胞内局在の点において全く異なる性質を発揮することを裏付けるものである．したがって，非晶質ナノシリカの安全性を確保するに当たっては，従来までの安全性試験に加えて，遺伝子や核機能を指標とした評価を実施する必要があると考えられた．われわれは現在，ナノシリカの核内移行性とDNA合成阻害，DNA傷害の発現機構の解明を目指して，nSP70曝露細胞の核分画をサンプルとしたプロテオーム解析を進めており（Fig. 3），2次元ディファレンシャル電気泳動法を用いた解析結果から，添加するシリカの粒子サイズの違いによって，発現量が変動する多数の核内タンパク質を見い出している．これらのタンパク質は，DNA合成阻害やDNA傷害の発現機序の解明に有用であることに加え，安全性を評価できるバイオマーカーとして活用できる可能性があることから，現在，質量分析による同定を試みているところである．以上のナノマテリアルの細胞内局在解析によって得られる局在部位／内部暴露量（*in vitro* トキシコキネティクス）に関する情報は，より精密なトキシコプロテオミクスを実行する上で有用な指針を与えるものと考えている．

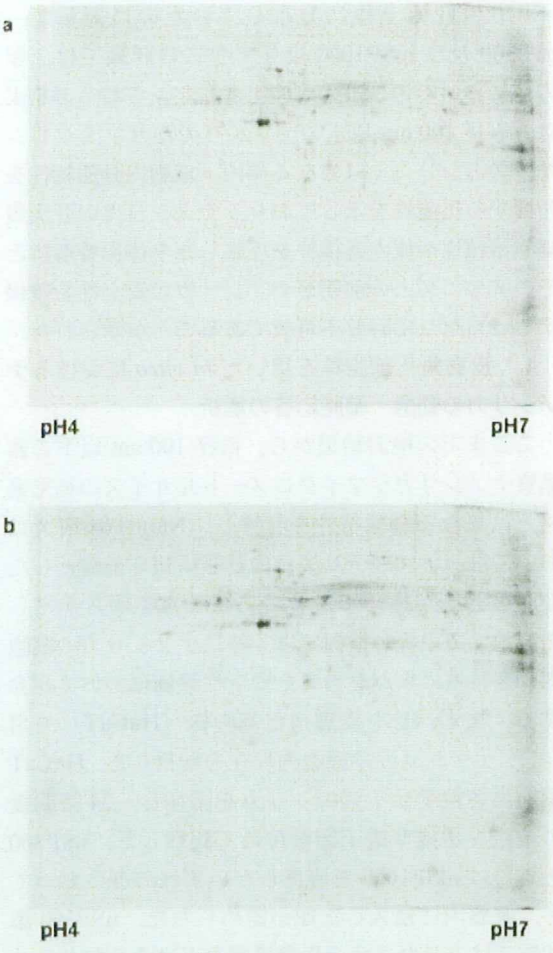

Fig. 3. 2D-DIGE Image of Fluorescently Labeled Nuclear Proteins from nSP70-treated HaCaT Cells

HaCaT cells were untreated or treated with nSP70 (100 μg/ml) for 24 h. Proteins were labeled with Cy3 (untreated, a), Cy5 (nSP70-treated, b), and Cy2 (mixture of untreated and nSP70-treated as internal reference (data not shown)) and separated on pH 4–7 IPG strips in the first dimension. This procedure was followed by separation on 12.5% SDS-polyacrylamide gels in the second dimension, and the gels were scanned with a Typhoon Trio+ fluorescent scanner.

5. おわりに

以上，各種バイオマーカーやイメージングを活用したトキシコキネティクス解析により，ナノメートルサイズ（100 nm以下）の非晶質ナノシリカが，皮膚透過性や体内動態／生体影響の点においてサブミクロン以上のサイズ（数百nm–数十μm）の従来素材とは異なる性質を発揮することを実証した．これらの結果は，ナノマテリアルの安全性確保・社会受容促進を達成するためには，ナノマテリアルをマイクロマテリアルとは別個の新素材として捉え，体内移行性や組織浸透性／蓄積性などの動態をより慎重に解析する必要があることを示している．過去のアスベストの例では，アスベストの利用が始まって40–50年で，悪性中皮腫や肺がんなどの問題が明らかとなった．[14–17] われわれはこれを大いに反省し，ナノマテリアルの使用が拡大しつつある今こそ，科学的根拠に基づきつつ，ナノマテリアルの安全性対策を講じなければならない．現在，経済開発協力機構（OECD）が世界規模でナノマテリアルの安全性評価ガイドラインの策定を進めており，ナノマテリアルの開発・実用化の規制に動いているが，ナノマテリアルが既にヒトの生活に密着していることや，ナノ産業の育成を考慮すると，慎重かつ適切な規制が不可欠であることは言うまでもない．現在，われわれは本稿で紹介したようなナノマテリアルのサイズに着目した検討のみではなく，表面性状や親／疎水バランス，形状と，動態や安全性との連関情報の収集，これらの情報を基盤としたナノマテリアルの安全性バイオマーカーの同定を推進している．将来的には，これらの研究を基盤として科学的根拠に基づいた安全なナノマテリアルの使用・設計指針の策定が実現するものと期待している．

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Size-dependent cytotoxic effects of amorphous silica nanoparticles on Langerhans cells

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Amorphous silica nanoparticles (nSPs), are widely used in medicines, cosmetics and food. However, due to their reduced particle size they are suspected to pose new risks induced by changes in biological reactivity and kinetics, which differ from those of bulk materials. In a previous study, we showed that silica particles with a diameter of 70 nm penetrated the stratum corneum (SC) of mouse skin and were taken up by living cells such as keratinocytes and Langerhans cells. To clarify the relationship between particle size, distribution and cellular response, we have evaluated size-dependent intracellular localization and cytotoxicity of silica particles, using the mouse epidermal Langerhans cell line XS52. On treatment with silica particles of diameters 70, 300, and 1000 nm, cellular uptake and cytotoxicity increased with reduction in particle size. These results suggest that smaller sized silica particles induced greater cytotoxicity against Langerhans cells, which was correlated with the quantity of particle uptake into the cells.

1. Introduction

The recent development of nanoscale engineering represents a current dynamic area of interdisciplinary research, incorporating nanomaterials (NMs) into a diverse product matrix such as diagnostics, food additives and cosmetics. Because amorphous silica nanoparticles (nSPs) and titanium oxide nanoparticles, etc. are colorless and reflect ultraviolet more efficiently than micro-sized particles, nSPs and titanium oxide nanoparticles are already used as cosmetic vehicles or functional ingredients in many cosmetics such as foundation creams and sunscreens. However, because NMs may possess novel properties, kinetics, and biological effects different from those of micro size bulk materials, their potential harmful effects on humans are raising concerns about their safety. Thus, there is an urgent need for risk assessment of NMs. To achieve this, it is most important to analyze the relationship between particle-size parameters, cellular distribution and biological effects, allowing prediction and avoidance of risk in using NMs.

In a previous study, we showed that silica particles with a diameter of 70 nm penetrated the stratum corneum (SC) of mouse skin and were taken up by living cells such as keratinocytes and Langerhans cells. So, to reveal the relationship between particle size, distribution, and cellular response, we evaluated size-dependent intracellular localization and cytotoxicity of silica particles, using the mouse epidermal Langerhans cell line XS52.

2. Investigations, results and discussion

To assess cellular uptake of nSPs, we observed XS52 cells treated with 100 μg/ml nSP70, nSP300 and mSP1000 using transmission electron microscopy (TEM). We found that nSP300 and mSP1000 were located in cytoplasm only (Fig. 1c and d), while nSP70 was surprisingly located in nucleus as well as cytoplasm (Fig.1a and b). Furthermore the quantity of silica particles taken up by the cells increased as particle size decreased. These results suggested that the uptake and localization of silica particles altered with particle size.

We next investigated biological effects of various-sized silica particles in XS52 cells. To assess the effect of treatments with nSPs on cellular proliferation, the [^{3}H]-thymidine incorporation assay was performed. As shown in Fig. 2, XS52 cell proliferation was dose-dependently inhibited by treatment with silica particles of all sizes. IC50 values for nSP70, nSP300 and mSP1000 were 4.2, 32.6, and 75.0 μg/ml, respectively. These results showed that the growth of XS52 cells was more strongly inhibited by smaller-sized nSP.

To study the mechanism responsible for the effects on XS52 cells treated with various-sized silica particles, we measured the quantity of lactate dehydrogenase (LDH) released. LDH is released into culture medium after the cellular membrane disruption that constitutes the last step of the in vitro cell death process. After 24 h of exposure (Fig. 3), no LDH release was observed in mSP1000-treated cells, while dose-dependent LDH

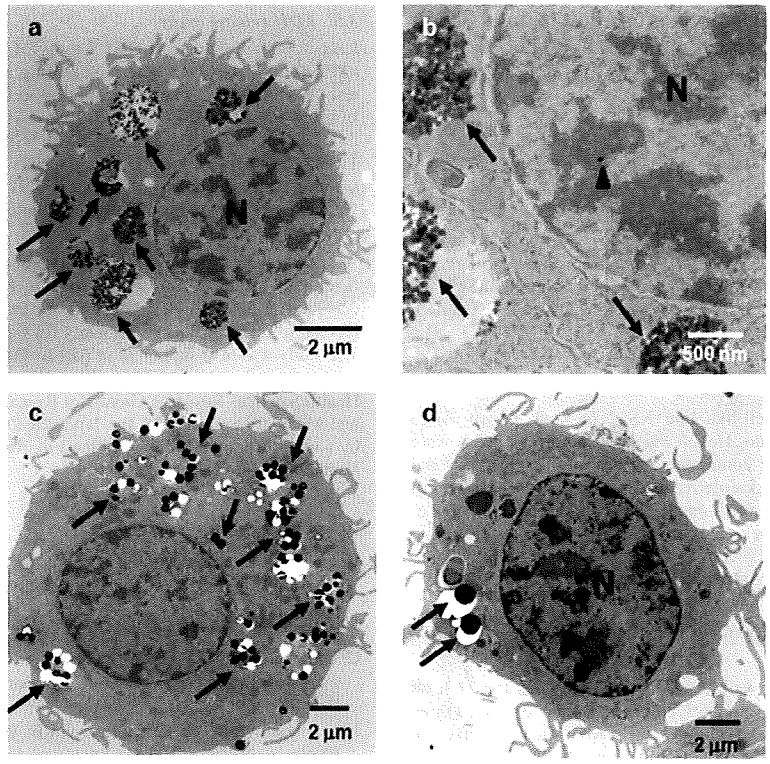

Fig. 1: Localization of silica particles in XS52 cells (arrows). Cells were treated for 24 h with nSP70 (a and b), nSP300 (c) and mSP1000 (d). nSP300 and mSP1000 were located in cytoplasm only. On the other hand, nSP70 was located in the nucleus as well as in cytoplasm (b, arrow head). Scale bars; 2 µm (a, c and d), 500 nm (b)

release was observed in nSP70- and nSP300-treated cells. The highest LDH release was recorded on treatment with 30 µg/ml nSP70 (193 ± 6.8% of control). This result therefore indicated that the cytotoxicity to XS52 cells may be due to cellular membrane damage. Consequently, it appears that the difference in the quantity of silica particles taken up by the cells may explain the size-dependent toxicity to XS52 cells.

