Research Article

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Differential genotoxicity of chemical properties and particle size of rare metal and metal oxide nanoparticles

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ABSTRACT: Nanoparticles of rare metal compounds are used in various products. However, their carcinogenicity and genotoxicity have not been sufficiently evaluated. The tumor-initiating and -promoting potentials of four rare metals, indium oxide (In_2O_3), dysprosium oxide (Dy_2O_3), tungsten oxide (WO_3) and molybdenum (Mo), with a well-defined particle diameter were evaluated. The mutagenicity of these rare metals was investigated by Ames test using five bacteria strains, and transformability of these rare metals was investigated by cell-transformation assay using v-Ha-ras-transfected BALB/c 3T3 cells (Bhas 42 cells). Nano-sized Dy_2O_3 showed strong mutagenesis in all five bacteria strains tested with and without metabolic activation, while micro-sized particles showed weak mutagenesis in two bacterial strains. Dy_2O_3 induced transformation colonies of Bhas 42 cell dose-dependently, although there was no difference in the number of transformed foci between nano-sized and micro-sized particles. Nano-sized In_2O_3 and WO_3 showed positive mutagenic response in TA1537 and TA98, respectively, whereas the micro-sized metal oxide particles showed no mutagenesis in the test bacterial strains. Both nano-sized and micro-sized In_2O_3 showed similar levels of transformability. However, nano-sized and micro-sized WO_3 did not show any transformability. Both nano-sized and micro-sized Mo particles showed neither mutagenesis nor transformability. These results suggest that mutagenicity of rare metals depends on their particle size, although transformability depends on their chemical components but not on their particle size. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: Ames test; genotoxicity; mutagenicity; nanoparticles; rare metal; metal oxide; transformation activity; tumor initiation; tumor promotion

INTRODUCTION

Recent studies have indicated that the toxicity of nanoparticles is greater than that of larger particles in the rat lung (Oberdörster *et al*., 2005). Dankovic *et al*. (2007) reported that the inhalation of ultrafine titanium dioxide (TiO_2, <100 nm particle size) induced lung tumors at lower mass concentration than fine TiO_2 (<2.5 μm particle size) *in vivo*, suggesting that some metal nanoparticles possess carcinogenic properties. The small size of nanoparticles facilitates not only uptake into cells and invasion into many tissues via blood and lymph circulation once incorporated into the body but also reaches into nuclear (Hackenberg *et al*., 2010). However, the relationship between carcinogenesis and particle physico-chemical characteristics of rare metals is not well understood.

Rare metals are composed of 47 sparse metal elements (Hampel, 1961). They possess unique chemical and physical properties that make them excellent industrial materials, especially in the field of alloys, electric magnetic materials and functional materials. Indium is widely used for conductive films of liquid crystal display. Dysprosium is used for magneto-optical memory disks and high-performance magnets. Tungsten and molybdenum are used for semiconductors. Little toxicological information is available for these rare metals, although their usage has increased. The rare metals by themselves are thought to be safe elements, whereas adverse health effects of the metal compounds have been reported. Homma *et al*. (2003) reported a case of interstitial pneumonia caused by occupational exposure of indium–tin oxide particles. Pneumoconiosis caused by inhaled cobalt and tungsten carbide particles was also reported (Enriquez *et al*., 2007). The genotoxicity of these rare metals is poorly understood.

The aim of this study was to evaluate the mutagenicity and transformation activity of nano-sized rare metals *in vitro* by the bacterial reverse mutation assay (Ames test) and Bhas 42 cell transformation assay, respectively. Three rare metal oxides and one elemental metal with defined particle diameter and components – indium oxide (In_2O_3), dysprosium oxide (Dy_2O_3), tungsten oxide (WO_3) and molybdenum (Mo) – were tested.

MATERIALS AND METHODS

Test Materials and Characterization of Rare Metal Nanoparticles in Suspension

Micro-sized and nano-sized (<100 nm particle size (BET)) indium (III) oxide (In_2O_3), dysprosium (III) oxide (Dy_2O_3), tungsten (VI) oxide (WO_3) and molybdenum (Mo) were purchased from

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Table 1. The numbers of revertant colonies induced by positive control reagents

	Direct		Metabolic activation	
	Pre-incubation time		Pre-incubation time	
Strains	20 min	8 h	20 min	8 h
TA98	209.0 ± 25.5	166.5 ± 2.1	1494.0 ± 108.9	876.0 ± 50.9
	(AF-2, 0.1 μg per plate)		(2AA, 10 μg per plate)	
TA100	488.5 ± 10.6	574.0 ± 18.4	1707.0 ± 26.9	584.0 ± 38.2
	(AF-2, 0.01 μg per plate)		(2AA, 10 μg per plate)	
TA1535	190.5 ± 20.5	308.5 ± 27.6	220.5 ± 4.9	174.0 ± 14.1
	(NaN_3, 2 μg per plate)		(2AA, 10 μg per plate)	
TA1537	1328.0 ± 60.8	2978.0 ± 140.0	275.0 ± 19.8	136.5 ± 10.6
	(9AA, 5 μg per plate)		(2AA, 10 μg per plate)	
WP-2 uvrA	295.5 ± 21.9	211.0 ± 21.2	120.5 ± 4.9	176.5 ± 10.6
	(AF-2, 0.02 μg per plate)		(2AA, 10 μg per plate)	

AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; NaN_3, sodium azide; 9AA, 9-aminoacridine hydrochloride hydrate; 2AA, 2-aminoanthracene. Data are expressed as means ± SD.

