3.8. Body Size Differences between Humans and Small Animals

What should always be kept in mind in medical research is that results from animal experiments can differ from actual clinical findings.[357−360] Traditionally, small animals have been used in most animal experiments. It remains unknown whether assessments of CNTs toxicity shown in vivo in small animals are reproducible in humans, which have larger organs. In particular, the toxicity of small particulate substances has not been controversial and may be negligible as the body size increases. Conversely, the effects on finer structures of individual organs may increase the toxicity.

Differences in blood vessel thickness depending on animal body size can impact the disposition of CNTs. Most blood vessels are thicker in humans than in small animals. However, the thickness and structure of the terminal microvessels are thought to be nearly the same in different animal species. Hence, the migration of CNTs from tissue to the bloodstream and the obstruction of blood vessels by CNTs transported via the bloodstream are reproducible in small animals. For this reason, CNTs biomaterials can be deemed safer in humans because of the greater thickness of their central blood vessels, provided that no problems have been revealed by in vivo kinetic studies in small animals. Kinetic differences in the transport of CNTs (used in DDSs and imaging) through blood vessels and its dependence on animal body size must fully be taken into consideration.

Because cell size is the same in humans and small animals, the relationship between CNTs and cells and the effects of CNTs on cells are nearly the same. Therefore, even for basic body reactions to a small particulate substance, the results of animal experiments are considered to be highly representative.

Although these differences depending on animal body size may be resolved to some extent by conducting studies in larger animals such as dogs, it is difficult to maintain constant experimental conditions, making evaluation of a wide variety of CNTs impossible in large animals. As with ordinary biomaterials, for which International Standards Organization (ISO) and other standards are already available, it is reasonable to commence clinical application of CNTs biomaterials, provided that no problematic findings are obtained from assessments in small animals. It should always be borne in mind, however, that adverse reaction assessments can yield results inconsistent with findings from animal experiments.

4. PRESENT STATUS OF RESEARCH INTO IN VITRO TOXICITY OF CNTs FOR BIOMATERIALS

Cells cultured to test for inhalation toxicity can be used to assess the in vitro toxicity of CNTs biomaterials.[361−365] A large number of studies have examined the use of macrophages to test for inhalation toxicity. Because macrophages play an important role in the in vivo response to CNTs implants, inhalation toxicity data obtained using this type of cell are relevant to toxicity assessment of CNTs biomaterials.[155]

Unlike drugs and other chemical substances, CNTs are nanosized particles possessing unique properties; therefore, special cautions should be exercised when investigating CNTs in vitro. For example, because CNTs are essentially hydrophobic and insoluble in water, a surfactant must be used as a dispersant in culture experiments.[329] One article reported that the chemical properties of such dispersants altered the toxicity of CNTs.[366−371] In addition, CNTs may adsorb phospholipids and albumin in the culture broth, which are recognized by and interact with cells.[372−374] Furthermore, attention should be paid to possible reactions between CNTs and test reagents.[91,191] One study concluded that photometric methods were unsuitable because CNTs absorb light.[375−377] These factors affect the results of in vitro studies, making their interpretation difficult.

4.1. Cellular Uptake of CNTs

Cellular uptake of CNTs has been investigated in many types of cells by many researchers, and different studies have reported widely variable results. For example, SWCNTs have been reported to be absorbed by RAW264.7 cells in some studies and not in others.[340,364,372,378] Firme et al. studied the mechanism of CNTs passage (e.g., endocytosis/phagocytosis and nanopenetration) through the cell membranes of many types of cells.[91] Endocytosis is a form of active uptake of small extracellular particles (diameter $\leq$100 nm), and phagocytosis is another form of active uptake in which relatively large particles enter immunocytes such as neutrophils, macrophages, and dendritic cells. On the other hand, nanopenetration is a form of passive uptake; some authors have hypothesized that chemically modified or molecule-adsorbing CNTs enter cells by nanopenetration.[75,107,157,379−384]

We examined the cellular uptake of pristine CNTs, and reported that the mechanism of this uptake depended on the type of cell and choice of dispersant. We also reported that nonimmunocytes also actively absorbed CNTs mainly through endocytosis/phagocytosis (Figure 6).[385,386] Other researchers likewise denied the role of nanopenetration in cellular uptake of SWCNTs.[387] Adhesion to cell surfaces has been observed even in cells that do not absorb CNTs; it remains unknown whether the molecules that facilitate CNTs adherence to cells and those that facilitate CNTs absorption are identical. It has been reported that cell membrane proteins are involved in the cellular uptake of CNTs.[384,388] Furthermore, these membrane proteins may bind specifically to CNTs.[80,389] However, it will be necessary to investigate the influence of protein-containing dispersants on this binding between membrane proteins and CNTs.[369,371,385] A recent report suggested that exposure to electromagnetic waves promotes CNTs entry not only into the cytoplasm of cells, but also into the nucleus.[390] In conclusion, much remains to be elucidated about the cellular uptake of CNTs and its underlying mechanism.

To clarify the mechanism underlying the cellular uptake of CNTs, a wide variety of approaches have been developed. For example, light scattering analysis was used to qualitatively assess the cellular uptake of CNTs; a fluorescence detection technique was used to study the cell trafficking of CNTs; and 3-D dark-field scanning transmission electron microscopy was used to examine ultrastructural localization of CNTs in appropriately prepared target cells.[368,391−393] Successful monitoring of the cellular uptake and intracellular behavior of CNTs would clarify the reactions between CNTs and cells in more detail. The mechanism behind the cellular uptake of CNTs and their intracellular behavior not only has a bearing on the cytotoxicity of CNTs, but also on their pharmacokinetics when used in DDSs; thus, much more of this research is expected.

4.2. Mechanism Behind the Cytotoxicity of CNTs

Many studies have assessed the cytotoxicity of CNTs. Some early studies found that CNTs and asbestos have equivalent cytotoxicity in macrophages and other cells.[75,76,394] Recent studies, however, found that CNTs have low cytotoxicity.[155]

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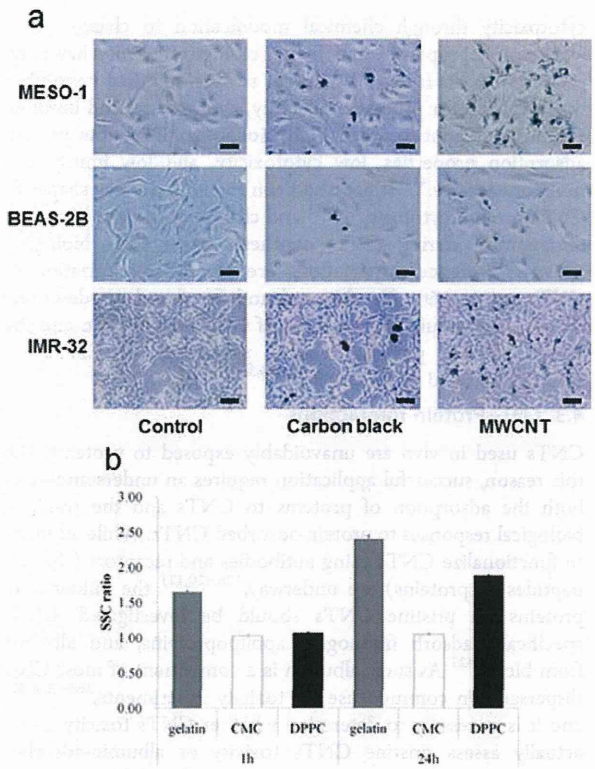

