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Comparative pulmonary toxicity study of nano-TiO_2 particles of different sizes and agglomerations in rats: Different short- and long-term post-instillation results

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ABSTRACT

Two intratracheal instillation experiments with nano-size titanium dioxide (TiO_2) particles of different sizes and agglomerations were conducted in rats to compare the biological responses induced by the different particles. In experiment 1, 5 mg/kg of nano-TiO_2 particles of different primary sizes was intratracheally instilled in rats. In experiment 2, a similar procedure was followed with 5 mg/kg of nano-TiO_2 particles of the same primary sizes but different agglomerations in liquid. Following the instillations, body and lung weight measurements, bronchoalveolar fluid (BALF) cells and inflammatory biomarkers assessment, and histopathological evaluations of the lungs and other tissues were conducted. Pulmonary inflammatory responses until 1 week post-instillation differed among the TiO_2 particle-exposed groups: that is, smaller particles induced greater inflammation in the short-term observations. With regard to the long-term effects (>1 week post-instillation), however, pulmonary inflammation remarkably recovered in all the TiO_2 particle-exposed groups, with no differences between the groups regardless of particle size. On the other hand, no clear relationship was observed between the TiO_2 particle-exposed groups with different agglomerations but the same primary size. These findings suggest that different evaluations can be derived on the basis of the observations up to 1 week post-instillation and those after 1 month post-instillation. In most of the current studies, the relationship between pulmonary responses and instilled particle sizes has been discussed only on the basis of the 24 h post-instillation results, which could be a misleading evaluation. Consequently, our findings indicate that both short- and long-term effects should be evaluated when assessing the toxicity of nanoparticles based on the results of intratracheal instillation studies.

1. Introduction

Engineered nanomaterials are being used widely with the development of nanotechnologies. However, there is growing concern regarding the risks to humans and the environment due to exposure during the production, use, consumption, and disposal of these products. In general, the term "nanomaterial" refers to a material with one, two, or three dimensions of roughly 1–100 nm in size and its aggregates (ISO, 2008). Although these materials are expected to fulfill various novel functions, the fact that their potential toxicities and the underlying mechanisms cannot be predicted by applying the present knowledge, because of their extremely small sizes, is a cause for concern.

For example, nano-size titanium dioxide (TiO_2) particles have greatly increased functions as catalysts due to the increase in surface area per particle weight. Further, they can be used as cosmetics due to increased clarity. On the other hand, the pulmonary responses induced by inhaled nanoparticles are considered to be greater than micron-sized particles because of the increased surface area per particle weight. Moreover, inhaled nanoparticles, after deposition in the lungs, largely escape the alveolar macrophage surveillance and gain greater access to the pulmonary interstitium by translocation from alveolar spaces through the epithelium (Donaldson et al., 2001; Oberdörster et al., 2005).

Oberdörster et al. (1992) and Renwick et al. (2004) have reported that rats intratracheally instilled with ultrafine (nano-size) TiO_2 particles have greater pulmonary inflammatory responses than those intratracheally instilled with fine (submicron-size) TiO_2 particles. Further, when the instilled doses were expressed in terms of particle surface area, the responses induced by the ultrafine and fine

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TiO_2 particles fell on the same dose–response curve (Oberdörster, 2000; Oberdörster et al., 2005; Donaldson et al., 2001). They therefore concluded that the greater inflammatory responses observed were due to the large surface area of the ultrafine TiO_2 particles. By intratracheal instillation studies of poorly soluble, low toxicity particles other than TiO_2 (such as carbon black and polystyrene latex) in rats, Brown et al. (2000, 2001) and Duffin et al. (2007) found that smaller particles produce greater pulmonary inflammatory responses. Some inhalation studies have been conducted to examine the relationship between the pulmonary responses and particle size. Following a 12-week inhalation study of rats exposed to nano-size (20 nm) and submicron-size (250 nm) TiO_2 particles, pulmonary inflammation was observed only the group exposed to the nano-size TiO_2 particles (Oberdörster et al., 1994). Bermudez et al. (2004) reported that nano-TiO_2 particles produce stronger inflammatory responses than micron-sized TiO_2 particles, by comparing the results of a 13-week inhalation study with nano- and micron-TiO_2 particles. These researchers suggested that the differences in the response were due to the differences in the surface area of the inhaled TiO_2 particles. Further, Tran et al. (2000) have reported that the responses of rats exposed to TiO_2 and $BaSO_4$ particles fell on the same dose–response curve when the dose was expressed in terms of particle surface area.