As reported elsewhere, we had shown that nSP70 penetrated the stratum corneum (SC) of mouse skin and was taken up by living Langerhans cells (Nabeshi et al. 2010). Furthermore, in the present study we showed that the difference in the quantity of silica particles taken up by the cells was linked to size-dependent toxicity and nSP70 taken up by Langerhans cells entered the nucleus. Thus, our previous and present results suggest that transdermal exposure to nSPs may (i) risk dysfunction of Langerhans cells, as shown by the cytotoxicity to XS52 cells, (ii) induce immune disruption by altering the immune response (Tinkle et al. 2003; Fifis et al. 2004) and (iii) induce dysfunction of the nucleus and genotoxicity via aggregation of intranuclear protein or inhibition of RNA transcription (Chen and von Mikecz 2005) following entrance of nSPs into the nucleus.

Collectively, the data obtained in this study offer highly useful information for prediction and avoidance of harmful effects mediated by nSPs used commercially in cosmetics. Thus, cor-

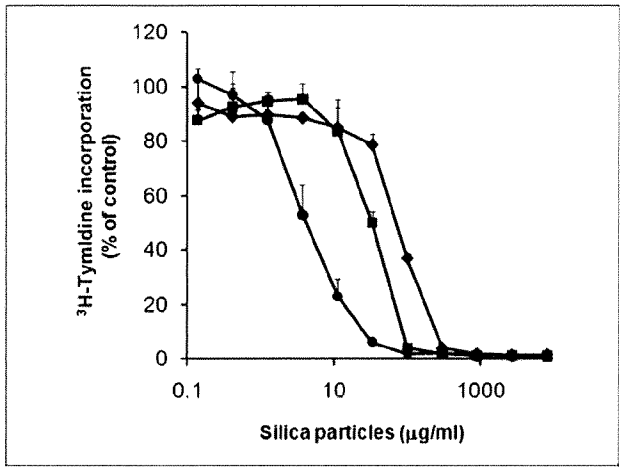

Fig. 2: Effect of various-sized silica particles on proliferation of XS52 cells. The proliferation of cells after incubation with nSP70 (circle), nSP300 (square) and mSP1000 (diamond) for 24 h was evaluated using the [^{3}H]-thymidine incorporation assay. The percentage increase in cell proliferation was calculated relative to the negative control. Data are presented as means ± SD

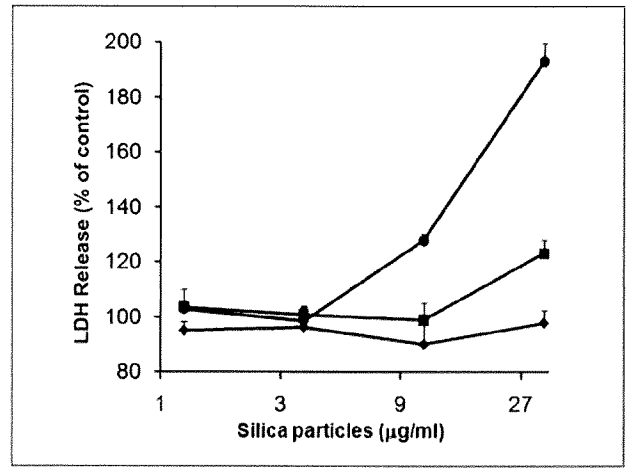

Fig. 3: Effect of silica particles on membrane damage. Cellular membrane damage in XS52 cells after incubation with nSP70 (circle), nSP300 (square) and mSP1000 (diamond) for 24 h was evaluated by the LDH release assay. The percentage cellular membrane damage was calculated relative to the negative (medium) controls. Data are presented as means ± SD

related analysis of physicochemical properties, harmful effects and biodistribution as performed in our study may offer valuable readouts for toxicity of nanomaterials and help to develop non-toxic nanomaterials in the future.

3. Experimental

3.1. Silica particles

Fluorescent (red-F)-labeled silica particle suspensions (25 mg/ml or 50 mg/ml) with a diameter of 70, 300 and 1000 nm (Micromod Partikeltechnologie GmbH, Rostock, Germany; designated nSP70, nSP300, mSP1000, respectively) were used in this study. In each case, silica particles were used after 5 min sonication and 1 min vortexing.

3.2. Cell culture

Cells from the Langerhans cell-like line XS52 (a kind gift of Akira Takashima, University of Toledo, Health Science Campus, Toledo) were expanded in complete medium containing 2 ng/ml murine GM-CSF and 10% culture supernatants from skin-derived stromal NS47 cells (a kind gift of Akira Takashima). Complete medium was RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% non-essential amino acids, 1% L-glutamine, 1 mM sodium pyruvate, 1% 2-mercaptoethanol, 10 mM HEPES buffer, and 1% Antibiotic-Antimycotic Mix stock solution.

3.3. Transmission electron microscopy (TEM) analysis of Langerhans cell line

XS52 cells were cultured with various-sized silica particles for 24 h on chamber slides, then fixed at 4 °C in 2.5% glutaraldehyde and washed three times in 0.1 M phosphate buffer (pH: 7.4); cells were then post-fixed in phosphate-buffered 1% osmium tetroxide for 60 min at 4 °C, dehydrated through a series of ethanol concentrations and embedded in EPON resin (TAAB, Watford, UK). Ultrathin sections were stained with lead citrate and examined under an electron microscope (Hitachi H-7650).

3.4. [^{3}H]-Thymidine incorporation assay

Proliferation of silica particle-treated XS52 cells was measured by [^{3}H]-thymidine incorporation assay. 1×10^4 cells were cultured with varying concentrations of nSPs for 18 h at 37 °C and [^{3}H] thymidine (1 μCi/well) was then added into each well. After a further 6 h, cells were harvested and lysed on glass fiber filter plates using a Cell harvester (PerkinElmer, MA, USA). The filter plates were then dried and counted by standard liquid scintillation counting techniques in a TopCounter (PerkinElmer, MA, USA).

3.5. LDH release assay

Lactate dehydrogenase (LDH) activity of XS52 cells exposed to nSP70, nSP300, mSP1000 was determined using a commercial LDH cytotoxicity test (WAKO, Osaka, Japan) according to the manufacturer's instructions. In brief, 5×10^3 cells were seeded into each well of a 96 well plate. After 24 h incubation, cells were treated with nSP70, nSP300, mSP1000 or 0.2% Tween 20 (positive control). After a further 24 h incubation period, 50 μl of medium overlying cells was used for LDH analysis. Absorption of light at 560 nm was measured using a spectrophotometer.

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Note

Histological analysis of 70-nm silica particles-induced chronic toxicity in mice

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ABSTRACT

Nano-sized silica is a promising material for disease diagnosis, cosmetics and drugs. For the successful application of nano-sized material in bioscience, evaluation of nano-sized material toxicity is important. We previously found that nano-sized silica particles with a diameter of 70 nm showed acute liver failure in mice. Here, we performed histological analysis of major organs such as the liver, spleen, lung, kidney, brain and heart in mice, chronically injected with 70-nm silica particles for 4 weeks. Histological analysis revealed hepatic microgranulation and splenic megakaryocyte accumulation in these 70-nm silica particles treated mice, while the kidney, lung, brain and heart remained unaffected. Thus, liver and spleen appear to be the major target organs for toxicity by the chronic administration of the 70-nm silica particles.

1. Introduction

Recent progress in nanotechnology, the act of reducing size from the microscale to the nanoscale, has provided us with dramatic changes in industrial manufacturing and medicine. It also offers many benefits to revolutionize biotechnology, such as synthesis of new drugs with targeted delivery and regenerative medicine [1]. Reducing particle size increases surface area and makes modification of unique physicochemical properties, such as high conductivity, strength, durability, and chemical reactivity possible [2]. Thus, the nanotechnology has led to novel materials and innovations in the industry, bioscience and medicine.

Nanomaterials are already being used in bioscience and medicine, such as electronics, sunscreens, cosmetics and medicine for the purposes of diagnosis, imaging and drug delivery. For example, nano-sized silica particles are intended for the systemic and local delivery of drugs [3]. However, the toxicity of the manufactured nano-sized particles has not been fully evaluated.

We previously found that nano-sized particles with a diameter of 70 nm caused acute liver failure, while micro-sized particles with a diameter of 300 or 1000 nm did not [4]. In this study, we performed histological analysis of chronic toxicity induced by intravenous administration of 70-nm silica particles (SP70) for 4 weeks into the major organs, such as liver, lung, spleen, kidney, brain and heart of mice.

2. Materials and methods

2.1. Materials

Nano-sized silica particles with a diameter of 70 nm were obtained from Micromod Partikeltechnologie GmnH (Rostock, Germany). The surface was not modified. The mean diameters of these particles analyzed by Zetasizer (Sysmex Co., Kobe, Japan) were determined to be 55.7 nm. The particles were spherical and nonporous, and were stored at 25 mg/ml in aqueous suspension. The suspensions were thoroughly dispersed with sonication before use and diluted in water. The dispersion of the particles was confirmed by electron microscopy (data not shown). Reagents used were of research grade.

2.2. Animals

The 8-week-old BLAB/c male mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), and housed in an environmentally controlled room at 23 ± 1.5 °C with a 12-h light/dark cycle. Mice had access to water and chow (Type MF, Oriental Yeast, Tokyo, Japan) *ad libitum*. Mice were intravenously

Abbreviations: SP70, 70-nm silica particles; HE, hematoxylin–eosin; ALT, alanine aminotransferase; HYP, hydroxyproline.

