Review

Dental Materials Journal 2011; 30(3): 245–263

Toxicity evaluations of various carbon nanomaterials

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Abstract

After the discovery of fullerene and carbon nanotubes, various carbon nanomaterials were discovered or synthesized. The carbon nanomaterials have remarkable properties, different from bulk materials with the same chemical composition, and are therefore useful for industrial applications. However, the toxicity of nanomaterials may also differ from that of the bulk materials; this difference poses a concern. The physical similarity of nanomaterials to asbestos has led to evaluations for toxicity by many researchers using various methods. In this review, we compile and compare the toxicity evaluations of each carbon nanomaterial.

Key Words: Carbon nanomaterials, Toxicity, Carbon nanotube

INTRODUCTION

Carbon is one of the most common elements, and graphite and diamond were long recognized as its allotropes. In 1985, the discovery of fullerene (a.k.a. C60, buckyball, or Buckminsterfullerene) by Kroto and Smalley was epochal as it introduced the third allotrope of carbon[1)]. Thereafter, carbon nanotubes (CNTs) were discovered by Iijima *et al.* in 1991[2)]. These new carbon nanomaterials had better properties than conventional materials and thus gained prominence. However, the toxicity of nanomaterials was concerned because of their surface area and reactivity. Especially, the physical similarity of CNTs to asbestos has led to evaluations for toxicity. In this review, the toxicity evaluations of each carbon nanomaterials using various methods were compiled and compared.

CARBON NANOMATERIALS

Fig. 1 shows typical carbon nanomaterials. Fullerene consists of more than sixty carbon atoms linked via hexagonal and pentagonal rings. Fig. 1a shows the structure of C60, which is composed of sixty carbon atoms. The existence of C60, whose diameter is as small as 0.71 nm, was predicted by Osawa[4)] in 1970, before its discovery by Kroto *et al.* A variation of fullerene, the higher fullerenes, which consist of more than 60 carbon atoms (e.g., C70, C76, C78, and C82), and the metal-encapsulated fullerenes, which encapsulate transition elements (e.g., Sc, Y, and lanthanides) in the fullerene cage, were also discovered.

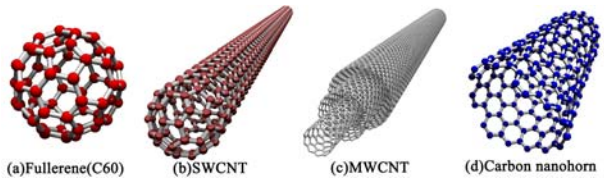

Fig. 1 Structure of various carbon nanomaterials.

CNTs are composed of a graphene sheet rolled up into a tubular structure. CNTs comprising a single layer are called single-walled carbon nanotubes (SWCNTs) (Fig. 1b), and those comprising multiple layers are called multi-walled carbon nanotubes (MWCNTs) (Fig. 1c). CNTs width ranges from a few to tens of nanometres, but their lengths range from less than a micrometer to a few millimeters. Other carbon nanomaterials were also discovered: carbon nanohorns[4)] consisting of cone-shaped tubular graphene (Fig. 1d), and carbon nanocapsules[5,6)] consisting of multi-layered polyhedrons of graphene. Fig. 2 shows the transmission electron microscope images of CNTs, bundled SWCNTs (2a), radially grown SWCNTs (2b), MWCNTs (2c), and carbon nanocapsules (2d).

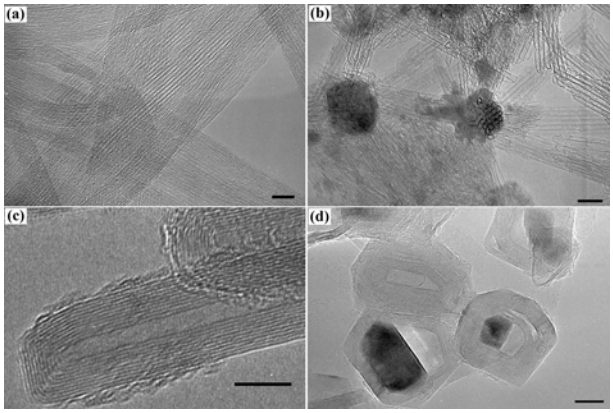

Fig. 2 TEM images of various carbon nanomaterials. (a) SWCNTs, (b) radial SWCNTs, (c) MWCNTs, and (d) carbon nanocapsules. (bar=10 nm)

TOXICITY EVALUATION OF CARBON NANOMATERIALS

The peculiar toxicity associated with nanomaterials that are different from bulk materials of the same chemical composition has been a concern. In particular, tubular materials with a high aspect ratio, e.g., CNTs, are suspected of showing asbestos-like toxicity because of their similarity in shape. Fig. 3 shows the trend of toxicity reports on carbon nanomaterials. The number of reports on the toxicity of carbon nanomaterials has increased since 2005. Initially, the reports focused mostly on fullerene and SWCNTs, but after 2008, the number

of reports on MWCNTs suddenly increased, as they are industrially useful. Details of the reports are described in the following sections. Also, reviews of the toxicity evaluation of carbon nanomaterials have been published[7-14].

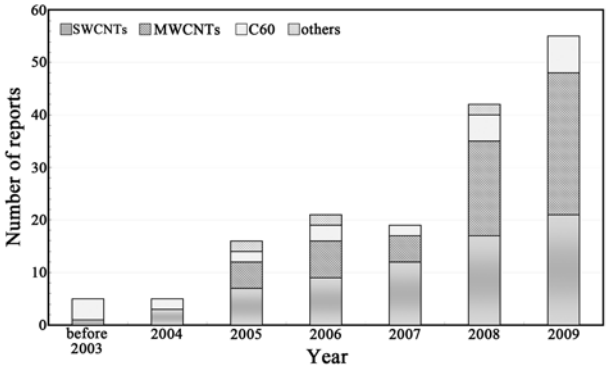

Fig. 3 Toxicity evaluation reports of carbon nanomaterials in recent years.

In this report, the following abbreviations are used.

C60: fullerene
CNTs: carbon nanotubes
SWCNTs: single-walled carbon nanotubes
MWCNTs: multi-walled carbon nanotubes
CB: carbon black
AC: activated carbon
i.p.: intraperitoneal injection
i.t.: intratracheal instillation
i.v.: intravenous injection
p.a.: pharynseal aspiration
s.c.: subcutaneous injection

Fullerene (C60)

Fullerene is the first carbon nanomaterial, and because it was studied for medical applications, its toxicity evaluations were performed from the beginning. Pure C60 is not water soluble; hence, OH groups were induced in fullerene (called "fullerol"), and its derivatives with larger additional groups were also synthesized. Table 1 shows the toxicity reports concerning fullerene and its derivatives. Cytotoxicity evaluations were conducted with various types of cells, and different results were reported by the different cells and treatments of fullerene. Jia *et al.*[20] applied non-treated C60 to macrophages and reported that the cytotoxicity of C60 was lower than that of SWCNTs and MWCNTs as shown in Fig. 4. Fiorito *et al.*[22] reported that the cellular uptake of C60 by macrophage cells was very low, as low as that of purified SWCNTs, and that their cytotoxicity was lower than that of graphite. In contrast, cytotoxic results were reported for C60 derivatives indicating that the toxicity level depended on the ligands[16-18]. In addition, it was reported that tetrahydrofuran (THF) used for the purification and dispersion of C60 remained in C60 aggregates after the treatment and enhanced the cytotoxicity[37]. These reports suggest that the specimen treatment process should also be considered in the toxicity evaluation.

