だけでは社会的混乱を招いてしまう。従って，今後，ナノマテリアルが社会から受容され，人類の豊かな暮らしに貢献するためには，適切なリスクマネジメントの実施によって安全性を確保した上で，そのメリットを最大限に享受することが重要である。本研究では，非晶質ナノシリカの皮膚吸収性・体内動態を明らかとした上で，粒子径と急性毒性・起炎性の連関を評価とした。さらに，最も重要なことであるが，適切な物性制御により起炎性を低減可能であることを明らかとした。すなわち，本研究は，単なるナノマテリアルの毒性研究（NanoTox研究）を目的としたものでなく，いかにして安全で安心，かつ有用なナノマテリアルを開発・実用化していくのかを視野にいれたナノマテリアルの安全科学研究（Nano-Safety Science研究）を実施したものである。今回は紙面の都合上，割愛させて頂いたが，我々は本項で紹介した検討に加えて，経肺／経口曝露時の体内吸収性・体内動態の解析や，脳神経／免疫／生殖発生学的なNano-Safety Science研究を推進しており，既に多くの興味深い知見を得つつある。今後は，ナノマテリアルの曝露実態の解明，定量的な体内動態評価や健康影響に及ぼす閾値追求など詳細な安全性評価を推進するとともに，安全かつ有効なナノマテリアルの開発・実用化支援をより強力に推進することが必要不可欠と考えられる。本稿では紙面の都合上，我々の知見の一例のみ紹介させて頂いたが，これらNano-Safety Science研究が，今後のより安全なナノマテリアルの創製・社会還元に繋がり，科学的根拠に基づいた情報発信・リスクコミュニケーションにより，リスクリテラシーが高まり，安全で安心な社会の構築などに貢献し得るものと期待している。

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Cytotoxicity of amorphous silica particles against macrophage-like THP-1 cells depends on particle-size and surface properties

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Recent studies have indicated that amorphous silica particles (SPs) show cytotoxicity against various types of cells, including macrophages. However, the mechanism of cell death has not been determined, and systematic investigations of the relationship between particle characteristics and cytotoxicity are still quite limited. Here, we compared the cytotoxicity of SPs of various sizes (30–1000 nm) and surface properties against differentiated THP-1 human macrophage-like cells. We found that 300 and 1000 nm SPs showed cytotoxicity against THP-1 cells, whereas 30, 50, and 70 nm SPs did not induce cell death. We demonstrated that 1000 nm SP showed strong cytotoxicity that depended on reactive oxygen species but was independent of caspases. Furthermore, we showed that surface modification of 1000 nm SPs dramatically suppressed their cytotoxicity. Our results suggest that systematic evaluation of the association between particle characteristics and biological effects is necessary for the creation of safe SPs.

1. Introduction

Amorphous (noncrystalline) silica particles (SPs) possess useful properties, including straightforward synthesis, relatively low cost, easy separation, high hydrophilicity, and facile surface modification. In addition, SPs are usually considered to have low toxicity, in contrast to crystalline silica, which can cause silicosis and some forms of lung cancer (Mossman and Churg 1998; Huaux 2007). Therefore, SPs have been used for many applications, including cosmetics, foods, medical diagnosis, cancer therapy, and drug delivery (Hirsch et al. 2003; Bharali et al. 2005; Roy et al. 2005; Bottini et al. 2007; Verraedt et al. 2009).

However, the increasing use of SPs has raised public concern about their safety. In fact, recent studies have found that SPs induce substantial lung inflammation and are cytotoxic against various cells, including macrophages (Wiethoff et al. 2003; Cho et al. 2007; Napierska et al. 2009). Thus, the safety and overall biological effects of SPs have been questioned (Akerman et al. 2002; Kirchner et al. 2005; Dostert et al. 2008). In addition, it has recently become evident that particle characteristics, including particle size and surface properties, are important factors in pathologic alterations and cellular responses (Albrecht et al. 2004; He et al. 2008; Waters et al. 2009). Therefore, investigation of the mechanisms of SP-induced inflammation and cytotoxicity and of the relationship between particle characteristics and cytotoxicity is important for the development of safe SPs.