Figure 6. Cellular uptake of pristine MWCNTs varies depending on the type of cell and the choice of dispersant. (a) Combined images from bright field images and phase-contrast photomicrographs obtained 24 h after exposure of human malignant pleural mesothelioma cells (MESO-1), human bronchial epithelial cells (BEAS-2B), and human neuroblasts (IMR-32) to carbon black (CB, 50 nm diameter) and MWCNTs. Both CB and MWCNTs were absorbed in the MESO-1 cells and BEAS-2B cells, and localized around the respective exposure sites, whereas in the case of the IMR-32 cells, both CB and MWCNTs adhered but failed to be absorbed. CB and MWCNTs were added at 1 μg/mL for the treatment of BEAS-2B cells, and 10 μg/mL for the treatment of the other cells. Scale bars = 50 μm. Reprinted with permission from ref 384. Copyright 2011 Nature Publishing Group. (b) A comparison of cellular uptake in BEAS-2B observed 1 and 24 h after exposure to MWCNTs dispersed using different dispersants. Cellular uptake was determined in terms of the intensity of side scattered light (SSC) from MWCNTs absorbed in the cells using a flow cytometer. The MWCNTs dispersed in gelatin or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were increasingly absorbed over time, whereas those dispersed in carboxymethylcellulose (CMC) were little absorbed in the cells. Reprinted with permission from ref 385. Copyright 2011 Dove Medical Press.

The reader of such in vitro cytotoxicity studies should be alert to the fact that CNTs above a certain level dose-dependently reduce cell counts regardless of cell type. This finding reflects a natural reaction of living cells to contact with foreign particulates such as CNTs. The issue is whether CNTs have a higher or lower degree of cytotoxicity than biologically safe substances.

The objective of the cytotoxicity study should also be noted. When safety is the aim of the CNTs biomaterials evaluation, concentrations in the toxic range (according to many reports; on the order of μg/mL) are used, which are much higher than the likely actual concentrations in vivo. Such high concentrations cannot occur in actual settings and can lead to an unreasonable emphasis on the toxicity. Rather, it would be more meaningful to determine the concentration at the lower limit of cytotoxicity and whether this lower limit can occur in vivo.

In addition, it should be well recognized that different types of cells can exhibit distinct responses even to the same kind of nanoparticles. This phenomenon was recently named the "cell vision" effect.[395] Exploring this effect will make it possible to clarify the mechanism for cytotoxicity. Mahmoudi et al. clarified the mechanism underlying this difference in cytotoxicity among the different cell types, and investigated the detoxification of nanoparticles.[396,397]

In all cases, when applying CNTs to biomaterials, their cytotoxicity to living organisms should be as low as possible, and by establishing the mechanism underlying their cytotoxicity, less cytotoxic CNTs can be found. A wide variety of studies to elucidate this mechanism are ongoing.[156,398,399]

4.2.1. Oxidative Stress. Oxidative stress is a focus of studies aimed at determining the mechanism underlying the toxicity of CNTs in vitro as well as in vivo. Some articles but not others have reported that CNTs may induce cytotoxic oxidative stress.[400] This cytotoxicity from oxidative stress has been attributed to the persistence of catalytic metals (Fe, Co, Ni, etc.) used in producing CNTs. Many studies have found that the cytotoxicity of CNTs increased with increase in metal content ratio.[72,368,401,402] Some CNTs contain in excess of 10% (w/w) metallic impurities, which can produce free radicals and thereby damage tissue.[263,400,403] This process can occur even after CNTs are phagocytosed by macrophage. For example, NADPH oxidase is intracellularly activated, and the resulting highly active superoxide radical kills bacteria and other pathogens. Residual Fe activates peroxides to produce hydroxyl (OH^-) radicals leading to oxidative effects on cellular proteins, lipids, and DNA. Residual Co can produce chromosome anomalies. However, a study found that Ni has no cytotoxic effects, but this finding needs to be investigated further.[75,290,404] Oxidative stress may be induced by aggregation of CNTs. Shvedova et al. found that CNTs have low in vitro cytotoxicity provided they are properly dispersed using appropriate procedures and their metallic impurities are removed.[155] Our study concluded that there was no correlation between the amount of oxidative stress from CNTs with low residual iron content and cell proliferative response or inflammatory reaction.[386,405] Carbon nanohorns, a type of carbon nanotubes without metallic impurities, were reported to be quite safe, with cytotoxicity less than 10% of the cytotoxicity of dust from road pavement.[406] However, it is unrealistic to expect that CNTs will contain absolutely no metallic impurities. Accordingly, an article discussed the limit of metallic impurity not affecting the redox properties of CNTs.[407] The susceptibility of CNTs to oxidation in the presence of metallic impurities was also analyzed.[408] In all cases, the lower was the level of metallic impurities, the lower was the level of induction of oxidative stress. Collectively, these available reports lead to the judgment that carbon purity level of 99% or more is not problematic.

On the other hand, it has long been suggested that when cells absorb CNTs, long fibers are left unabsorbed and induce oxidative stress.[281] This phenomenon is known as frustrated phagocytosis. A recent report stated that CNTs that are shorter than a given length are absorbed and not toxic, whereas longer CNTs are not absorbed but are toxic.[409−412] Consequences such as carcinogenesis may stem from prolonged inflammation

due to frustrated phagocytosis in the thoracic cavity lasting long after CNTs are inhaled (Figure 7). Cytotoxicity due to frustrated phagocytosis in the context of use of CNTs as biomaterials is discussed in section 6.2.2.

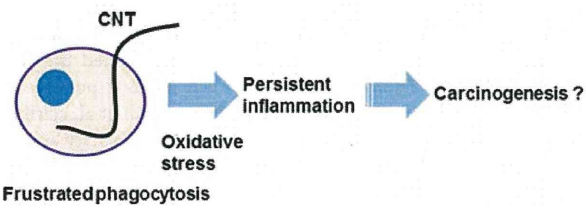

Figure 7. A schematic diagram showing a hypothesized mechanism of carcinogenesis due to frustrated phagocytosis. If left unabsorbed, long CNTs in cells can produce oxidative stress and induce inflammation. It has been suggested that a long period of persistent inflammation in the thoracic cavity following inhalation of CNTs can lead to carcinogenesis. Currently, research into the inhalation toxicity of CNTs is facing a problem with the determination of the margin of inhalation exposure that does not cause persistent inflammation.

In September 2012, the National Institute of Standards and Technology (NIST) in the U.S. reported a finding that is completely inconsistent with findings that SWCNTs protect DNA from oxidative stress.[413] Hence, no consistent conclusion has been reached concerning oxidative stress. Collectively, previous studies using many types of cells under a wide variety of conditions have led to a near consensus that CNTs do not induce oxidative stress if their aggregability and length are limited.[155] A recent study showed that chemical treatment with, for example, triethylene glycol can reduce the likelihood of aggregation in biological fluids and toxicity of even long CNTs.[414]

4.2.2. Effects on Immunity. The second issue concerns the interactions of CNTs with immunocompetent cells, including cellular uptake and subsequent intracellular transport. As such, immunocompetent cells bear a direct relationship to the safety of CNTs in vivo. Of course, pristine CNTs (because they lack antigen-presenting protein) do not cause immune reactions other than those to a foreign substance. Hence, if localized inflammation is brief, immune reactions should resolve. However, immunocompetent cells may absorb CNTs because of their nanosize, may not absorb some CNTs completely because of their fibrous form, and may orchestrate the development of an inflammatory response to residual metals and other factors in CNTs. Keeping these possibilities in mind, it is necessary to understand how immunocompetent cells respond to CNTs. Many in vitro studies have reported no response of immunocompetent cells to very pure and very short CNTs.[155,415] For example, CNTs did not have a remarkable effect on antigen-presenting cells (APCs) such as mouse macrophages (RAW 264.7 cells) and mouse bone marrow-derived dendritic cells (bmDCs).[416] An article reported that CNTs did not induce inflammatory cytokines in macrophages, whereas residual metals did.[85,401,402] If CNTs are shown to escape surveillance by immunocompetent cells, this finding will provide strong evidence for high safety of CNTs as biomaterials. Of course, it is theoretically impossible that pristine CNTs cause autoimmune disease.

