Evaluation of CNT toxicity by comparison to tattoo ink

The absence of an optimal nano-sized reference material has been the biggest obstacle in evaluating the safety of carbon nanotubes as biomaterials. In this study, black tattoo inks, which have a long history of use by humans, are shown to be suitable reference materials composed of nano-sized carbon black particles. We have also demonstrated that multi-walled carbon nanotubes have comparable basic safety properties to those of tattoo inks when used as biomaterials.

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Carbon nanotubes (CNTs)[1,2] are being used to develop new biomaterials in medical applications such as drug delivery and *in vivo* imaging systems[3-6], which are often used in the treatment of cancer. CNTs have been reported to function as good scaffolding for bone tissue and nerve regeneration[7,8]. Moreover, CNTs are being studied in combination with existing biomaterials to improve mechanical strength and durability[9].

Biocompatibility and toxicity are the most important issues surrounding the application of CNTs as a biomaterial for implantation in the human body. The toxicity of CNTs is currently being studied worldwide; however, the majority of these studies focus on the inhalation of CNTs[10-12]. In industry, CNTs are most likely to be inhaled; ergo, the urgent need to study its toxicity on inhalation. However, we believe that a completely different approach should be used to evaluate the toxicity of CNTs as a biomaterial, specifically, toxicity tests for implantation, cytotoxicity, carcinogenicity, genotoxicity, and accumulation in organs. To date, only a limited number of studies have tested the effects of CNTs following implantation, and the results of these studies were inconsistent[13-16]. This is because there was no known reference material for nano-sized carbon particles with proven biological safety; thus, standards for toxicity evaluation had not been established[6].

Black tattoo inks have a long history of being nonreactive and have been used on large numbers of people[17,18]. We are the first to use black tattoo inks as a reference material to evaluate the most basic biological toxicity features in long and short term implantation tests, cytotoxicity tests, and cytokine production assays of cultured macrophages.

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Experimental

Tattoo inks

We used two commercial black tattoo inks: Tattoo carbon black-1 (TCB-1) (Sumi-Black, Unique Tattoos, Subiaco, Australia) and Tattoo carbon black-2 (TCB-2) (Lining-Black, Classic Ink, Victoria, Australia). The appearances of TCB-1 and TCB-2 were visually inspected in glass dishes. The liquids were weighed, dropped on dishes, and then dried in an oven at 70 °C for three and ten hours, respectively. The inks were dried further in a vacuum oven at 50 °C for four hours in order to completely remove the entrapped solvents. The solid content of TCB-1 and TCB-2 was 78.7 % and 7.85 %, respectively. These dried TCB-1 and TCB-2 samples were examined by scanning electron microscope-energy dispersive x-ray spectroscopy (SEM-EDS) and the constituents were detected and quantified. The Raman spectra of TCB-1 and TCB-2 were measured using a microprobe Raman system, using industrial, general carbon black as a control. TCB-1, TCB-2, and the industrial carbon black were also observed by transmission electron microscope (TEM). Exothermal peak temperatures of TCB-1 and TCB-2 were measured in the atmosphere by thermogravimetric analysis (TGA) with a heating speed of 10 °C/min.

Multi-walled CNTs

We employed a commercial multi-walled CNT (MWCNT) manufactured using a chemical vapor deposition method, with an average diameter and length of 100 nm and 10 μm, respectively, and carbon purity of at least 99.9 %. The conditions for sterilization were 121 °C for 15 minutes in an autoclave. Tween 80 or polyvinyl alcohol (PVA) was used as a surfactant to disperse the MWCNTs in solution. The SEM images, Raman spectra, TEM images, and TGA results of MWCNTs were observed and measured using methods similar to those used for the tattoo inks.

Subcutaneous implantation tests with mice

The MWCNT material was added at a concentration of 4.0 mg/ml to physiological saline mixed with 0.1 vol% Tween 80 by sonication for 30 minutes using an ultrasonic homogenizer. Mixtures of TCB-1 and TCB-2 (4.0 mg/ml) were also prepared by the same method. The positive control was prepared by mixing zinc dibutyldithiocarbamate (ZDBC) at 4.0 mg/ml in physiological saline with 0.1 vol% Tween 80.