On the basis of a review of these studies, Maynard and Kuempel (2005) stated that surface area is the most relevant exposure metric for poorly soluble, low toxicity particles. The U.S. National Institute of Occupational Safety and Health (NIOSH, 2005) has supported the conclusion, and in November 2005, proposed a recommended exposure limit (REL) of 0.1 mg/m^3 for ultrafine TiO_2 particles (primary particle size <100 nm), which is 15 times lower than that for fine TiO_2 particles (i.e., 1.5 mg/m^3). In a recently published paper by researchers from NIOSH (Sager et al., 2008), it was stated that pulmonary response differ according to particle sizes and that the differences in the responses can be explained by the differences in particle surface area doses.

In contrast, it has also been reported that the pulmonary toxicities on exposure to these types of particles did not depend upon particle size or surface area. Warheit et al. (2006) found no significant differences in the pulmonary effects of intratracheally instilled TiO_2 particles of different sizes, surface areas, and crystallinity in rats, although there were significant differences in particle surface area (i.e., up to 30 times). Further, following intratracheal instillation studies in rats exposed to TiO_2 and crystalline silica with different crystallinities, Warheit et al. (2007a,b) concluded that the surface chemistry is a considerably more important factor determining pulmonary toxicity than particle surface area. Regarding nano- and micron-sized crystalline silica, Kajiwara et al. (2007) compared pulmonary inflammation up to 6 months post-instillation between rats intratracheally instilled with normal Min-U-Sil 5 crystalline silica particles (geometrical mean of particle size = 1.8 μm) and those instilled with the supernatant (obtained by centrifugation) of these particles (geometrical mean of particle size = 0.74 μm). Their conclusion was that pulmonary inflammation is greater in the rats exposed to the micron-sized particles than those exposed to the supernatant.

As mentioned earlier, the relationship between toxicity and particle size is still not clear. One of the reasons is considered to be differences in not only the size but also the crystallinity, surface chemistry, and purity of the test samples in most of the studies conducted so far. For example, ultrafine and fine TiO_2 particles had different crystalline structures in the studies conducted by Bermudez et al. (2002, 2004) and Sager et al. (2008). For the ultrafine TiO_2 particles, both groups used 80% anatase and 20% rutile TiO_2 particles manufactured by Degussa; however, for the micron-sized TiO_2 particles, they used 100% rutile TiO_2 particles manufactured by either DuPont (Bermudez et al., 2002) or Sigma–Aldrich (Sager et al., 2008). Further, in some studies, the characterizations of the administered substances were not sufficient. Moreover, in most of the studies, only the 24-h post-instillation results were compared and assessed; only Warheit et al. (2006, 2007a, 2007b) and Kajiwara et al. (2007) assessed the results up to 3 months post-instillation. These differences make identification of the factors affecting toxicity very difficult.

However, to assess the toxicity of nanoparticles and manage their risks, it is important to understand whether nanoparticles are more toxic than micron-sized particles. Therefore, in this study, two intratracheal instillation experiments with nano-TiO_2 particles of different primary sizes and agglomerations but the same manufacturer, manufacturing method, and crystalline structure (100% anatase) were conducted in rats to compare the biological responses in the lungs and other tissues of rats exposed to the different particle types. The results of these two experiments were then used to evaluate the relationships between pulmonary toxicity and particle size or agglomerations.