4.2.3. Attempts To Lessen the Cytotoxicity. As stated above, various methods for minimizing the cytotoxicity of CNTs have been studied. For example, reducing nanotube cytotoxicity through chemical modification to change physicochemical properties and hence biological activity has been proposed. A library of 80 different surface-modified nanotubes was screened for protein bindability, cytotoxicity, and immune responses. Nanotubes had high biocompatibility, low protein adsorption properties, low cytotoxicity, and low immunostimulatory activity.[417] It has also been found that some shapes of CNTs are not cytotoxic,[309,418] and change of the graphitization temperature during CNTs synthesis alters their biological activity.[405] Hence, expectations are for the minimization of CNTs cytotoxicity. To this end and for the above-described reasons, the cellular mechanisms of CNTs recognition and the effects of the physicochemical properties of CNTs on cytotoxicity need to be clarified.[396,397,419]

4.3. CNT–Protein Interactions

CNTs used in vivo are unavoidably exposed to proteins. For this reason, successful application requires an understanding of both the adsorption of proteins to CNTs and the resulting biological responses to protein-adsorbed CNTs. While attempts to functionalize CNTs using antibodies and receptors (that are peptides or proteins) are underway,[176,420,421] the influence of proteins on pristine CNTs should be investigated. CNTs specifically adsorb fibrinogen, apolipoproteins, and albumin from blood.[422] As such, albumin is a component of most CNT dispersants in common use for toxicity experiments,[366−368,370] and it is necessary to determine whether CNTs toxicity assays actually assess pristine CNTs toxicity or albumin-adsorbed CNTs toxicity. Examination of the mode of adsorption to SWCNTs by plasma proteins fibrinogen, γ-globulin, transferrin, and bovine serum albumin using an atomic force microscope was reported, and protein binding reduced SWCNTs cytotoxicity.[423] However, the SWCNTs used in this experimental study contained many metals such as Cr, Fe, Mo, and Co, and their effect must also be taken into account.

The phenomenon in which various proteins coat the nanoparticle surface has recently been termed "protein corona" formation.[424] The protein corona is influenced by a wide variety of factors, including temperature, protein concentration, gradient concentration, protein source, and physicochemical properties of nanoparticles. The protein corona has also been reported to have major impacts on the biological reactions of cells and living organisms. For example, nanoparticles on cells and living organisms were shown to lose activity when their surface is partially covered by protein.[355,356,425−429] As such, the protein corona may determine the fate of CNTs in living organisms. In addition, changes on the nanoparticle surface caused by formation of the protein corona can alter the effects of chemically modified CNTs. Shannahan et al. compared the proteins coating MWCNTs with SWCNTs, and those coating modified with unmodified, which revealed a difference in protein composition between SWCNTs and MWCNTs and an increase in the variety of component proteins as a result of modification with COOH groups.[430] Functional deterioration of chemically modified nanoparticles has been repeatedly shown to occur; there is an urgent need to determine whether the same phenomenon can occur in CNTs.

On the other hand, to explain the decreased cytotoxicity of protein-bound CNTs, a recent study hypothesized that the human body developed a biological system mediated by protein binding to deal with exposure to numerous nanoparticles (i.e., developed a defensive mechanism against nanoparticles).[431]

Table 1. Proteins of Human Monoblastic Leukemia Cells (THP-1) Changed by Exposure to CNTs As Determined by Proteomic Analysis[a]

gene ontology term	proteins
biosynthetic process	heat shock protein β-1, elongation factor 1-δ, DNA mismatch repair protein Msh2, 6-phosphogluconate dehydrogenase decarboxylating, triosephosphate isomerase
signal transduction/cell communication	elongation factor 1-δ, DNA mismatch repair protein Msh2, 14-3-3 protein γ, serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B α isoform, protein DJ-1
carbohydrate metabolic process	6-phosphogluconate dehydrogenase decarboxylating, triosephosphate isomerase, serine/threonine-protein phosphatase PP1-α catalytic subunit, α-ketoglutarate dehydrogenase, neutral α-glucosidase AB
nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	DNA mismatch repair protein Msh2, 6-phosphogluconate dehydrogenase decarboxylating, triosephosphate isomerase, DNA damage-binding protein 1
protein metabolic process	actin related protein 2/3 complex subunit 2, serine/threonine-protein phosphatase PP1-α catalytic subunit, serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B α isoform, DNA damage-binding protein 1
catalytic process	6-phosphogluconate dehydrogenase decarboxylating, triosephosphate isomerase, α-ketoglutarate dehydrogenase, DNA damage-binding protein 1
multicellular organismal development	DNA mismatch repair protein Msh2, triosephosphate isomerase, 14-3-3 protein γ, serine/threonine-protein phosphatase PP1-α catalytic subunit
response to stress	heat shock protein β-1, DNA mismatch repair protein Msh2, DNA damage-binding protein 1, protein DJ-1
cell differentiation	heat shock protein β-1, DNA mismatch repair protein Msh2, 14-3-3 protein γ
cell cycle	DNA mismatch repair protein Msh2, serine/threonine-protein phosphatase PP1-α catalytic subunit, DNA damage-binding protein 1
transport	14-3-3 protein γ, protein DJ-1
cell death	heat shock protein β-1, DNA mismatch repair protein Msh2
organelle organization and biogenesis	actin related protein 2/3 complex subunit 2, DNA mismatch repair protein Msh2
translation	heat shock protein β-1, elongation factor 1-δ
lipid metabolic process	triosephosphate isomerase

[a]Adapted with permission from ref 463. Copyright 2011 Elsevier.

This suggests that CNTs research may elucidate the body's defensive mechanism, which is unclear.

4.4. Mutagenicity, Genotoxicity, and Apoptotic Potential of CNTs

Assessments of the mutagenicity and genotoxicity of CNTs are also important in vitro safety studies.[432−441] This is because the results from these assessments reflect the carcinogenicity of CNTs. Relatively common approaches include the Ames test, comet assay, and micronucleus test.