Subcutaneous pockets in 6 week old male ddY mice prepared by creating a 10 mm incision in the back skin and infusing 10 μl of the test solution into the pocket with a micropipette. Twenty mice were each included in the negative control (infused only with physiological saline with 0.1 vol% Tween 80), MWCNT, TCB-1, TCB-2, and positive control groups. Five mice from each group were euthanized at 1, 4, 12, and 24 weeks after surgery and the skin and subcutaneous tissue of the infused area was harvested as one block. The sample was fixed with neutral formalin (20 %), embedded in paraffin, sectioned (4 μm thick), and stained with hematoxylin and eosin. Plates were then prepared and observed using an optical microscope.

All animal experimentation procedures were carried out in compliance with the guidelines of the institutional animal care committee of Shinshu University.

Cytotoxicity tests

Colony-forming assays were performed using the V79 cell (Chinese hamster lung fibroblast). Liquid culture media consisted of Eagle's minimum essential medium (MEM) mixed with 0.292 g/L L-glutamine, 2.2 g/L sodium hydrogen carbonate, 0.11 g/L sodium pyruvate, and 5 vol% fetal bovine serum. Additionally, 4 mg/L antibiotic-antimycotic and 0.1 wt% PVA, as a surfactant, were added to the culture.

The MWCNT material was added to the liquid culture at concentrations of 12.5, 50, 200, 400, 800, and 1600 μg/ml and sonicated for 30 minutes to prepare the solution. The highest concentration of MWCNT (1600 μg/ml) that allows its uniform dispersion was used for our study. At higher concentration, the density of MWCNTs results in the production of floating particles that cannot be dispersed. The same concentrations of TCB-1 and TCB-2 solutions were prepared using the same procedure. Positive control liquid was prepared by adding ZDBC at concentrations of 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 μg/ml. Cell suspensions were diluted with a liquid culture medium and seeded into 12-well plates (100 cell/ml, 1.0 ml/well). The liquid culture was extracted after 24 hours, and 1.0 ml of each of the negative control (liquid culture media), MWCNT, TCB-1, TCB-2, and positive control liquid was added (n = 6 for each group). The liquid culture was replaced at day three, following the same procedure, and was evaluated at day six. Giemsa staining was performed after formalin fixation, and colony numbers were counted visually.

Cytokine production assay of cultured macrophages

Six week old male ddY mice were euthanized and bilateral tibias were harvested. Both ends of the bone were cut, and the liquid culture was circulated to the diaphysis by a syringe attached to a 26-gauge needle for bone marrow cell collection. The culture solution was prepared by adding 0.292 g/L L-glutamine, 10 vol% fetal bovine serum, and 4 mg/L antibiotic-antimycotic to αMEM and 0.1 wt% PVA.

Cell solutions (3 ml) were seeded on a 6 cm petri dish, and 30 000 units of macrophage colony stimulating factor (M-CSF) were added. The supernatant was collected after a 20 hour cultivation period, before macrophage adhesion, and was centrifuged at 1500 rpm for 7 minutes to collect the cells. The liquid culture was added at a concentration of 1.25×10^5 cell/ml and seeded onto a 48-well plate at 400 μl/well. Then, 4000 units of M-CSF were added[19,20] to the bone marrow cell-derived macrophage culture in order to prevent the emergence of apoptotic cells caused by the absence of M-CSF for at least 24 hours. After culturing for three days, the liquid culture was extracted, and the negative control (liquid culture media), MWCNT, TCB-1, TCB-2, or positive control liquid and 4000 units of M-CSF were added. In the MWCNT solution, MWCNTs were added at

concentrations of 12.5, 50, 200, 400, 800, and 1600 μg/ml, and the solution was sonicated for 30 minutes. TCB-1 and TCB-2 solutions of the same concentrations were prepared following the same procedure. Positive control liquid was prepared by adding lipopolysaccharide (LPS) at 100 ng/ml. The liquid cultures were harvested after 24 hours, and interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α were measured by enzyme-linked immunosorbent assay (ELISA) (n = 3 for each group) using a Quantikine kit. The liquid cultures were removed from the plates on which MWCNTs, TCB-1, and TCB-2 had been added. The plates were then washed with PBS and examined through phase contrast microscopy.

Statistics

Colony inhibition was compared among the MWCNT, TCB-1, and TCB-2 groups using one-way analysis of variance followed by Dunnett's multiple comparison test. The two-sided significance level was defined as $p = 0.05$. Analysis was performed by using SPSS 14.0 statistical package for Windows.