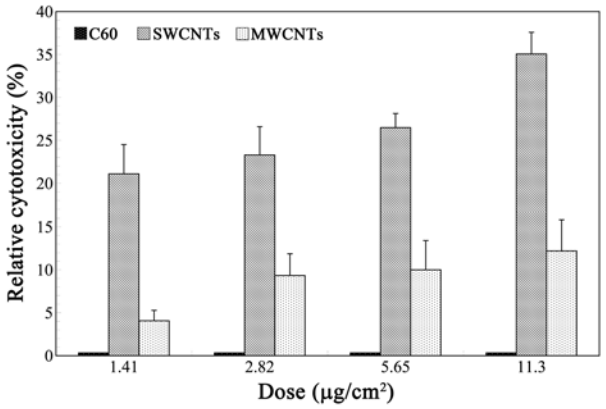

Fig.4 Comparison with cytotoxicity to alveolar macrophage among C60, SWCNTs and MWCNTs at different dosage[20].

Animal experiments on C60 toxicity were conducted using rats and fishes. Chen *et al.*[15] administered polyalkylsulfonated C60 dispersion orally, intraperitoneally, and intravenously. No lethal damage was observed by oral administration, but the median lethal dose (LD_{50}) was estimated as 600 mg/kg in intraperitoneal administration. C60 injected intraperitoneally or intravenously accumulated in the kidney and induced nephropathy. The inhalation toxicity of airborne nanomaterials and nanoparticles was also a concern; therefore, the intratracheal administration[28] or aerosol inhalation[29] was also examined. Sayes *et al.*[28] reported no lethal damage for intratracheally administered C60 and fullerol. Baker *et al.*[29] observed no gross or microscopic lesions at necropsy after inhalation of C60 aggregate aerosol. Protein concentration increased in the bronchoalveolar lavage fluid (BALF), but the toxicological effect was minimal. *In vivo* toxicological studies using fishes[19,27,32] reported significant lipid peroxidation in the brain, a decrease in the hatching rate of the zebra fish embryo, and fin malformation. Such C60 toxicity could be decreased by a dose of antioxidant, thus the damage was presumed to be caused by the generation of peroxides or free radicals. In contrast, the antioxidant effect of C60 was also reported[38], which suggests that the toxicity mechanism of C60 should be studied in detail.

Single-walled carbon nanotubes (SWCNTs)

SWCNTs were first synthesized by an arc-discharge deposit on a carbon electrode, but the mass production method of chemical vapor deposition (CVD) using hydrocarbons as the source was developed later. Metallic nanomaterials (e.g., Fe, Co, Ni, and Y) act as catalysts, and SWCNTs grow on those catalysts. Therefore, raw SWCNT products contain the metallic catalyst residue as well as amorphous carbons. The metallic catalyst residue could be removed by acid treatment, and the effect of such residues should be considered in the toxicity evaluation of SWCNTs. The toxicity reports on SWCNTs are listed in Table 2.

In the cytotoxicity evaluation of SWCNTs, human alveolar epithelial cells (A549), human keratinocyte cells (HaCaT), and macrophages are

Table 1 Toxicity evaluation of fullerene (C60)

Material	Method	Concentration	Result	Author	Ref.
polyalkylsulfonated C60	Animal exp.(rat)	0, 2500 mg/kg（oral） 0-1000 mg/kg(ip) 0-100 mg/kg（iv）	Nontoxic for oral administration. LD_{50} for i.p. injection was 600 mg/kg	Chen (1998)	15
dendritic and malonic C60	Cytotoxicity（human T-lymphocyte）	50 μM	Dendritic C60 inhibited cell growth, malonic C60 had light effect.	Rancan (2002)	16
water soluble C60	Cytotoxicity（HepG2, LLC-PK1, MCF-7）Hemolysis assay	0-80 μM(hemolysis assay）	Polycationic pyrrolidine derivative of C60 indicated cytotoxicity and hemolytic activity.	Bosi (2004)	17
water soluble C60(C60(OH)24 etc.)	Cytotoxicity（HepG2, HDF）	0-2400 ppb	Increase of water soluble functional group on C60 decreased cytotoxicity.	Sayes (2004)	18
C60	Animal exp.（largemouth bass）	0.5, 1 ppm	Significant lipid peroxidation was found in brain and gill.	Oberdörster (2004)	19
C60, SWCNTs, MWCNTs	Cytotoxicity（guinea pig alveolar macrophage）	0-226 μg/cm^2	C60 showed no cytotoxicity. Cytotoxicity of SWCNTs was higher than MWCNTs.	Jia (2005)	20
$C60(OH)_{24}$	Cytotoxicity（HepG2, HDF, NHA）	0.24-2400 ppb	LC50=2-50 ppb（depend on cell type）	Sayes (2005)	21
C60, SWCNTs, graphite	Cytotoxicity（murine macrophage cell line:J 774）	15-60 μg/ml	No cytotoxicity for C60, SWCNTs	Fiorito (2006)	22
C60	Cytotoxicity（human monocyte macrophage）	0-10 μg/ml	Cytotoxicity of C60, SWCNTs was lower than graphite.	Porter (2006)	23
$C60(OH)_{24}$	Cytotoxicity（human vascular endothelial cell）	0-100 μg/ml	Cytotoxicity >100 μg/ml	Yamawaki (2006)	24
C60-based amino acid	Cytotoxicity（HEK）	0-0.4 mg/ml	IL-1β, IL-6, IL-8 activities increased >0.04 mg/ml of C60 derivative.	Rouse (2006)	25
C60, $C60(OH)_n$	Cytotoxicity(L929, C6, U251)	C60 : 0.01-1 μg/ml C60(OH)n : 10-1000 μg/ml	LC50 of C60: 0.25 μg/ml LC50 of C60(OH)n: 800-1000 μg/ml	Isakovic (2006)	26
C60, $C60(OH)_{16–18}$	Animal exp.（zebrafish embryo）	C60 : 1.5 mg/ml $C60(OH)_{16-18}$: 50 mg/ml	$C60(OH)_{16-18}$: no toxicity C60 : embryo and larval development delayed C60 toxicity was mitigated by antioxidant.→Free radical-induced toxicity was suggested.	Zhu (2007)	27
C60, $C60(OH)_{24}$	Animal exp.（rat, i.t.）	0.2-3 mg/kg	C60, $C60(OH)_{24}$ produced transient inflammation and no significant lung toxocity.	Sayes (2007)	28
C60 aggregate（55 nm, 0.93 μm）	Animal exp.（rat, aerosol inhalation）	2.22, 2.35 mg/m^3	No change was observed by both diameter of C60 aggregates. Protein concentration in BALF was increased by smaller aggregate. Lung half-lives were 26, 29 days.	Baker (2008)	29
C60	Cytotoxicity（CHO, MDCK）	0-113.7 mg/L	LD50=33 mg/L	Han (2008)	30
$C60(OH)_{22-26}$	Cytotoxicity（HLE B-3）	0-50 μM	No cytotoxicity under dark condition. Phototoxicity observed under UV irradiation with 5 μM of C60.	Roberts (2008)	31
C60, $C60(OH)_{16–18}$	Animal exp.（zebrafish embryo）	0-300 ppb	Mortality and the incidence of fin malformations and pericardial edema increased >200 ppb in dark. Light activated toxicity.	Usenko (2008)	32
serum albumin complexed with tris(dicarboxymethylene) C60	Cytotoxicity（LY80）	100 μM	No cytotoxicity in dark, but cell numbers reduced by half with visible light irradiation.	Qu (2008)	33
SWCNTs, MWCNTs C60, graphite	Microbial cytotoxicity（*E.coli, P.aeruginosa, B.subtilis, S.epidermis*）	Cell culture on carbon nanoparticle-coated filter	SWCNTs: highest cytotoxicity for all bacteria. C60: toxic for *E.coli* and *P.aeruginosa* . Cytotoxicity of MWCNTs and graphite was moderate.	Kang (2009)	34
liposome-C60 complex	Photo-toxicity（Balb/3T3）, Mutagenicity（*S. typhimurium*）	0.49-1000 μg/ml	No cytotoxicity and mutagenicity under UV irradiation.	Kato (2009)	35
C60, CB, SWCNTs, MWCNTs	Animal Exp.(*Drosophia*)	larval: 0, 100, 1000 μg/g in food adult: culture in dry particle filled capsule	larval=no toxicity for larval; adult=reduce locomotor function & survivorship by CB, SWCNTs but low toxicity for C60 & MWCNTs.	Liu (2009)	36
C60, $C60(OH)_{18}$	Cytotoxicity（RAW264.7）	5 μM	THF treated C60, $C60(OH)_{18}$ generated cytotoxicity.→Cytotoxicity is related to the residual THF.	Kovochich (2009)	37