The Ames test, also known as the reverse mutation test, is to quantify reverse mutation (i.e., restoration of amino acid biosynthesis capability in bacteria originally deprived of that capability through mutation). Ames test studies with *Salmonella typhimurium* and other test strains have often shown that neither SWCNTs nor MWCNTs are mutagenic. A mutagenesis study showed that the frequency of mutations in mammalian cells (Chinese hamster pulmonary fibroblasts) is not altered by MWCNTs.[438,442−445]

The comet assay is a technique used to detect DNA damage in individual cells, enabling separate determination of early disorders induced at the DNA level, repair kinetics, and residual disorders. For this reason, comet assays have been performed on many types of cells exposed to SWCNTs and MWCNTs. CNTs induced DNA damage in some studies but not in others. The prevailing opinion is that any DNA damage caused by CNTs is mediated by reactive oxygen species (ROS).[446−449]

The purpose of the micronucleus test is to detect damage to the gene of interest in animal cells following administration of a test substance. Cells containing micronuclei can serve as an index of gene damage. Micronucleus test studies to assess the toxicity of SWCNTs and MWCNTs in many types of cells have yielded mixed results.[399,404,442]

Some studies of apoptosis induction by CNTs found induction of apoptosis signals in macrophages and other cells to induce apoptosis signals, while others did not find any sign of apoptosis induction.[318,378,450−452] Many cells incorporating CNTs underwent G1 phase arrest.[430] We reported that iron-rich MWCNTs caused nonapoptotic cell death.[453] On the other hand, other experiments found that highly pure MWCNTs caused apoptosis-like cell death, suggesting that the CNTs impurities have a major effect on apoptosis.[386]

In conclusion, the mutagenicity and genotoxicity of CNTs remain unclear; some studies judged CNTs to be mutagenic or genotoxic and others did not.[89,432,437,443,454−456] Results varied and depended on the cell type even within the same study.[442] In cases where genotoxicity was observed, authors hypothesized metals-induced oxidation of the DNA or suggested other hypotheses.[457] Variable results and conclusions are attributable to variable test conditions such as the dispersibility of CNTs in solution and the amount of CNTs used, as well as the amount of CNTs impurities, but not the form of CNTs (all studies assessed particulate substances). There is no current evidence in CNTs of high purity, although carcinogenicity from mutagenicity or genotoxicity calls for vigilance.[155] Further investigation will be necessary in different cell types to determine whether cells incorporating CNTs undergo apoptosis.

4.5. Cellular Signaling Events

Microarray or proteomics studies of cell signaling events induced by CNTs have been reported.[458] In a microarray study using human embryonic kidney cells exposed to SWCNTs for 2 days, decreased expression of cyclins and *cdks* (a gene affecting the G1 phase of the cell cycle) and increased expression of apoptosis-related genes were demonstrated.[318] Other researchers exposed foreskin cells to SWCNTs, and found that the expression of HMOX1, HMOX2, ERCC4, and HSPE1 and that of ATM, CCNC, DNAJB4, and GADD45A more than doubled when determined using stress and toxicity arrays and RT-PCR, respectively.[459] Using reporter gene assays of MWCNT-exposed bronchial epithelial cells, MWCNTs activated the transcription factor NF-κB to induce increased phosphorylation of p38, ERK1, and HSP27 in the MAP kinase pathway and the

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production of inflammatory cytokines.[369] Activation of NF-κB in macrophages was also reported.[460] We examined the effects of MWCNTs on cellular signaling events in osteoclasts and showed that MWCNTs suppressed osteoclast differentiation by inhibiting the nuclear migration of the transcription factor NFATc1.[217] In conclusion, the influences of CNTs on cell signaling events are important to the understanding of cellular function, and further research will be needed.

Proteomics-based studies have been conducted using keratinocytes and hepatoma cells. Results have shown changes in expression of proteins related to metabolism, stress, redox, cytoskeleton formation, apoptosis, etc., in both types of cell.[461,462] Our proteomics analysis under low-cytotoxicity conditions using monoblastic leukemia cells that do not absorb MWCNTs confirmed these changes in proteins (Table 1).[463] Such comprehensive analyses of cell signaling events increase understanding of the essential features of cellular change.[464] It is hoped that research activities will identify the pathways on which CNTs have a direct impact, and make major contributions to the assessment of the cytotoxicity of CNTs.

4.6. Choice of Cells

To date, cytotoxicity studies have often been conducted using fibroblasts and macrophages such as RAW cells. However, cellular reactions to CNTs depend on the type of cell,[396,397] and it can be thought that the reactions are specific for the organ bearing the target cells. For example, a study comparing the cytotoxicity of CNTs in the liver, spleen, and lung found that CNT-induced oxidative stress dose-dependently increased toxicity in the liver and lung, but not in the spleen.[465] We must clarify the mechanism underlying the reactions of different cell types and organs to CNTs. Because biological reactions to CNTs vary among types of cells and organs, toxicity studies using cells from likely sites of use will be needed before CNTs can be clinically applied.

For example, in a study assessing CNTs for use in nerve regeneration, human neuroblastoma cells and primary mouse neurons were exposed to MWCNTs, and their reactions were examined for effects on cell survival, oxidative stress, and apoptosis.[70] Another study examined the effects of CNTs on heart cells, specifically on impulse conduction characteristics, myofibril structure, and reactive oxygen species production in the patterned growth strands of neonatal rat ventricular cardiomyocytes. CNTs particles had much less effect than diesel exhaust particles and titanium dioxide nanoparticles.[466] To assess the use CNTs as a possible bone tissue regeneration scaffold, we examined in detail their effects on osteoblasts (bone-forming cells) and osteoclasts (bone-absorbing cells), as described in section 2.3.2.[217,218]

5. REFERENCE MATERIALS FOR SAFETY EVALUATION OF CNTs AS BIOMATERIALS

The safety of CNTs for biomaterial application remains unknown because toxicity studies have yielded inconsistent or even contradictory results as stated above. Moreover, no nanoparticle reference material has been shown to be safe to use in living organisms. All biomaterials are essentially foreign to living organisms, and hence exhibit some toxicity to living organisms. Of concern is the level of toxicity; the biological safety of CNTs cannot be assessed without conducting a toxicity study using as a reference substance that has already been recognized as safe to use in living organisms.

For example, in 2010, the cytotoxicity, genotoxicity, and apoptosis-inducing potential of MWCNTs was examined in human fibroblasts. Physiological saline admixed with a dispersant served as the only negative control. Results showed that MWCNTs exhibited dose-dependent toxicity in all dose groups as compared to the negative control, and that the cell survival rate decreased dramatically due to DNA damage, triggering pathways leading to programmed cell death. Hence, the conclusion was reached that CNTs are highly toxic. It should be noted, however, that it is scientifically incorrect to assess the toxicity of CNTs merely by comparing the results obtained in the presence and absence of CNTs. The solution (containing a dispersant) used in the reported study cannot serve as a reference for toxicity assessment. This study showed nothing more than that the experimental system used worked well, and no conclusion regarding CNTs toxicity can be drawn.

For researchers in this field, identification of an appropriate reference material for toxicity studies, which is presently unavailable, is a top priority. Kostarelos et al. pointed this out in 2009 in their review published in *Nature Nanotechnology*.[68] The reference substance must be a nanosized particulate with established biological safety. A substance can be judged as safe to use in living organisms only if it is shown to be equally or less toxic than its reference material. To render a judgment on the functioning of an experimental system, a conventional chemical substance can be used as a feasible alternative for the positive-control reference material. However, no best negative-control reference material has been found, so the safety of CNTs as biomaterials remains indeterminable.

5.1. Why Is There No Substance That Can Serve as a Reference for CNTs?

Researchers have been seeking a substance with many of the same properties as CNTs. Such references do not actually exist. Without a reference, CNTs cannot be used as biomaterials. From a broader viewpoint, any nanosized particulate substance should be considered to be a reference candidate. In fact, reference materials are specified for bulk biomaterials on the basis of this broad concept. For example, in cytotoxicity testing of bulk materials, a high-density polyethylene film serves as the negative reference material for the extraction method, and a polyurethane film containing zinc diethyldithiocarbamate (ZDEC) serves as the positive reference material. For the direct contact method, a plastic sheet for tissue culture serves as the negative reference material, and ZDEC-containing polyurethane serves as the positive reference material. These substances are specified in the ISO 10993-5 Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity (2009).[467] Hence, it is internationally accepted that a reference for a bulk biomaterial should be a bulk material of totally different nature. There is no rationale for viewing particulate materials as the only exception.

