doses were quite different.

The presently utilized dose of MWCNT was also far lower than that of Takagi *et al.* (2008a); ours being 0.24 mg/rat, 0.85 x 10^8 particles/rat, 1 mg/kg body weight and 3.62 x 10^8 particles/kg body weight, whereas theirs being 3 mg/mouse, 10.65 x 10^8 particles/mouse, 120 mg/kg body weight and 426 x 10^8 particles/kg body weight. Takagi *et al.* (2008a) achieved the 87.5% incidence of mesotheliomas within 25 weeks, which is not so different from the incidence of 85.7% and the earliest onset at the end of week 37 of the present study. Assuming the higher sensitivity of animals (because of the genetical modification; not considering possible species difference) and the 120-times higher dose as a weight per unit body weight basis in the *p53* gene deficient mice study (Takagi *et al.*, 2008a) than in the present study, it is suggested that MWCNT is capable of exerting its carcinogenicity by its substantially low dose level. In fact, Takagi *et al.* (2008b, 2008c) preliminarily described that MWCNT seemed to induce mesotheliomas in their model even at a dose of 3 μg/mouse, 1,000 times lower than their previous dose as a weight basis. In any case, the exact dose-dependency of the carcinogenicity of MWCNT must be critically evaluated, when the data of ongoing detailed studies becomes available.

The *p53* gene deficient mice study (Takagi *et al.*, 2008a) has been faced with criticism in terms of its methodology; low relevance to supposed human situation and highly artificial conditions in a certain sense (Donaldson *et al.*, 2008; Ichihara *et al.*, 2008). It is easily imagined the present study will have to deal with a similar criticism. One must understand, however, that this is the first step of the hazard identification stage in which the presence or absence of a hazard must be assessed under the most severe exposure conditions in the most sensitive animal species/models (Takagi *et al.*, 2008a, 2008b and 2008c). Human relevant conditions must of course keep in mind but are well capable of being assessed in the later steps/stages of the risk assessment processes. Needless to say, a chemical with a serious hazard can be without a high risk, if such a hazard occurs only under the human irrelevant conditions, or a risk can be properly managed. On the other hand, the nature of MWCNT used in the *p53* gene-deficient mice study (Takagi *et al.*, 2008a), in which agglomerates were present in association with fibrous/rod-shaped particles, was another target of criticism (Donaldson *et al.*, 2008; Ichihara *et al.*, 2008). It is well known that nanomaterials can exist as either truly nanometer-scale materials, over-nanometer scale materials or their mixture, and physical, chemical and biological characteristics may differ among such different states of a particular substance, which is one of the important issues for the risk assessment of nanomaterials. Potential hazard of a newly introduced substance, however, should principally be assessed at first using its sample as is, because its exposure will occur in such a state. This is why the *p53* gene deficient mice study (Takagi *et al.*, 2008b) and the present study were performed using the MWCNT sample as is. Influence of different states, including scale, on biological effects of MWCNT should then be assessed in the later steps/stages of the risk assessment processes.

In conclusion, the present data identifies a carcinogenic hazard of MWCNT. While such a hazard was detected under the particular condition, the obtained fact will serve as one of the indispensable evidences to be used for the risk assessment crucial for not only protection and improvement of human health and welfare, but also safe and acceptable development and prevalence of MWCNT and similar upcoming materials.

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