Table 2 Toxicity evaluation of single-walled carbon nanotubes (SWCNTs)

Material	Method	Concentration	Result	Author	Ref.
CNTs containing soot	Animal exp.(guinea pig, i.t.)	5 mg/guinea pig	no toxicity	Huczko (2001)	39
CNTs containing soot	Animal exp.(rabbit eye instillation, human patch test for skin)	not shown	no toxicity	Huczko (2001)	40
SWCNTs(Fe)	Cytotoxicity(human keratinocyte(HaCaT))	0.06-0.24 mg/ml	Cell viability decreased with 0.24 mg/ml.	Shvedova (2003)	41
FITC-labeled SWCNTs	Cytotoxicity(human fibroblast 3T6, murine fibroblast 3T3)	1-10 μM	Cell survival rate was 90% at 5 μM and 20% at10 μM.	Pantarotto (2004)	42
SWCNTs(1.4 nmφ, L>1 μm; amorphous carbon, Ni, Co), quartz	Animal exp.(mouse, i.t.), Cytotoxicity(lung parenchymal cell)	0-5 mg/kg	SWCNTs produced non-dose-dependent granulomas and transient inflammation.	Warheit (2004)	43
SWCNTs(Ni, Fe), purified SWCNTs, CB, quartz	Animal exp.(mouse, i.t.)	0.1, 0.5 mg/mouse	SWCNTs induced granulomas and inflammation dose-dependently and stronger than quartz.	Lam (2004)	44
SWCNTs(1 nmφ, 1 μmL)	Cytotoxicity(mouse macrophage-like cell)	3.8 μg/ml	Uptake of SWCNTs was visualized with NIR fluorescence.	Cherukuri (2004)	45
functionalized SWCNTs (1-5 nmφ, 0.1-1 μmL)	Cytotoxicity(HL60, 3T3, Chinese hamster ovary cell)	0.05 mg/ml	The functionalized SWCNTs were uptaken to the cells without toxicity by endocytosis.	Kam (2004)	46
SWCNTs	Cytotoxicity(HEK293)	0.78-200 μg/ml	SWCNTs decreased cell proliferation and adhesion in dose-dependent. SWCNTs induce G_1 arrest and apoptosis.	Cui (2005)	47
SWCNTs(Fe) CB, SiO_2	Animal exp.(mouse, i.t.)	0-40 μg/mouse	SWCNTs induced granulomas and inflammation dose-dependently and stronger than quartz and CB.	Shvedova (2005)	48
SWCNTs	Cytotoxicity(human keratinocyte(HaCaT), HeLa, A549, L1299)	0-20 μg/ml	SWCNTs showed cytotoxicity more than 0.5 μg/ml and activate NF-kB pathway.	Manna (2005)	49
SWCNTs(1.4 nmφ, L≒1 μm) MWCNTs(10-20 nmφ, L=0.5-40 μm), C60	Cytotoxicity(Guinea pig alveolar macrophage)	0-226 μg/cm^2	Cytotoxicity of SWCNTs was observed >0.38 μg/cm^2. Cytotoxicity of MWCNTs was lower than SWCNTs.	Jia (2005)	20
C60, SWCNTs, Graphite	Cytotoxicity(murine macrophage cell line:J 774)	15-60 μg/ml	C60 and SWCNTs showed no cytotoxicity.	Fiorito (2006)	22
functionalized SWCNTs (f-CNTs)	Cytotoxicity(B, T lymphocyte, macrophage)	1-10 μg/ml	f-CNTs were uptaken in cells. Highly water soluble f-CNTs didn't influence the cell activity.	Dumortier (2006)	50
SWCNTs(Co, Ni)	Cytotoxicity(A549, ECV304, NR8383)	50 μg/ml	Cytotoxic effect was observed by A549 with MTT assay (50%) but no toxicity was detected by WST-1.	Wörle-Knirsch (2006)	51
functionalized SWCNTs	Cytotoxicity(HDF)	0-2 mg/ml	Degree of functionalization increased, f-CNTs became less cytotoxic. f-CNTs were less cytotoxic than surfactant stabilized SWCNTs.	Sayes (2006)	52
SWCNTs(2 nmφ, 500 nmL), MWCNTs(50 nmφ, 5 μmL), CB, AC	Cytotoxicity(HDF)	0-100 μg/ml	Cell survival rate: MWCNTs>CB>AC>SWCNTs	Tian (2006)	53
SWCNTs	Animal exp.(rabbit, i.v.)	75 μg/rabbit	After 24 hrs., SWCNTs concentrated in liver, but no acute toxicity.	Cherukuri (2006)	54
SWCNTs(Ni, Y), Crocidolite	Cytotoxicity(MSTO-211H)	7.5-30 μg/ml	Suspended SWCNT-bundles were less cytotoxic than agglomarated SWCNTs and asbestos.	Wick (2007)	55
SWCNTs(0.4-1.2 nmφ, Fe=2.3 mg/g)	Cytotoxicity(V79) Ames test	0-96 μg/cm^2	Cell viability decreased to 70% with 96 μg/ml, but no mutations were observed.	Kisin (2007)	56