Essentially, the unfavorable criticism of nanosized fibrous particulate substances is due largely to the fact that asbestos causes cancer and other diseases. Because CNTs resemble asbestos in size and shape, their toxicity has created a stir in the media.[281,282] It should be noted, however, the inhalation toxicity of CNTs is distinct from the toxicity of CNTs biomaterials. Recently, inhaled spherical titanium oxide particles were reported to be carcinogenic;[468] however, if the judgment is made on the basis of shape and size only, no spherical nanoparticles could be used as biomaterials, and almost all nanoparticles would be inapplicable to biomaterials.

It is obvious to everyone that this claim makes no sense. Even if fibrous nature, thin and long shape, and large aspect ratio are problematic, we should keep in mind that carbon fiber biomaterials have long been used for Achilles tendon repair and other clinical purposes with absolutely no coincidence of carcinogenicity.[96,469,470] In conclusion, the most reasonable approach is to assess the toxicity of CNTs by focusing on biological reactions to nanosized particulate substances.

5.2. Biomaterials Comprising Artificial Nanosized Particles

The second reason for the inability to find a best reference material is that no nanosized particulate substance has been used as a biomaterial. This issue bears not only on CNTs, but also on a wide variety of nanoparticles, and research into biological application of nanoparticles has recently been rapidly growing. Some pharmaceuticals anchored to nanosized particles are already in clinical application. For example, abraxane, a nanoparticle substance prepared by conjugating the anticancer agent paclitaxel with albumin, degrades in the body, releasing the anticancer agent. Such conventional nanosized particles are specifically used as DDSs by making the best use of their biodegradability, and cannot be viewed in the same way as nanoparticulate biomaterials that are poorly degraded in the body.[301]

To date, only four kinds of artificial materials have been used in living organisms: chemical substances, materials with biodegradability, bulk materials lacking biodegradability, and micrometer-sized or larger particulate substances. Nanosized particulates have not been used in the body. Chemical substances, biomaterials with biodegradability, and bulk biomaterials have been used in the human body since ancient times, and many such substances have proven to be safe. For this empirical reason, researchers have been able to use these substances as references. When these substances were used as biomaterials for the first time, no scientific toxicity testing was needed. Those substances found over time to be safe to use in the human body remain in use today. Toxicity studies using some of these substances as references have been conducted to demonstrate the safety of other substances in the same category, and then using the other substances thus judged to be safe as references, the safety of still other similar substances has been demonstrated. Through this process, numerous substances have been made available for clinical application. The internationally accepted ISO standards dealing with safety evaluation are currently serving very well and have also emerged from this historical precedent.[467] The standard reference materials are known biologically safe substances rather than new reference materials evaluated to be safe for humans. Micrometer-sized or larger particulate biomaterials, for example, granular hydroxyapatite, have never posed a major problem even though they were subjected to the same safety evaluation process as conventional biomaterials.[471−473] Because CNTs and other nanosized particulate substances fall into a different category of biomaterials than micrometer-sized or larger particulate substances, the use of conventional bulk biomaterials and hydroxyapatite particles as reference materials for them is controversial. Because nanosized particulate substances have not been used in the human body, there is no implicit reference with established safety.[474]

For these reasons, obtaining a reference with confirmed biosafety in the human body for use in toxicity studies of CNTs appears to be impossible. From a broader perspective, however, otherwise unknown nanoparticles may be discovered. We considered that highly pure carbon black could serve as a reference for CNTs, because it is the primary component of the black ink used in tattoos, and also because black tattoo inks have long been injected into human bodies and are currently used by a tremendous number of people worldwide. Evidence showing that black tattoo inks are composed of nanosized carbon black particles is described below, with an overview of the biological safety of CNTs using carbon black as a reference.

5.3. Safety Evaluation of CNTs Using Nanosized Carbon Black Particles as a Reference

5.3.1. Nanosized Carbon Black Particles in Tattoo Ink. Two commercially available black tattoo inks (Sumi-Black, Unique Tattoos, Subiaco, Australia; Lining-Black, Classic Ink, Victoria, Australia) were purchased and extensively analyzed for components. Each was dried, and the resulting solid product was morphologically examined by scanning electron microscopy (SEM); particles with a nearly uniform diameter of several tens of nanometers were found to have accumulated (Figure 8a). After SEM examination, the particles were subjected to an elemental analysis using energy dispersive X-ray spectroscopy (EDS). Results showed that both inks had a C content of about 99.5 wt % and different impurity profiles, with trace amounts of Na and S detected and attributable to the surfactant added. A Raman analysis using common industrial carbon black (Vulcan XC 72, Cabot, Boston, MA) as a control revealed that Raman shift of both black tattoo inks was nearly the same as that of the control (Figure 8b). Furthermore, transmission electron microscopy (TEM) revealed that the particles in black tattoo inks had nearly the same shape as those of ordinary carbon black (Figure 8c). These findings identified the particles in tattoo inks as pure carbon black (i.e., nanosized carbon particles) as with MWCNTs.[97]

In 2012, on the other hand, a report titled "Chemical Substances in Tattoo Ink" was released from Denmark.[475] Concerning a research project implemented by the Danish Technological Institute in cooperation with Bispebjerg Hospital and the National Food Institute, Technical University of Denmark, the report explicitly described carbon black as the principal component of black tattoo ink, and toxicity assessments of carbon black found no biological safety problem.

An extremely large number of humans have received black tattoos since ancient times, and this practice has caused no major problems; tattoos are popular even today. Hence, carbon black can be described as a biomaterial that has been proven by historical evidence to be safe for use in the human body. As such, the nanosized carbon particles used in black tattoos, as with CNTs, are very pure carbon black; thus, carbon black should be considered as a good reference material for CNTs.

5.3.2. Comparison of Characteristics of CNTs and Carbon Black. To use the biologically safe carbon black tattoo ink as a reference material for CNTs, both substances should share some characteristics. Despite their considerably different characteristics, current reference materials for bulk materials have been used as international standards, and safety assessments have been conducted with no major problems. This has become feasible because of the large amount of data compiled throughout the long history of biomaterials research. However, references for nanoparticle biomaterials remain to be found. The accuracy of safety evaluation will be increased by using substances with similar characteristics in the beginning.

The characteristics (including composition, size, shape, and surface chemistry of the reference material used for CNTs [i.e.,

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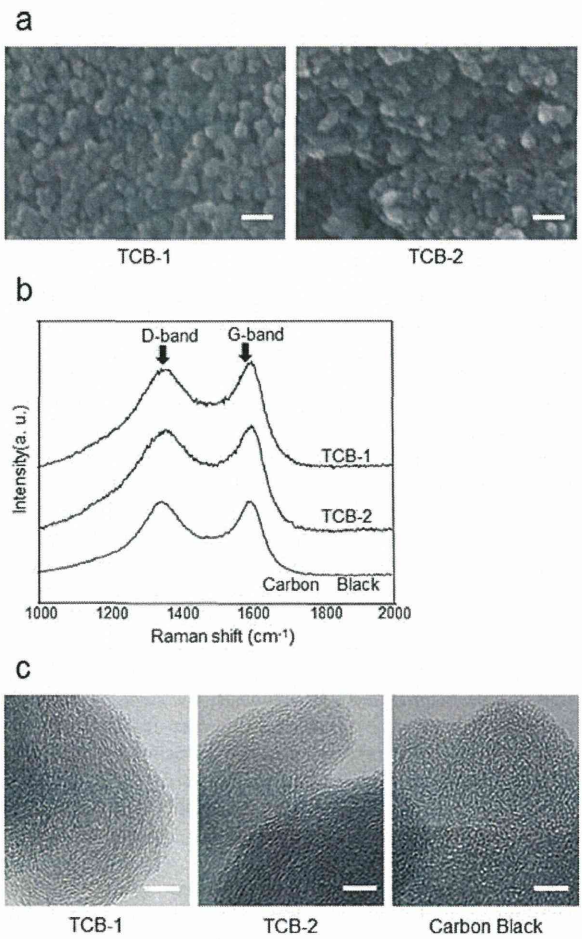

