Time-course Analysis of Pulmonary Inflammation Induced by Intratracheal Instillation of Fine Crystalline Silica Particles

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Research

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Abstract

Background: Crystalline silica is an important cause of serious pulmonary diseases. The relationship between this hazard and various physicochemical parameters of crystalline silica has been studied for more than a century, and the toxic potential of silica is known to be associated with its surface properties. However, no *in vivo* dataset has yet been accumulated to clarify the relevance of this, especially regarding longer term effects. To provide new insights into these longer term effects, we performed single intratracheal instillation testing of five different crystalline silicas and assessed the time-course changes in pulmonary inflammation, lung burden, and thoracic lymph node loads. The tested silicas were prepared from two commercial products [Min-U-Sil5 (MS5, US Silica Company) and SIO07PB (SPB, Kojundo Chemical)] by using three different pretreatment steps: centrifugation (C), grinding (G), and surface dissolving (D). The five types of silica particles—MS5, MS5_C, SPB_C, SPB_G, and SPB_D—were suspended in purified water and intratracheally instilled into male F344 rats. Bronchoalveolar lavage, lung burden analysis, and histopathological examination were performed 3, 28, and 91 days after instillation.

Results: MS5 and MS5_C provoked similar biphasic inflammation that differed in severity: MS5 induced granuloma generation 91 days after instillation, whereas the smaller particles of MS5_C elicited only macrophage aggregation. SPB_C induced severe and persistent inflammation, whereas SPB_G and SPB_D induced only slight and transient acute inflammation. All of the silicas were dose-dependently retained in the lung. Silicas that induced prolonged responses were translocated to the thoracic lymph nodes as early as 28 days after instillation. Those that induced transient inflammation were removed effectively from the lung with very little lymph node retention. No quantitative association was identified between lung retention and physicochemical properties, although common features of the retained materials seemed to be reduced solubility, similar surface properties, and relatively small particle.

Conclusion: Our key findings suggest that prolonged silica toxicity occurs through lymph node retention. Although the main drivers of translocation to the lymph nodes have not yet been clarified, we provide *in vivo* evidence of the contributions of at least silica solubility, surface properties, and particle size.

Background

Nanosafety has become a major human health concern. Because nanoscale materials have novel physicochemical properties and can be used to achieve innovative functionalities, they are being applied in a variety of fields of technology. However, because these novel physicochemical properties can cause unexpected health hazards, numerous studies have been conducted to investigate the relationships between physicochemical properties and hazard potential (1,2). The most likely exposure route for such materials is inhalation, and the Organisation for Economic Co-operation and Development has established inhalation toxicity testing guidelines to accommodate the evaluation of manufactured nanomaterials (3,4). However, a much simpler delivery method—intratracheal instillation—is also available for hazard identification or comparative toxicity evaluation of such materials (5).

Silicon dioxide (SiO_2), which is one of the world's most abundant naturally occurring compounds, is used in many products in our everyday lives. However, SiO_2 occurring naturally as crystalline silica is one of the most important causes of occupational disease in the form of silicosis, especially in mining industry workers, and it

can be a factor in the development of tuberculosis, chronic bronchiolitis, chronic obstructive pulmonary disease, and lung cancer (6,7). The International Agency for Research on Cancer has classified crystalline silica as a Group 1 carcinogen (carcinogenic to humans) (8). The toxicity mechanisms of crystalline silica have been investigated for more than a century, although they are very complicated and are not yet completely understood. Clouter et al. (9) reported that the hazard potential of crystalline silica is associated with the material's hemolytic activity *in vitro*, and Warheit et al. (10) revealed *in vivo* that hemolytic activity was correlated with the severity of pulmonary injury, rather than with particle size or surface area. The physicochemical property associated with the hemolytic potential was then revealed *in vitro* to be the surface silanol heterogeneity (11,12).

Here, we performed single intratracheal instillation of silicas in male F344 rats and examined the time-course changes in pulmonary inflammation, lung burden, and thoracic lymph node load. Intratracheal instillation is considered useful for hazard identification and comparative toxicity evaluation (5,13,14). The inflammatory responses and lung burden were measured in the acute (Day 3), subacute (Day 28), and subchronic (Day 91) phases.

As test silicas, we chose the commercially available products Min-U-Sil5 (MS5, US Silica Company, Frederick, MD, USA) and SIO07PB (SPB, Kojundo Chemical, Saitama, Japan). MS5 is representative of respirable crystalline silica particles that have been widely used in toxicological studies (6). To obtain particles of a smaller diameter, we centrifuged and smaller particles were collected to yield MS5_C ("C" means centrifuged). Unlike in the case of MS5, little toxicological information is available for SPB. The particle diameter of raw SPB powder is large, at about 800 nm, and so it is not of respirable size. To prepare dispersions of particles of respirable size, we centrifuged SPB suspension and yielded much smaller particles as SPB_C; moreover, SPB was ground by ball milling to yield SPB_G ("G" means ground) and was then dissolved by alkaline treatment to yield SPB_D ("D" means dissolved). Here, we characterized the physicochemical properties of the five tested silicas (MS5, MS5_C, SPB_C, SPB_G, and SPB_D). Additionally, we investigated the association between physicochemical parameters and hazard potential.

Methods

Silica preparation

We used fine commercial powders of Min-U-Sil5 (MS5, US Silica Company, Frederick, MD, USA) and SIO07PB (SPB, Kojundo Chemical, Saitama, Japan). To investigate the contribution of different physicochemical properties to the pulmonary toxicity of these materials, MS5 was dispersed well in purified water (endotoxin-free deionized water) by ultrasonication (42 kHz, 180 W) for 3 h. The mixture was then centrifuged at 100*g* for 15 min, and the smaller particles in the supernatant were collected to yield MS5_C. The particles of raw SPB powder were too large to be respirable, so we performed a similar dispersion and centrifugation procedure to yield respirable SPB_C. Additionally, grinding is an efficient method of producing smaller particles. Raw SPB powder was therefore ground in a planetary ball mill (PM100, Retsch, Haan, Germany) for 30 min at 450 rpm; SPB_G suspension was then obtained by using the dispersion and centrifugation procedures described above. Consequently, the specific surface area of SPB_G was larger than that of SPB_C, although both the primary and secondary particle sizes were comparable. The percentage crystallinity of SPB_G, as judged from XRD (Ultima

IV, Rigaku Co., Tokyo, Japan) measurements, was decreased because of mechanical damage from the milling: the α -quartz particles were likely partially converted to an amorphous component (38), presumably in the surface layer. Therefore, to remove the surface amorphous component, the SPB_G particulates were dissolved by using hot NaOH aqueous solution (1 N, 60 °C, 16 h), and the smallest particle-size sample, SPB_D, was obtained.