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injected with vehicle or the particles twice a week for 4 weeks. On day 3 after the last injection, the mice were sacrificed, and the serum and organs were recovered. The experimental protocols conformed to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

2.3. Histological analysis

The liver, spleen, lung, kidney, brain and heart were removed and fixed with 4% paraformaldehyde. After sectioning, thin sections of tissues were stained with hematoxylin and eosin for histological observation.

2.4. Biochemical analysis

Serum alanine aminotransferase (ALT) was measured using a commercially available kit according to the manufacturer's protocol (Wako Pure Chemical, Osaka, Japan).

2.5. Hydroxyproline (HYP) assay

Hepatic HYP content was measured by Kivirikko's method with some modification [5]. Briefly, liver tissue was hydrolyzed in 6 M HCl at 110 °C for 24 h. The resultant supernatant was neutralized with 8 N KOH, and then 2 g of KCl and 1 ml of 0.5 M borate buffer were added, followed by a 15-min incubation at room temperature and further incubation for 15 min at 0 °C. Chloramines-T solution was then prepared and added. After additional incubation for 1 h at 0 °C, 2 ml of 3.6 M sodium thiosulfate was added, followed by incubation at 120 °C for 30 min. Next, 3 ml of toluene was added with incubation for a further 20 min at room temperature. After centrifugation, 2 ml of the resultant supernatant was added to Ehrlich's reagent, followed by incubation for 30 min at room temperature. Subsequently, absorbance was measured at 560 nm.

2.6. Statistical analysis

Statistical analysis was performed by Student's *t*-test. The level of significance was set at $p < 0.05$.

3. Results and discussion

We previously found that intravenous administration of SP70 induced liver injury through a single administration [4]. To investigate the chronic toxicity of SP70, 10 or 30 mg/kg of SP70 was intravenously injected into mice twice a week for 4 weeks at which point the livers were not injured or injured by the single injection, respectively [4]. During chronic administration, no significant differences were observed in the body weight between the vehicle and the SP70-treated group (Fig. 1) and no abnormal behaviors were detected (data not shown). Therefore, SP70 treatment did not show apparent toxicity in mice at the low dose.

Next, we performed histological analysis of tissues that are enriched with reticuloendothelial system (RES) such as the liver, spleen, and lungs and non-RES organs such as the heart, kidney and brain. As shown in Fig. 2A and B, treatment with SP70 induces hepatic microgranulation and increases splenic megakaryocyte accumulation. In contrast, the remaining RES organ, the lung, and all the non-RES organs did not show tissue injury with SP70 treatment (Fig. 2B–F). Thus, we examined a serum biochemical marker of liver injury, ALT, to confirm liver injury. SP70 treatment significantly elevated serum ALT levels (Fig. 3A), but a renal injury marker, blood urea nitrogen, was not elevated by these treatments

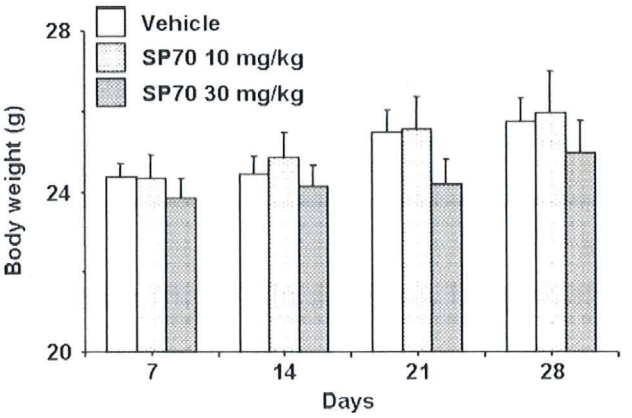

Fig. 1. Body weight changes in mice treated with SP70 for 4 weeks. Mice were intravenously administered SP70 at 0, 10 or 30 mg/kg twice a week for 4 weeks. The body weights of the treated mice were monitored on days 7, 14, 21 and 28. Each point represents mean ± SEM ($n = 5–7$).

(data not shown). Chronic hepatic injury causes liver fibrosis, finally leading to hepatic carcinoma. The chronic treatment with SP70 also elevated a marker of fibrosis, HYP, in the liver (Fig. 3B). Taken together, chronic SP70 treatment appears to injure the liver and spleen.

As innovative materials cover wide fields from industry to life science, nanomaterials have potential to improve the quality and performance of many consumer products as well as medical therapies. Thus, it is very critical in the field of nanotechnology to also assess the risk of nano-sized materials. As the use of nano-sized silica particles in cosmetics and the application in pharmaceutical research, e.g., drug delivery and molecular imaging [3,6] are increasing, we evaluated the toxicity of nano-sized silica particles. We have already found that SP70 causes acute liver injury in mice [4]. In the present study, we evaluate the influence of chronic administration of SP70 for 4 weeks on major organs by histological analysis. As the nano-sized particles are taken into RES organs such as the liver, lung and spleen, we expected that all the RES organs would be injured by chronic SP70 exposure. However, histological abnormalities in the lung were not observed. Kim et al. found that 50-nm silica particles were distributed into all the RES organs, but the amount of the distributed particles into the lung was smaller than that into the liver and spleen [7]. Therefore, the lack of histological abnormalities in the lung may be due to a lower distribution of SP70.

The underlying mechanism for histological injury in the liver and spleen remains to be elucidated. We previously found that the serum levels of inflammatory cytokines (interleukin-6 and tumor necrosis factor-α) were elevated by SP70 [4]. Uptake of SP70 by macrophages in the liver and spleen may cause the release of the cytokines from the macrophage, leading to histological abnormalities. Macrophage receptor with collagenous structure (MARCO), CD204 and CD36 are all macrophage silica particle receptors [8–10]. CD36 is expressed in macrophages of BALB/c mice [10].

In the present study, there is no observation of histological injury in lung, kidney, brain and heart. Regulation of liver and spleen injuries may be critical for the safe application of these nano-sized silica particles. Future analysis is necessary to determine tissue distribution of SP70. Extensive studies are also required to provide the basis for a new class of nanomaterials for drugs, proteins, and gene delivery applications. We are developing materials and methods to control the bio-distribution of these nano-sized silica particles.

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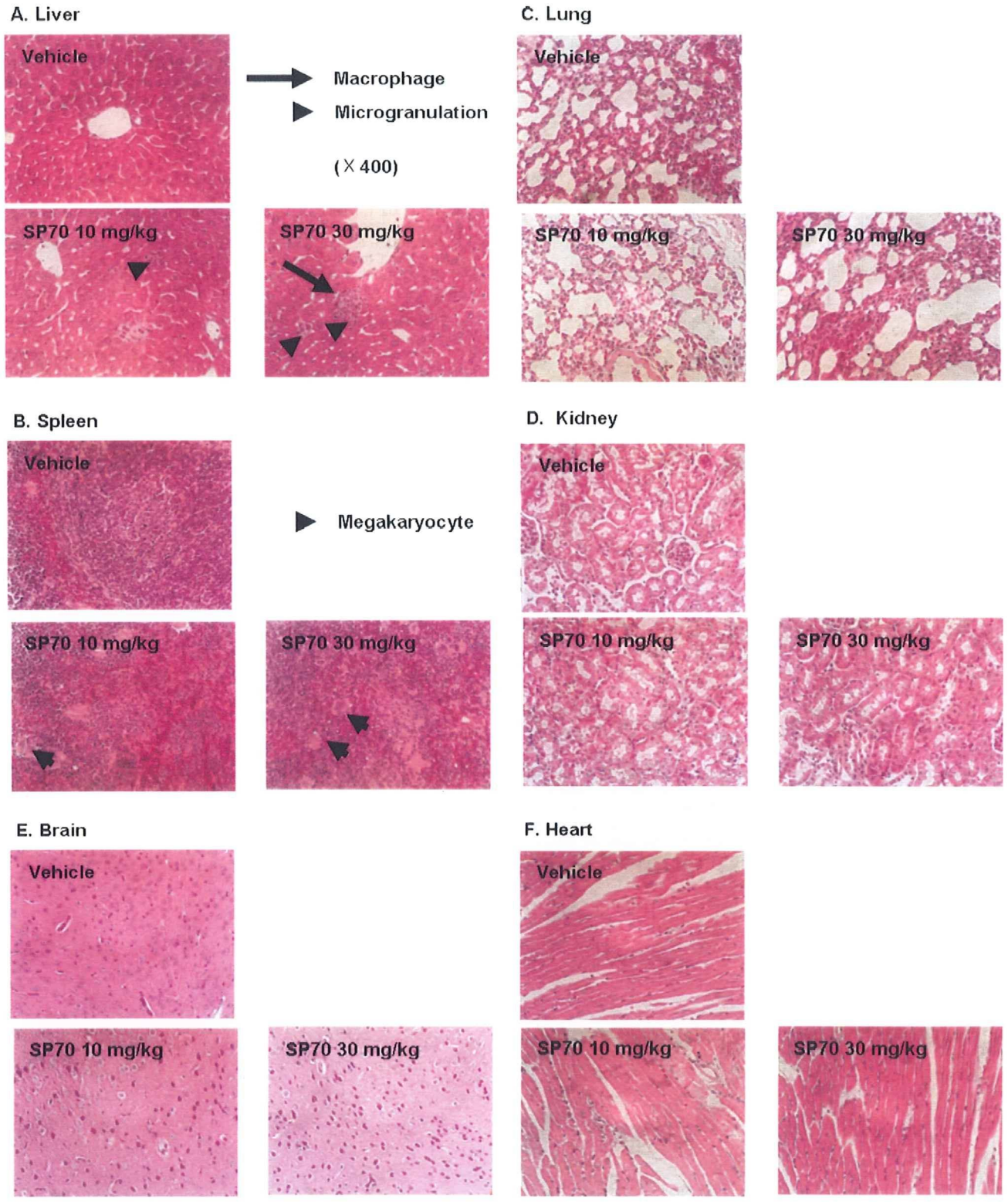

Fig. 2. Histopathological evaluation of the organs from SP70-treated mice. After chronic treatment with SP70 for 4 weeks, liver (A), spleen (B), lung (C), kidney (D), brain (E) and heart (F) were recovered and fixed with paraformaldehyde, followed by staining with hematoxylin and eosin. The arrowheads in (A) and (B) indicate microgranulation in the liver and accumulation of megakaryocyte in the spleen, respectively. The tissue sections were observed under a microscope at 400×. The pictures are representative of at least four independent sections.