Figure 8. Having historically been proven safe to the human body, tattoos comprise nanosized highly pure carbon black, and hence serve well as a reference material for evaluating the safety of CNTs, which likewise occur in the form of nanosized carbon particles. (a) Scanning electron microscopy (SEM) images of tattoo carbon black-1 (TCB-1) and tattoo carbon black-2 (TCB-2) prepared by drying two different tattoos. TCB-1 and TCB-2 were found to have accumulated in the form of generally regular particles having a diameter of about 30−50 nm, and generally irregular particles having a diameter of about 50 nm, respectively. (b) Raman analysis of TCB-1, TCB-2, and ordinary carbon black. TCB-1 and TCB-2 exhibited nearly the same Raman shift pattern as with ordinary carbon black. D-band, turbostratic amorphous; G band, graphite crystal. (c) Transmission electron microscopy (TEM) images of TCB-1, TCB-2, and ordinary carbon black. These three substances were found to have nearly the same particle shape. Reprinted with permission from ref 97. Copyright 2011 Elsevier.

carbon black]) were compared to those of CNTs per se (Table 2).[476] Both substances are highly pure carbon particulates of similar size (i.e., they are nanosubstances, entities internationally recognized as being not less than 100 nm in one or more of the three dimensions).[477,478] CNTs and carbon black have distinct shapes: fibrous particles and spherical particles, respectively. Although various classifications of surface chemistry are available, the most common practice is to characterize surfaces as hydrophilic or hydrophobic. The surfaces of CNTs are hydrophobic, and carbon black particles, without surface treatment, are essentially hydrophobic. Hence,

Table 2. Comparison of Characteristics of CNTs and Carbon Black

characteristic	CNT	carbon black
composition	high-purity carbon	high-purity carbon
size	nanosized	nanosized
shape	fibrous particle	spherical particle
surface chemistry	hydrophobic	hydrophobic

three of the four representative characteristics of particulate substances are shared; therefore, it is reasonable to use carbon black as a reference for CNTs. Although some researchers may disagree based on the distinction between fibrous and spherical particles, no reference can have exactly the same characteristics as the test substance. Considering the absence of any other appropriate reference, it is very fortunate that carbon black with high similarity to CNTs has a long history of use in living organisms and demonstrated safety in the human body. Problems stemming from the fibrous nature of some nanoparticles are discussed in section 6.2. Despite these problems, it can be concluded that fibrous nanoparticles pose no hazard at sites of CNTs implantation if inflammation is not persistent.

All experiments can be performed using mass as an index because CNTs and carbon black are both highly pure carbon. For particulate substances, it is often difficult to measure the number and volume of particles; therefore, the ability to use mass as the simplest index is an obvious advantage in the evaluation of CNTs safety. Carbon black can also serve as a reference for the evaluation of the biological safety of other nanosubstances used as biomaterials. However, mass cannot be used as an index for safety comparison when density varies; therefore, another index such as particle count will have to be used, making the procedure more complicated and difficult to perform, and even reducing its accuracy.

Research into application of non-CNT carbon-based nanomaterials to biomaterials has also progressed steadily, although there are fewer non-CNTs studies than CNTs studies. The use of fullerenes or graphene for DDSs and imaging has been studied, and their biological safety has been evaluated.[479,480] Additionally, nanosized carbon fibers, which traditionally have not been nanosized, are now available thanks to recent technical advances. Although carbon fiber products are promising candidates as nanobiomaterials because of their history of clinical use as biomaterials, their safety needs to be evaluated because of their nanosize,[214,481] and carbon black can serve as an appropriate reference.

5.3.3. Safety Test. A skin implantation test with MWCNTs was conducted using carbon black tattoo ink as a reference material. Results showed that MWCNTs induced acute but mild inflammation reactions in subcutaneous tissue, which resolved early. The subcutaneously implanted MWCNTs were shown to be absorbed initially by macrophages and remained in the macrophages for a long time. These short- to long-time histological reactions to the MWCNTs were found to be very similar to the histological reactions to the carbon black tattoo ink particles (Figure 9). This finding shows that when implanted in vivo, MWCNTs (as with tattoo ink particles) exhibit good tissue affinity at the implantation site and stay intact in macrophages for a long time.[97]

We then conducted a colony formation assay to determine the in vitro cytotoxicity of MWCNTs using carbon black tattoo ink particles as a reference. Both MWCNTs and carbon black tattoo ink particles inhibited colony formation in a concen-