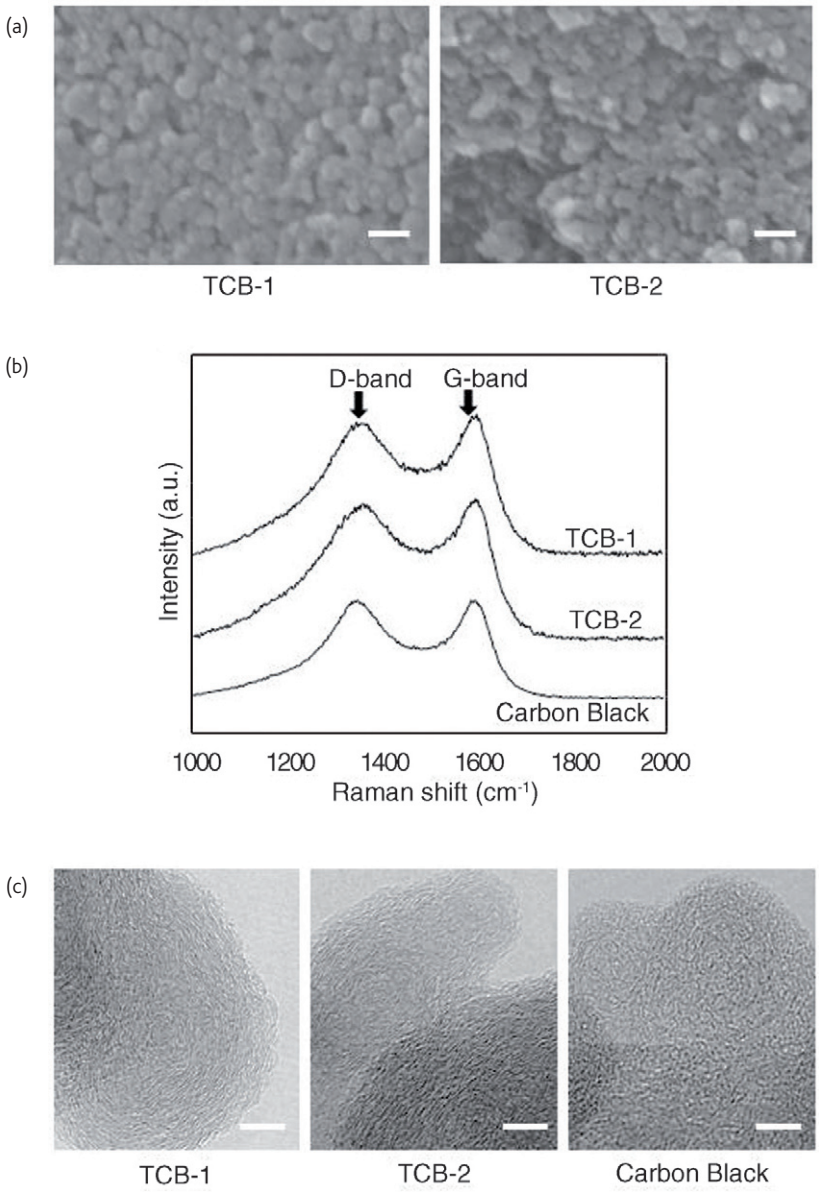

Fig. 1 Black tattoo inks are composed of nano-sized carbon black particles of high purity. (a) SEM images of two kinds of tattoo inks: TCB-1 and TCB-2. Almost uniform particles of approximately 40 nm in diameter were observed for TCB-1, while relatively non-uniform particles of approximately 50 nm in diameter were observed for TCB-2. Scale bar, 100 nm. (b) Raman spectra of TCB-1, TCB-2, and general carbon black. TCB-1 and TCB-2 showed almost the same Raman shift as general carbon black. D band: defect-induced mode; G band: E_{2g2} graphite mode. (c) TEM images of TCB-1, TCB-2, and general carbon black, showing nearly identical particle morphologies. Scale bar, 5 nm.