Sigma-Aldrich Co. (St Louis, MO, USA). Only Mo was an elemental metal particle while the rest of the particle was metal oxide, because nano-sized molybdenum oxide was not commercially available. Rare metal particles were suspended in ultrapure water at a concentration of 0.2 mg ml^{-1}, and the solutions were sonicated for 5 min. The solutions containing nano-sized rare metal particles were then filtered with sterile Acrodisc syringe filters with Supor membrane (5 μm pore size, Pall Corporation, Port Washington, NY, USA). After filtration, the size distribution and ζ-potential of nano-sized rare metals were evaluated by dynamic light scattering photometer (DLS; DLS-7000, Otsuka Electronics Co. Ltd, Osaka, Japan) and Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK), respectively.

Ames Test

The mutagenicity of rare metals was evaluated by the revised bacterial reverse mutation assay (Ames test) (Maron and Ames, 1983). *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP-2 uvrA strains were used for detecting mutagens that caused frameshift mutations and base-pair substitutions (Aiub *et al*., 2002). Tests were performed with and without metabolic activation using rat liver S9 fraction (S9 mix, Kikkoman Corporation, Chiba, Japan) according to the OECD guidelines. In order to increase the sensitivity of detection of mutagens, the pre-incubation time was increased from the 20 min normally used in the Ames test to 8 h. A suspension of the particles was diluted with 50 mM phosphate buffer (pH 7.4) to 20, 40 and 80 μg ml^{-1} since our preliminary data showed growth inhibition of bacteria strains at metal concentrations more than 156 μg ml^{-1} (data not shown). These metal particle suspensions were pre-incubated with each tester strains for 20 min or 8 h at 37 °C with [metabolic activation (+); metabolic activation method] or without [metabolic activation (−); direct method] 5% S9 microsomal preparation. After pre-incubation, 1 ml of the incubation mixture was added to the top agar solution with 0.5 mM L-histidine and 0.5 mM D-biotin for *Salmonella typhimurium* or with 0.5 mM L-tryptophan for *Escherichia coli* strain and was poured onto a minimal glucose agar medium plate (Tesmedia AN, Oriental Yeast Co. Ltd, Tokyo, Japan). The plate was incubated for 48 h at 37 °C and apparent revertant colonies were counted. Test results were considered to show significant mutagenic activity when a 2-fold increase in the number of the revertant colonies in treated plates over the solvent control plates was observed. The test was performed in duplicate for each concentration of particle suspension. *trans*-2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2, Wako Pure Chemical Industries, Ltd, Osaka, Japan) for TA98, TA100 and WP-2 uvrA, sodium azide (NaN_3, Wako) for TA1535 and 9-aminoacridine hydrochloride hydrate (9AA, Sigma-Aldrich) for TA1537 strains were used as positive controls of the test without metabolic activation, and 2-aminoanthracene (2AA, Wako) for all tester strains was used as positive control of the test with metabolic activation. The numbers of revertant colonies in TA98, TA100, TA1535, TA1537 and WP-2 uvrA induced by each positive

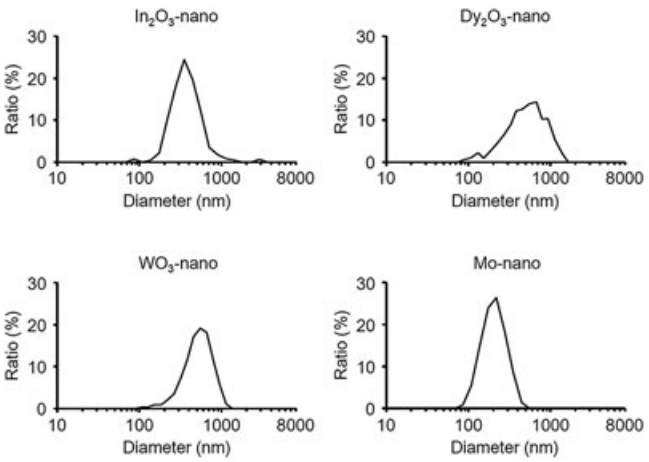

Figure 1. Size distribution of nano-sized rare metals in suspension. Size distribution of nano-sized In_2O_3 (In_2O_3-nano), Dy_2O_3 (Dy_2O_3-nano), WO_3 (WO_3-nano) and Mo (Mo-nano) in suspension was measured by dynamic light scattering photometer in the range from 10.0 nm to 7355.4 nm. Values are the mean of three experiments.