Material	Method	Concentration	Result	Author	Ref.
SWCNTs, AC	Cytotoxicity(smooth muscle cell)	0-0.1 mg/ml	SWCNTs surpressed the cell proliferation higher than 0.05 mg/ml, but AC showed no surpression.	Raja (2007)	57
SWCNTs 2 types ・0.8-1.2 nmφ(Fe) ・1.2-1.5 nmφ(Ni, Y) CB	Cytotoxicity(HaCaT, BEAS-2B, A549)	0-400 μg/ml	EC50 was depend on cell type. EC50 for A549 was higher than 400 μg. HiPco® SWCNTs were more reactive.	Herzog (2007)	58
SWCNTs, MWCNTs CB, quartz	Cytotoxicity(A549, rat alveolar macrophage; NR8383)	5-100 μg/ml	No acute toxicity. Purified SWCNTs didn't generate intracellular reactive oxygen species→effect of metal traces.	Pulskamp (2007)	59
SWCNTs(purified; 1-4 nmφ)	Animal exp.(mouse, p.a.)	0, 40 μg/mouse	Vitamin E-deficient mouse showed higher sensitivity to SWCNT-induced accute inflammation and enhanced profibrotic responses.	Shvedova (2007)	60
SWCNTs, MWCNTs, graphite	Cytotoxicity(SaOS2)	Cell culture on carbon nanoparticle-coated filter	Cell proliferation : SWCNTs＞MWCNTs＞graphite	Aoki (2007)	61
SWCNTs	Cytotoxicity(HepG2)	10 μg, 1 mg/ml	Depletion of micronutirients (folate) adsorbed on SWCNTs caused the cytotoxic effect.	Guo (2007)	62
SWCNTs, diesel exhause particles(DEP), TiO_2	Cytotoxicity(neonatal rat ventriicular cardiomyocytes: NRVM)	SWCNTs:0.25-50 μg/ml	SWCNTs showed low toxicity than DEP and TiO_2.	Helfenstein (2008)	63
SWCNTs(L<500 nm)	Bacteria cytotoxicity(*T. thermophila*)	0.9-14.6 μg/ml	SWCNTs inhibited bacterivory > 3.6 μg/ml.	Ghafari (2008)	64
SWCNTs(PEG modified)	Animal exp.(mouse, i.v.)	100 μM	SWCNTs persisted in liver and spleen for 4 months without apparent toxicity.	Schipper (2008)	65
SWCNTs(Fe, Ni+Y)	Cytotoxicity(A549)	0-0.8 mg/ml (suspension and filtered medium)	Cell viability was decreased by >0.1 mg/ml of SWCNTs suspension and >0.8 mg/ml of tis filtered medium.	Casey (2008)	66
SWCNTs, graphite	Animal exp.(mouse, i.v.)	40 μg-1 mg/mouse	SWCNTs were accumulated in lung, but low inflammation, no change in immunological indicators and no apoptosis was observed→low toxicity	Yang (2008)	67
SWCNTs(Ni, Y)	Cytotoxicity(chicken neuronal, glial cell)	0-30 μg/ml	Neural cell numbers surpressed by 30 μg/ml of SWCNTs, but no effect on neurite outgrowth. Agglomerated SWCNTs were more cytotoxic.	Belyanskaya (2009)	68
SWCNTs(8 nmφ, L<5 μm) CB, SiO_2, ZnO(10-20 nmφ)	Cytotoxicity(Primary mouse embryo fibroblast)	5-100 μg/ml	SWCNTs were moderately cytotoxic than ZnO, but induced more DNA damage.	Yang (2009)	69
SWCNTs, MWCNTs C60, graphite	Microbial cytotoxicity(*E.coli, P.aeruginosa, B.subtilis, S.epidermis*)	Cell culture on carbon nanoparticle-coated filter	SWCNTs showed highest cytotoxicity for all bacteria. C60 was toxic for E.coli and P.aeruginosa. Cytotoxicity of MWCNTs and graphite was moderate.	Kang (2009)	34
C60, CB, SWCNTs, MWCNTs	Animal Exp.(*Drosophia*)	larval: 0, 100, 1000 μg/g in food adult: culture in dry particle	larval=no toxicity for larval; adult=reduce locomotor function & survivorship by CB, SWCNTs but low toxicity for C60 & MWCNTs.	Liu (2009)	36
SWCNTs, CB (pristine, acid-functionalized(AF))	Animal exp.(mouse, inhalation)	10, 40 μg/mouse	40 μg of AF-SWCNTs and CB increased pulmonary neutrophils. Acid functionalization increased pulmonary toxicity.	Tong (2009)	70
SWCNTs(0.8-1.2 nmφ, 0.1-1 μm), MWCNTs (80 nmφ, 10-20 μm), CB	Animal exp.(mouse, p.a.)	40 μg/mouse	CNTs activated the lung and systemic acute response.	Erdely (2009)	71
SWCNTs(4 nmφ, 0.5-100 μm), MWCNTs(15 nmφ, 0.5-200 μm), CB	Animal exp.(mouse, s.c., intranasal injection)	200, 400 μg/mouse	IgE in serum and inflammatory cells in BALF were increased by CNTs. MWCNTs were toxic than SWCNTs.	Nygaard (2009)	72

widely used because of their relationship with respiratory, dermatological, and immunological toxicity. The studies examined cell types and estimation methods, and the results varied with the purity of SWCNTs. The effect of the residual metals in SWCNTs was reported by Pulskamp *et al.*[59]: the purified (catalytic metal removed) SWCNTs showed a low generation of reactive oxygen species. Wörle-Knirsch *et al.*[51] reported that the MTT assay of A549 showed a decrease in cell viability upto 40% after adding SWCNTs, but no viability decrease was observed by WST-1 assay as shown in Fig. 5. The difference suggested that the toxicity representation depended on the assay method. Pertaining to the dependence on cell types, Herzog *et al.*[58] reported that EC_{50} (50% reduction concentration in cell viability) of SWCNTs for A549 was higher than 400 μg/ml, which is tens of times higher than those for human bronchial epithelial cells (BEAS-2B) and HaCaT. SWCNTs are less soluble in water than C60, and surface modification improves water solubility and guides the functional modification with organic ligands. Dumortier *et al.*[50] studied the toxicity of various derivatives of SWCNTs with B and T lymphocytes and macrophages. Highly water soluble modified SWCNTs were taken up into the cells without affecting cell viability, which suggested that the low agglomeration of modified SWCNTs caused low toxicity. In previous reports, cellular uptake of SWCNTs was also observed by fluorescence microscopy[45,46], but the uptake of SWCNTs was not related to cytotoxicity. Surfactants are also widely used to improve the water solubility of SWCNTs. Wick *et al.*[55] demonstrated that SWCNTs dispersed with a surfactant had a suppressed cytotoxicity. Therefore, the agglomeration of SWCNTs would strongly affect cytotoxicity.