Here we demonstrate that SPs exhibit cytotoxicity against THP-1 human macrophage-like cells in a size-dependent man-

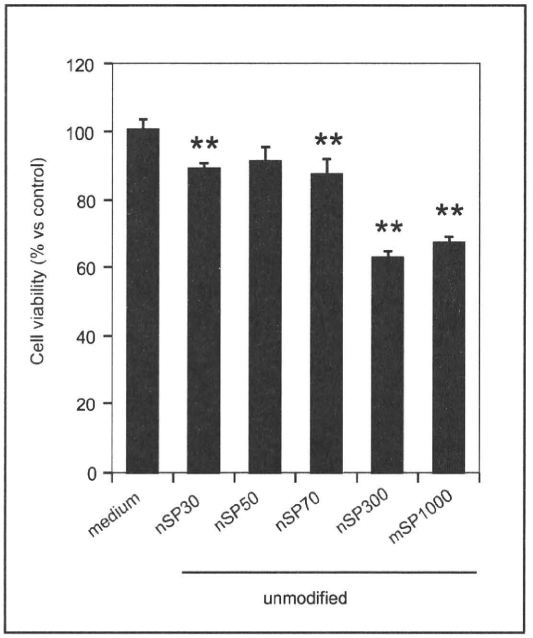

Fig. 1: Correlation between SP particle size and cytotoxicity against macrophage-like cells. PMA-primed THP-1 cells were treated with 100 μg/mL unmodified SPs for 24 h, and cell viability was evaluated by means of the standard methylene blue assay. The data represent the mean ± SD ($n = 5$; $^{**}P < 0.01$ versus value for medium control)