Table 2. Characterization of metal compounds used in this study

Characteristics	Dy_2O_3-nano	Dy_2O_3-micro	In_2O_3-nano	In_2O_3-micro	WO_3-nano	WO_3-micro	Mo-nano	Mo-micro
Purity[a]	>99.9%	>99.99%	>99.9%	>99.99%	NA	>99%	>99.8%	>99.9%
Diameter[a]	< 100 nm (BET) < 50 nm (XRD)	NA	< 100 nm (BET) < 50 nm (XRD)	NA	< 100 nm (BET) < 50 nm (XRD)	10.5 µm	< 100 nm (BET) < 100 nm (TEM)	4.51 µm
Mean diameter in solution[b]	565.2 nm	>7 µm	391.0 nm	>7 µm	545.5 nm	>7 µm	213.2 nm	>7 µm
Zeta potential[c]	50.6 mV	NM	−31.6 mV	NM	−54.4 mV	NM	−42.2 mV	NM
Primary crystallite size[a]	6.0 nm	NA	17.0 nm	NA	15.9 nm	NA	NA	NA
Surface area[a]	14.1 $m^2 g^{-1}$	NA	28.0 $m^2 g^{-1}$	NA	9.7 $m^2 g^{-1}$	NA	NA	NA
Impurities[a]	Ag: 120 ppm Zn: 1.6 ppm	Mg: 1.4 ppm	Ca: 320.8 ppm Al: 78.6 ppm Mg: 78.6 ppm Cu: 49.9 ppm B: 29.2 ppm	Cu: 10.1 ppm Fe: 5.9 ppm Na: 5.3 ppm Mg: 5.0 ppm Ca: 2.1 ppm Zn: 0.4 ppm Ni: 0.3 ppm	NA	Zr: 4480 ppm K: 442 ppm	Ca: 148 ppm Fe: 68.6 ppm Sn: 34.1 ppm	Ba: 3.0 ppm

NA, not available; NM, not measured.
[a]Manufacturer's specification.
[b]Measured by DLS.
[c]Measured by Zetasiser.

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control and those induced by metabolic activation are shown in Table 1, demonstrating that assay systems functioned as designed.

Bhas 42 Cell Transformation Assay

To investigate tumor promotion activity of rare metals, a Bhas 42 cell transformation assay was performed (Ohmori *et al*., 2004). Bhas 42 cells were established from BALB/c 3 T3 cells transfected with v-Ha-ras oncogene, and are regarded as initiated cells in the two-stage carcinogenesis model. Bhas 42 cells were cultured in DMEM/F12 medium supplemented with 5% fetal bovine serum in six-well plates (4×10^4 cells per well, day 0). After cultivation for 3 days, medium was replaced with fresh medium containing rare metals (0–5 μg ml^{-1}). The culture medium was changed on days 7 and 10 for fresh medium containing rare metals, and was then changed on days 14 and 17 for fresh medium without rare metals. On day 21, cells were fixed with methanol for 30 min and stained with 2.5% Giemsa solution for 20 min. Transformed colonies were defined by morphological characteristics: deep basophilicity, dense multi-layering of cells, random orientation of cells at the edge of colonies and more than 50 cells within a focus.

Data Analysis

Two-way ANOVA with Fisher's protected least significant difference was employed to compare the mean values between two groups. A *P*-value of <0.05 was considered to indicate a statistically significant difference.

RESULTS

In order to characterize the physicochemical properties of rare metals, size distribution of nano-sized rare metals in suspension was measured by DLS (Fig. 1). Although all nano-sized rare metals were denoted as less than 100 nm particle size (BET) by the manufacturer, the results show a size distribution ranging between 85.8 and 2881.3 nm with a mean diameter at 391.0 nm for nano-sized In_2O_3, between 85.8 and 1132.3 nm with a mean diameter at 565.2 nm for nano-sized Dy_2O_3, between 106.5 and 1134.1 nm with a mean diameter at 545.5 nm for nano-sized WO_3, and between 85.8 and 442.2 nm with a mean diameter at 213.2 nm for nano-sized Mo, suggesting that all tested metals were aggregated in suspension. Nanoparticles were not detected in micro-sized In_2O_3 or micro-sized Mo in suspension, and only a trace amount of nanoparticles was detected in micro-sized Dy_2O_3 and micro-sized WO_3, measured by DLS (data not shown), therefore we concluded that the mean diameter of micro-sized rare metal particles in solution was more than 7 μm, the upper detection limit of DLS.

The stability of nano-sized rare metal suspensions against aggregation was evaluated by surface charge measurement. The ζ-potential of nano-sized rare metal suspensions showed a range from −54.4 to 50.6 mV, indicating that all nano-sized rare metals dispersed well in water suspensions. Characterization of metal compounds used in this study is summarized in Table 2.

The effect of pre-incubation time on the number of revertant colonies in Ames test was evaluated to optimize the assay protocol. TA98 strain was pre-incubated with nano-sized or micro-sized WO_3 (40 μg per plate) for 20 min and 1, 2, 4, 8, 16 or 24 h (Fig. 2). The number of revertant colonies induced by nano-sized