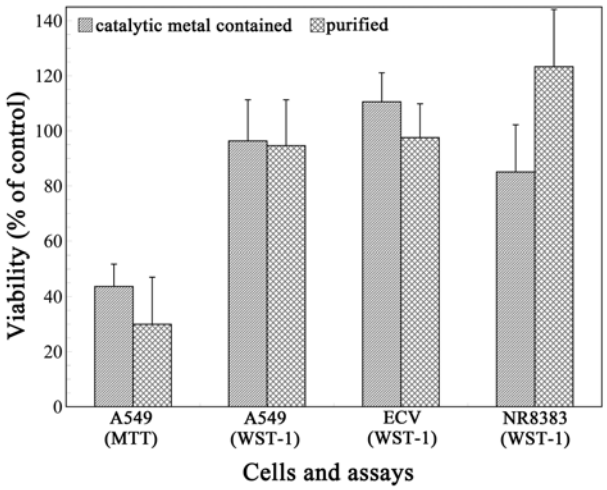

Fig.5 Comparison with various cell viability estimated with MTT and WST-1 assay. Cells were incubated for 24 h with SWCNTs (50 μg/ml)[51].

In animal toxicity evaluations, Huczko *et al.*[39,40] first reported that SWCNTs did not induce any abnormalities by intratracheal instillation, eye instillation, and the patch test for skin. However, neither the properties of tested SWCNTs nor the physiological representations were reported. Warheit *et al.*[43] evaluated the acute lung toxicity of intratracheally instilled SWCNTs in rats. SWCNTs produced non-dose-dependent series of multifocal granulomas. In the BALF biomarkers and lung cell proliferation tests, quartz particles produced a significant increase in pulmonary inflammation and cytotoxicity, but SWCNTs produced transient inflammation and cytotoxicity. However, Lam *et al.*[44] reported that SWCNTs induced dose-dependent epithelioid granulomas and interstitial inflammation stronger than those induced by quartz in a similar evaluation. The toxicity of SWCNTs occurred irrespective of whether it contained the residual catalytic metals or not. Shvedova *et al.*[60] demonstrated that vitamin E-deficient mice were associated with a higher sensitivity to SWCNT-induced acute inflammation and enhanced profibrotic responses. Vitamin E is generally known as the major antioxidant, and this report suggested that the toxicity of SWCNTs was caused by oxidative stress and was reduced by the antioxidant protection by vitamin E. Intravenously injected SWCNTs were mainly concentrated in the liver and spleen, but the absence of acute toxicity in the target organs was reported[54,64].

Multi-walled carbon nanotubes (MWCNTs)

The number of toxicity evaluations for MWCNTs has increased in recent years, as shown in Table 3. The mass production process using catalytic chemical vapor deposition (CCVD), developed by Endo *et al.*[99], reduced the cost of MWCNTs drastically, and the application of MWCNTs in industry, e.g., in batteries, was realized. As a result, the toxicity evaluation of MWCNTs became significant.

MWCNTs comprise several layers of graphene tubes stacked in concentric circles, and their diameter is tens of nanometers, which is thicker than SWCNTs. Fig. 6 shows the SEM images of MWCNTs with different diameters. Thin MWCNTs with 20 nm diameter (Fig. 6(a)) appeared flexible, while thick MWCNTs with 100 nm diameter (Fig. 6(b)) appeared needle shaped and different from the thin MWCNTs. In the toxicity evaluation of MWCNTs, such differences in diameter and shape should be addressed.

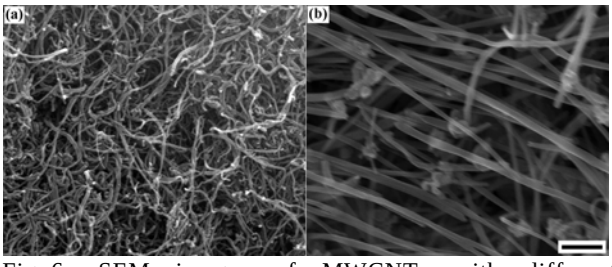

Fig. 6 SEM images of MWCNTs with different diameters. (bar=500 nm)

In the cytotoxicity tests, a dose-dependent decrease in the viability or survival rate was reported[74,78]; whereas, opposite results, such as lower toxicity of MWCNTs compared to that of

SWCNTs[19,53] or no cytotoxicity of MWCNTs[81,82], were also reported. One reason for this difference was that the variation in shape and size of MWCNTs affected water dispersivity, agglomeration, and their effect on the cells. Bottini *et al.*[80] and Vittorio *et al.*[95] reported that purified and surface oxidized MWCNTs with acid treatment suppressed cell viability. Wang *et al.*[96] reported that MWCNTs with smaller diameters showed less cytotoxicity. These results suggest that the cytotoxicity of MWCNTs was strongly affected by their size, purity, and surface conditions. Pertaining to the dependence of toxicity on cell types, Soto *et al.*[86] reported that macrophages showed similar EC_{50} for MWCNTs, asbestos (chrysotile), and CB; in contrast, human lung epithelial cells (A549) showed low EC_{50} for MWCNTs compared with asbestos and CB as shown in Fig. 7. Simon-Deckers *et al.*[88] also reported higher cytotoxicity for MWCNTs compared with oxide particles, and cellular uptake of MWCNTs was observed. Thus, the toxic effect of MWCNTs for the respiratory system should be an area of concern.

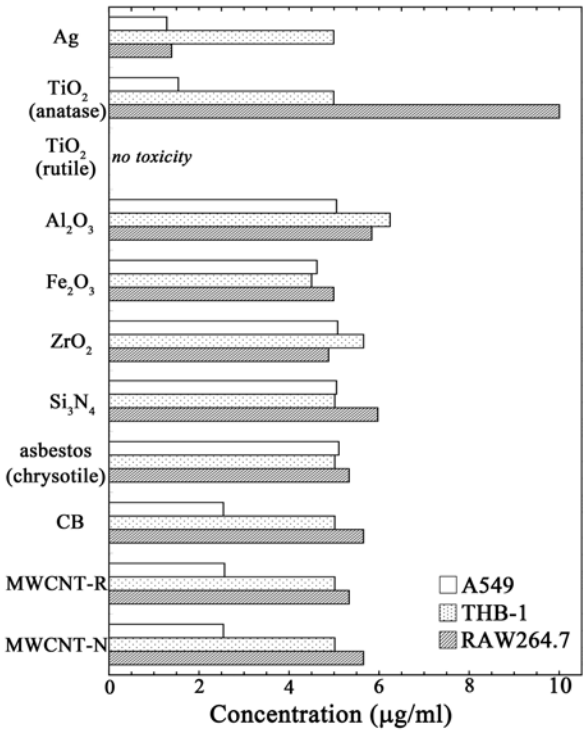

Fig. 7 EC_{50} of various nanoparticulate materials for human epithelial cell (A549), human macrophage (THB-1) and murine macrophage (RAW264.7)[86]. MWCNT-N has smaller diameter (5-30 nm) and larger specific surface area (218 m^2/g) than MWCNT-R (10-30 nm, 16 m^2/g).