Characterization of the tested silica particles

Particle size

Primary particle size was calculated from 500 particle images taken by using FE-SEM (Hitachi S4800, Hitachi High-Technologies Corporation, Tokyo, Japan: see Fig. 1). Because primary particle-image recognition was difficult owing to the strong agglomeration of SPB_D, in the case of this silica the particle size was calculated from the results of specific surface area measurements assuming that the particles were simple spherical shapes (see following section). The secondary particle sizes of the silicas in water suspension were measured by means of dynamic light scattering (Zetasizer Nano ZS; Malvern Instruments Ltd., Egham, UK).

Specific surface area

Specific surface area was measured with a Gemini VII surface area analyzer (Shimadzu, Kyoto, Japan). Volumes of nitrogen gas absorbed to well-dried samples were measured. Specific surface areas were then calculated by using Brunauer–Emmett–Teller theory, taking into consideration the vapor pressure.

Zeta potential and point of zero charge

The zeta potential of the silica suspension was measured by means of laser doppler electrophoresis with the same instrument used for the particle size measurement (Zetasizer). Each 2 mg/mL suspension was prepared and the point of zero charge was measured or estimated by using HCl to titrate the pH close to 1 (see Fig. 2). The data were summarized as mean values of duplicate measurements.

Crystallinity percentage

It is well known that crystalline silica is easily deformed to amorphous silica by mechanical damage; therefore, we needed to determine the crystallinity percentages. Conventional XRD estimation methods that use amorphous halos and crystalline reflections are poor, because the halos of nanosized particles are too broad and weak to analyze. Crystallinities were therefore estimated by using internal standard samples (39). The values indicate mass-basis crystalline component percentages.

In advance, reference XRD data (a series of integrated intensities of 18 peaks) were obtained from a 1:1 powder mixture of standard reference materials of α -quartz (SRM 1878a) and α -alumina (SRM 676a) produced by the National Institute of Standards and Technology (NIST Gaithersburg, MD, USA), with respective certified purities

in the crystalline phase of 93.7% and 99.02%. These reference data were then used to derive the unknown degree of crystallinity of a targeted crystalline silica sample from XRD data (the same series of peaks) collected from a 1:1 mixture of the α -quartz powder and NIST's α -alumina (SRM 676a).

Solubility analysis

Time-course changes in the solubility of the tested silica in saline or ALF were assessed (see Fig. 3). ALF was prepared by using the method described in a previous study (30). Ten milliliters of suspension (2.0 mg/mL) and 30 mL of ALF were mixed and shaken at 200 rpm. At each time point, 2.5 mL of the fluid was sampled and filtered at 6000g for 30 min with an ultrafilter (10,000 MW; VS2032, Sartorius Stedim Biotech GmbH, Göttingen, Germany) to separate the dissolved Si ions. Ultrapure water (1 mL) was then added to the ultrafilter, and the samples were centrifuged at 6000g for 15 min; these steps were performed twice. HNO₃ (0.5 mL) and HF (0.05 mL) were added to 0.1 or 0.5 mL of the filtered solutions, left for 6 h, and then diluted to appropriate concentrations with ultrapure water or 5% HNO₃ solution. The Si contents of the samples were then determined by inductively coupled plasma-sector field mass spectrometry (ICP-SFMS) using a Finnigan ELEMENT XR (Thermo Fisher Scientific Inc., MA, USA) at time points of 0.5, 1, 2, 4, 8, 24, 32, 48, 168, and 336 h.

Animal experiment

F344/DuCrIj rats (Charles River Laboratories Japan, Inc., Kanagawa, Japan; male, 12 weeks old) were used. The animals were housed in barrier-system animal rooms maintained at 21 to 25 °C and a relative humidity of 40% to 70%, with 10 to 15 air changes each hour and a photoperiod of 12 h of light each day.

One vehicle control group (purified water) and three dose groups were set up for each silica. The dose levels of the MS5 derivatives were set at 0.67, 2, and 6 mg/kg, whereas the SPB derivatives were dosed at 0.22, 0.67, and 2 mg/kg; 10 rats per dose were used for each material. The doses were set according to the results of preliminary studies: a single intratracheal instillation study was performed by using MS5 and SPB_C. We confirmed that evident inflammation was inducible by 3 days post-instillation, and we then set the highest doses tolerable. Details of the intratracheal instillation procedure were given in our previous study (40). Briefly, with the guidance of a laryngoscope, each dose suspension or vehicle was intratracheally instilled via the oral cavity under isoflurane anesthesia (Day 0). The rats' clinical condition was then examined daily, and body weights were measured once or more weekly. On Days 3, 28, and 91, the animals were anesthetized and then humanely killed by being bled from the abdominal aorta. BALF and lung burden analysis were then performed in half the animals in each group (n = 5), and histopathological examination was performed in the other half (n = 5).

BALF examination

Detailed procedures of the BALF examination were also given in our previous study (40). After euthanasia of each rat, the whole lung was lavaged twice with 7 mL of saline (n = 5). Note that, because of a technical error, the BALF could not be obtained from one rat 28 days after it had received MS5_C at 2 mg/kg group (n = 4). Total cell counts (ADVIA 120 hematology analyzer, Siemens Healthcare Diagnostics, Tarrytown, NY, USA) were

determined. Then smear slides were prepared and Giemsa stained, and a differential cell count of 200 cells was performed. Additionally, total protein content and lactate dehydrogenase activity were measured (Clinical Analyzer 7170, Hitachi Science Systems, Ibaraki, Japan).