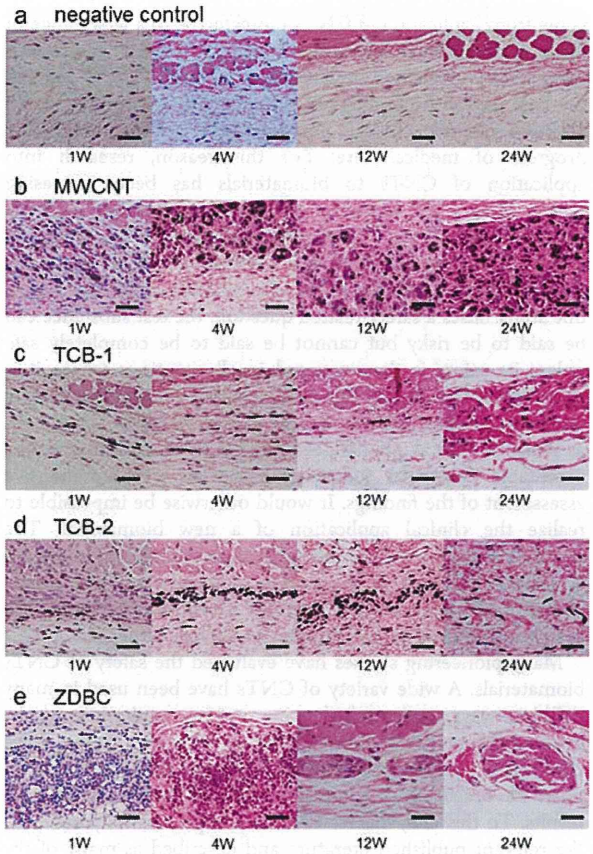

Figure 9. Histological reactions to subcutaneously implanted MWCNTs are very similar to those to carbon black, showing good tissue compatibility. Hematoxylin-eosin staining. Scale bars = 20 μm. TCB-1, Tattoo carbon black-1; TCB-2, Tattoo carbon black-2 (see Figure 8). (a) Histological images from a negative control group (NC) of male ddY mice at 6 weeks of age receiving an injection of 10 μL of physiological saline and a surfactant given to a pocket created in subcutaneous tissue in the back. At 1 week of treatment, the subcutaneous tissue had been repaired nearly completely. Repair was complete at 4 weeks. No change was observed at 12 and 24 weeks. (b) Histological images of subcutaneous tissue from a group receiving an injection of 10 μL of MWCNT solution (4.0 mg/mL). Most particles were found to have been absorbed in macrophages at 1 week. In the areas around the injection site, accumulated fibroblasts, neutrophils, and lymphocytes were found, with weak inflammatory reactions observed. At 4 weeks, the MWCNTs remained incorporated in macrophages, and the inflammatory reactions around the injection site had resolved. The macrophages that had absorbed MWCNTs turned into multinucleated giant cells, creating an appearance like foreign-body granuloma. The histological profiles obtained at 12 and 24 weeks did not differ from the profile obtained at 4 weeks. (c) Histological images of subcutaneous tissue from a group receiving an injection of 10 μL of TCB-1 solution (4.0 mg/mL). At 1 week, most particles were found to have been absorbed in macrophages in the subcutaneous tissue, and as in the MWCNT group, accumulated fibroblasts, neutrophils, and lymphocytes were found, with weak inflammatory reactions observed. At 4 weeks, the inflammatory reactions around the injection site had resolved as in the MWCNT group. The histological profiles obtained at 12 and 24 weeks were similar to the profile obtained at 4 weeks. (d) Histological images of subcutaneous tissue from a group receiving an injection of 10 μL of TCB-2 solution (4.0 mg/mL). All histological profiles obtained at 1, 4, 12, and 24 weeks were similar to those obtained with the TCB-1 solution. (e) Histological images of subcutaneous tissue from a group receiving an injection of 10 μL of zinc dibutyldithiocarbamate (ZDBC) solution (4.0 mg/mL). At 1 week, accumulation of many types of inflammatory cells such as fibroblasts, neutrophils, lymphocytes, and plasma cells was observed, and intense inflammatory reactions had been induced over a wide area, with fat necrosis and nuclear debris formation observed. No accumulation of macrophages was observed. Even at 4 weeks, inflammatory cells remained and inflammatory reactions persisted, although the inflammation was going to disappear. At 12 and 24 weeks, the inflammatory reactions had resolved, and the subcutaneous tissue had been repaired into scar tissue with fibrosis. Reprinted with permission from ref 97. Copyright 2011 Elsevier.

tration-dependent fashion. At higher concentrations, colony counts were higher with exposure to MWCNTs than with exposure to carbon black tattoo ink particles (Figure 10). These findings demonstrated that the cytotoxicity of MWCNTs was not greater than that of carbon black tattoo ink particles.[97] When assessing the cytotoxicity of nanoparticles, the colony formation assay yields numerical results, and is currently considered to be the best (most sensitive and reproducible) method of toxicity assessment.[402]

Furthermore, we conducted a carcinogenicity test of CNTs in a trancegenic rasH2 mouse[482−485] using tattoo carbon black tattoo ink as a reference material. The rasH2 mouse has recently also been used in studies of bulk biomaterials.[486−488] We implanted MWCNTs or tattoo carbon black subcutaneously. Results showed that no neoplasms were produced because of implantation of MWCNTs. In the group with carbon black implanted as a reference, one animal died but had apparent tumors on histopathological examination (Figure 11, Table 3). The 75 mg/kg dose of MWCNTs implanted in this study was considerably higher than the doses that had been used in previous implantation studies in ordinary mice.[89,91,155,304] In summary, the above-described test for assessing the carcinogenicity of subcutaneously implanted CNTs by in the trancegenic animals for the first time revealed no carcinogenesis from CNTs as well as tattoo carbon black tattoo ink.[98]

The aforementioned tests showed that nanosized carbon black particles (a tattoo ink component) could be used as a reference for safety evaluation of CNTs. The in vivo implantation test, cytotoxicity test, carcinogenesis test (in transgenic mice), and other tests all found that the toxicity of CNTs is equal to or less than that of carbon black tattoo ink. If a safety test using a substance with verified biological safety as a reference material finds that CNTs exhibit a level of toxicity equivalent to, or lower than, that of the reference material, then it can be concluded that CNTs are safe. We are currently conducting mutagenesis and genotoxicity tests with highly pure carbon black as a reference material. So far, our results show that CNTs are as safe as carbon black particles under the experimental conditions used in the studies.

6. DISCUSSION AND PERSPECTIVE

6.1. Available Safety Evaluations Relevant to CNTs as Biomaterials

In this Review, studies on using CNTs as biomaterials have been reviewed, and currently available in vivo and in vitro studies on the evaluation of CNTs safety as biomaterials have been described separately. It is clear that many benefits will

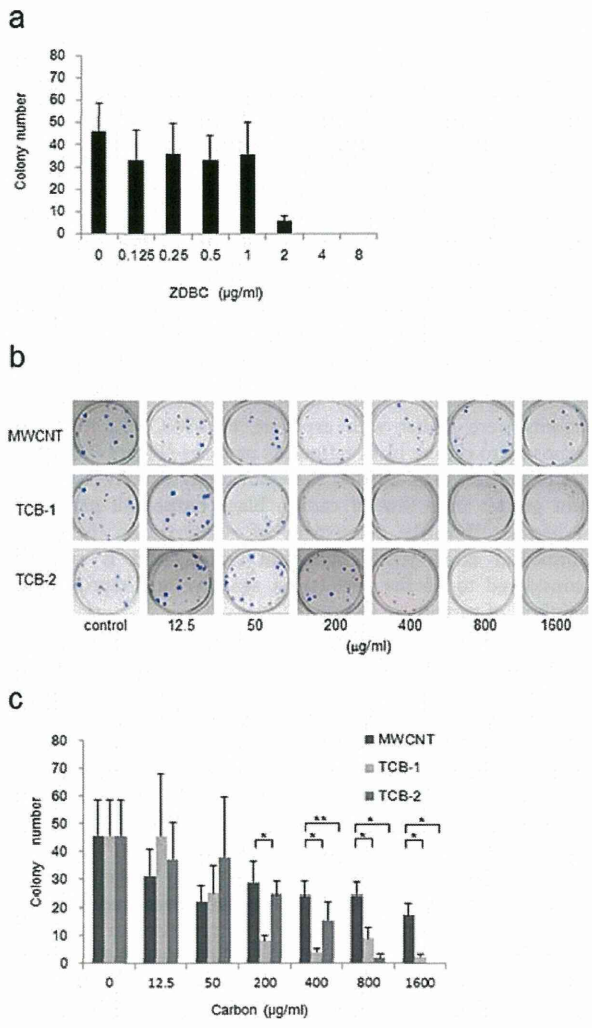

Figure 10. The cytotoxicity of MWCNTs is not higher than that of carbon black. TCB-1, Tattoo carbon black-1; TCB-2, Tattoo carbon black-2 (see Figure 8). (a) Appropriateness of cytotoxicity assessment in colonization test. The colonization capacity of V79 cells (Chinese hamster lung fibroblast cell line JCRB0603) decreased as the concentration of the positive control ZDBC increased. The concentration for 50% colony count reduction (IC_{50}, reference value range: 1−4 μg/mL) was found to be between 1 and 2 μg/mL, confirming that the cytotoxic action of the test substance was properly assessed. (b) Macroscopic photographs showing colonization test results. Colony counts of V79 cells cultured using a culture broth alone and those cultured in the presence of MWCNT solution, TCB-1 solution, and TCB-2 solution were compared. The concentrations in the solutions were 12.5, 50, 200, 400, 800, and 1600 μg/mL, respectively. (c) Colony counts versus carbon concentrations in MWCNT, TCB-1, and TCB-2 solutions. MWCNTs inhibited the colonization in a concentration-dependent fashion, and TCB-1 and TCB-2 likewise inhibited the colonization in a concentration-dependent fashion. When comparing colony counts, MWCNTs produced significantly higher colony counts than TCB-1 at concentrations of 200 μg/mL or more, and than TCB-2 at concentrations of 400 μg/mL or more. Error bars indicate standard deviations (n = 6); *, $p < 0.001$; **, $p = 0.016$. Reprinted with permission from ref 97. Copyright 2011 Elsevier.