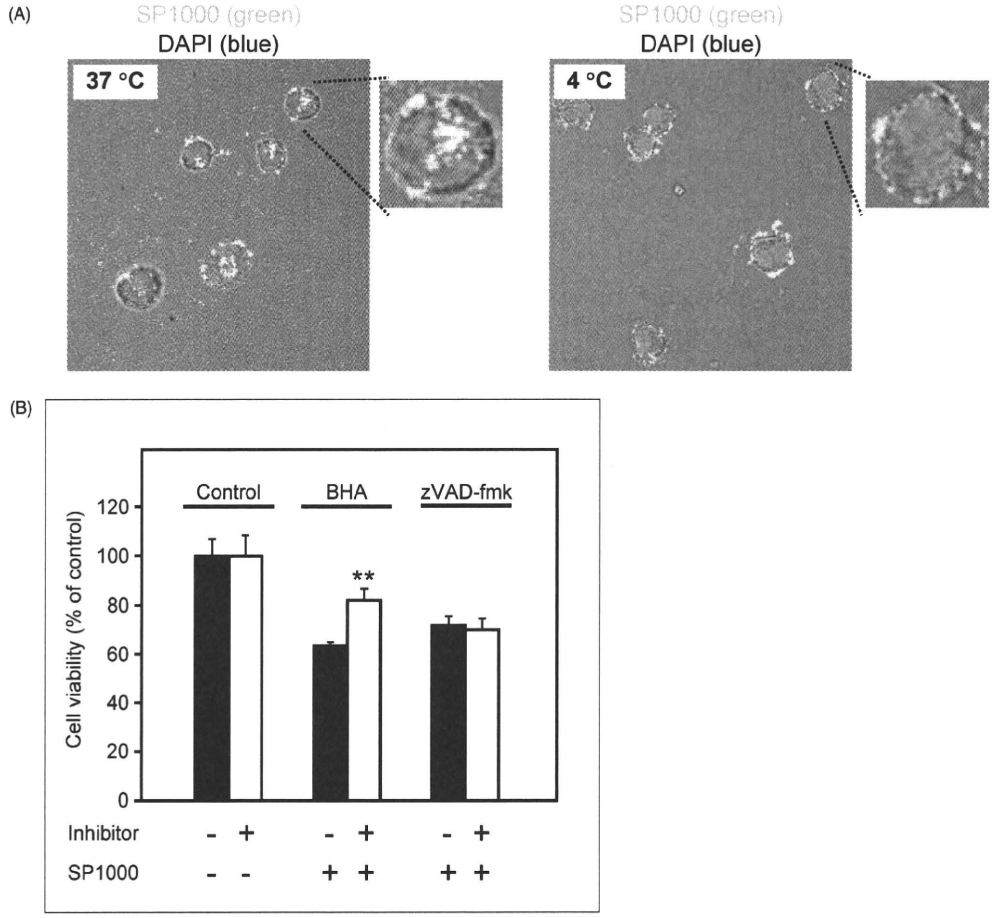

Fig. 2: ROS and caspase dependence of mSP1000-induced cell death. (A) Confocal microscopy images of the ingestion of mSP1000. FITC-conjugated mSP1000 (green) were added to the PMA-primed THP-1 cells at 100 μg/mL. Cells were incubated for 6 h at 37 °C (left) or 4 °C (right). The nucleus was stained with DAPI (blue). (B) Effect of a caspase inhibitor and an ROS scavenger on cytotoxicity of mSP1000. PMA-primed THP-1 cells were treated with 100 (g/mL SP1000s for 24 h in the presence or absence of BHA (150 μM) or zVAD-fmk (60 μM). Cell viability was measured by means of the methylene blue assay. The data represent the mean ± SD ($n = 4$; $^{**}P < 0.01$ versus value for inhibitor [-] control)

ner. Furthermore, we show that SPs with diameters of 1000 nm induce the production of reactive oxygen species (ROS), which triggers THP-1 cell death. We also demonstrate that surface modification of SPs with various functional groups significantly suppresses SP cytotoxicity.

2. Investigations, results and discussion

In this study, we examined whether the size and surface characteristics of SPs are correlated with their cytotoxicity. We also investigated the mechanism by which SPs induce the death of macrophage-like THP-1 cells, with the goal of providing information for the creation of novel safe SPs.

2.1. Amorphous silica particles induce cell death in a size-dependent manner

We used five SPs with diameters between 30 and 1000 nm (nSP30, nSP50, nSP70, nSP300, and mSP1000); the mean secondary particle diameters of the SPs measured by means of a Zetasizer were 33, 44, 79, 326, and 945 nm, respectively (data not shown). To compare the cytotoxicities of the SPs with different diameters, we examined their cytotoxicity against macrophages, which are the first line of defense against infection or injury from various inhaled agents. We incubated phorbol 12-myristate 13-acetate (PMA)-primed human macrophage-like THP-1 cells with SPs and analyzed the levels of cell viability. Twenty-four hours after the incubation, we found that nSP300 and mSP1000 induced marked cytotoxicity, whereas nSP30, nSP50, and nSP70 showed no cytotoxicity (Fig. 1). These results indicate that the particle size of the SPs was intimately involved in their biological effects.

2.2. mSP1000-induced cytotoxicity depends on ROS but not on caspases

Macrophages remove inhaled agents including foreign particles by means of their phagocytic activity. To confirm that THP-1 cells took up mSP1000, we treated THP-1 cells with fluorescein-5-isothiocyanate (FITC)-conjugated mSP1000 at 37 °C or 4 °C. We visually confirmed that mSP1000 were ingested into THP-1 cells at 37 °C, whereas only adsorption of mSP1000 on the cellular surface was detected at 4 °C (Fig. 2A). These results