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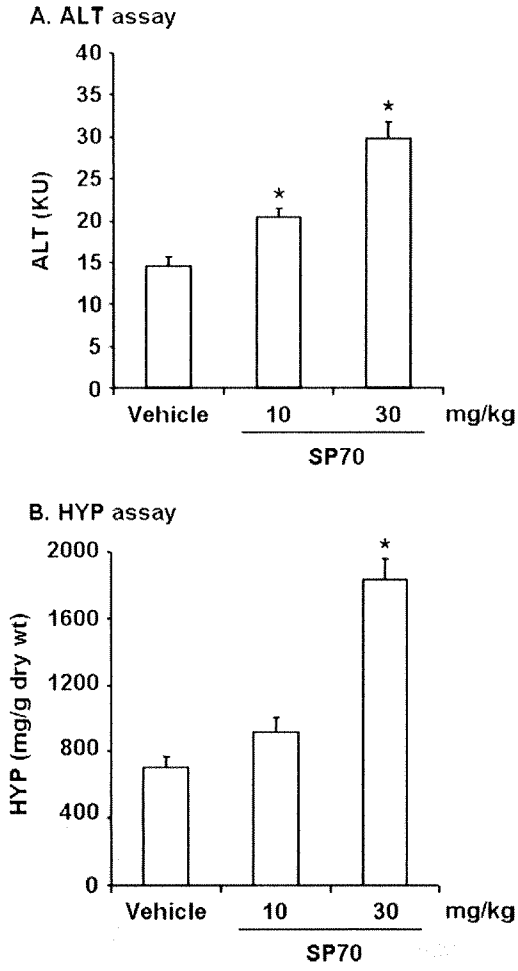

Fig. 3. Biochemical analysis of liver injury. After chronic SP70 treatment for 4 weeks, the serum and liver were collected. Then, the serum alanine aminotransferase (A) and hepatic hydroxyproline (HYP) levels (B) were measured as described in Section 2. Data are shown as mean ± SD ($n = 4$). The results are representative of at least three independent experiments. *Significant difference from the vehicle-treated group ($p < 0.05$).

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We thank all members of our laboratory for their useful comments and discussion. This study is partly supported by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan.

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Research paper

Silica nanoparticles as hepatotoxicants

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ABSTRACT

Nano-size materials are increasingly used in cosmetics, diagnosis, imaging and drug delivery, but the toxicity of the nano-size materials has never been fully investigated. Here, we investigated the relationship between particle size and toxicity using silica particles with diameters of 70, 300 and 1000 nm (SP70, SP300, and SP1000) as a model material. To evaluate acute toxicity, we first performed histological analysis of liver, spleen, kidney and lung by intravenous administration of silica particles. SP70-induced liver injury at 30 mg/kg body weight, while SP300 or 1000 had no effect even at 100 mg/kg. Administration of SP70 dose-dependently increased serum markers of liver injury, serum aminotransferase and inflammatory cytokines. Repeated administration of SP70 twice a week for 4 weeks, even at 10 mg/kg, caused hepatic fibrosis. Taken together, nano-size materials may be hepatotoxic, and these findings will be useful for future development in nanotechnology-based drug delivery system.

1. Introduction

The recent development of technology for reducing material size has provided innovative nanomaterials. Nanomaterials are engineered structures with at least one dimension of 100 nm or less, and have unique physicochemical properties with regard to size, chemical composition, surface structure, solubility, shape and aggregation. Nanomaterials have been widely used in microelectronics, catalysts, ultra-sensitive molecular sensing and imaging probes, pharmaceutical agents and cosmetics. Thus, the development of reduced particle size from the macro to the nanoscale provides benefits to a range of industrial and scientific fields. However, materials that are inert in bulk form may be toxic in nano-size forms, and it is thus essential to understand the biological activities and potential toxicity of nanomaterials [1–3].

The influence of inhalation of nanomaterials on human health has been widely investigated. Occupational exposure to quartz, mineral dust particles and asbestos induce inflammation, fibrosis and cytotoxicity in the lung [3]. In animal models, inhaled nanoparticles do not locally remain in the lung, and pass into blood flow, resulting in distribution to distant organs, such as the liver, kidney, brain and heart [4–7]. Moreover, biomedical applications for diagnosis and therapeutic purposes will require intravenous, subcutaneous or intramuscular administration [8–10]. Thus, it is necessary to confirm the influence of nanomaterials in systemic flow on various organs.

Silica nanoparticles have been applied to diagnosis and drug delivery [4,11], and intraperitoneal administration of silica nanoparticles results in the biodistribution of the nanoparticles to diverse organs, such as the liver, kidney, spleen and lung [4]. Both micro- and nano-size silica particles are also commercially available. In the present study, we investigated the influence of nanomaterials on major organs, such as the liver, kidney, spleen and lung using silica particles as a model material. When silica particles with a diameter of 70, 300 or 1000 nm were intravenously injected, only the 70-nm particles led to acute and chronic liver injury.

2. Materials and methods

2.1. Materials

Silica nanoparticles with a diameter of 70, 300 or 1000 nm were purchased from Micromod Partikeltechnologie GmbH (Rostock,

Abbreviations: SP70, 70 nm silica particles; SP300, 300 nm silica particles; SP1000, 1000 nm silica particles; ALT, aminotransferase; BUN, blood urea nitrogen; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; $GdCl_3$, gadolinium chloride; CPA, cyclophosphamide; LSEC, liver sinusoidal endothelial cells; MARCO, macrophage receptor with collagenous structure.

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Germany). The average size of the silica particles was determined to be 75.7, 311 and 830 nm by Zetasizer (Sysmex Co., Kobe, Japan). The particles were spherical and nonporous. The particles were stocked at 25 mg/ml (70 nm) and 50 mg/ml (300 and 1000 nm) in aqueous suspension. The stock solutions were suspended using vortex mixer for 5 min before use. The resultant solutions did not show aggregation of the particles by electron microscopy analysis. Reagents used in this study were of research grade.

2.2. Animals

BALB/c male mice (8 wk) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), and were housed in an environmentally controlled room at 23 ± 1.5 °C with a 12-h light/12-h dark cycle. Mice had free access to water and commercial chow (Type MF, Oriental Yeast, Tokyo, Japan). Mice were intravenously injected with the silica particles at 10–100 mg/kg body weight. The experimental protocols conformed to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

2.3. Histological analysis

The liver, kidney, spleen and lung were removed and fixed with 4% paraformaldehyde. After sectioning, thin tissue sections of tissues were stained with hematoxylin and eosin for histological observation. Liver sections were stained with Azan-Mallory for observation of liver fibrosis.

2.4. Biochemical assay

Serum alanine aminotransferase (ALT) levels and blood urea nitrogen (BUN) were measured using a commercially available Transaminase-CII kit and Blood Urea Nitrogen-B Test Wako (WAKO Pure Chemical, Osaka, Japan), respectively. Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured with an ELISA kit (BioSource International, CA, USA).

2.5. Gadolinium chloride assay

For Kupffer cell blockage of phagocytosis and partial depletion in the liver, mice were injected intravenously with gadolinium chloride ($GdCl_3$) at 10 mg/kg body weight at 30 and 6 h prior to intravenous administration of nanoparticles [12,13]. Blood was then recovered 24 h after injection of nanoparticles for ALT assay.

2.6. Cyclophosphamide assay

Disruption of liver sinusoidal endothelial cells was carried out by intraperitoneal injection of 300 mg/kg body weight cyclophosphamide (CPA) at 24 h prior to administration of nanoparticles [14,15]. Blood was recovered at 24 h after injection of nanoparticles for ALT assay.

2.7. Hepatic hydroxyproline content

Hepatic hydroxyproline content was assayed by Kivirikko's method, with some modification [16]. Briefly, liver tissue was hydrolyzed in 6 M HCl at 110 °C for 24 h in a glass tube. After centrifugation, the resultant supernatant was neutralized with 8 N KOH, and 2 g of KCl and 1 ml of 0.5 M borate buffer were then added, followed by incubation for 15 min at room temperature and further incubation for 15 min at 0 °C. Chloramine-T solution was then prepared and added. After additional incubation for 1 h at 0 °C, 2 ml of 3.6 M sodium thiosulfate was added, followed by incubation at 120 °C for 30 min. Next, 3 ml of toluene was added with incubation for a further 20 min at room temperature. After centrifugation, 2 ml of the resultant supernatant was added to Ehrlich's reagent, followed by incubation for 30 min at room temperature. Subsequently, the absorbance was measured at 560 nm.

2.8. Statistical analysis

Statistical analysis was performed by two-way ANOVA, followed by Student's *t*-test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Liver injury by 70-nm silica nanoparticles

We initially investigated the acute toxicity of silica particles with diameters of 70 (SP70), 300 (SP300) or 1000 nm (SP1000) at maximal dose of 100 mg/kg. Intravenous injection of SP70 at 50 and 100 mg/kg was often lethal, but mice injected with SP300 and SP1000 survived. Fig. 1 shows hematoxylin–eosin staining of the liver, spleen, lung and kidney in silica particle-injected mice. We found no toxicity in any of these organs in SP300 or SP1000-injected mice at 100 mg/kg, and we found no abnormalities in the spleen, kidney and lung in SP70-injected mice at 30 mg/kg (Fig. 1A–D). However, degenerative necrosis of hepatocytes in the liver was observed in SP70-injected mice, thus suggesting that SP70 is toxic to the liver (Fig. 1A).

Next, in order to confirm the hepatotoxicity of SP70, we examined serum ALT activity, a biochemical marker of liver injury. Consistent with the histological data, injection of SP70 elevated serum ALT levels 35-fold over control values at 30 mg/kg, but injection of SP300 or SP1000 had no effect even at 100 mg/kg (Fig. 2A). Elevation of blood urea nitrogen, a biochemical marker of kidney injury, was not observed (Fig. 2B). Serum levels of inflammatory cytokine IL-6 and TNF-α were markedly elevated to 1124 and 80 pg/ml, respectively, in SP70-treated mice at 3 h (Fig. 2B and C). Slight elevation of serum IL-6 levels was observed in SP300- and SP1000-injected mice (28 and 32 pg/ml, respectively), while no elevation of TNF-α was observed. The IL-6 levels seen in SP300- or SP1000-treated mice were insufficient for liver injury. To investigate dose dependency of SP70-induced liver injury, we also investigated serum ALT and inflammatory cytokine levels at 12 h after SP70 administration. ALT, IL-6 and TNF-α levels were elevated in a dose-dependent manner after SP70 injection, and significant increases were observed with doses as low as 20 mg/kg (Fig. 3A–C). Taken together, these data suggest that 70-nm silica particles are toxic to the liver.