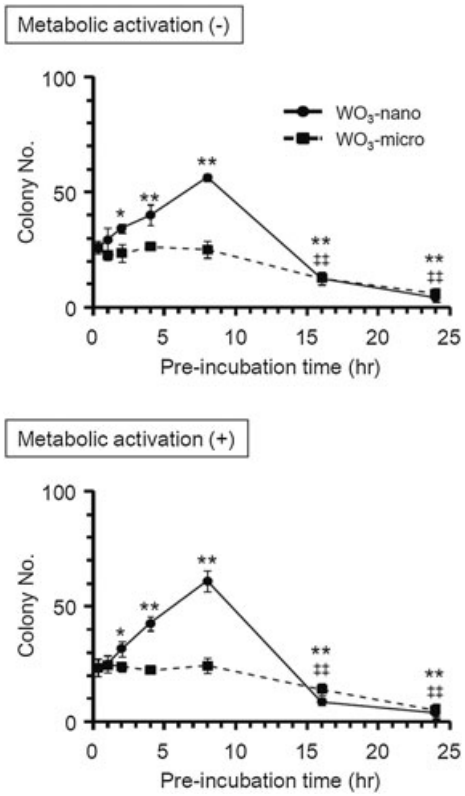

Figure 2. Effect of pre-incubation time on the number of revertant colonies. TA98 strain was pre-incubated with nano-sized or micro-sized WO_3 (40 μg per plate) for 20 min and 1, 2, 4, 8, 16 or 24 h. The numbers of revertant colonies in TA98 strain induced by nano-sized (solid line) and micro-sized (dashed line) WO_3 with [metabolic activation (+)] or without [metabolic activation (−)] metabolic activation are shown. The data are means ± SD of three experiments in duplicate. * $P < 0.05$, ** P 0.01 (WO_3-nano, pre-incubation time vs 20 min incubation). ‡‡ $P < 0.01$ (WO_3-micro, pre-incubation time vs 20 min incubation).

WO_3 increased time-dependently up to 8 h of pre-incubation, indicating that a longer pre-incubation time enhanced the sensitivity for detecting mutagens. However, prolongation of pre-incubation time beyond 8 h decreased the number of revertant colonies, suggesting the induction of bacterial cell death. Similar effect of prolonged pre-incubation was observed on the number of revertant colonies with both direct method [metabolic activation (−)] and metabolic activation method [metabolic activation (+)]. From these data, we conducted the Ames test with 20 min and 8 h pre-incubation in the subsequent experiments.

We selected four rare metals, Dy_2O_3, In_2O_3, WO_3 and Mo, widely used in advanced industrial applications, to evaluate their mutagenicity. The mutagenicity of these rare metals and metal oxides with defined particle diameters and components was tested by Ames test using five bacteria strains. Nano-sized Dy_2O_3 increased the number of revertant colonies dose-dependently in all strains tested with and without metabolic activation, although micro-sized Dy_2O_3 increased the number of revertant colonies only at the highest concentration (80 μg per plate) in TA98 and TA1535 strains (Fig. 3). Pre-incubation for 8 h induced a larger number of revertant colonies as compared with that pre-incubated for 20 min in nearly all strains. The number of revertant colonies was larger in the group tested without metabolic

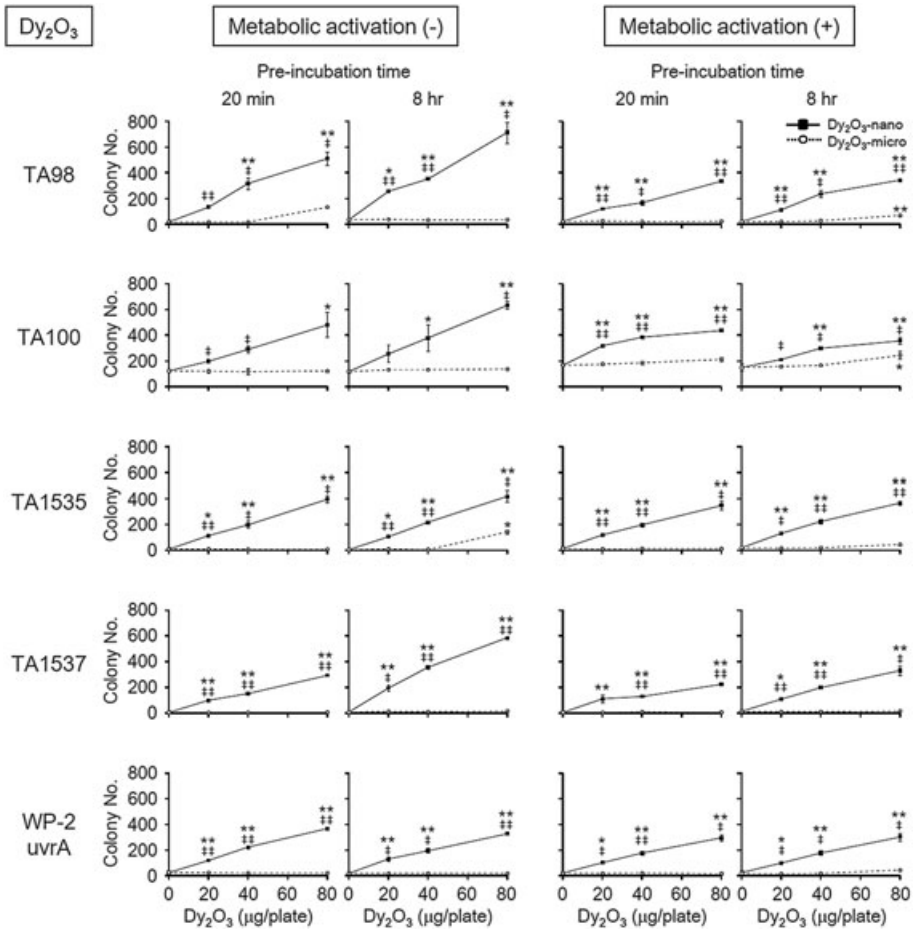

Figure 3. Numbers of revertant colonies in bacteria strains induced by Dy_2O_3. The numbers of revertant colonies in TA98, TA100, TA1535, TA1537 and WP-2 uvrA strains induced by nano-sized (solid line) and micro-sized (dashed line) Dy_2O_3 with [metabolic activation (+)] or without [metabolic activation (−)] metabolic activation are shown. The data are means ± SD of three experiments in duplicate. * $P < 0.05$, ** $P < 0.01$ (vs 0 μg per plate Dy_2O_3). ‡ $P < 0.05$, ‡‡ $P < 0.01$ (nano- vs micro-sized particles).

activation as compared with the metabolic activation group in TA98, TA100 and TA1537 strains.