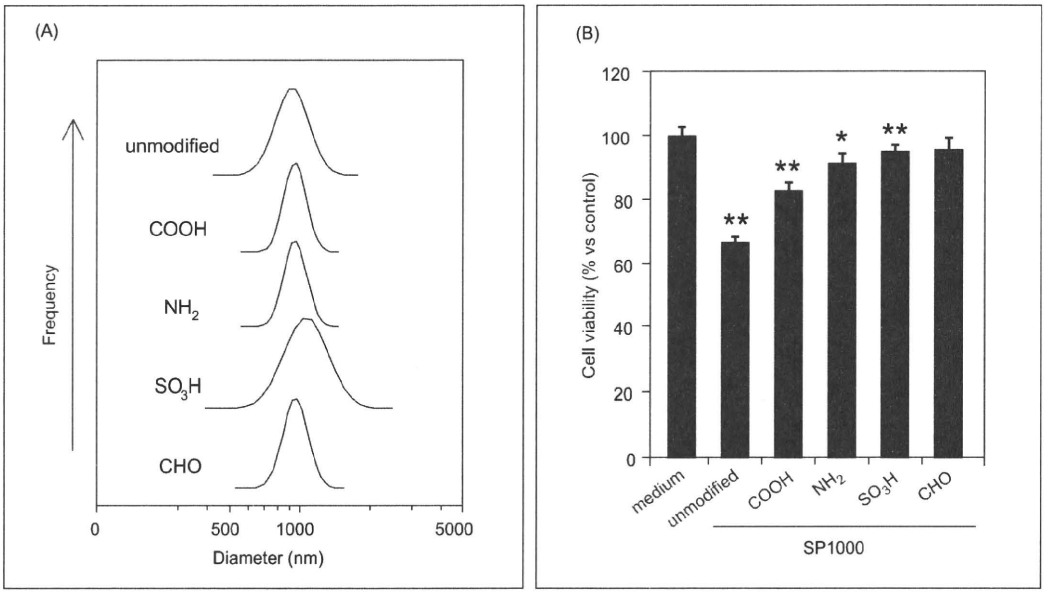

Fig. 3: Correlation between surface modification of mSP1000s and cytotoxicity against macrophage-like THP-1 cells. (A) Particle size distributions of unmodified and surface-modified mSP1000s. Particle size distributions were measured with a Zetasizer 3000HS after sonication at a particle concentration of 300 (g/mL in H_2O. (B) Cytotoxicity of surface-modified mSP1000s. PMA-primed THP-1 cells were treated with the surface-modified mSP1000s at 100 μg/mL for 24 h. After the stimulation, cell viability was measured by means of the standard methylene blue assay. The data represent the mean ± SD ($n = 5$; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for medium control)

indicate that mSP1000 were recognized and taken up into THP-1 cells by energy-dependent phagocytosis.

We next examined the mechanism of mSP1000-induced cytotoxicity. To determine whether there was an association between caspases and mSP1000-induced cell death, we treated cells with mSP1000 in the presence or absence of zVAD-fmk, a broad caspase inhibitor (it inhibits caspase-1, -3, -4, and -7). We found that zVAD-fmk did not affect the mSP1000-induced cytotoxicity, which indicates that the cytotoxicity was independent of caspases (Fig. 2B). Recently, four kinds of cell death pathways were reported: apoptosis, necrosis, pyroptosis, and pyronecrosis (Ting et al. 2008). Apoptosis and pyroptosis are dependent on the activity of caspases, whereas necrosis and pyronecrosis are independent of caspases. Therefore, our results suggest that mSP1000-induced cell death might have been necrosis or pyronecrosis. Both pathways elicit substantial inflammation, whereas apoptosis is a non-inflammatory cell death that does not affect the area around the dying cells (Ting et al. 2008). Therefore, we suspected that SP-induced cell death might be associated with inflammatory responses induced by mSP1000. However, the stimulation of macrophages with materials such as silica is known to induce ROS production (Msiska et al. 2009). Excessive production of ROS itself causes irreversible cellular injuries and contributes to the pathogenesis of several inflammatory diseases (Cross et al. 1994; Terman et al. 2006). To determine whether ROS were involved in mSP1000-induced cell death, we stimulated THP-1 cells with mSP1000 in the presence of a broad ROS scavenger, butylated hydroxyanisole (BHA), and found that the scavenger significantly inhibited the cytotoxicity of mSP1000 (Fig. 2B). These results indicate that ROS played an important role in the mSP1000-induced cell death and that the cytotoxicity induced by mSP1000 depends on ROS production but is independent of caspases, which suggests that mSP1000-induced cell death is inflammatory necrosis or pyronecrosis.