In the toxicity evaluation of MWCNTs, many reports compared MWCNTs with asbestos because of their similarity in shapes[76,77,86,87,91]. Muller *et al.*[76] reported that intratracheally instilled MWCNTs in rats remained in the lungs after 60 days and induced inflammation, fibrotic reactions, and granulomas. The tissue reaction and cytotoxicity of MWCNTs were similar to those of chrysotile. Poland *et al.*[87] reported the effect of fiber length on toxicity. As shown in Fig. 8, unlike short fibers, long fibers of MWCNTs and amosite induced inflammation and granulomas in the abdominal cavity; thus, the tissue response was dependent on the length of such fibrous materials. Takagi *et al.*[91] administered MWCNTs and crocidolite intraperitoneally to p53 heterozygous mice, which are sensitive to asbestos, and it was observed that MWCNTs induced mesothelioma earlier than crocidolite. These reports suggest that MWCNTs have a toxicity similar to or higher than asbestos. However, MWCNTs are more cohesive than asbestos; thus, agglomerated granules of MWCNTs would be easily generated after instillation or injection into animals. In addition, the exposure to nanomaterials is different from the experimental procedures, e.g., intratracheal instillation or abdominal injection. Therefore, the above toxicity evaluations did not always show the pulmonary toxicity, which may be similar to asbestos.

In an evaluation of environmental influence, Lin *et al.*[94] evaluated the toxicity for insects (drosophila). MWCNTs showed low toxicity for larval and adult drosophila. Kang *et al.*[34] reported the microbial cytotoxicity for SWCNTs, MWCNTs, and C60, and the tendency was different between the Gram-positive and Gram-negative microbes. Lin *et al.*[94] reported that MWCNTs suppress the cell viability of plant cells (*Arabidopsis*). These results suggest that the environmental influence of MWCNTs should be a concern, especially because mass production of MWCNTs has started in recent years.

Other carbon nanomaterials

Other carbon nanomaterials, carbon nanocapsules, carbon nanofibers (CNFs), and carbon nanohorns, were evaluated for toxicity, as shown in Table 4. The authors reported on evaluation of toxicity of metal-encapsulating carbon nanocapsules (MECNCs)[100]. Decrease in cell viability and increase in LDH release increased was observed to increase higher than 1 μg/mL (ppm) of MECNCs as shown in Fig. 9a. Fig. 9b shows the effect of MECNCs on the cell proliferation of rat fibroblast. The proliferation was suppressed dose-dependently and the effect of MECNCs was the same as that of TiO_2 at 1 ppm. These effects were probably caused by the physical stimulation from the aggregated particles of the carbon nanocapsules. CNFs have a structure similar to MWCNTs. MWCNTs consist of cylindrical graphene sheets that are grown parallel to their long axis; whereas, most CNFs have graphene sheets in a direction not parallel to their long axis. Hat-stacked carbon nanotubes (H-CNFs), which have cone-shaped graphene sheets, were evaluated as having no specific toxicity in the cytotoxicity test and animal experiments[101,102]. Carbon nanohorns, which are horn-shaped graphene tubules, showed slight cytotoxicity but no mutation, and no stimulation for the eyes, skin, and respiratory tract was observed[103,104].

Table 3 Toxicity evaluation of multi-walled carbon nanotubes (MWCNTs)

Material	Method	Concentration	Result	Author	Ref.
SWCNTs (1.4 nmφ, L≒1 μm) MWCNTs (10-20 nmφ, L=0.5-40 μm), C60	Cytotoxicity (Guinea pig alveolar macrophage)	0-226 μg/cm^2	Cytotoxicity of SWCNTs was observed >0.38 μg/cm^2. Cytotoxicity of MWCNTs was lower than SWCNTs.	Jia (2005)	20
MWCNTs (5-20 nmφ, 20-40 μmL)	Cytotoxicity (SaOS2)	Cell culture on MWCNTs-coated filter	Higher cell proliferation on MWCNTs coated filter	Aoki (2005)	73
MWCNTs (200 nmφ)	Cytotoxicity (HaCaT)	0.1-0.4 mg/ml	Cell viability decreased until 70% and IL-8 release increased.	Monteiro-Riviere (2005)	74
MWCNTs (20-40 nmφ, L=220 nm, 825 nm)	Cytotoxicity (THP-1) Animal exp.(rat, s.c. implantation)	5-500 ng/ml	Cytotoxicity of MWCNTs was lower than LPS and no length dependence; Degree of inflammatory response was slight in shorter MWCNTs.	Sato (2005)	75
MWCNTs (10 nmφ; L=0.7, 5.9 μm) CB, chrysotile	Animal Exp.(rat, i.t.) Cytotoxicity (peritoneal macrophage)	Animal exp.: 0.5-5 mg/rat Cytotoxicity: 20-100 μg/well	MWCNTs remained in lung after 60 days; induced inflammation, fibroic reaction and granulomas; similar response to chrysotile.	Muller (2005)	76
MWCNTs, SWCNTs, chrysotile, CB	Cytotoxicity (RAW264.7)	0-10 μg/ml	Cell viability decreased >2.5 μg/ml without material dependence.	Murr (2005)	77
MWCNTs, carbon nano-onion(MWCNOs)	Cytotoxicity (HSF42, IMR-90)	0.06-6 μg/ml	Cell number decreased by MWCNTs dose-dependently and increased apoptosis/necrosis.	Ding (2005)	78
SWCNTs (2 nmφ, L=500 nm) MWCNTs(50 nmφ, L=5 μm), CB, AC	Cytotoxicity (HDF)	0-100 μg/ml	Cell survival rate: MWCNTs>CB>AC>SWCNTs	Tian (2006)	53
MWCNTs (20 nmφ), CNFs (150 nmφ), CB	Cytotoxicity (lung tumor cell; H596, H446, Calu-1)	0.002-0.2 μg/ml	Decrease of cell proliferation = CB>CNFs>MWCNTs	Magrez (2006)	79
MWCNTs (20-40 nmφ, L=1-5 μm), CB	Cytotoxicity (human T lymphocytes)	40, 400 μg/ml	Oxidized MWCNTs surpressed cell viability less than 20% at 400 μg/ml and more toxic than pristine MWCNTs	Bottini (2006)	80
MWCNTs (φ= 1-3 nm, 2-4 nm, 1-5 nm)	Cytotoxicity (HUVEC)	0.5-0.9 μg/ml	No cytotoxicity	Flahaut (2006)	81
MWCNTs (Co)	Cytotoxicity (human osteoblastic line hFOB 1.19, human fibroblastic line HS-5)	25 μg/ml	Viability was slightly decreased with MWCNTs.	Chłopek (2006)	82
MWCNTs (20-40 nmφ, L=450 μm) N-doped MWCNTs (50 nmφ, L=300 μm)	Animal exp.(mouse; i.t., i.p., nasal and oral administration)	1-5 mg/kg	N-doped MWCNTs are less harmful than MWCNTs or SWCNTs.	Carrero-Sanchez (2006)	83
SWCNTs, MWCNTs CB, quartz	Cytotoxicity (A549, rat alveolar macrophage; NR8383)	5-100 μg/ml	No acute toxicity. Purified SWCNTs didn't generate intracellular reactive oxygen species→effect of metal traces.	Pulskamp (2007)	59
SWCNTs, MWCNTs, graphite	Cytotoxicity (SaOS2)	Cell culture on carbon nanoparticle-coated filter	Cell proliferation : SWCNTs＞MWCNTs＞graphite	Aoki (2007)	61
MWCNTs	Cytotoxicity (mouse ES cell)	0, 5, 100 μg/ml	MWCNTs increased mutation frequency by 2-fold.	Zhu (2007)	84
MWCNTs (50 nmφ, L=10 μm)	Animal exp.(mouse, i.t., inhalation)	intratracheal= 0.05 mg/mouse inhalation=32.61 mg/m^3	i.t. induced inflammation to the lining wall of bronchi and destruction of alveolar netted structure. Inhalation induced proliferation and thickening of alveolar walls.	Li (2007)	85