2. Materials and methods

2.1. Sample preparation

In the first experiment (experiment 1), three different sizes of anatase TiO_2 particles (Ishihara Sangyo Kaisha, Ltd. Osaka, Japan) were used. The product names of these particles are ST-01, ST-21, and ST-41, corresponding to the primary particle sizes (grain sizes) of approximately 5, 23, and 154 nm. In this paper, these particle types are referred to as ultrafine (UF), superfine (SF), and fine (F) TiO_2, respectively. To maintain the best dispersion state, 1–2 mg/mL disodium phosphate (DSP) was added as a dispersant to all the TiO_2 particle solutions, followed by agitation in an UAM015 agitating bead mill (Kotobuki Industries Co., Ltd., Tokyo, Japan) at 10–12 m/s for 2 h with 15-μm zirconium oxide (ZrO_2) beads. Subsequently, the supernatant was recovered by centrifugation at 8000 × *g* for 1 h (in the case of UF TiO_2), or the filtrate was recovered by filtration through a filter of 1-μm pore size (in the case of SF TiO_2 and F TiO_2). These solutions were intratracheally instilled after dilution with distilled water or concentration to a particle concentration of 5 mg/mL by using an evaporator.

In the second experiment (experiment 2), only ST-01 particles (5-nm primary particle size) were used. By varying the preparation method, three different agglomeration states of TiO_2 particles in 2–13 mg/mL of DSP solution were obtained. In this paper, these particle types are referred to as UF1, UF2, and UF3 TiO_2, in order of lower agglomeration. For preparing the UF1 TiO_2 particles, ST-01 particles were agitated using an UAM015 agitating bead mill at 12 m/s for 2 h with 15-μm ZrO_2 beads. Subsequently, the supernatant was recovered by centrifugation at 16,000 × *g* for 1 h. To prepare the UF2 TiO_2 particles, ST-01 particles were dispersed by sonication for 2 min, and then, the filtrate was recovered by filtration through filters of 1- and 0.1-μm pore sizes. To prepare the UF3 TiO_2 particles, ST-01 particles were agitated in an UAM015 agitating bead mill at 15 m/s for 1.5 h with 50-μm ZrO_2 beads, the filtrate was recovered by filtration through a filter of 1-μm pore size, and then the supernatant was recovered by centrifugation at 1000 × *g* for 1 h. The DSP concentrations were different in each TiO_2 sample solution, ranging from 2 mg/mL (UF1 TiO_2) to 13 mg/mL (UF3 TiO_2).

In both experiments, 2 mg/mL DSP solution was used as the negative control (vehicle). Pulmonary effects induced by 13 mg/mL DSP solutions (same as that in the case of UF3 TiO_2 solutions) were examined by additional experiments: pulmonary responses due to the administration of the 13 mg/mL and 2 mg/mL DSP solutions were similar (data not shown). Further, in experiment 2, Min-U-Sil 5 crystalline silica particles (US Silica Co., Berkley Springs, WV) dispersed in 2 mg/mL of DSP solution was used as the positive control. Min-U-Sil 5 crystalline silica is known to produce continuous pulmonary inflammation in the lungs (Warheit et al., 2007a; Kajiwara et al., 2007).

2.2. TiO_2 particle characterization

Size distributions of each TiO_2 particle in DSP solution after sample preparation (i.e., secondary particle size distributions) were measured by the dynamic light scattering (DLS) method (Microtrac UPA150; Nikkiso Co., Ltd., Tokyo, Japan). Moreover, morphologies of the TiO_2 particles in the prepared solutions were observed by transmission electron microscopy (TEM).

2.3. Experimental animals

In both experiments, 8-week-old male Crl: CD (SD) rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were used. Their mean body weight was in the range of 279–335 g at intratracheal instillation.