Revertant colonies were not observed when all strains were pre-incubated with nano- or micro-sized In_2O_3 for 20 min (Fig. 4). Nano-sized In_2O_3 increased the number of revertant colonies of only TA1537 with 8 h pre-incubation, although micro-size particles did not increase the number of revertant colonies. The number of revertant colonies of TA1537 increased dose-dependently when the cells were exposed to up to 80 μg per plate of nano-sized In_2O_3 with metabolic activation. However, without metabolic activation, a significant increase of the number of revertant colonies of TA1537 induced by nano-sized In_2O_3 was observed only at the concentration of 40 μg per plate. Nano-sized In_2O_3 particles greater than 40 μg per plate induced bacterial cell death in the group with 8 h pre-incubation.

Similar to the case of In_2O_3, nano- or micro-sized WO_3 did not increase the number of revertant colonies with 20 min pre-incubation for all strains tested (Fig. 5). However, the number of revertant colonies of TA98 increased in the group treated with nano-sized WO_3 and 8 h pre-incubation, although there was no change in the number of revertant colonies in the group treated with micro-sized WO_3 particles. The number of revertant colonies of TA98 increased in the group treated with nano-sized WO_3 and with metabolic activation dose-dependently at the concentration up to 80 μg per plate. Without metabolic activation, the number of revertant colonies in TA98 increased at the concentration up to 40 μg per plate while the number of revertant colonies decreased at 80 μg per plate, suggesting the toxic effect of nano-sized WO_3 at higher concentrations. The number of revertant colonies of TA1535 increased slightly but dose-dependently in the group treated with nano-sized WO_3 and with metabolic activation and 8 h pre-incubation. The number of colonies of TA1535 treated with WO_3 at the concentration of 80 μg per plate was significantly higher than that of the control (2.4-fold over control level). In contrast, no changes were seen in the number of revertant colonies of all strains treated with nano- or micro-sized Mo tested with either 20 min or 8 h pre-incubation, or with/without metabolic activation (data not shown).

Promotion activity of rare metals was evaluated by Bhas 42 cell transformation assay (Fig. 6). Nano-sized In_2O_3 increased transformed colonies significantly at the concentration up to 1 μg ml^{-1}, while the number of transformed colonies was higher in the group treated with micro-sized In_2O_3 at the concentration up to 2 μg ml^{-1}. Both nano- and micro-sized Dy_2O_3 also increased transformed colonies significantly at concentrations up to 2 μg ml^{-1}. However, the number of transformed colonies induced

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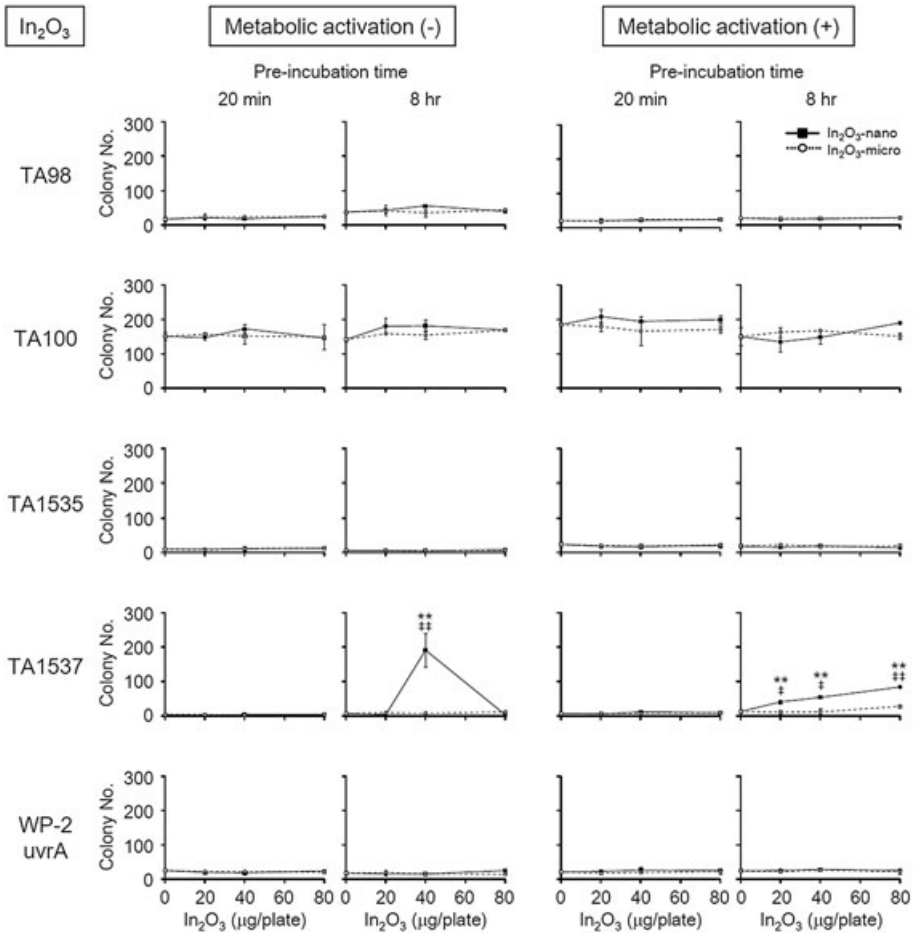