Lung burden and lymph node load analyses

After the lavage, the SiO₂ contents of the lungs and thoracic lymph nodes (parathymic lymph node and bilateral posterior mediastinal lymph nodes) were analyzed. The lungs and lymph nodes were homogenized in 2 mL of ultrapure water (Milli-Q Advantage A10 Ultrapure Water Purification System, Merck Millipore, Billerica, MA). HNO₃ (68%, 0.5 mL), H₂SO₄ (98%, 0.1 mL), and H₂O₂ (35%, 0.1 mL) were added to 0.75 g of homogenized lung tissues and whole lymph nodes. The acidified samples were heated to 180-200 °C for 20 min in a microwave sample preparation instrument (ETHOS 1, Milestone Srl, Milano, Italy; or Speedwave 4, Berghof, Mühlhausen, Germany). After the samples had been cooled to 40 °C, HF (38%, 0.1 mL for tissue samples) was added and the samples were then left for 6 h. The acid-treated samples, diluted to appropriate concentrations with ultrapure water or 5% HNO₃ solution, were examined by ICP-SFMS (Finnigan ELEMENT XR; Thermo Fisher Scientific Inc.). Additionally, the contents of the BALF samples were measured and combined to the SiO₂ contents of the lung to calculate the lung burden.

Pathological examination

The lungs, trachea, parathymic lymph nodes, posterior mediastinal lymph nodes, liver, kidneys, spleen, and brain were removed. The lungs, liver, kidneys, spleen, and brain were weighed, and the relative weights were calculated on the basis of body weight. All organs or tissues were fixed in 10% (v/v) neutral phosphate-buffered formalin solution. Paraffin-embedded specimens were prepared, sectioned, stained with hematoxylin and eosin, and examined histopathologically. The histological changes and terminology of the lung and lymph nodes underwent peer-review externally by two expert pathologists.

Statistical analysis

For body weights, organ weights, the results of the BALF examinations, lung burden and lymph node loads, StatLight software (Yukms Co., Ltd., Tokyo, Japan) was used for statistical analyses. For body weights and organ weights, Bartlett's test for homogeneity of variance was conducted. When the variances were homogeneous at a significance level of 5%, we applied Dunnett's test; otherwise, we applied a non-parametric Dunnett's test. For BALF, lung burden, and lymph node loads, Bartlett's test was conducted. When the variances were homogeneous at a significance level of 1%, including logarithmic and square-root transformations, we applied Dunnett's test; otherwise, we applied Steel's test. Statistical significances were judged at the 5% probability level.

Results

Scanning electron micrographs

We prepared field-emission scanning electron microscopy (FE-SEM) images of the tested silicas (Fig. 1). FE-SEM revealed that MS5, MS5_C, SPB_C, and SPB_G had clumping particulates, whereas the SPB_D particles fused with each other to such an extent that we could no longer identify representative particulate shapes or particle sizes (see following section.)

Characterization of the tested silica particles

Particle size

The primary particle sizes of the tested silicas were calculated from the SEM images (MS5, MS5_C, SPB_C, and SPB_G) or from the specific surface area (all the silicas) (Table 1). Secondary particle sizes in the dose suspensions, as measured by dynamic light scattering, ranged from 58.8 to 481 nm in the following order: SPB_D << MS5_C < SPB_C < SPB_G << MS5. As intended, these sizes were considered to be in the respirable range (15).

Specific surface area

The specific surface area values, as measured by means of N₂ gas absorption, are shown in Table 1. The specific surface areas of the tested silicas ranged from 6.1 m²/g to greater than 25 times more at 153.6 m²/g, in the following order: MS5 < SPB_C < MS5_C < SPB_G < SPB_D.

[Please place Table 1 here.]

Zeta potential and point of zero charge

We plotted a zeta potential titration curve, which reflects the distribution of surface acidic moieties (Fig. 2). MS5 and MS5_C, as well as SPB_C and SPB_G, had similar curves, implying that they had similar surface properties. The titration curve of SPB_D differed slightly from those of the others but was fairly close in pattern to those of SPB_C and SPB_G. The pH points at which the zeta potential was zero (i.e. the point of zero charge) were calculated as 1.31 for SPB_G and 1.23 for SPB_D (Table 1). Those for MS5, MS5_C, or SPB_C could not be determined (<1), although they were estimated as the following order: MS5 ≈ MS5_C < SPB_C.

Crystallinity percentage

The mass-basis rates of the crystalline silica contents were calculated by using the X-ray diffraction (XRD) method (Table 1). Crystallinity percentages ranged from 59.1% to 89.0% in the following order: SPB_G < SPB_D < MS5_C < SPB_C < MS5.

Solubility

We determined the solubilities of the silica materials in artificial lysosomal fluid (ALF) and physiological saline (Fig. 3). MS5 was slightly soluble in either solution, whereas MS5_C was almost insoluble. This indicated that the insoluble compartment of MS5 was collected as MS5_C during the centrifugation pretreatment. Among the SPB derivatives, SPB_G was the most soluble in ALF and in saline; the solubilities of SPB_C and SPB_D to ALF were similar to each other, and that of SPB_C to saline was higher than that of SPB_D after long time mixing.

Animal experiment

Actual dose and initial lung burden

The actual doses of the silica suspensions are shown in Table 2. Three days after instillation, between 49.2% and 85.3% of the nominal dose was deposited in the lungs, except in the case of MS5_C (2.0 mg/kg nominal dose; 123.8%). This may have been caused by installation of an incorrect volume, although the dose level can still be regarded as an intermediate.

[Please place Table 2 here.]

Clinical signs and body weights

No mortalities or abnormal clinical signs were observed in either control animals or treated animals after instillation of any of the silica materials. No marked treatment-related body weight changes (Additional file 1) were noted in any of the control or treated animals throughout the observation periods.