Material	Method	Concentration	Result	Author	Ref.
MWCNTs（30 nmφ, L=0.1-3 μm） chrysotile, CB, oxides	Cytotoxicity（RAW264.7, THB-1, A549）	5 μg/ml	Macrophages : Cytotoxicity of MWCNTs was similar to others; A549 : EC_{50} of MWCNTs was lower than others.	Soto (2007)	86
MWCNTs（15-100 nmφ, L=1-56 μm） amosite	Animal Exp. (mouse, peritoneal cavity injection)	50 μg/mouse	Long fiber of MWCNTs and amosite induced inflammation and granulomas in the abdominal cavity, but not induced by short fibers.	Poland (2008)	87
MWCNTs（10-160 nmφ; L<3.5 μm, <12 μm） TiO_2, Al_2O_3	Cytotoxicity（A549）	0.1-100 μg/ml	Cytotoxicity of MWCNTs was higher than oxides; Not depend on length of CNTs, but on residual metals; Cellular uptake of MWCNTs was observed.	Simon-Deckers (2008)	88
MWCNTs（11.3 nmφ, L=0.7 μm）	Animal Exp.（rat, i.t.） Cytotoxicity（MCF-7）	Animal exp.0.5, 2 mg/rat Cytotoxicity: 10-50 μg/ml	MWCNTs induced micronuclei both *in vivo* and *in vitro*.	Muller (2008)	89
MWCNTs(different surface defect)	Animal Exp.（rat, i.t.） Cytotoxicity（MCF-7）	Animal exp.: 0.5, 2 mg/rat Cytotoxicity: 10-50 μg/ml	Heat treated MWCNTs at 600℃（purification) and 2400℃（anneal the structural defects) showed low stimulation to lung	Muller (2008)	90
MWCNTs（70-170 nmφ, L=1-19 μm） crocidolite	Animal Exp.（p53+/- mouse, abdominal injection）	3 mg/mouse	MWCNTs induced mesothelioma earlier than crocidolite	Takagi (2008)	91
SWCNTs, MWCNTs C60, graphite	Microbial cytotoxicity(*E.coli, P.aeruginosa, B.subtilis, S.epidermis*)	Cell culture on carbon nanoparticle-coated filter	SWCNTs showed highest cytotoxicity for all bacteria. C60 was toxic for *E.coli* and *P.aeruginosa*. Cytotoxicity of MWCNTs and graphite was moderate.	Kang (2009)	34
MWCNTs（68 nmφ, pristine L=2-164 μm, purified L=4-65 μm）	Cytotoxicity（human monocyte-derived macrophage）	0-20 μg/ml	Cell survival rate and viability were decreased dose-dependently; MWCNTs entered into cytoplasm and nuclei were observed.	Cheng (2009)	92
MWCNTs（30-40 nmφ）	Cytotoxicity（MC3T3-E1）	Cell culture on MWCNTs-coated dish	Compared with collagen-coated dish, slightly low cell proliferation and strong cell adhesion were observed on MWCNTs-coated dish	Terada (2009)	93
MWCNTs（9.5 nmφ, L=1.5 μm）	Cytotoxicity（*Arabidopsis* T87）	0-600 mg/L	Smaller MWCNTs agglomerates decreased cell viability and SOD activity.	Lin (2009)	94
MWCNTs（40 nmφ, long=2 μm, short=0.5 μm）	Cytotoxicity（neuroblastoma; SH-SY5Y）	5-500 μg/ml	EC_{25} of Long, short, acid-treated MWCNTs were 48, 34, 18 μg/ml; Acid-treated MWCNTs (water soluble) increased cytotoxicity.	Vittorio (2009)	95
C60, CB, SWCNTs, MWCNTs	Animal exp.(*Drosophia*)	larval: 0, 100, 1000 μg/g in food adult: culture in dry particle	larval=no toxicity for larval; adult=reduce locomotor function & survivorship by CB, SWCNTs but low toxicity for C60 & MWCNTs.	Liu (2009)	36
SWCNTs（0.8-1.2 nm φ,L= 0.1-1 μm） MWCNTs（80 nmφ, L=10-20 μm）, CB	Animal exp.(mouse, p.a.)	40 μg/mouse	CNTs activated the lung and systemic acute response.	Erdely (2009)	71
SWCNTs（4 nmφ, L=0.5-100 μm） MWCNTs（15 nmφ, L=0.5-200 μm） CB	Animal exp.(mouse, s.c., intranasal injection)	200, 400 μg/mouse	IgE in serum and inflammatory cells in BALF were increased by CNTs. MWCNTs were toxic than SWCNTs.	Nygaard (2009)	72
MWCNTs	Cytotoxicity（guinea pig alveolar macrophages）	0-20 μg/ml	MWCNTs in smaller diameter showed less cytotoxicity.	Wang (2009)	96
MWCNTs（50 nmφ, L=3.9 μm）	Animal exp.(mouse, p.a.)	10-50 μg/mouse	MWCNTs induced increase of biomarkers in BALF and plumonary fibrosis.	Porter (2010)	97
MWCNTs（5-15 nmφ, L=0.1-10 μm, Al_2O_3, Fe, Co）	Animal exp.（rat, inhalation）	0-2.5 mg/m^3	>0.5 mg/m^3: glanulomatous inflammation and lipoproteinosis in lung; 0.1 mg/m^3: minimal granulomatous inflammation.	Ma-Hock (2009)	98

Table 4 Toxicity evaluation of other carbon nanomaterials

Material	Method	Concentration	Result	Author	Ref.
Metal-encapsulating carbon nanocapsules (MECNCs)	Cytotoxicity（rat fibroblast, alveolar macrophage）	0.01-10 μg/ml	Cell viability decreased and cytokines release increased more than 1 μg/ml of MECNCs addition.	Uo (2005)	100
H-CNF（Hat-stacked carbon nanofibers）	Cytotoxicity（THP-1）	5-500 ng/ml	Carboxylated H-CNF dose-dependently induced TNF-α release and NF-κB activity. Surfactant (CHAPS) treatment increased cytotoxicity.	Sato (2005)	101
H-CNF（Hat-stacked carbon nanofibers）	Animal exp.（rat, s.c. implantation）	powder implantation	No severe inflammation	Yokoyama (2005)	102
MWCNTs, Cabon nano-onion(MWCNOs)	Cytotoxicity（HSF42, IMR-90）	0.06-6 μg/ml	Cytotoxicity of MWCNTs >MWCNOs with activating different gene expression.	Ding (2005)	78
MWCNTs（20 nmφ）, CNFs（150 nmφ）, CB	Cytotoxicity（lung tumor cell; H596, H446, Calu-1）	0.002-0.2 μg/ml	Decrease of cell proliferation = CB>CNFs>MWCNTs	Magrez (2006)	79
Single-walled carbon nanohorns	Cytotoxicity（hamster lung fibroblast cell） Ames test, Animal exp.（rat, rabbit, guinea pig）	Cytotoxicity:0.31 3-2.5 mg/ml	No cytotoxicity, mutagenecity, plumonary toxicity, irritation for skin and eye.	Miyawaki (2008)	103
Single-walled carbon nanohorns	Cytotoxicity（hamster lung fibroblast cell）	0-300 μg/ml	Cell survival rate decreased >100 μg/ml.	Matsuoka (2009)	104