come from application of CNTs biomaterials to a wide range of important medical services, including cancer treatment, regenerative medicine, implants, and DDSs.[180,489−491] Making the best use of the findings of these application studies would improve current clinical practices and ensure remarkable progress of medical care. For this reason, research into application of CNTs to biomaterials has been increasing rapidly (Figure 1); however, no clinical application of CNTs has been realized yet[77] because the evidence for the biological safety of CNTs as biomaterials is not definitive. It is easy to say that certain new materials pose risks to living organisms; when one study raises a safety-related question, the test substance can be said to be risky but cannot be said to be completely safe unless its safety is demonstrated in all situations where it is likely to be used. Safety cannot be assured without conducting numerous studies, and this is practically impossible. Therefore, as many studies as possible are needed to make a reasonable, acceptable consensus judgment based on a comprehensive assessment of the findings. It would otherwise be impossible to realize the clinical application of a new biomaterial. The accumulated research into the application of CNTs biomaterials is already sufficient to make such a judgment. The primary objective of this Review is to logically determine whether CNTs can be safely used as biomaterials in clinical settings.

Many pioneering studies have evaluated the safety of CNTs biomaterials. A wide variety of CNTs have been used in many different ways, and studied using various methodologies by many different researchers. Therefore, different studies have often yielded inconsistent results. However, the right judgment must be based on a comprehensive assessment of all such results. To this end, this Review has comprehensively reviewed the relevant published literature and described as many of the latest findings as possible. We conclude that the number of studies reporting the biological safety of CNTs as biomaterials is increasing and that most of the recently published reviews have concluded that CNTs are very safe.[91,99,191,326,492] Taken together, the findings suggest that CNTs will find clinical application as biomaterials through a stepwise process involving appropriate methods and sites of use.

6.1.1. In Vivo Studies. As stated above, no reported in vivo studies have found that CNTs are associated with life-threatening or otherwise serious toxicities such as carcinogenicity. Furthermore, recently reported studies for the most part have shown that inflammation in response to highly pure CNTs implanted in the body was not intense and resolved quickly.[58,89,91,155,304] The organ(s) sites of CNTs accumulation after transport through the bloodstream, the response of tissues and cells to the accumulation, and period of CNTs accumulation are important issues. No study has reported any problem resulting from intravenous injection of CNTs.[91,191,310] However, more accurate techniques must be developed for monitoring the behavior and disposition of CNTs intravenously injected in large amounts for use in DDSs and imaging. Bearing in mind that CNTs can enter the pulmonary circulation when inhaled, the development of such techniques is ongoing worldwide.[86,144,306,312−315] Hence, the distribution of CNTs after passage through the bloodstream will be revealed in the near future. At present, researchers should refrain from clinically applying CNTs to sites with abundant blood supply until adequate data are available to verify its safety.

On the other hand, it is necessary to determine whether CNTs topically used as biomaterials enter the bloodstream. Because CNTs are particles, they should be limited by size from

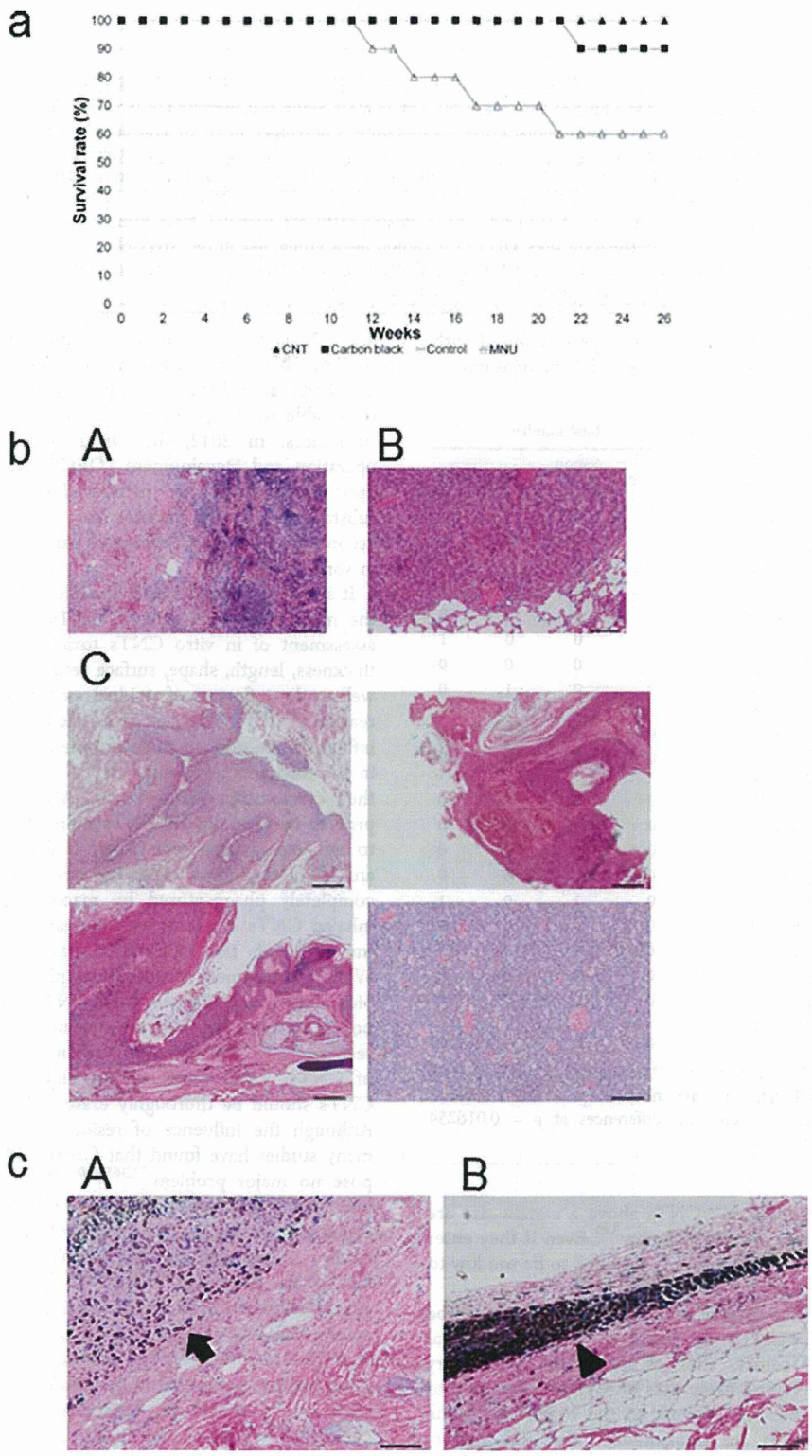

Figure 11. In a subcutaneous implantation test using cancer-developing transgenic rasH2 mice, MWCNTs were found to be not carcinogenic; their carcinogenicity was determined to be not higher than that of carbon black. (a) Changes over time in survival rate of rasH2 mice. All mice in the MWCNT group were alive at 26 weeks. In the carbon black group, 1 animal died at 22 weeks, and at 26 weeks, 9 of the 10 animals were alive. In the solvent group, all mice were alive at 26 weeks. In the *N*-methyl-*N*-nitrosourea (MNU) group, 1 animal died at 13, 14, 17, and 22 weeks each; 6 of the 10 animals were alive at 26 weeks. (b) Histological images of tumor masses in various organs of cancer-developing mice. (A) A tumor mass was