Bronchoalveolar lavage fluid (BALF) examinations

We examined the BALF cytological parameters (Fig. 4). Note that statistical significances are indicated only for neutrophil numbers and macrophage numbers: those for the other parameters are shown in Additional Table 1. In the cases of MS5 and MS5_C, dose-related significant increases in neutrophil numbers compared with in the controls were observed on Day 3. As the neutrophil numbers increased, their percentages of the total number of cells increased (dashed lines), and conversely the macrophage percentages decreased (solid lines). By Day 28 the neutrophil infiltration had largely subsided in the MS5 and MS5_C groups, although by Day 91 it had recurred in the MS5 groups. Nevertheless, slight but significant neutrophil infiltration (compared with in the controls) was present in the high-dose MS5_C group on Days 28 and 91. Among the SPB derivatives, SPB_C at all dose levels had induced very severe neutrophil infiltration by Day 3, and the response was still persisting on Day 91, without recovery. Note that macrophage numbers in the SPB_C groups were significantly lower than in the controls on Days 3 (all groups) and 28 and 91 (only in the medium-dose group). The decrease on Day 3 was attributable to the SPB_C instillation, because macrophage alteration was also observed in the histopathological examination (see following section). In contrast, the decreases on Days 28 and 91 may have been incidental because dose response was unclear. Whereas after the SPB_G and SPB_D treatments, only slight and transient neutrophil infiltration to an extent significantly greater than in the controls was detected on Day 3, and only in the high-dose groups. In the high-dose SPB_D group, macrophage numbers were significantly higher than in the controls.

BALF chemistry parameters, including total protein content and lactate dehydrogenase activity, showed responses similar to those of the cytological parameters (Additional files 2 and 3). No significant changes compared with in the controls were noted in any dose group at any time point in the SPB_G treatments.

Lung burden and lymph node load

We examined time-course changes in lung burden and thoracic lymph node loads (Fig. 5). All of the silicas were dose-dependently retained in the lung at all time points and were excreted from the lung over time with the exception of SPB_C (Fig. 5, solid lines). The slowly excretion of SPB_C seemed to be aligned with the persistent inflammatory responses detected in the BALF of the same animals, and with the results of the histopathological examinations (see following section). Conversely mean amounts retained in the lung increased in the following groups in the following time periods: medium dose of MS5, from Days 28 to 91; medium dose of SPB_C, from Days 3 to 28; and low dose of SPB_C, from Days 3 to 28. These results may have been caused by technical variations at instillation, but importantly, they reflect low efficacies of clearance of MS5 and SPB_C.

On Day 28 the lymph node loads of MS5 and MS5_C (high and medium doses) and SPB_C (all three doses) were significantly greater than the loads on Day 3 or in the vehicle controls. On Day 91 the lymph node loads of MS5 (all doses), MS5_C (high and medium doses), SPB_C (all three doses), and SPB_D (high dose) were significantly greater than those on Day 3 or in the vehicle controls (Fig. 5, dashed lines). Otherwise, there was little change at any of the doses of SPB_G or at the medium and low doses of SPB_D.

Thus the silica particles that induced only transient inflammation—namely, SPB_G and SPB_D (see Fig. 4)—were removed from the lung effectively via pathways other than lymph node drainage, whereas those that induced prolonged inflammation—namely, MS5, MS5_C, and SPB_C—tended to remain in the lung for at least 91 days after instillation, during which period they were slowly translocated to the thoracic lymph nodes.

Pathological examination

Organ weight

We determined absolute and relative (g/100 g body weight) lung weights (Figs. 6 and 7). With administration of the MS5 derivatives, absolute and relative lung weights were significantly increased in the 6 mg/kg groups on Day 3 compared with in the controls, and the absolute weight of animals receiving 2 mg/kg of MS5_C was also significantly increased. No significant changes were noted on Days 28 or 91, although absolute weight in the high-dose MS5 group was about 1.10 times that of the corresponding control on Day 91. Among the SPB derivatives, significant increases in absolute or relative weights, or both, were observed as follows on Day 3: animals receiving SPB_C at 0.67 or 2 mg/kg or SPB_D at 2 mg/kg; Day 28: those receiving SPB_C at 0.22, 0.67, or 2 mg/kg; and Day 91: those receiving SPB_C at 0.67 or 2 mg/kg, or both. On Day 3, the relative weight of animals receiving SPB_D at 0.22 mg/kg was significantly decreased, although this was likely incidental.

Histopathological examination

Histopathological findings in the lung are summarized in Table 3, and representative changes are shown in Figures 8 and 9 and Additional file 4. On Day 3, inflammatory cell infiltration was observed in the high-dose MS5 and MS5_C groups (Fig. 8A, 8B). By Day 28, the inflammatory responses had tended to recover: slight alveolar macrophage aggregation only was seen (Fig. 9A, 9C); this was also the case on Day 91 for MS5_C (Fig. 9D). However, in the MS5-treated group, nodular granulomas had been generated by Day 91 (Fig. 9B). Among the groups that received SPB derivatives, moderate inflammatory cell infiltration and degeneration and necrosis of alveolar macrophages were observed in the high-dose SPB_C group on Day 3 (Fig. 8C). The inflammation induced in the SPB_C group remained active on Days 28 and 91 and was accompanied by macrophage degeneration (Fig. 9E, 9F, arrowheads) and type II cell hyperplasia. Only slight to mild inflammatory cell infiltration was observed in the high-dose SPB_D group on Day 3 (Fig. 8E), and no marked changes were noted in the SPB_G groups (Fig. 8D). No neoplastic lesions were noted in any silica group at any of the doses at any time point.

[Please place Table 3 here.]

Histological findings in the posterior mediastinal lymph nodes are summarized in Table 4, and representative changes are shown in Figure 10 and Additional file 5. Granuloma formation was seen in the MS5, MS5_C, and SPB_C groups on Day 28 and thereafter (Fig. 10). Severity and incidence tended to increase over time (Table 4). No abnormal changes were noted in the SPB_G or SPB_D groups.

[Please place Table 4 here.]

No toxicologically relevant lesions were noted in the other organs or tissues examined at any time point (Additional files 6–8).

Discussion

Crystalline silica is a cause of the most prevalent occupational disease in mining industry workers, and numerous toxicological studies have been conducted over more than a century (6,8). The toxic mechanism has not yet been concluded although, some predictors has been figured out until now. The hazard potential is known to associated with the surface hemolytic activity (9,10), and the hemolytic activity is associated with the surface silanol distribution (16). Turci et al. (12) estimated that there is heterogeneity of the surface silanol acidic sites, and that this is reflected in the behavior of the zeta potential titration curve. Generally, in water suspensions, silica particles are negatively charged. As the pH value decreases, the zeta potential of homogeneous silanol silica sharply approaches zero (12). In contrast, that of heterogeneous quartz silanol gradually moves toward zero, and these heterogeneous silanol surfaces are a more potent cause of cellular stress in macrophages (12). Accordingly, here, we examined the zeta potential behavior of five silica suspensions. Although we were unable clearly to distinguish their silanol disorder statuses, we surmised from the zeta potential patterns that the surface properties of the tested silicas differed between the MS5 derivatives and the SPB derivatives.