2.3. mSP1000-induced cell death is suppressed by surface modification with functional groups

To assess the correlation between surface modification and SP cytotoxicity, we used mSP1000 modified with various surface functional groups (-COOH, -NH_2, -SO_3H, and -CHO). The mean secondary particle diameters of unmodified mSP1000 was 945 nm, and the corresponding values for the modified particles were 1022, 958, 1023, and 969 nm, respectively (Fig. 3A). We compared the cytotoxicity of the modified and unmodified particles against THP-1 cells and found that mSP1000-induced cytotoxicity was suppressed by the surface modification (Fig. 3B). Interestingly, we confirmed that all the surface-modified mSP1000 were taken up equally into the cells (data not shown). We expect that surface modification can be used as a novel method to create safe SPs.

In summary, we confirmed that the cytotoxicity of SPs depended on particle size and surface properties. We confirmed that mSP1000-induced cell death was dependent on ROS production but independent of caspases. We believe that this information will be useful for the creation of novel safe SP-based materials.

3. Experimental

3.1. Materials and reagents

We used SPs with diameters between 30 and 1000 nm (nSP30, nSP50, nSP70, nSP300, and mSP1000), and mSP1000s with various surface functional groups (-COOH, -NH_2, -SO_3H, and -CHO). The SPs (Sicaster) were purchased from Micromod Partikeltechnologie (Rostock/Warnemünde, Germany). PMA and BHA were purchased from Sigma (St. Louis, MO). zVAD-fmk was purchased from Merck Calbiochem (Darmstadt, Germany).

3.2. Cell treatment

THP-1 cells (human acute monocytic leukemia cell line) were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan) supplemented

with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics at 37 °C. Treatment of THP-1 cells with PMA reportedly induces differentiation to a macrophage phenotype (Hoff et al. 1992; Rutault et al. 2001).

3.3. *Size distribution of silica particles*

The size distributions of the SP were measured with a Zetasizer 3000HS (Malvern, Worcestershire, UK) after sonication at a particle concentration of 300 μg/mL in H_2O.

3.4. *Cytotoxicity of various silica particles*

THP-1 cells (1.5×10^4 cells/well) were seeded in 96-well plates, differentiated to macrophages by incubation with 0.5 (M PMA for 24 h, and then washed once with incubation medium. After the PMA priming, cells were treated with 100 (g/mL SPs for 24 h. The cytotoxicity of the SPs against THP-1 cells was assessed by means of the standard methylene blue assay. In brief, after the SP treatment, cells were fixed with 100 μL of 2.5% glutaraldehyde for 15 min and stained with 100 μL of 0.05% methylene blue for 15 min. Then, the cells were lysed with 200 μL of 0.33 N HCl. The $OD_{655-415}$ was measured using a multiwell spectrophotometer (Molecular Devices, Inc., Tokyo, Japan).
For the inhibitory assays, PMA-primed THP-1 cells were pre-incubated with BHA (150 μM) or zVAD-fmk (60 μM) for 30 min and then treated with 100 μg/mL SPs for 24 h in the presence or absence of each inhibitor.

3.5. *Laser scanning confocal microscopy analysis*

THP-1 cells (1.0×10^5 cells/well) were seeded on Lab-Tek II Chambered Coverglass (Nunc, Rochester, NY), differentiated to macrophages by incubation with 0.5 μM PMA for 24 h, and treated for 6 h with 100 μg/mL mSP1000s. Then the cells were washed and fixed with 4% paraformaldehyde and mounted with Prolong Gold with 2-(4-amidinophenyl)-1*H*-indole-6-carboxamidine (DAPI, Invitrogen, Carlsbad, CA) for nuclear staining. Fluorescence was observed with a laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

3.6. *Statistical analysis*

All results are presented as means ± standard deviation (SD). Differences were compared using Student's *t*-test or Scheffé's method after analysis of variance (ANOVA).