Figure 4. Numbers of revertant colonies in bacteria strains induced by In_2O_3. The numbers of revertant colonies in TA98, TA100, TA1535, TA1537 and WP-2 uvrA strains induced by nano-sized (solid line) and micro-sized (dashed line) In_2O_3 with [metabolic activation (+)] or without [metabolic activation (−)] metabolic activation are shown. The data are means ± SD of three experiments in duplicate. ** $P < 0.01$ (vs 0 μg per plate In_2O_3). ‡ $P < 0.05$, ‡‡ $P < 0.01$ (nano- vs micro-sized particles).

by nano-sized In_2O_3 and Dy_2O_3 was lower than in the group treated with these nanoparticles at the concentration of 5 μg ml^{-1}. Growth inhibition of Bhas 42 cells was observed when more than 10 μg ml^{-1} of rare metals were added (data not shown), while a lower number of transformed colonies was observed in the group treated with the nano-sized In_2O_3 and Dy_2O_3 at the concentration of 5 μg ml^{-1}, which may be attributed to toxicity of these metal oxides. There was no difference in the number of transformed colonies between nano-sized and micro-sized metals, suggesting that the particle size of metals was not affected by their promotion activity. In contrast, WO_3 or Mo did not increase the number of transformed colonies in the concentration range of 0.01–5 μg ml^{-1} for either nano-sized or micro-sized particles.

DISCUSSION

In the two-stage carcinogenic model, a primary tumor is established through the initiation and promotion process (Neyman and Scott, 1967; Whittemore and Keller, 1978). In this study, we estimated the tumor initiation potential of rare metals by Ames test and tumor promotion potential by Bhas 42 cell transformation assay. The data are summarized in Table 3. Interestingly, particle size dependency on mutagenicity was observed in Ames test using three rare metal oxides, Dy_2O_3, In_2O_3 and WO_3, although the transformability of these metal compounds was independent of their particle size. Comparing the mutagenicity of nano- and micro-sized Dy_2O_3, the number of revertant colonies induced by nano-sized Dy_2O_3 was consistently larger than that induced by the same concentration (micrograms per plate) of micro-sized Dy_2O_3, incubated with bacterial cultures (Fig. 3). Nano-sized In_2O_3 and WO_3 also showed positive mutagenic responses in TA1537 and TA98, respectively, whereas micro-sized In_2O_3 or WO_3 did not show any mutagenesis in the tested bacterial strains (Fig. 4 and 5). These results suggest that mutagenic potential of nano-sized Dy_2O_3, In_2O_3 and WO_3 is greater than that of micro-sized Dy_2O_3, In_2O_3 and WO_3. It was reported that nanoparticles was permeable to cell membrane and translocate into nucleus (Feldherr *et al*., 1992), suggesting that nano-sized Dy_2O_3, In_2O_3 and WO_3 might cause genetic damage directly to exhibit their mutagenicity. On the other hand, our data showed that there was no difference in the number of transformed foci between nano-sized and micro-sized Dy_2O_3 and In_2O_3 confirmed by Bhas 42 cell transformation assay (Fig. 6). This indicates that the particle size of rare metals did not influence their transformability. Dy_2O_3 and In_2O_3 showed transformability, while WO_3 or Mo did not show any transformation