MS5—one of the silica products most frequently tested in toxicological research (6,8)—is well known to cause biphasic inflammation, consisting of an early-stage response and later granuloma formation, in rats (17). These granulomas, which are similar to those seen in silicosis, can be induced in rats by inhalation of, or

intratracheal exposure to, MS5 (18–20), as occurred in our study. Interestingly, our results revealed that granuloma formation in the lung was suppressed by centrifugation pretreatment: MS5_C did not cause granuloma formation in this organ. The zeta potential analysis revealed that the surface properties of MS5 and MS5_C were similar to each other, so the contributions of their surface silanols are presumably comparable. In the lymph nodes however, granulomatous changes were detected in both the MS5- and MS5_C-treated groups. This is because the lymphatic system is a major route of excretion of exogenous materials, as determined by toxicity studies (21). Especially in the case of crystalline silica, Friedetzky et al. (22) have reported that lymph node silicosis, which is characterized by disorganization within the lymph nodes, occurs before the pulmonary lesions. Correspondingly, here, the amount of translocated MS5_C was much less than that of MS5 (see Fig. 5), and this was confirmed by histological grading (Table 4 and Fig. 10). This implies that pulmonary granulomas are generated when a silica accumulates beyond the capacity or threshold of the draining lymph nodes. Because the initial lung burdens of MS5 and MS5_C were comparable to each other, less MS5_C than MS5 was translocated to the lymph nodes. One possible cause of the difference is the occurrence of another clearance pathway from the lung, namely the mucociliary escalator (23). Mucociliary transport occurs predominantly for undissolved and relatively small particles (24). It is conceivable that the less soluble and smaller MS5_C was excreted to a much greater extent than MS5 via the mucociliary system. Therefore, our results suggest that efficient mucociliary excretion leads to suppression of the pulmonary granuloma formation induced by MS5 derivatives; moreover, a key finding in MS5 derivative toxicity is lymph node accumulation.

Among the SPB derivatives, SPB_G and SPB_D were excreted rapidly from the lung with only minor translocation to the lymph nodes, but SPB_C was not rapidly excreted. As mentioned above, efficient mucociliary transport—a main excretion pathway other than the lymphatic system—may be involved in excretion, predominantly for undissolved and relatively small particles. Accordingly, the rapid excretion of SPB_D—the smallest and less soluble particles—seemed reasonable, although those of SPB_C and SPB_G were less straightforward. These two silicas were of comparable particle sizes, but SPB_C was less soluble than SPB_G (see Fig. 3). Therefore, SPB_C could be expected to be excreted effectively via the mucociliary escalator, but in fact it was predominantly excreted to the lymph nodes (see Fig. 5). One possible mechanism of the reduced mucociliary clearance is the serious macrophage toxicity that was specifically detected in SPB_C-treated animals. Mucociliary excretion is triggered by macrophage engulfment of foreign bodies (24,25), but in SPB_C-treated animals the macrophages showed moderate to severe degeneration or necrosis, or both, with decreasing numbers (see Fig. 4). In this situation, macrophage function cannot be maintained. The mechanism by which silica is toxic to alveolar macrophages is triggered by lysosomal membrane destabilization and rupture, which eventually causes cell death and necrosis (26–28). Pavan and Fubini (29) have reported that this membrane destabilization is caused by surface silanol heterogeneity. In light of this hypothesis, macrophage toxicity should also occur in SPB_G-treated animals, because SPB_C and SPB_G have similar surface properties (see Fig. 2). However, SPB_G induced barely detectable neutrophil infiltration in the BALF examination. Another marked difference between the properties of SPB_C and SPB_G was their solubility in ALF: SPB_G was much more soluble (see Fig. 3). This may have contributed to the detoxification of SPB_G. Previously we reported in an intratracheal instillation study that soluble NiO particles were rapidly excreted from the lung via the urine and digestive tract (30). NiO instillation was accompanied by severe pulmonary inflammation, possibly because of the toxic potential of Ni⁺ per se, but in the case of silica instillation the dissolved ion form is regarded as non-toxic (31,32). Thus, SPB_G may be disorganized in the lysosomes and then excreted without

translocation to the lymph nodes, possibly via the digestive tract or in the urine. In contrast, SPB_C was aggressively translocated to the lymph nodes, despite the severe macrophage toxicity. It is uncertain whether the lymph node accumulation was the cause or the result of macrophage toxicity, but notably SPB_C—a silica that induced prolonged toxicity—was also accumulated in the lymph nodes.

Taken together, our results showed that a key finding in prolonged silica toxicity was translocation to the thoracic lymph nodes. Unfortunately, some dose groups did not show time-dependent translocation, so unlike in our previous work (30) we were unable to determine a translocation constant. This may have been because of the low clearance rates of MS5 and SPB_C. Comparison of the properties of the effectively excreted silicas (SPB_G and SPB_D) and the poorly cleared ones (MS5, MS5_C, and SPB_C) revealed a distinct difference in two parameters, namely the point of zero charge and the crystallinity (see Table 1). The points of zero charge of the safer two silicas were able to be determined (SPB_G, 1.31; SPB_D, 1.23), but they were incalculable for the others, in the following order: $MS5 \approx MS5_C < SPB_C < 1$. This may reflect differences in the surface properties under acidic conditions, such as intralysosomal after engulfment by macrophages, and it may thus be one possible predictor of toxicity. However, considering the severity and specificity of the macrophage toxicity in the SPB_C treatments, the contribution of properties associated with the point of zero charge is likely to be limited.