3.2. Involvement of Kupffer cells in SP70-induced liver injury

Kupffer cells are large liver macrophages, and are localized within the liver sinusoidal cells. Kupffer cells play a role in defense against various particles and substances entering the liver through the portal circulation [17]. Indeed, Kupffer cells clear virus particles from the bloodstream by phagocytosis [18–20]. The phagocytosis of parasites by Kupffer cells is accompanied by the release of pro-inflammatory cytokines that act as a paracrine signal to neighboring hepatocytes, and induce chemotaxis and aggregation of neutrophils. $GdCl_3$ inhibits phagocytosis by Kupffer cells and transiently eliminates Kupffer cells [12], and $GdCl_3$ has thus been widely used to investigate the roles of Kupffer cells in the liver [21,22]. To investigate the involvement of Kupffer cells in particle-induced liver injury, we evaluated the effects of $GdCl_3$ on nanoparticle-induced liver injury. As shown in Fig. 4A, pre-injection of $GdCl_3$ prior to injection of SP70 elevated serum ALT levels 5.5-fold

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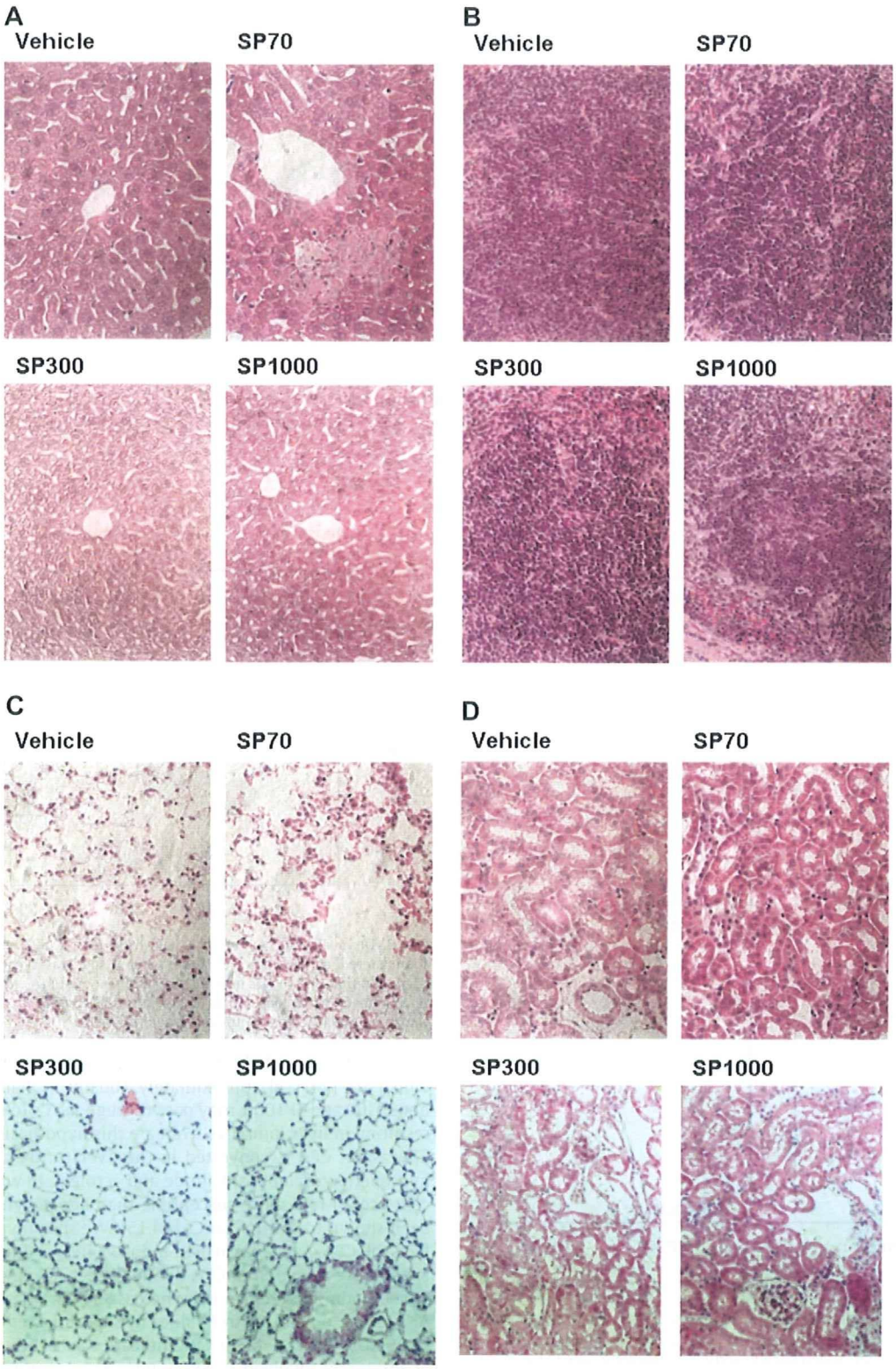

Fig. 1. Histological analysis of tissues in silica particle-treated mice. Silica particles with diameters of 70 (SP70), 300 (SP300) or 1000 nm (SP1000) were intravenously administered to mice at 30, 100 and 100 mg/kg, respectively. At 24 h after administration, tissues of liver (A), spleen (B), lung (C) and kidney (D) were collected, and fixed with 4% paraformaldehyde. Tissue sections were stained with hematoxylin and eosin and observed under a microscope. Data are representative of at least four mice.

in the SP70-injected group. In contrast, pre-injection of $GdCl_3$ did not affect ALT levels in the SP300- or SP1000-administered group. Taken together, these results indicate that phagocytosis of SP70 by Kupffer cells may attenuate liver injury, but the release of proinflammatory cytokines from Kupffer cells is not associated with SP70-induced liver injury.

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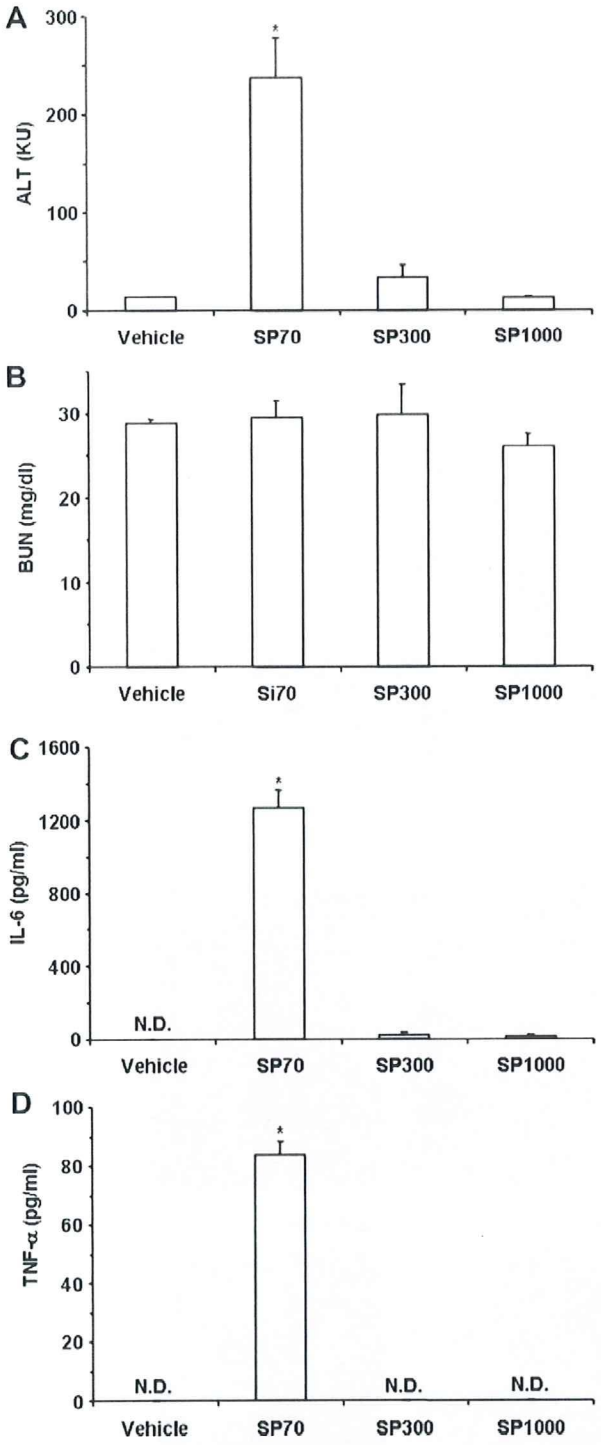

Fig. 2. Biochemical analyses of liver injury in silica particle-injected mice. SP70, SP300 or SP1000 was intravenously injected to mice at 30, 100 or 100 mg/kg, respectively. Blood was recovered at 3 and 24 h of the injection. Serum ALT (A) and BUN (B) at 24 h and IL-6 (C) and TNF-α (D) levels at 3 h were measured using a commercially available kit, as described in Section 2. Data are means ± SEM ($n = 4$). *Significant difference vs. vehicle-treated group ($p < 0.05$).

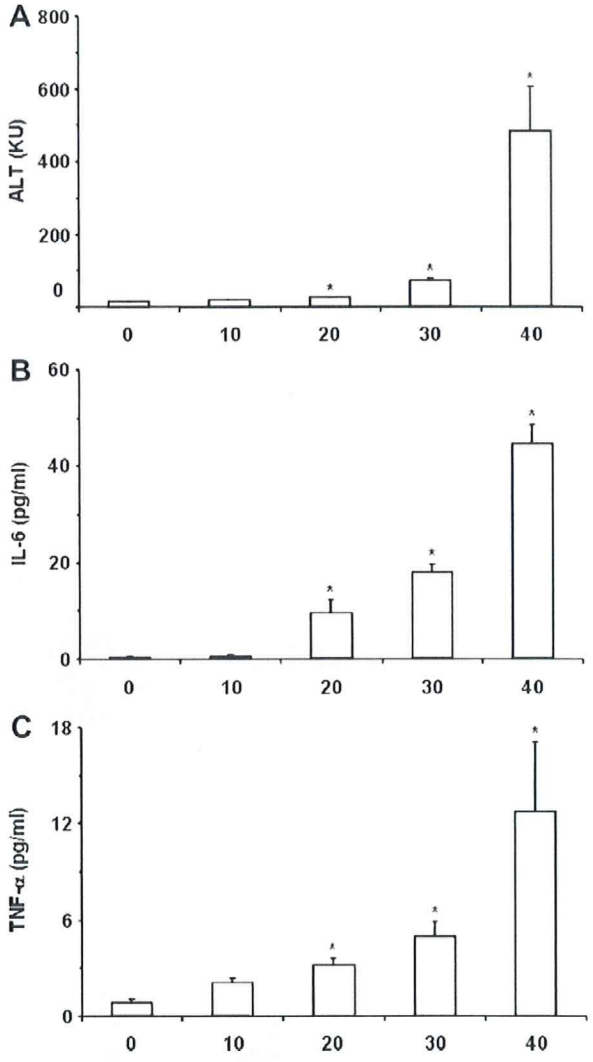

Fig. 3. Dose dependency of SP70 on liver injury. SP70 were intravenously administered at the indicated doses, and blood was recovered at 12 h after administration. Serum was used for measurement of ALT (A), IL-6 (B) and TNF-α (C), as described in Section 2. Data are means ± SEM ($n = 4$). *Significant difference compared with the vehicle-treated group ($p < 0.05$).