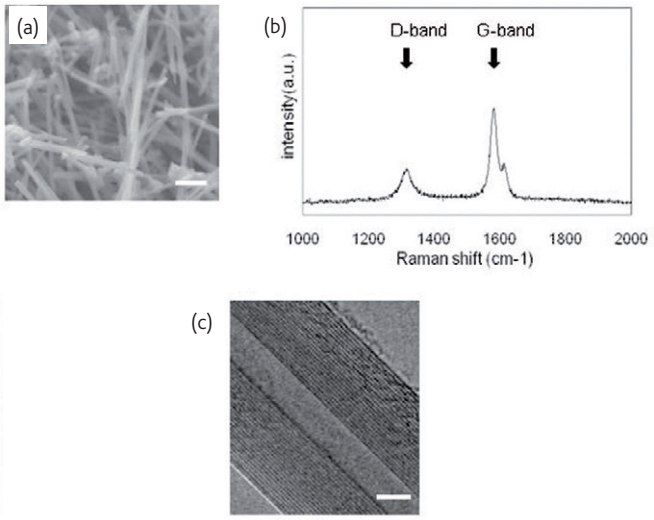

Fig. 2 Characterization of MWCNTs. (a) SEM images of MWCNTs revealed a fibrous morphology with an approximate diameter and length of 100 nm and 10 μm, respectively. Scale bar, 400 nm. (b) Raman spectra of MWCNTs. (c) TEM images of MWCNTs revealed multiple walls. Scale bar 5nm.

Results

The morphology of the black tattoo ink particles was observed by SEM. TCB-1 showed accumulation of almost uniform particles approximately 40 nm in diameter, while TCB-2 showed accumulation of relatively non-uniform particles approximately 50 nm in diameter (Fig. 1a). The particles underwent elemental analysis by SEM-EDS. TCB-1 consisted of C (99.6 wt%) and Na (0.4 wt%). TCB-2 consisted of C (99.5 wt%), Na (0.1 wt%), and S (0.4 wt%). No other elements were observed in either sample. Impurities such as Na and S were considered to be from the added surfactants and they were found only in trace amounts. Comparative Raman studies on TCB-1 and TCB-2 were performed using the industrial carbon black (which is used as a filler for rubber products) as a control. TCB-1 and TCB-2 showed a Raman feature similar to that of the general carbon black, where the G band at 1582 cm^{-1} originated from the E_{2g2} graphite mode and the D band at 1360 cm^{-1} was a defect-induced mode (Fig. 1b). The R values (defined as the integrated intensity of the D band [I_D] divided by the intensity of the G band [I_G]) for TCB-1 and TCB-2, and general carbon black were comparable, at approximately 0.95 and 1.0, respectively. Observation by TEM revealed that the morphologies of TCB-1 and TCB-2 particles were almost the same as that for general carbon black (Fig. 1c). These results showed that the black tattoo inks were composed of nano-sized carbon black particles.

The SEM image, Raman spectra, and TEM image of MWCNTs are shown in Fig. 2. The exothermal peak temperatures, measured by TGA, for TCB-1, TCB-2, and MWCNTs were 500 °C, 620 °C, and 780 °C, respectively. These results indicated that MWCNTs possessed a lower reactivity than the carbon black used for tattoos.

Subcutaneous implantation of the MWCNTs in mice was the first safety test performed. All of the mice survived the test period and the skin in the implanted area recovered early in the test period without any abnormal reactions, including necrosis or tumor formation. Histological analysis of the negative control group (saline group) revealed that the subcutaneous tissue had almost and completely recovered at 1 and 4 weeks, respectively, remaining unchanged at 12 and 24 weeks (Fig. 3a). In the MWCNT group, the majority of the particles were incorporated into macrophages at 1 week and peripheral tissue showed a weak inflammatory reaction with the accumulation of fibroblasts, neutrophils, and lymphocytes. At 4 weeks, MWCNT particles were still incorporated in macrophages, which became multinucleated giant cells in a state of foreign-body granuloma, and the inflammation had been suppressed. Tissue reactions at 12 and 24 weeks were unchanged from that at 4 weeks (Fig. 3b). The TCB-1 and TCB-2 group showed a similar tissue reaction. At 1 week, the majority of particles had been incorporated into macrophages, and, as in the MWCNT group, fibroblasts, neutrophils,