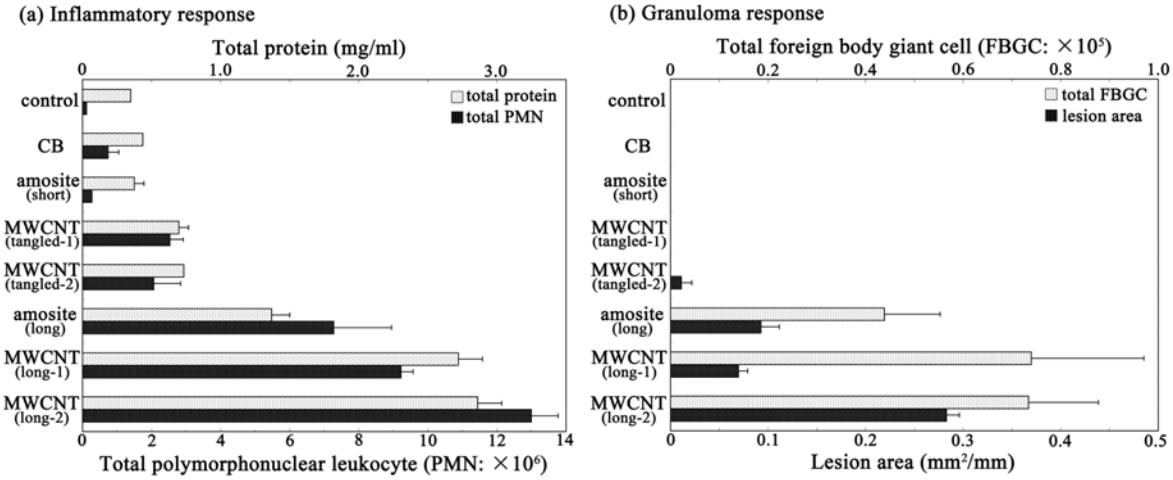

Fig. 8 Comparison with inflammatory and granuroma response for short, tangled and long fibrous materials[87]. The length of MWCNTs are tangled-1=1-5 μm, tangled-2=5-20 μm, long-1=13 μm (mean) and long-2=56 μm (max.).

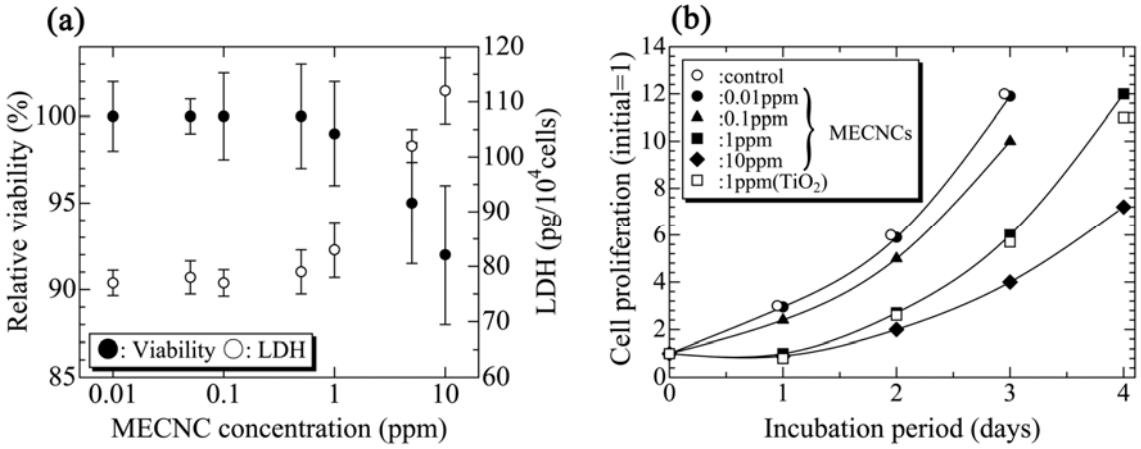

Fig. 9 (a)The cell viability and the LDH release of the rat fibroblasts for various concentrations of MECNCs; (b)The cell proliferation for various concentrations of MECNCs and TiO_2 at 1 ppm.

ACTUAL EXPOSURE OF CARBON NANOMATERIALS COMPARED TO TOXICITY EVALUATIONS

Exposure to nanomaterials usually occurs by inhalation of airborne particles. However, the experimental setup for airborne particle inhalation is difficult. Thus, alternative methods such as the intratracheal instillation of particles suspended in saline droplets have been widely used. However, to understand the difference between the experimental and actual conditions is significant. Recently, animal inhalation tests of airborne CNTs in actual conditions were reported[70,85,98]; the inhalation induced an increase of pulmonary neutrophils, thickening of alveolar walls, and granuloma formation.

Typical amounts of CNTs inhaled in routine working conditions were reported to be less than 1 μg/kg/day for SWCNTs (Maynard *et al.*[105]) and several μg/kg/day for MWCNTs (Han *et al.*[106]). In similar inhalation experiments for animals, Ma-Hock *et al.*[98] estimated the amount inhaled by rats that are exposed to 2.5 mg/m^3 of CNTs in air for 90 days to be higher than 1000 μg at the maximum. This value is several times higher than actual routine exposures, thus indicating that the above inhalation tests simulated severe exposure conditions compared to actual conditions. Therefore, it is difficult to estimate the exact toxicity of carbon nanomaterials.

CONCLUSION

Many toxicity evaluations have been conducted for various carbon nanomaterials, and different results have been reported by different methods. In the reports that suggested toxicity of carbon nanomaterials, the experimental conditions of exposure to nanomaterials often generated a higher load than actual exposures. Therefore, it is difficult to specify the exact toxicity of carbon nanomaterials. CNT's resemble asbestos in shape; therefore, a concern with CNTs is that they may have a similar toxicity. However, CNTs are quite flexible compared to asbestos, which has a needle-like shape and stiffness, and the chemical properties are also different. Hence, the toxicity of CNTs should be discussed carefully and separately from that of asbestos. Because the toxicity of carbon nanomaterials has not been deduced or disproven, these materials should be handled like any other hazardous materials under the precautionary principle, and unnecessary exposure to humans and the environment should be avoided.

ACKNOWLEDGEMENT

This work was supported by Grant-in-Aid for Challenging Exploratory Research No. 21659449, from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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