3.3. Involvement of liver sinusoidal endothelial cells in SP70-induced liver injury

Sinusoidal endothelium forms a barrier between the bloodstream and hepatocytes, preventing passage of particles. Liver sinusoidal endothelial cells (LSECs) are perforated by fenestrations, which are pores of approximately 100 nm in diameter. SP70, but not SP300 and SP1000, may pass through LSECs to the hepatocytes, resulting in liver injury. To evaluate this hypothesis, we performed CPA assay. CPA is converted in the liver to toxic metabolites, 4-hydroperoxycyclophosphamide and acrolein, to which endothelial cells are 20-fold more susceptible than hepatocytes [14]. CPA has been shown to disrupt LSECs [14,15]. We thus investigated the effects of CPA on nanoparticle-induced liver injury. As shown in Fig. 4B, pre-injection of CPA did not affect ALT levels in SP300- or SP1000-administered mice, whereas CPA dramatically decreased ALT levels to near control values in SP70-injected mice (from 235 to 29 KU). These data on CPA indicate that LSECs may be directly or indirectly involved in SP70-induced liver injury, but may not be a barrier against SP70.

3.4. Chronic toxicity of SP70

Finally, we investigated the effects of SP70 on chronic liver injury. SP70 was injected into mice every 3 days for 4 weeks at 10 or 30 mg/kg. The lower dose (10 mg/kg) did not cause acute liver

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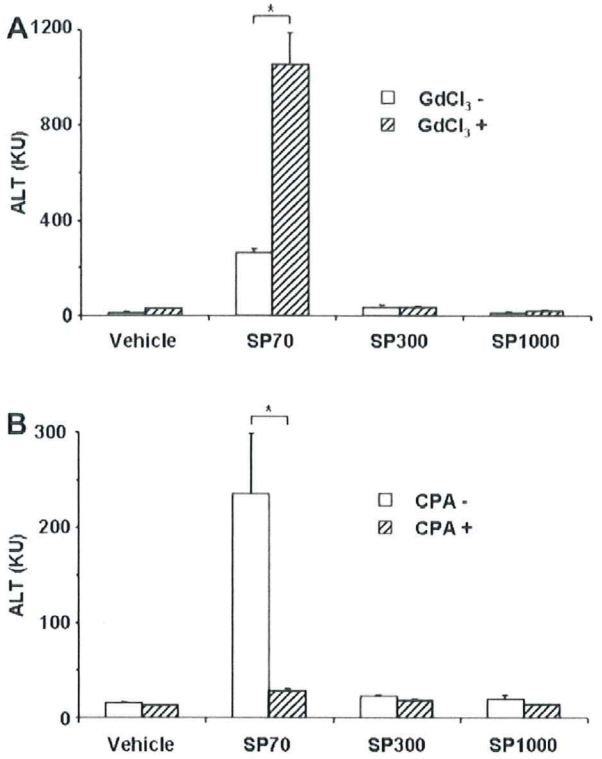

Fig. 4. Pharmaceutical analysis of SP70-induced liver injury. (A) $GdCl_3$ assay. Vehicle or $GdCl_3$ (10 mg/kg) was intravenously injected into mice at 30 h or 6 h prior to treatment with silica particles (SP70, 30 mg/kg; SP300, 100 mg/kg; SP1000, 100 mg/kg). At 24 h after particle administration, blood was recovered, and the resultant serum was used for ALT assay. Data are means ± SEM ($n = 4$). *Significant difference between vehicle- and silica particle-treated groups ($p < 0.05$). (B) CPA assay. Vehicle or CPA (300 mg/kg) was intraperitoneally injected to mice at 24 h prior to treatment with silica particles. At 24 h after administration of particles, blood was recovered, and the resultant serum was used for ALT assay. Data are means ± SEM ($n = 4$). *Significant difference between vehicle- and silica particle-treated groups ($p < 0.05$).

failure (Fig. 3A). Histological analysis demonstrated that chronic exposure of SP70-induced denaturation of hepatocytes in a dose-dependent manner (Fig. 5A). Serum ALT levels were also elevated by SP70 administration (Vehicle, 14.3 KU; SP70, 24.8 and 42.1 KU at 10 and 30 mg/kg, respectively) (Fig. 5B). Liver fibrosis is a symptom of chronic liver injury, and thus, we investigated liver fibrosis. Collagen, which is accumulated in the fibrotic liver, was stained with Azan reagent, and blue-stained regions were observed in SP70-treated, but not vehicle-treated, liver sections (Fig. 5C). Elevated hydroxyproline content parallels the extent of fibrosis, and we investigated the hydroxyproline contents in the SP70-treated mouse liver. Injection of SP70 significantly elevated hepatic hydroxyproline contents 1.6- and 3.5-fold over control values, at 10 mg/kg and 30 mg/kg, respectively (Fig. 5D). These data indicate that chronic administration of SP70 causes liver fibrosis, even at doses that are non-toxic in a single injection.

4. Discussion

In the present study, we evaluated the acute toxicity of silica particles with a diameter of 70, 300 or 1000 nm, and we found that 70-nm silica particles injure the liver, but not the spleen, lung or kidney. Moreover, chronic administration of 70-nm silica particles caused liver fibrosis, even at doses that were non-toxic in a single injection.

Surface area is a critical factor for toxicity of nano-size particles in the liver. The numbers of particles of SP70, 300 and 1000 are 2.8×10^{12}, 3.5×10^{10} and 9.5×10^{8} particles/mg, respectively.

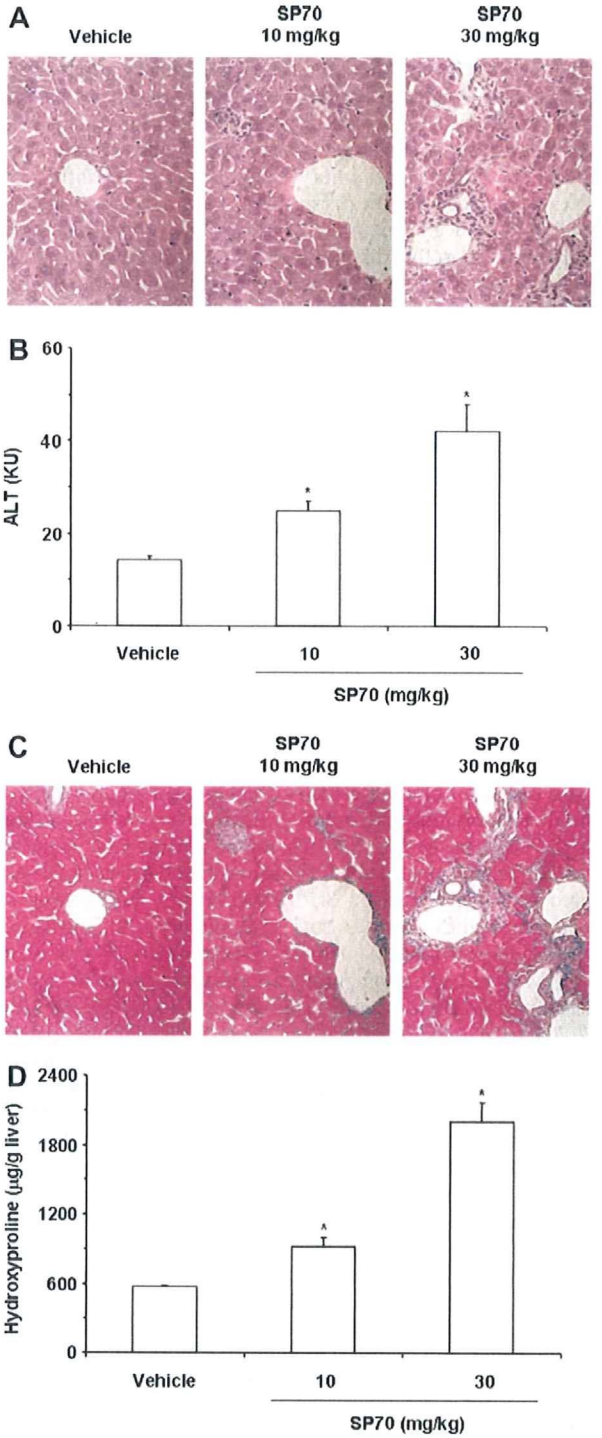

Fig. 5. Effect of SP70 on chronic liver injury. Mice were subjected to repeated administration of SP70 (10 or 30 mg/kg) every 3 days for 4 weeks. At 3 days after the last administration, mice were sacrificed. Tissues of livers were fixed with 4% paraformaldehyde, and liver sections were then stained with hematoxylin and eosin (A) or Azan (C). Hydroxyproline levels in the liver were assayed as described in Section 2 (C). Serum samples were used for measurement of ALT (B). (A and C) Data are representative of at least eight mice. (B) Data are means ± SEM ($n = 4$). *Significant difference vs. vehicle-treated group ($p < 0.05$).

The surface area of SP300 at 100 mg/kg, at which SP300 was not toxic, is similar to that of SP70 at 30 mg/kg, at which SP70 was toxic. Difference in the surface area may not affect the different toxicities in the liver between SP70 and 300.