The crystallinities (on a mass-basis ratio of crystalline silica to the total silica weight) of the tested silicas were in the following order: SPB_G 59.1% < SPB_D 60.9% < MS5_C 70.3% < SPB_C 77.8% < MS5 89.0% (see Table 1). The toxicity of amorphous silica has not yet been clarified, although mechanical damage such as grinding can cause partial conversion of crystalline to amorphous silica in the surface layer of the particles, thus weakening toxicity (29). Here, conversion seemed to have occurred to some extent in SPB_G, as suggested by the low crystallinity percentage. However, the amorphous component was not concentrated in the surface layer of SPB_G, because that of the alkaline-treated SPB_D was not dramatically increased but remained comparable. Moreover, the intensity of the inflammatory response did not correspond to the crystallinity order. Therefore, crystallinity was unlikely to have contributed to the toxic potential.

Additionally, we analyzed the contribution of other quantitative parameters—initial lung burden, lung burden on the basis of secondary particle size, and lung burden on the basis of specific surface area—to BALF neutrophil numbers on Day 3 (Fig. 11). Neutrophil numbers showed significant increases in the case of all the tested silicas (see Fig. 4): the BALF and initial lung burden data were obtained from identical animals so as to enable us to precisely analyze the dose responses. Seemingly, neutrophil infiltration was dependent on mass-basis (mg/kg) dose levels (Fig. 11A) rather than on a secondary particle basis (mg/kg/nm) (Fig. 11B) or specific surface area basis (m²/kg) (Fig. 11C) in the MS5, MS5_C, SPB_G, and SPB_D groups. Notably, SPB_C induced excessive neutrophil infiltration, and the neutrophils may have been recruited by factors different from those for the others silicas, such as cytokines leaked from degenerated or ruptured alveolar macrophages (33).

Both absolute and relative weights increased dose-dependently in the cases of MS5, MS5_C, SPB_C, and SPB_D treated animals on Day 3. Notably, in our study, no treatment-related body weight losses occurred (Additional file 1), so the absolute and relative weight values tended to be aligned with each other. Porter (19) also reported that lung weight increased with inhalation of MS5, reflecting the pulmonary inflammatory status. Wahlström et al. (34) reported the usefulness of lung weight measurement in inhalation toxicity studies, and they showed some correlation between the weight increases (fold change) and histological inflammatory changes, including

perivascular inflammation, macrophage infiltration, alveolitis, pneumonitis, and pneumonia. Our results also indicated that lung weight responded closely to acute pulmonary inflammation (Day 3) as detected in the BALF examination and histopathology. Additionally, significant increases of the lung weights remained in the SPB_C-treated groups on Days 28 and 91—with persist inflammation. Notably, on Day 91, MS5-treated animals showed neutrophil infiltration and even protein leakage into the alveoli (Fig. 4 and Additional file 2), indicating the presence of pulmonary edema with granuloma formation, but the lung weight change was not significant. Nevertheless, on a fold-change basis, the absolute lung weights of the low-, medium-, and high-dose MS5-treated animals were 1.02, 1.04, and 1.10 times those of the corresponding controls. Therefore, lung weight may be useful as a nonspecific indicator of the inflammation induced by intratracheal instillation.

In terms of dosing method, care needs to be taken in conducting instillation studies and in interpreting their results. Instillation bypasses the upper respiratory tract, so coarse particles that would normally be filtered out in the nasal cavity, can reach the alveoli. This therefore leads to excessive deposition compared with that in real-life inhalation exposure (13). Also, the dose rates we used here were very much higher than the slow build-up seen in occupational settings (35). Therefore, instillation data cannot be used directly in human risk assessment, and the method must be regarded as a hazard identification method (5). Although real-life dose rates cannot be replicated in intratracheal instillation studies, our intention was to apply finely dispersed silica suspensions, all of which were within a respirable size (i.e. secondary particle size $< 0.5 \mu\text{m}$) (15). Notably, silica does not induce toxic lesions in the nasal cavity, so our nasal bypass delivery method does not raise concern about underestimation of the effects on the upper respiratory tract. Regarding the dose levels, the silica concentration of lung tissue paraffin blocks from silicosis patients has been reported at 2.24 mg/mg lung (36). The highest dose applied here was 6 mg/kg, in the high-dose MS5 and MS5_C groups; this was converted to about 1.25 mg/lung (calculated from a provisional body weight of 250 g at dosing, and an absolute lung weight of 1 g)—much less than in the silicosis patients. Therefore, the dose levels that we used here were not excessive. An additional limitation of instillation studies has recently been raised. Holzwarth et al. (37) reported that some instillation studies are not able to achieve nominal dose amounts because of the unexpected retention of dosing syringes; the authors pointed out that numerous misleading results have been published. We agree with this perception, and we therefore believe that it is very important to analyze the lung burden at all time points, as we did here. We believe that our data, taken together, are sufficiently reliable and will help to further understanding of the toxicity of crystalline silica.

Our reliable intratracheal instillation study revealed variable hazard profiles of crystalline silica. Additionally, we revealed that translocation to the lymph node is important for predicting silica toxicity by using a commonly tested MS5 derivative. Furthermore, to the best of our knowledge, this is the first report to show variable SPB-derivative toxicity. Although the main drivers of the translocation from lung to lymph nodes have not yet been clarified, our data suggested that silica solubility, surface properties, and particle size may be contributors. To gain a comprehensive understanding of silica toxicity we need further, well-planned investigations.

Conclusions

Here, we evaluated the comparative toxicities of five different silica particles, including a classical MS5 derivative and novel SPB derivatives. Variable toxicity profiles were noted, and the key finding of translocation to the lymph nodes, which involved prolonged pulmonary toxicity, was observed. Although further knowledge

needs to be accumulated for a comprehensive understanding of these complex silica toxicities, we believe that our results give valuable insights, not only into the mechanisms of silica toxicity but also into the design of future tests by particle toxicologists.

Abbreviations

ALF: Artificial lysosomal fluid; BALF: Bronchoalveolar lavage fluid; FE-SEM: Field-emission scanning electron microscopy; ICP-SFMS: Inductively coupled plasma-sector field mass spectrometry; MS5: Min-U-Sil5; NIST: National Institute of Standards and Technology; SPB: SI007PB; XRD: X-ray diffraction.