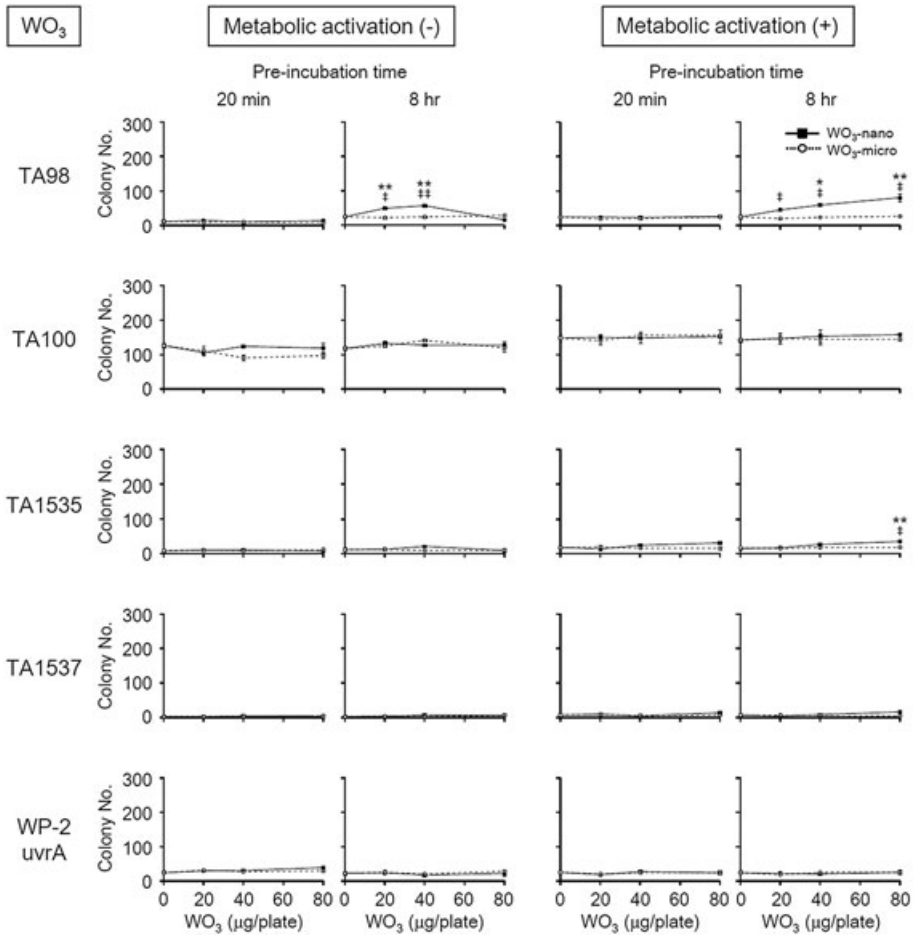

Figure 5. Numbers of revertant colonies in bacteria strains induced by WO_3. The numbers of revertant colonies in TA98, TA100, TA1535, TA1537 and WP-2 uvrA strains induced by nano-sized (solid line) and micro-sized (dashed line) WO_3 with [metabolic activation (+)] or without [metabolic activation (−)] metabolic activation are shown. The data are means ± SD of three experiments in duplicate. * $P < 0.05$, ** $P < 0.01$ (vs 0 μg per plate WO_3). ‡ $P < 0.05$, ‡‡ $P < 0.01$ (nano- vs micro-sized particles).

activity. These results suggest that chemical components but not particle size of rare metals influence transformability. Tumor promotion is mediated by various cytosolic signal transduction, i.e. protein kinase C pathway (Zani *et al*., 1988). Although the mechanism of tumor promotion induced by metal oxides is unknown, Dy_2O_3 and In_2O_3 may affect cytosolic signal transduction elements to interfere with the homeostatic regulatory system, causing abnormal cell proliferation. Our results suggest that nano-sized Dy_2O_3 and In_2O_3 can translocate into cytosol and nuclear to exhibit both mutagenicity and transformability, although micro-sized Dy_2O_3 and In_2O_3 translocation may be restricted to cytosol and exhibit transformability only. This is the first report to compare the mutagenicity and transformability of rare metal compounds with respect to their particle size. In summary, the mutagenicity of rare metals was dependent on their particle size while transformability was independent on their particle size. The detailed molecular mechanism of mutagenicity and transformability induced by rare metals in relation to their particle size will be investigated in the near future.

It is suggested that carcinogenicity of chemical compounds is related to their solubility (Jaurand, 1991; Ivask *et al*., 2010). All metal compounds used in our experiments have a very low solubility in buffer solutions. Although metal nanoparticles may have low solubility in aqueous media, particles may reach close proximity to nucleotides and interfere with gene functions. As shown in Figs 4 and 5, nano-sized In_2O_3 and nano-sized WO_3 showed mutagenicity only when pre-incubated for 8 h with bacteria strains, although they did not increase the number of revertant colonies with 20 min pre-incubation. A longer pre-incubation time might enhance the solubility of nano-sized In_2O_3 and nano-sized WO_3 in reaction buffers, and a trace amount of solubilized metal nanoparticles might exhibit their mutagenic activities via generating oxidative DNA damage (Emerit *et al*., 2001), or these nanoparticles might bind to nucleoprotein, nucleotide or functional proteins owing to longer contact time. Although high concentrations of metal nanoparticles show growth inhibition of tester strains, prolonged pre-incubation time with a lower concentration of the metal particles increased the sensitivity of detection of mutagenic effects. Both nano-sized and micro-sized Mo particles showed neither mutagenesis nor transformability. Only Mo was an elemental metal particle while the rest of the particle was metal oxide, suggesting that elemental Mo particle was more difficult solubilize in buffer solutions than other tested metal oxide particles.

Concerning the size dependency of metal toxicity, this study shows that some nano-sized rare metals were mutagenic, although

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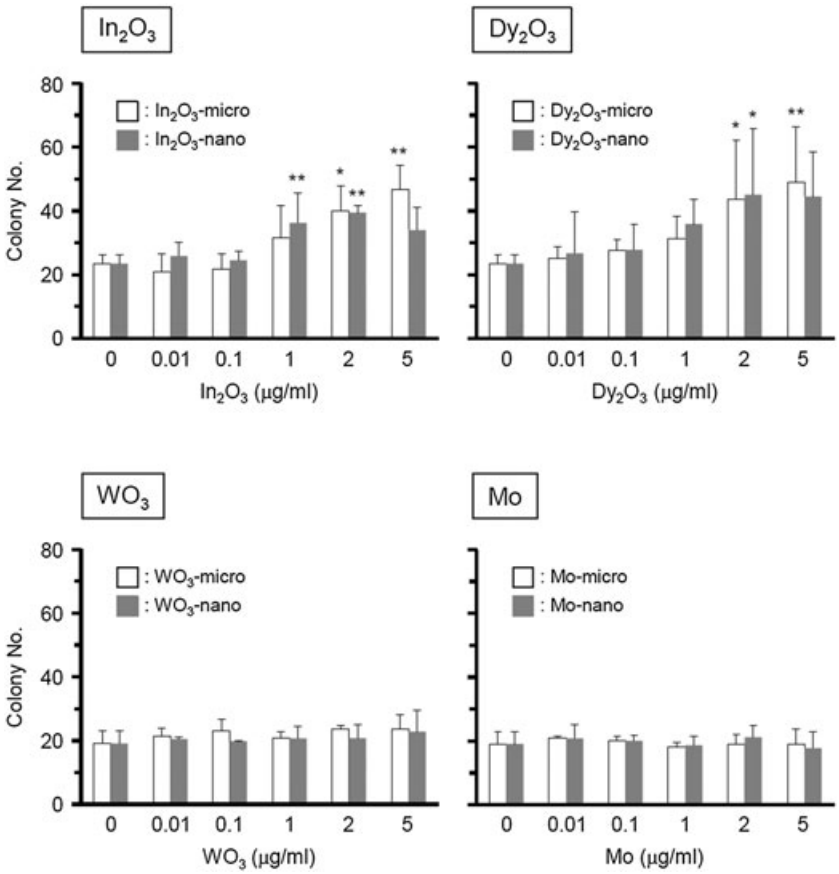