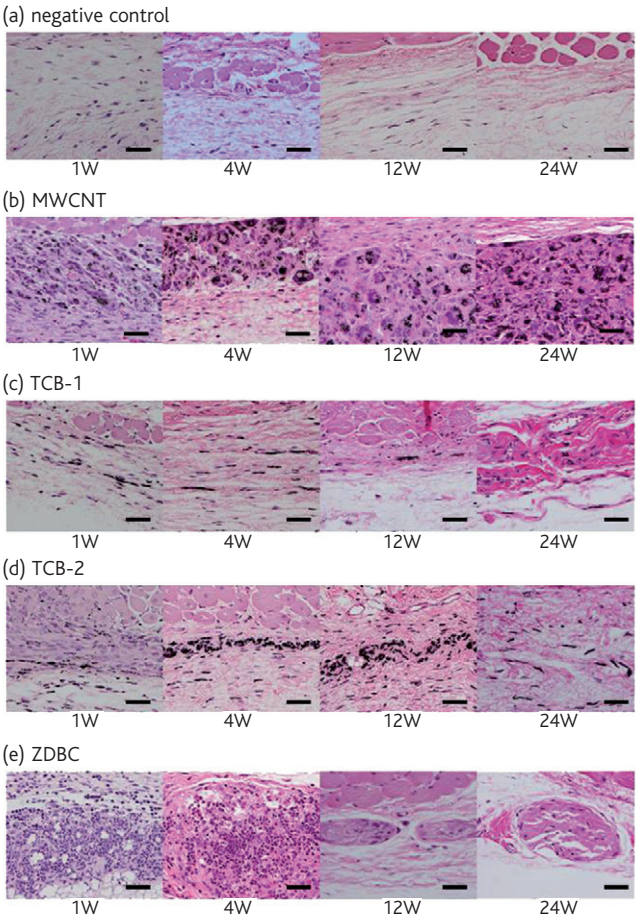

Fig. 3 Tissue reaction to MWCNTs implanted in subcutaneous tissue. Tissues reacted to MWCNTs and black tattoo ink particles similarly. Hematoxylin and eosin staining, scale bars, 20 μm. (a) Subcutaneous tissue images from the negative control group. (b) Subcutaneous tissues infused with 10 μl MWCNT solution (4.0 mg/ml). (c) Subcutaneous tissues were infused with 10 μl TCB-1 solution (4.0 mg/ml). (d) Tissues infused with 10 μl TCB-2 solution (4.0 mg/ml). (e) Tissues infused with 10 μl ZDBC solution (4.0 mg/ml).