Declarations

Ethics approval and consent to participate

All the *in vivo* experiments were preapproved by the Institutional Animal Care and Use Committee of our laboratory. All procedures were conducted in compliance with the guidelines for the animal experiment at the test facility.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

1. Kobayashi, Y. T., and Y. O. conducted animal experiments. T. S, K. Kawaguchi, K. Koga, and K. U. prepared silica suspensions and analyzed physicochemical properties. N. S. performed lung burden and solubility analyses. S.A., T. Kayashima , M. N., and N. I. contributed to the study design and interpretation of data and

provided valuable advice for writing of the manuscript. T. Kobayashi prepared the majority of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1: Physicochemical property of tested silica.

	Primary particle size*	Secondary particle size**	Specific surface	Point of zero charge [pH]	Crystallinity rate [%]
	Mean ± S.D. [nm]	Mean: Min – Max [nm]	area [m ² /g]		
MS5	264 ± 207 (371)	481: 372 – 696	6.1	< 1	89.0
MS5_C	170 ± 91 (97.2)	220: 193 – 262	23.3	< 1	70.3
SPB_C	218 ± 126 (120)	222: 199 – 254	18.8	< 1	77.8
SPB_G	251 ± 109 (67.0)	231: 199 – 250	33.8	1.31	59.1
SPB_D	-(14.7)	58.8: 55.3 – 62.8	153.6	1.23	60.9

*(): Calculated from the surface area. S.D.: Standard deviation.

Table 2: Initial lung burden of silica particles.

	Nominal dose [mg/kg]	Actual dose [mg/kg]	Lung burden at Day 3 [mg/kg/lung]*	Initial fraction reaching the alveolar region [%]**
MS5	0.67	0.62	0.48 ± 0.018	77.4
	2.0	1.9	1.1 ± 0.32	57.9
	6.0	5.8	4.6 ± 0.48	79.3
MS5_C	0.67	0.75	0.64 ± 0.10	85.3
	2.0	2.1	2.6 ± 0.35	123.8***
	6.0	6.4	4.5 ± 1.9	70.3
SPB_C	0.22	0.20	0.11 ± 0.050	55.0
	0.67	0.62	0.32 ± 0.15	51.6
	2.0	1.9	1.3 ± 0.42	68.4
SPB_G	0.22	0.21	0.16 ± 0.0054	76.2
	0.67	0.62	0.40 ± 0.18	64.5
	2.0	1.9	1.1 ± 0.42	57.9
SPB_D	0.22	0.20	0.10 ± 0.011	50.0
	0.67	0.65	0.32 ± 0.12	49.2
	2.0	1.9	0.97 ± 0.19	51.1

*: Mean ± 1 S.D.. **: Lung burden at Day 3 / actual dose.

***: Greater than nominal dose: this may be caused by incorrect instillation.

Table 3 Summary of histopathological examination of the lung.

MS5			Day 3			Day 28					
Day 91											
Dose level			0.67	2	6				0.67	2	6
0.67 2			6			[mg/kg]					
Infiltration/ inflammatory	±	0/5	0/5	3/5				0/5	0/5	0/5	
0/5 1/5				0/5							
cells/ alveolar space	+	0/5	0/5	2/5				0/5	0/5	0/5	
0/5 0/5				4/5							
Infiltration/ inflammatory	±	0/5	0/5	2/5				0/5	0/5	0/5	
0/5 0/5				0/5							

cells/ perivascular								
Aggregation/ foamy cells	±	0/5	0/5	0/5		0/5	1/5	3/5
0/5 0/5				1/5				
	+	0/5	0/5	0/5		0/5	0/5	0/5
0/5 0/5				4/5				
Granulation tissue	+	0/5	0/5	0/5		0/5	0/5	0/5
0/5 0/5				3/5				
	++	0/5	0/5	0/5		0/5	0/5	0/5
0/5 0/5				2/5				
MS5_C		Day 3				Day 28		
Day 91								
Dose level		0.67	2	6		0.67	2	6
0.67 2				6	[mg/kg]			
Infiltration/ inflammatory	±	0/5	4/5	0/5		0/5	0/5	0/5
0/5 0/5				0/5				
cells/ alveolar space	+	0/5	0/5	5/5		0/5	0/5	0/5
0/5 0/5				0/5				
Infiltration/ inflammatory	±	1/5	0/5	1/5		0/5	0/5	0/5
0/5 0/5				0/5				
cells/ perivascular	+	0/5	0/5	1/5		0/5	0/5	0/5
0/5 0/5				0/5				
Aggregation/ foamy cells	±	0/5	0/5	0/5		0/5	0/5	1/5
0/5 0/5				4/5				
	+	0/5	0/5	0/5		0/5	0/5	3/5
0/5 0/5				1/5				
SPB_C		Day 3				Day 28		
Day 91								
Dose level		0.22	0.67	2		0.22	0.67	2
0.22				0.67	2	[mg/kg]		
Infiltration/ inflammatory	±	0/5	4/5	0/5		1/5	0/5	0/5
1/5 2/5				0/5				
cells/ alveolar space	+	0/5	1/5	0/5		4/5	5/5	1/5
1/5 3/5				4/5				
	++	0/5	0/5	5/5		0/5	0/5	3/5
0/5 0/5				1/5				
Infiltration/ inflammatory	±	1/5	0/5	3/5		1/5	1/5	0/5
0/5 0/5				0/5				
cells/ perivascular	+	0/5	0/5	0/5		3/5	0/5	1/5
0/5 0/5				0/5				
	++	0/5	0/5	0/5		1/5	0/5	0/5
0/5 0/5				0/5				

Degeneration and necrosis/ 1/5 1/5	±	0/5	2/5	0/5		1/5	0/5	1/5
alveolar macrophage 0/5 4/5	+	0/5	0/5	5/5		3/5	3/5	0/5
	++	0/5	0/5	0/5		0/5	1/5	3/5
	+++	0/5	0/5	0/5		0/5	0/5	1/5
Aggregation/ foamy cells 0/5 4/5	+	0/5	0/5	0/5		0/5	5/5	5/5
	++	0/5	0/5	0/5		0/5	0/5	0/5
Hyperplasia/ type II cells 0/5 4/5	+	0/5	3/5	1/5		0/5	0/5	1/5
	++	0/5	1/5	4/5		0/5	0/5	3/5
SPB_G			Day 3				Day 28	
Day 91								
Dose level		0.22	0.67	2		0.22	0.67	2
0.22				0.67	2	[mg/kg]		
Aggregation/foamy cells 0/5 0/5	+	0/5	0/5	0/5		0/5	0/5	0/5
SPB_D			Day 3				Day 28	
Day 91								
Dose level		0.22	0.67	2		0.22	0.67	2
0.22				0.67	2	[mg/kg]		
Infiltration/ inflammatory 0/5 0/5	±	1/5	0/5	0/5		0/5	0/5	0/5
cells/ alveolar space 0/5 0/5	+	0/5	0/5	5/5		0/5	0/5	0/5
Infiltration/ inflammatory 0/5 0/5	±	0/5	0/5	5/5		0/5	0/5	0/5
cells/ perivascular 0/5 0/5	+	0/5	1/5	0/5		0/5	0/5	0/5