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There are highly specialized endothelial cells, LSECs, in the liver, and these separate sinusoidal blood from hepatocytes. Passage of particles through LSECs is the first step for translocation from the bloodstream to hepatocytes. LSECs have fenestrations with a diameter of 100 nm, and the liver injury seen with 70-nm silica particles may be due to the particle size. We investigated the role of LSECs in the particle-induced liver injury using CPA, a disruptor of LSECs [13–15]. Unexpectedly, the disruption of LSEC did not cause SP300- and SP1000-induced liver injury. These results were consistent with the previous report that disruption of LSECs by CPA did not affect the hepatocyte transduction of a lentivirus vector with a diameter of 120–200 nm larger than the fenestrations of LSECs [13]. In contrast, SP70-induced liver injury was dramatically suppressed by disruption of LSECs, and pores in the LSEC may be responsible for the hepatic toxicity of SP70. Spaces, called the space of Disse, exist between LSEC and hepatocytes [23]. Particles entering into these spaces can avoid efflux into the blood flow in the sinusoids of the liver, resulting that this may enhance interaction between the particles and hepatocytes. Thus, the Disse spaces between LSECs and hepatocytes may be responsible for the liver injury caused by SP70.

Resident macrophages in the liver, Kupffer cells play a pivotal role in defense against foreign particles by eliminating such particles via phagocytosis [17]. $GdCl_3$ has been widely used to block phagocytosis by Kupffer cells and to deplete Kupffer cells [12,13,20,21]. Inactivation of Kupffer cells had no effect on SP300 and SP1000 treatment, whereas pre-treatment with $GdCl_3$ led to increased liver injury by SP70. There is no evidence that $GdCl_3$ exerts any direct toxic effects on hepatocytes, LSECs, or on other cells in the liver [24]. Thus, the elevation of SP70 toxicity may be caused by the depletion of Kupffer cells. Depletion of Kupffer cells enhanced the transgene activity of adenovirus vectors with a similar size with SP70 in the liver [22]. Therefore, inhibition of phagocytosis of Kupffer cells may enhance the interaction between SP70 and hepatocytes by increase in SP70 moving into the Disse spaces. Inhalation of silica particles causes lung injury [25], and alveolar macrophages function as a defense against inhaled agents, including viruses and environmental particles, via phagocytosis [26]. Macrophage receptor with collagenous structure (MARCO), CD204 and CD36 are reported to be the receptors for inert particles [26–30]. Uptake of silica particles through MARCO or CD204 induces cytotoxicity in alveolar macrophages, leading to lung fibrosis [30,31]. Alveolar macrophages from BALB/c do not express MARCO and CD204, and silica particles are taken up through CD36 [30]. Thus, uptake of SP70 by Kupffer cells through CD36 might not trigger liver injury. In this study, we found that chronic administration of SP70 caused liver fibrosis, even at 10 mg/kg body weight, at which level acute liver injury was not observed after a single injection. Nano-size particles-induced continuous inflammation in the liver will cause liver fibrosis leading to hepatic cancer.

Further evaluation of relationship between toxicity and variety of sizes, shapes, and chemical modification on the surface of particles is needed, and the future studies based on these data will provide very useful information on future development of drug delivery system using nano-size materials.

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Influence of 70 nm silica particles in mice with cisplatin or paraquat-induced toxicity

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In the pharmaceutical industry, nano-size materials are designed as drug carriers and diagnosis probes. Interactions between nano-size materials and chemicals need investigating. Here, we investigated whether nano-size materials affect chemical-induced toxicity using silica particles, which have been widely used in cosmetics and drug delivery and have diameters of 70 (SP70), 300 (SP300) and 1000 (SP1000) nm, a popular anti-tumor agent, cisplatin, and a widely used herbicide, paraquat. Mice were treated with either cisplatin (100 μmol/kg, intraperitoneally) or paraquat (50 mg/kg, intraperitoneally), with or without intravenous silica particle administration. All treatments were non-lethal and did not show severe toxicity, except for injection with both cisplatin and SP70, which were lethal. When mice received with paraquat and/or the silica particles, synergistic enhanced toxicity was observed in both paraquat- and SP70-treated mice. These synergic effects were not observed with either Si300 or 1000 treatment. Our findings suggest that further evaluation on the interaction between nano-size materials and chemicals is critical for the pharmaceutical application of nanotechnology.

1. Introduction

Nano-size materials are typically defined as engineered structures having at least one dimension of 100 nm or less. Changing from micro- to nano-size in materials expands surface area. In addition, nano-sized materials may have unique physicochemical properties due to their small size, chemical composition, surface structure, solubility, and shape. Recent development of nano-size particles from the micro- to nano-scale provides us with new tools, not only for industrial use such as electronics and catalysts, but also for pharmaceutical use such as cosmetics, diagnostic imaging and drug delivery (Caruthers et al. 2007; Vallet-Regi et al. 2007; Bartlett et al. 2007; Medarova et al. 2007). Although an expanded surface area of nano-size materials is advantageous, wide surface area can be accompanied by increasing interactions with biological tissues, cells, proteins, and nucleic acids, leading to toxic effects on humans (Nel et al. 2006; Oberdorster et al. 2005; Fischer and Chan, 2007). It is rare for a human to be exposed to only nano-size materials; we are often exposed to nano-size materials as well as other substances, such as xenobiotics and pharmaceutical agents. Nano-silica particles are intended for cosmetics and systemic and local delivery of drugs (Vallet-Regi et al. 2007). Previously, we found that intravenous administration of 70 nm, but not 300- and 1000 nm, silica particles caused liver injury (Nishimori et al. in press). Taken together, the synergistic effect of nano-size materials with other toxic substances should be evaluated, as there are few studies to date.

In this study, we investigated the synergistic effect of 70 nm silica particles with chemicals using cisplatin, a widely used anti-tumor agent (Ozols and Young 1991; Hartmann et al. 1999; Witjes 1997), and paraquat, one of the most widely used and highly toxic herbicides (Vandenbogaerde et al. 1984), providing evidence for synergistically enhanced toxicity.

2. Investigations and results

We previously found that intravenous administration of 70 nm size silica particles (SP70) caused liver failure, but 300- (SP300) and 1000- (SP1000) nm size particles did not (Nishimori et al. in press). Here, we investigated whether interaction between chemicals and silica particles occurred. To avoid direct interactions between chemicals and silica-particles before administration and absorption, we injected chemicals and silica-particles intraperitoneally and intravenously, respectively. Administration of cisplatin has been shown to cause adverse effects such as hepatic and renal failure (Lu and Cederbaum 2006; Ramesh et al. 2007). Indeed, serum levels of biochemical markers for hepatic and renal injury were elevated by cisplatin as shown in Fig. 1A and B, respectively. Co-treatment with cisplatin and SP300/1000 did not exhibit severe toxicity, whereas co-administration with cisplatin and SP70 showed lethal toxicity. Although SP70 did not show hepatic toxicity at 20 mg/kg, co-administration of SP70 with cisplatin caused the death of 5 of the 8 mice (Fig. 1). The surviving

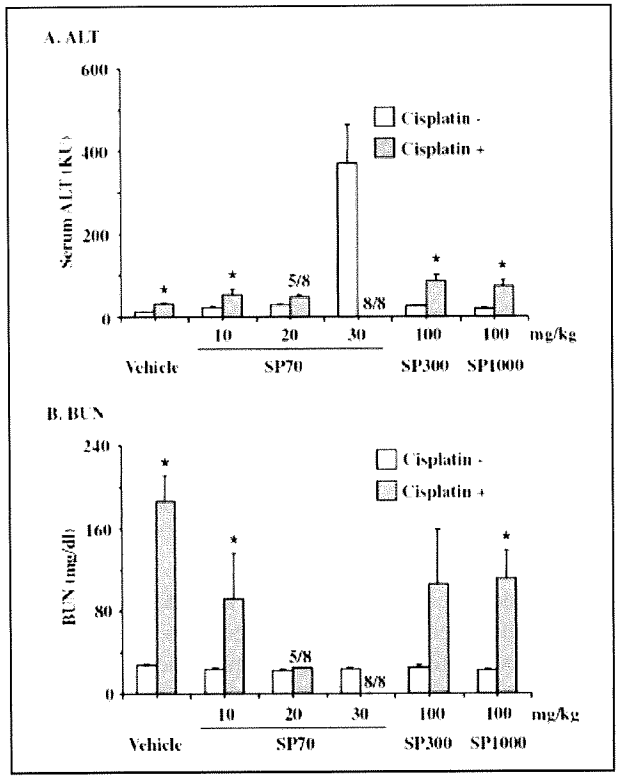

Fig. 1: Effect of SP70 on cisplatin-induced toxicity
Mice were injected with cisplatin at 0 (open column) or 100 μmol/kg (filled column) and each silica particle (SP70, 70 nm particles; SP300, 300 nm particles; SP1000, 1000 nm particles) at the indicated dose, intraperitoneally and intravenously, respectively. At 24 h post-injection, the serum was recovered. ALT (A) and BUN (B) levels were assayed as described in the Materials and methods. Five of 8 and 8 of 8 mice died in the 20 mg/kg SP70/cisplatin and 30 mg/kg SP70/cisplatin-injected group, respectively. Data are representative of three independent experiments. Data are mean ± SEM (n = 4–12). *Significant difference between vehicle and cisplatin-treated group ($p < 0.05$)

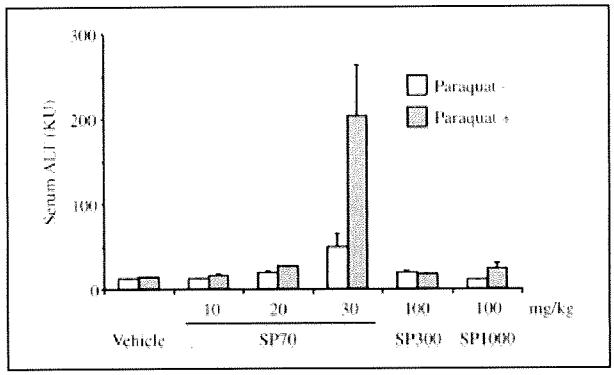

Fig. 2: Effect of SP70 on paraquat-induced toxicity
Mice were injected with paraquat at 0 (open column) or 50 mg/kg (filled column) and silica particles (SP70, SP300 or SP1000) at the indicated dose, intraperitoneally and intravenously, respectively. At 24 h post-injection, the serum was recovered. ALT levels were assayed as described in the Materials and methods. Data are representative of two independent experiments. Data are the mean ± SEM (n = 4)

mice did not show abnormal ALT and BUN levels. SP70 did not show a lethal effect on mice at 30 mg/kg, but all mice injected with SP70 (30 mg/kg) died. We next investigated the interaction between paraquat and silica particles. Co-administration of paraquat (50 mg/kg) and silica particles did not elevate serum ALT, and SP70 showed synergistic elevation of serum ALT levels from 48.9 to 203.4 KU (Fig. 2). Synergistic effects of paraquat on SP300 or 1000 were not observed.