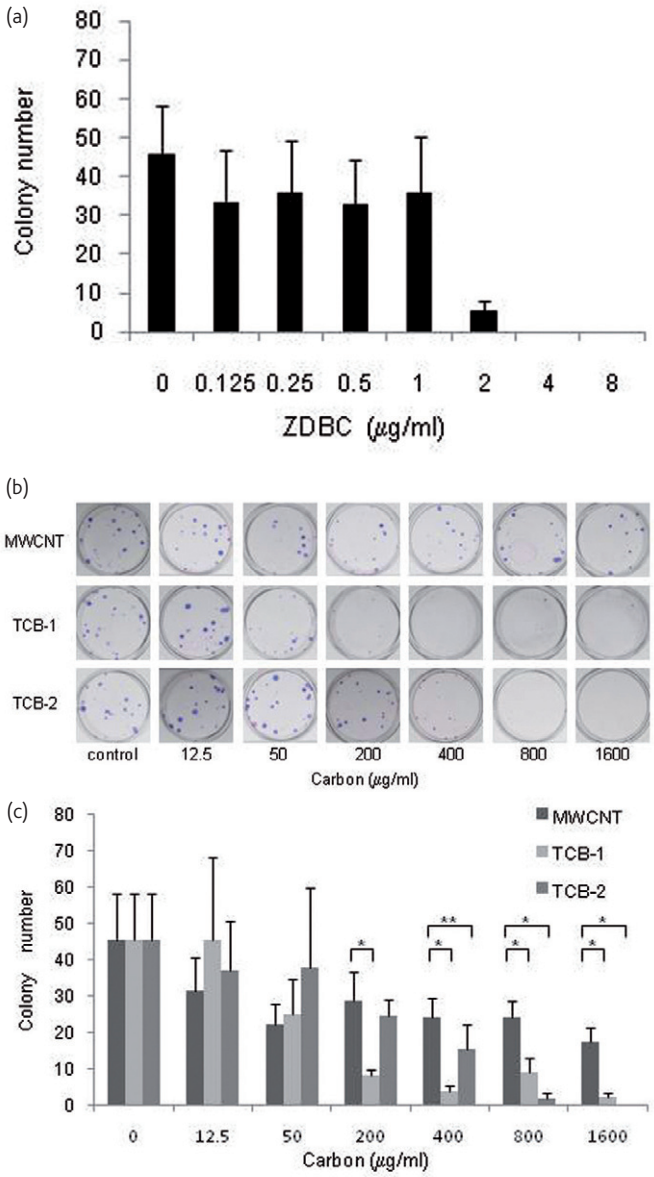

*Fig. 4 Cytotoxicity of MWCNTs was lower than that of the carbon black for tattoo ink. (a) Validity of cytotoxicity evaluation by colony forming assay. Colony formation of V79 cells was reduced at 2 μg/ml concentration in the presence of ZDBC. The IC 50 (standard value: 1–4 μg/ml) was between 1 and 2 μg/ml, verifying test validity. Error bars, SD; n = 6. (b) Direct contact photos of colony forming assay. Colony formation of V79 was compared among culture medium alone (negative control), MWCNT, TCB-1, and TCB-2 solutions. Concentration of each solution was 12.5, 50, 200, 400, 800, and 1600 μg/ml. (c) Colony formation of MWCNT, TCB-1, and TCB-2 solutions by concentration. MWCNTs inhibited colony formation in a concentration-dependent manner, as did both TCB-1 and TCB-2. Error bars, SD; n = 6. *, $p < 0.001$. **, $p = 0.016$.*

and lymphocytes were observed. The inflammatory reaction was slightly weaker than that in the MWCNT group. Inflammation around the tissue was suppressed by 4 weeks, as in the MWCNT group, and tissue images similar to those at 4 weeks were observed at 12 and 24 weeks (Fig. 3c, d). In the positive control ZDBC group, a large number of various inflammatory cells, including fibroblasts, neutrophils, lymphocytes, and plasma cells, had accumulated by 1 week, and strong inflammation over a wide area had developed with fat necrosis and nuclear debris. Macrophage accumulation was not observed. At 4 weeks, inflammatory cells were still present with inflammation, though it had resolved slightly. At 12 and 24 weeks, inflammation was suppressed and subcutaneous tissue had recovered as fibrotic scar tissue (Fig. 3e). Overall, MWCNTs induced mild acute inflammatory reactions in subcutaneous tissue but the inflammation was quickly suppressed. Furthermore, implanted MWCNTs were incorporated into macrophages quickly and remained there for a long period. The short and long term tissue reactions to MWCNTs were almost the same as those to TCB-1 and TCB-2, showing good local tissue compatibility.

Next, we conducted an *in vitro* cytotoxicity test, a colony-forming assay, to determine the toxicity level of MWCNTs as a biomaterial. Addition of the positive control material ZDBC decreased colony formation at 2 μg/ml and prevented colony formation at 4 μg/ml or higher. The concentration at which 50 % of colonies formed are inhibited (IC 50, standard value: 1 – 4 μg/ml) was shown to be between 1 and 2 μg/ml. Thus, this test was validated as appropriate for evaluating cytotoxicity (Fig. 4a). The MWCNTs inhibited colony formation in a concentration dependent manner. TCB-1 and TCB-2 results were similar to those of the MWCNTs. In the TCB-2 group, colony formation was not observed at 1600 μg/ml. The MWCNTs showed a significantly larger colony number, at ≥ 200 μg/ml, than TCB-1. At ≥ 400 μg/ml, the MWCNT group showed a significantly larger colony number than the TCB-2 group (Fig. 4b, c). Thus, MWCNTs were thought to have lower cytotoxicity than those of TCB-1 and TCB-2.

Finally, we exposed the MWCNTs to macrophages *in vitro* to study their reaction. Phase-contrast microscopy analysis of the macrophages cultured with MWCNTs, TCB-1, or TCB-2 at 50 μg/ml revealed that the macrophages had become blackened by the carbon particles but that their nuclei had not (Fig. 5a). These results suggested incorporation of MWCNT, TCB-1, or TCB-2 particles in the macrophage cytoplasm. Results of ELISA showed high values for the positive control group for IL-1β, IL-6, and TNF-α. In the MWCNT group, IL-1β, IL-6, and TNF-α were not detected at the highest concentration of 1600 μg/ml, the upper limit for particle dispersion in solution. The TCB-1 and TCB-2 groups displayed similar results (Fig. 5b). It was thus concluded that the MWCNTs, as well as the carbon black tattoo ink, were incorporated into the macrophage cytoplasm, but did not increase inflammatory cytokine production.