±: slight, +: mild, ++ moderate, +++: severe

Table 4. Summary of histopathological examination of posterior mediastinal lymph nodes.
MS5
Day 3
Day 28
Day 91

Dose level		0.67	2	6		0.67	2	6
0.67 2				6	[mg/kg]			
Granuloma formation	±	0/5	0/5	0/5		0/5	0/5	0/5
		0/5 1/5		0/5				
	+	0/5	0/5	0/5		0/5	0/5	0/5
		0/5 3/5		0/5				
	++	0/5	0/5	0/5		0/5	0/5	0/5
		0/5 0/5		5/5				
MS5_C		Day 3				Day 28		
Day 91								
Dose level		0.67	2	6		0.67	2	6
0.67 2				6	[mg/kg]			
Granuloma formation	±	0/5	0/5	0/5		0/5	0/5	0/5
		0/5 3/5		0/5				
	+	0/5	0/5	0/5		0/5	0/5	0/5
		0/5 0/5		1/5				
	++	0/5	0/5	0/5		0/5	0/5	0/5
		0/5 0/5		4/5				
SPB_C		Day 3				Day 28		
Day 91								
Dose level		0.22	0.67	2		0.22	0.67	2
0.22				0.67 2	[mg/kg]			
Granuloma formation	±	0/5	0/5	0/5		0/5	2/5	0/5
		4/5 1/5		2/5				
	+	0/5	0/5	3/5		0/5	0/5	3/5
		0/5 4/5		0/5				
	++	0/5	0/5	2/5		0/5	0/5	2/5
		0/5 0/5		3/5				
±: slight, +: mild, ++ moderate, +++: severe.								

Figures

Figure 1

Scanning electron micrographs of tested silica particles.

Figure 2

Zeta potential titration curve of silica particles. Zeta potential was measured in duplicate and mean values were plotted.

Figure 3

Dissolution rates of silica particles. Each 2.0-mg/mL suspension was mixed with saline or artificial lysosomal fluid and assessed at each time point (0.5, 1, 2, 4, 8, 24, 32, 48, 168, and 336 h).

Figure 4

Bronchoalveolar lavage fluid cytological analyses. Top panels: Day 3; middle: Day 28; bottom: Day 91. Solid lines: macrophage percentage; dashed lines: neutrophil percentage. Uncapped error bars: 1 S.D. for total cell count. Capped error bars: 1 S.D. for macrophage number and neutrophil number. Macrophage and neutrophil numbers were statistically analyzed vs. corresponding control values (one-tailed). *: $P < 0.05$ (Dunnett's test); **: $P < 0.01$ (Dunnett's test); ##: $P < 0.01$ (Dunnett's test after logarithmic transformation); ##: $P < 0.05$ (Dunnett's test after square-root transformation); †: $P < 0.05$ (Steel's test); all compared with vehicle controls.

Figure 5

Lung burden and translocation to thoracic lymph nodes. Bars: 1 S.D. Solid lines indicate lung burden. Dashed lines indicate total thoracic lymph node (LN) load. A: Statistical significance of lung burden compared with vehicle control (A: $P < 0.05$; AA: $P < 0.01$). B: Significantly different from initial lung burden (Day 3) (B: $P < 0.05$; BB: $P < 0.01$). a: Statistical significance of LN load compared with vehicle control (a: $P < 0.05$; aa: $P < 0.01$). b: Significantly different from initial LN load (Day 3) (b: $P < 0.05$; bb: $P < 0.01$). (N): Non-parametric analysis. Each panel shows high-dose, medium-dose, and low-dose groups from top to bottom.

Figure 6

Absolute lung weights (g) on Days 3, 28, and 91 after instillation. *: $P < 0.05$ (Dunnett's test); **: $P < 0.01$ (Dunnett's test) compared with vehicle control.

Figure 7

Relative lung weights [g/100 g body weight (B.W.)] on Days 3, 28, and 91 after instillation. *: $P < 0.05$ (Dunnett's test); **: $P < 0.01$ (Dunnett's test) compared with vehicle control.

Figure 8

Representative histopathological changes in the lung 3 days after intratracheal instillation of silicas. Arrowheads: degenerated or necrotic alveolar macrophages. Hematoxylin and eosin stain.

Figure 9

Representative histopathological changes in the lung at 28 or 91 days after intratracheal instillation of silicas. A: aggregation of alveolar macrophages; B: granuloma formation and aggregation of alveolar macrophages. C: aggregation of alveolar macrophages; D: aggregation of alveolar macrophages. E: inflammatory cell infiltration in alveolar spaces and degeneration and necrosis of alveolar macrophages (arrowheads); F: inflammatory cell infiltration in alveolar spaces and degeneration and necrosis of alveolar macrophages (arrowheads). Hematoxylin and eosin stain.

Figure 10

Representative histopathological changes in the posterior mediastinal lymph nodes (28 or 91 days after intratracheal instillation of silica). A: slight granuloma formation (arrowheads, also in the two panels directly below). Hematoxylin and eosin stain.

Figure 11

Dose-response analysis of BALF infiltrating neutrophil numbers on Day 3. Neutrophil numbers were plotted to compare the initial lung burden; lung burden on a secondary particle size basis—horizontal line of B shows (lung burden [mg/kg] / secondary particle size [nm]); and lung burden on a specific surface area basis—horizontal line of C shows (lung burden [mg/kg] $\times$ specific surface area [m²/mg]).