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Leading Opinion

Acute phase proteins as biomarkers for predicting the exposure and toxicity of nanomaterials☆

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ABSTRACT

Recently, nanomaterials have become an integral part of our daily lives. However, there is increasing concern about the potential risk to human health. Here, we attempted to identify biomarkers for predicting the exposure and toxicity of nanomaterials by using a proteomics based approach. We evaluated the changes of protein expression in plasma after treatment with silica nanoparticles. Our analyses identified haptoglobin, one of the acute phase proteins, as a candidate biomarker. The results of ELISA showed that the level of haptoglobin was significantly elevated in plasma of mice exposed to silica nanoparticles with a diameter of 70 nm (nSP70) compared to normal mice and those exposed to silica particles with a diameter of 1000 nm. Furthermore, the other acute phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA) were also elevated in plasma of nSP70 treated mice. In addition, the level of these acute phase proteins was elevated in the plasma of mice after intranasal treatment with nSP30. Our results suggest that haptoglobin, CRP and SAA are highly sensitive biomarkers for assessing the risk of exposure to silica nanoparticles. We believe this study will contribute to the development of global risk assessment techniques for nanomaterials.

1. Introduction

With the recent development of nanotechnology, nanomaterials such as silica nanoparticles are beginning to be used on a global scale. In comparison to conventional materials with submicron size, nanomaterials display unique properties such as high levels of electrical conductivity, tensile strength and chemical reactivity [1]. Nanomaterials have already been used in various fields such as electronic engineering, cosmetics and medicine [2,3]. Because nanotechnology is emerging as a leading industrial sector, humans will be increasingly exposed to a wide range of synthetic nanomaterials with diverse properties.

The increasing use of nanomaterials has raised public concerns about the potential risks to human health [4–6]. For example, it is reported that carbon nanotubes induce mesothelioma-like lesions in mice in a similar way to crocidolite asbestos [7]. Other reports showed that exposure to titanium dioxide particles induce inflammatory responses and lung injury in mice [8,9]. In addition, our group showed that silica nanoparticles with a diameter of 70 nm can penetrate mouse skin and enter the circulatory system (unpublished data). Furthermore our group demonstrated that silica nanoparticles induce severe liver damage after systemic administration [10–12]. However, current knowledge of the potential risk of nanomaterials is considered insufficient. Indeed, concerns about the potential dangers of nanomaterials have led the World Health Organization and the Organization for Economic

☆ *Editor's Note*: This paper is one of a newly instituted series of scientific articles that provide evidence-based scientific opinions on topical and important issues in biomaterials science. They have some features of an invited editorial but are based on scientific facts, and some features of a review paper, without attempting to be comprehensive. These papers have been commissioned by the Editor-in-Chief and reviewed for factual, scientific content by referees.

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Co-operation and Development to call for an urgent and detailed evaluation of their safety. Therefore, it is extremely important to progress these safety evaluations in order to facilitate the development of nanomaterials that are harmless to humans, because nanomaterials have the potential to improve the quality of human life. In particular, it is hoped that a risk assessment system can be developed to estimate or predict the safety and toxicity of nanomaterials.

Molecular biomarkers, obtained from biological samples such as blood, urine and tissue, constitute an objective indicator for correlating against various physiological conditions or variation of disease state [13,14]. By using biomarkers, we are able to predict not only the present disease and clinical condition but the risk of acquiring disease in the future. Nowadays, biomarkers that act as predictors of cancer have already been developed and are commonly used in clinical practice [14]. Furthermore, such an approach is capable of predicting adverse effects of drugs and medicines [15,16]. By contrast, studies of biomarkers for nanomaterials have barely advanced. These biomarkers would represent the unity of local and systemic physiological responses induced as a result of the exposure. Therefore, biomarkers for nanomaterials will be invaluable for predicting their potential toxicity and establishing strategies for the safe development of nanomaterials production and use.

Here we attempted to develop potential biomarkers of nanomaterials using a proteomics analysis with the aim of developing safe forms of nanomaterials.