Discussion

Tattooing is an ancient art that is still practiced worldwide. While some tattoo inks have been associated with allergies, skin inflammation, and keratoacanthomas, these adverse reactions are rare, with the ink color being responsible[17,18]. Generally, black tattoo inks are safe for use on the human body. The black tattoo inks used in this study were found to consist of nano-sized particles with high carbon purity. While

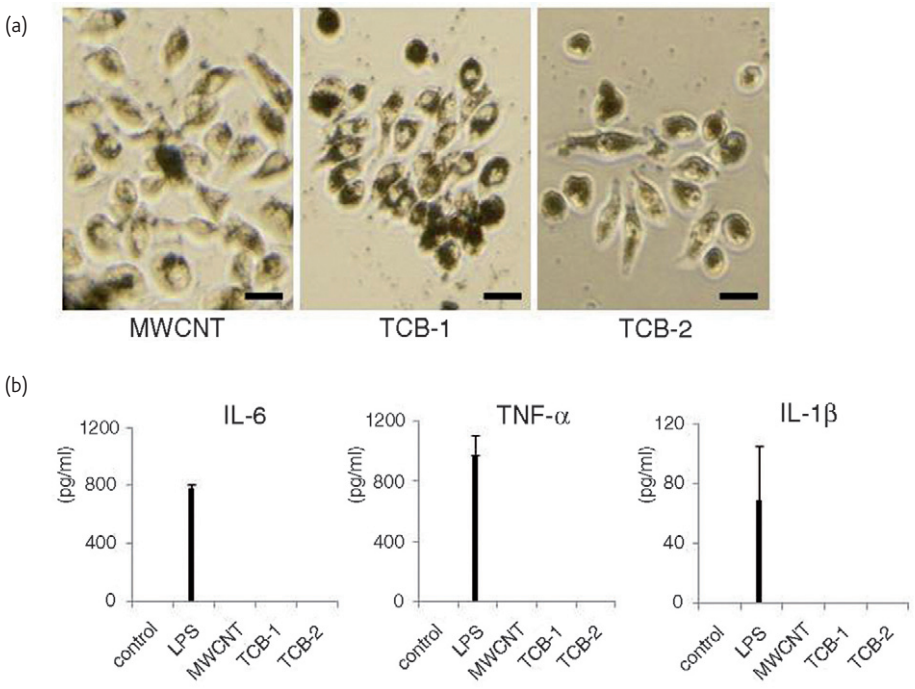

Fig. 5 MWCNTs incorporated in macrophages did not induce inflammatory cytokine. (a) Phase-contrast microscopic images of macrophages treated with 50 μg/ml solutions of MWCNTs, TCB-1, and TCB-2 for 24 hours. Particles of MWCNTs, TCB-1, and TCB-2 were incorporated in macrophage cytoplasm. Scale bar, 20 μm. (b) Inflammatory cytokine production of macrophages treated with MWCNTs, TCB-1, and TCB-2. LPS (100 ng/ml) was added to the culture after 24 hours. Error bars, SD; n = 3.

both are carbon based and have similar particle size, CNTs differ from tattoo carbon black in shape, aspect ratio, structure, and surface areas. Therefore, we believe that tattoo carbon black particles are a possible reference material in biological safety evaluations for same-mass comparison with CNTs.

Our comparison with the reference materials has shown that highly pure commercial MWCNTs have comparable tissue compatibility and cytotoxicity. The safety tests were performed in accordance with the international standard ISO10993. Nano-sized materials such as CNTs, which are smaller than cells, may act on cell surfaces or be incorporated into cells *in vitro*[21]. Therefore, to study the cytotoxicity of MWCNTs upon cell contact, we used a colony-forming assay because it currently has the best sensitivity and repeatability[22]. In contrast to the colony-forming assay that we performed, the majority of cytotoxicity evaluations, including trypan blue exclusion test or MTT test, employ absorbance or fluorescence. Staining materials such as MTT, WST-1, neutral red, or alamar blue are reported to potentially interact with carbon materials; thus, cytotoxicity testing using these stains could lead to an incorrect evaluation of the cytotoxicity[23].

MWCNTs did not induce inflammatory cytokines from cultured macrophages. Recently, Palomäki *et al*. also reported that pure CNTs did not induce cytokine secretion from mouse macrophages (RAW264.7 cell line)[24]. However, it has been indicated that CNTs stimulate the expression of inflammatory cytokines in macrophages[25]. This discrepancy in our results is attributable to the amount of residual metal catalyst in the CNT samples. Thus, pure CNTs might not target macrophages. This *in vitro* macrophage reaction supports the *in vivo* results of subcutaneously implanted MWCNTs being incorporated into macrophages at an early stage and remaining there, stably, for a long period, without inducing a severe inflammatory reaction.