3. Discussion

In this study, we investigated the combined effects of chemicals on nano-size particle-induced toxicity, and found cisplatin and paraquat had synergistic toxic effects with silica particles with a diameter of 70 nm.

One characteristic of nano-size materials is their large surface area, and one explanation for the differing synergistic effects of nano- and macro-size particles is this difference in surface area. Indeed surface areas of SP300 and 1000 are 0.229 and 0.0068 per gram of particles relative to that of SP70, respectively. Lethality and ALT levels in cisplatin and paraquat, respectively, were observed with the injection of SP70 at 20 mg/kg, but not SP300 at 100 mg/kg, at which point the surface area of SP300 is almost equal to that of SP70. Therefore, the influence surface area has on the additive toxicity may be negligible. We previously found that differing hepatotoxicity among SP70, 300 and 1000 might be due to the accumulation of SP70 in the Disse space between liver sinusoidal endothelial cells and hepatocytes (Nishimori et al. in press). Differences in silica particle dynamics may be responsible for the synergistic effects of SP70. The profile of absorbed proteins on silica particles differed with particle size, and the amount of absorbed proteins was dependent on surface area; i.e., smaller particles absorbed more proteins on the surface (Dutta et al. 2007). The most abundant serum protein is albumin, and chemicals absorbed into the systemic circulation are often absorbed onto albumin. The structurally altered albumin is rapidly cleared from the circulation by a scavenger receptor (Demoy et al. 1999; Jansen et al. 1991; Kamps et al. 1997). The albumin-chemical complexes may be absorbed onto SP70, resulting in the aggregation of chemicals onto SP70 particles through albumin. Lipid-coated aggregation of cisplatin increased the cytotoxicity to 1000-fold compared with free drugs (Burger et al. 2002). The aggregated particles containing high dose of chemicals might be taken up, leading to enhanced toxicity. We will perform further biochemical and comprehensive analyses, such as proteome and genome assays, to determine the mechanism of these synergistic effects.

This report indicates synergistic toxicity of a nano-size silica particle with chemical agents. Further evaluation of such interactions between nano-size materials and pharmaceutical agents for future pharmaceutical application of nanotechnology are necessary.

4. Experimental

4.1. Materials

Silica particles with a diameter of 70, 300, or 1000 nm were obtained from Micromod Partikeltechnologie GmnH (Rostock, Germany). The size distribution of the particles was analyzed by a Zetasizer (Sysmex Co., Kobe, Japan), and mean diameters were 55.7, 296, and 989 nm, respectively. The particles were spherical and nonporous, and stored at 25 mg/ml (70 nm) and 50 mg/ml (300 and 1000 nm) in aqueous suspension. The suspensions were thoroughly dispersed with sonication before use and diluted in water. An equal volume of solution was injected in each treatment. Paraquat and cisplatin were dissolved in saline and stored at −20 °C before use. All reagents used were of research grade.

4.2. Animals

The 8-week-old BALB/c male mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). They were maintained in controlled

environment (temperature: 23 ± 1.5 °C; light: 12 h light/dark cycle) with free access to standard rodent chow and water. The mice were given 1 week to adapt before commencing. The experimental protocols conformed to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

4.3. Biochemical analysis

Serum alanine aminotransferase (ALT) and blood urea nitrogen (BUN) were measured using commercially available kits according to the manufacturer's protocols (WAKO Pure Chemical, Osaka, Japan).

4.4. Statistical analysis

Statistical analysis was performed by Student's t-test. $P < 0.05$ considered statistically significant.

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—Reviews—

トキシコキネティクス／トキシコプロテオーム解析による ナノマテリアルの生物学的影響評価

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Evaluation of Biological Influence of Nano-materials using Toxicokinetic and Toxicoproteomic Approach

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The recent development of nanotechnology has facilitated a dramatic reduction in the size of materials. Nanomaterials are nanometer-sized materials with specific physicochemical properties that are different from those of the bulk material of the same composition. Such properties make them very attractive for cosmetic and medical applications. However, nanoparticles can act on living cells or bodies at the nano-level resulting in biologically undesirable as well as desirable effects. Thus, reduction in particle size from the micro- to nano-scale not only provides benefits to diverse scientific fields but also poses potential risks to the environment and to human health. Although significant resources are aimed at exploiting the desirable properties of nanoparticles for applications in medicine or cosmetics there are only limited attempts to evaluate potentially undesirable effects *in vivo*. Thus, there is a pressing need for a careful consideration of the benefits and side effects to the use of nanoparticles in medicine and cosmetics. In recent years, the majority of toxic biological response induced by nanomaterials (Nanotoxicity) has focused on cell culture systems. However, data from these studies will require verification from *in vivo* experiments using animals. An understanding of Toxicokinetics (the relationship between the physical properties of the nanomaterials and their *in vivo* behavior) would provide a basis for evaluating undesirable effects. Moreover, toxicoproteomics may identify predictive bio-markers for examining nanotoxicity. In this review article, we describe the assumptions and challenges in the field of nanotoxicity and describe advances for studying nanotoxicity of nanosilicas using toxicokinetics/toxicoproteomics both *in vivo* and *in vitro*.

Key words——nanomaterial; nanosilica; toxicokinetics; toxicoproteomics

1. はじめに

ナノテクノロジーは，物質をnm（ナノメートル）レベルで制御することにより，その物質の機能や特性を飛躍的に向上させることを目的とした超微細加工技術であり，近年，広範な産業技術分野に革新的発展をもたらし得るキーテクノロジーとして脚光を浴びている．ナノテクノロジーを駆使して創製される新素材（ナノマテリアル）は，医療・香粧品開発分野においても技術革新をもたらす素材として注目され，現在，産官学連携の下でナノテクノロジーを活用した新規医薬／香粧品用素材の開発及び新用途探索が精力的に推進されている．医療分野では，既にナノスケールの酸化鉄粒子（Super Paramagnetic Iron Oxide; SPIO）や半導体ナノ粒子（Quantum Dot; QD）などを活用したイメージング診断技術の進展が目覚ましく，[1-5] また，われわれの研究グループを含めた数多くの研究者によって各種ナノマテリアルを駆使したドラッグデリバリーシステムの開発なども精力的に進められている．[6-9] さらに，

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化粧品に含まれる酸化チタンや酸化亜鉛，シリカといった無機顔料は，粒子の大きさが小さいほど機能性（透明感・使用性・紫外線防御能）が高まることが経験的に知られており，1960–70年代からナノサイズの無機顔料が既に化粧品に配合されてきた．このように，特に医薬・香粧品領域ではナノテクノロジーの急速な進歩に伴ってナノマテリアル含有製品の開発に一層拍車が掛かっている．[10,11] その一方で，ナノマテリアルの生産増加においては他のすべての化学物質と同様に，生体影響を考慮する必要性が徐々に表面化しつつある．[12] しかしながら，こういったナノマテリアルの生体影響／毒性解析研究は途に就いたばかりであり，ナノマテリアルの人体に対する有害性に関する情報収集は世界的にみても圧倒的に立ち遅れているのが現状である．ナノ顔料配合化粧品が既に実用化・販売されていることに加えて，国家レベルでナノマテリアルの産業応用が推進され，ナノマテリアルへの人体曝露を避け得ない状況にある現代では，特に人体に対するナノマテリアルの生物学的影響に関する評価及び情報収集と，それらの情報を基盤としたナノマテリアルのリスク予測法の開発が急務となっている．

ナノマテリアルとは，そもそもどのような“構造・性質”を持つ素材として定義されているのであろうか？　種々の機関・報告で一定した見解が得られておらず，国際的に同意の得られた定義が存在していないのが現状であるが，少なくとも構造の面では“1つの次元の大きさが1–100 nmの素材”として幅広く定義することが多い．すなわち，一次元がナノスケール（他の二次元へ広がりを有する）の材料は薄膜・塗膜，二次元がナノスケール（残る一次元へ広がりを有する）の材料はナノワイヤやナノチューブ，三次元がナノスケールの材料はナノ粒子のことを指す．場合によっては，ナノ粒子を使用（投与）した時点で構成分子に分解する不安定なナノ粒子（リポソームやマイクロ・ナノ乳液）と，不溶性ナノ粒子（酸化チタン，フラーレン，QD）とに分類することや，定義上はナノマテリアルではないものの，ナノ粒子の“凝集体や塊”を互いにナノマテリアルがつながったナノ構造材料として定義することもある．一方で，性質の面では一次元以上の外形寸法あるいは内部構造がナノスケールであり，ナノ領域の寸法を有しない同じ組成の材料と比べて新規な性質を有する材料であると定義されることが多い．一般に，ナノサイズの物質は従来までの物質と比べて，1）電子状態，2）化学的性質（表面積の増大など），3）力学的性質，4）電気的性質，5）磁気的性質，6）光学的性質が変化する可能性が指摘されている．[12–14] 例えば，QDやSPIOはイメージング試薬として利用されているが，QDは粒子の大きさに応じて色調が変化すること，SPIOは超常磁性を発揮することなど，これらはナノサイズ特有の性質を利用した素材である．このように，ナノサイズのマテリアルは従来までのバルクサイズの素材にはない未知の特性を発揮する可能性を秘めており，こういったナノマテリアル特有の構造・性質が今までは想像もできなかった新たな医療／香粧品素材の創出や使用用途の開拓を期待させる所以である．しかしながら，逆にナノマテリアルのリスク評価の観点からみると，ナノマテリアルの個々の未知特性や凝集体・塊を形成した際の予測困難な性質変化は，リスク評価基準の作成（標準化）を妨げる主要因となっている．これらの背景から，国際標準化機構（ISO）においては，ナノマテリアルの国際標準化活動を促進するために技術委員会を設置するなど種々の取り組みを進めている．しかし，ナノマテリアルの構造・性質に関して詳細な情報が蓄積されていない現状において，むやみに標準化を推し進めることは，ナノマテリアルの危険性ばかりを浮き彫りにしてナノテクノロジーの社会からの拒絶を招く可能性があるため，その後の産業応用の阻害要因になり得ることも考慮すべきである．こういったナノマテリアルの物性／構造的な多様性は，ナノマテリアルの生物学的影響に単一の回答が存在しないことを意味しているかもしれない．しかし，産業界の未来を切り開く“光”となり得ると期待されているナノマテリアルを活用した豊かな社会を実現するためには，ナノマテリアルの安全性確保のためのリスク評

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