Figure 6. Tumor-promoting activity of rare metal compounds in the Bhas 42 cell transformation assay. The numbers of transformed colonies in Bhas 42 cells induced by nano-sized (solid bar) and micro-sized (open bar) In_2O_3, Dy_2O_3, WO_3 and Mo are shown. The data are means ± SD of six experiments in duplicate. * $P < 0.05$, ** $P < 0.01$ (vs 0 μg ml^{-1}).

micro-sized rare metals were slightly mutagenic or nonmutagenic. Mutagenic potentials of nano-sized Dy_2O_3, nano-sized In_2O_3 and nano-sized WO_3 were much greater than their micro-sized counterparts. Although the size-dependent toxicity of particles has been widely proposed, most *in vitro* studies on particle toxicity have been conducted only using nanoparticles without comparison to their micro-sized counterparts. Few studies employed both nanoparticles and their bulky counterparts. Karlsson *et al*. (2009) recently reported that cupric oxide (CuO) nanoparticles induced cytotoxicity, mitochondrial damage and DNA damage against human lung epithelial cell line A549 significantly more strongly than CuO micrometer particles. However, they mentioned in the paper that all metal nanoparticles used in the study were aggregated in solution and the mean size of these particles was 200–1600 nm. Our results also indicated the aggregation of metal nanoparticles in suspension (Fig. 1). The mean diameter of nano-sized In_2O_3, nano-sized Dy_2O_3, nano-sized WO_3 and nano-sized Mo in suspension was 391.0, 565.2, 545.5 and 213.2 nm, respectively, whereas the size distribution of these particles demonstrated only trace amounts of nanoparticle (<100 nm diameter) in all test suspension. Suspensions with nano-sized rare metals contained particles of less than 5 μm diameter, because of filtration with the Supor membrane. However, suspensions with micro-sized rare metal contained particles of more than 7 μm diameter, estimated by DLS measurement (Table 2). We did not observe clear correlation between the size of aggregates and mutagenicity. These fractions containing nano-sized particles might influence the induction of mutagenicity. The detail molecular mechanism of mutagenicity induced by these metal nanoparticles will be investigated further.

Table 3. Summary of Ames test and transformation assay results using rare metal compounds

Compounds	Mutagenicity		Transformability	
	Nano-sized particle	Micro-sized particle	Nano-sized particle	Micro-sized particle
Dy_2O_3	++ (5/5)	+ (2/5)	+	+
In_2O_3	+ (1/5)	− (0/5)	+	+
WO_3	+ (1/5)	− (0/5)	−	−
Mo	− (0/5)	− (0/5)	−	−

Mutagenicity and transformability of rare metals were evaluated by Ames test and Bhas 42 cell transformation assay, respectively. The ratio of bacterial strains with positive response is indicated in parentheses.

Regarding the *in vivo* carcinogenicity of rare metal compounds, the toxicological properties have not been fully investigated. For indium compounds, it was reported that long-term inhalation of indium phosphide in F344/N rats and B6C3F1 mice induced alveolar/bronchiolar adenoma and carcinoma (National Toxicology Program, 2001). Repeated intratracheal

instillation of indium arsenide into hamster lungs induced alveolar/bronchiolar cell hyperplasia with squamous cell metaplasia, suggesting that it is carcinogenic (Yamazaki *et al*., 2000). Recently, the carcinogenicity of metal nanoparticles was reported. Inhalation of ultrafine TiO_2 produced lung tumors in rats at lower concentrations than fine TiO_2 (Dankovic *et al*., 2007). The small size of nanoparticles facilitates invasion into cells and may cause genetic damage leading to neoplastic transformation. Our results demonstrated that nano-sized Dy_2O_3 and nano-sized In_2O_3 possessed tumor-initiating and -promoting properties, suggesting the potential carcinogenicity of these nanoparticles.

In conclusion, our results show that nano-sized rare metals – nano-sized Dy_2O_3, nano-sized In_2O_3 and nano-sized WO_3 – were mutagenic, although micro-sized rare metals were weakly or not mutagenic. Moreover, In_2O_3 and Dy_2O_3 exhibited transformation activity, although size dependency of transformation activity was not observed. These data indicate that physical and chemical properties of rare metal and metal oxide particles are important for assessing the mutagenic and transformation activity leading to their genotoxicity. *In vivo* studies to confirm the carcinogenicity of rare metal nanoparticles is recommended.

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