The effect of intraperitoneal CNT administration on macrophage response revealed that the inflammatory reaction caused by long CNTs continued for a long time; however, the inflammatory reaction caused by short CNTs resolved quickly[10]. This difference was due to incomplete phagocytosis, where the macrophage could not completely incorporate the CNTs, resulting in toxicity. One previous study reported 20 μm long (at least) CNTs as potentially toxic when inhaled[12]. This threshold of the risk associated with fiber length should be applied to biomaterials, since CNTs embedded in the living body are endocytosed by macrophages; thus, it might be best to avoid the use of long CNTs in biomaterials. The mean MWCNT length used in our study was 10 μm, and our samples contained few CNTs with minimum lengths of 20 μm. The CNT characteristics that are targets for antigen presentation by macrophages require further study.

We used pure CNTs without chemical modification to evaluate their basic safety. However, the actual application of CNTs as biomaterials will use CNTs that have been chemically modified. For example, n-doped MWCNTs are reported to be less toxic than pure MWCNTs via the inhalation toxicity test and toxicity tests in *Entamoeba histolytica*, which incorporated CNTs. These suggest that n-doped MWCNTs

Instrument citation

Scanning electron microscope and energy dispersive x-ray spectroscope (SEM-EDS), JSM-7000F and JED-2300, JEOL, Tokyo, Japan
Microprobe Raman system, R-XN1-532, Kaiser, Ann Arbor, MI, USA
Transmission electron microscope, JEM-2010, JEOL
Thermal analysis equipment, Thermo plus EVO2 TG8120, Rigaku, Tokyo, Japan
Phase contrast microscope, IX71, Olympus, Tokyo, Japan
Ultrasonic homogenizer, HA380A, AS ONE, Osaka, Japan
Quantikine kit, R&D Systems, Minneapolis, MN, USA
Industrial general carbon black, Vulcan XC 72, Cabot, Boston, MA, USA
Commercial multi-walled CNTs, VGCF-S; Showa Denko, Tokyo, Japan
Tween 80, Polysorbate80, NOF, Tokyo, Japan
Polyvinyl alcohol, 160-03055, Wako, Osaka, Japan
V79 cell, Chinese hamster lung fibroblast: JCRB0603, Cell bank, Tokyo, Japan
Antibiotic-antimycotic, GIBCO15240, Invitrogen, Carlsbad, CA USA
Macrophage colony stimulating factor (M-CSF), Leukoprol, Kyowa Hakko, Tokyo, Japan
Lipopolysaccharide (LPS), L2880, Sigma-Aldrich, St. Louis, MO, USA
Male ddY mice, Japan SLC, Hamamatsu, Japan

are safer biomaterials[26,27]. Reference materials that provide a clear standard for safety evaluation are warranted to develop safer and more effective CNTs made by adding various chemical modifications.

This report is significant because it is the first of its kind to validate the possible use of carbon black tattoo inks as a reference material for studying CNT safety. However, the quality of tattoo ink substances is not standardized across products. The next step will be to develop international standards for nano-sized carbon black. Evaluation with a safety standard will reveal important aspects of the tissue or cell responses to CNTs, and scientifically detailed experiments for biological safety will become possible in combination with other tests.

Moreover, we have revealed the previously unknown basic safety of MWCNTs, partly by comparison to this reference material. Because CNTs have a thin and long fibril morphology and can easily aggregate, further evaluation of carcinogenicity and genotoxicity is warranted. Furthermore, CNTs may accumulate in the liver or spleen to induce toxicity. These other tests should be considered before drawing conclusions on CNT safety. Moreover, sufficient evaluation should be performed to confirm the biological safety of many types of CNTs, as they vary in their manufacturing method, diameter, length, and impurities.

Conclusions

A few studies have indicated that there are some risks associated with CNT implantation[15,24], while others have shown CNTs to have low toxicity[4,16,23]. The biggest obstacle in evaluating CNT toxicity has been the absence of a nano-sized reference material to use as a safety standard for comparison[6]. We have been the first to show that black tattoo inks, which have been used on countless human bodies over millennia, are composed of nano-sized carbon black particles, and are, therefore, a possible safety reference material for biological toxicity evaluation. The clinical use of CNTs will lead to diverse applications and help advance present day medicine[3,5,6,28,29]. We believe that the results of this pilot study can be used as a basis for the application of CNTs as an innovative biomaterial.

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