2. Materials and methods

2.1. Materials

Silica particles were purchased from *Micromod* Partikeltechnologie (Rostock/Warnemünde, Germany). The silica particles with diameters of 30, 70, 300 and 1000 nm (nSP30, nSP70, nSP300 and mSP1000, respectively), and nSP70 with surface functional groups such as carboxyl group and amino group (nSP70-C and nSP70-N, respectively) were used in this study. The silica particles were sonicated for 5 min and vortexed for 1 min prior to use.

2.2. Animals

Female BALB/c mice were purchased from Nippon SLC, Inc (Shizuoka, Japan) and used at 6–8 weeks of age. All of the animal experimental procedures in this study were performed in accordance with the National Institute of Biomedical Innovation guidelines for the welfare of animals.

2.3. Blood sample collection

For administration of silica particles through an intravenous route, BALB/c mice were treated with nSP70, nSP300, mSP1000, nSP70-C, nSP70-N or saline at 0.8 mg/mouse. At various times (6 h, 24 h, 3 day and 7 day) after treatment of these silica particles, blood samples were collected. For administration of silica particles through an intranasal route, BALB/c mice were treated with nSP30, nSP70 or saline intranasally at 0.5 mg/mouse. Blood samples were collected 24 h after the treatment of these silica nanoparticles.

2.4. Analysis of biomarkers for nanomaterials using a proteomics approach

BALB/c mice were treated with 0.8 mg/mouse nSP70 or saline intravenously. After 24 h, blood samples were collected and plasma was harvested by centrifuging blood at 12000 rpm for 15 min. Proteo prep (Sigma–Aldrich; Saint Louis, MO) was used to remove albumin and immunoglobulins from the plasma according to the manufacturer's instructions. Plasma samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue staining. Plasma diluted into aliquots corresponding to 10 μg protein were mixed with an equal volume of Laemmli sample buffer (BIO-RAD, Tokyo, Japan) containing 5% 2-mercaptethanol and boiled for 5 min prior to electrophoresis. Electrophoresis was performed at 15 mA for 10 min (stacking) followed by separation (600 V, 40 mA, 100 W) for approximately 45 min, using Precision Plus Protein Kaleidoscope molecular weight markers (BIO-RAD) as standards.

2.5. Identification of candidate proteins as biomarkers

Bands of interest were excised from the gel and then destained with 50% acetonitrile (ACN)/25 mM NH_4HCO_3 for 10 min, dehydrated with 100% ACN for 10 min, and then dried using a centrifugal concentrator. Next, 8 μl of 20 μl/ml trypsin solution (Promega, Madison, WI) diluted 5-fold in 50 mM NH_4HCO_3 was added to each gel piece and then incubated overnight at 37 °C. We used three solutions to extract the resulting peptide mixtures from the gel pieces. First, 50 μl of 50% (v/v) ACN in 0.1% aqueous trifluoroacetic acid (TFA) was added to the gel pieces, which were then sonicated for 30 min. Next, we collected the solution and added 80% (v/v) ACN in 0.1% TFA. Finally, 100% ACN was added for the last extraction. The peptide solution were dried and resuspended in 10 μl of 0.1% formic acid. The resulting peptide mixture was then analyzed by nano-flow liquid chromatography/tandem mass spectrometry (LC/MS; maXis, Bruker Daltonik GmbH, Bremen, Germany).

2.6. Measurement of acute phase proteins

Plasma levels of haptoglobin, C-reactive protein (CRP) and serum amyloid A (SAA) were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (Life Diagnostics, Inc.; West Chester, PA), according to the manufacturer's instructions.

2.7. Statistical analyses

All results are expressed as means ± SD. Differences were compared by using the Bonferroni's method after analysis of variance (ANOVA).

3. Results

3.1. Identification of biomarkers of nanomaterials

We used silica particles as a model nanomaterial because it is one of the most common nanomaterials to have been developed. Silica particles are increasingly being used as additives in cosmetics and foods [17,18]. It is predicted that the global market for silica particles will soon grow to $2 billion and a ton of silica particles is currently produced worldwide every year. Here, we used silica particles with a diameter of 30, 70, 300 and 1000 nm (nSP30, nSP70, nSP300 and mSP1000, respectively). The mean secondary particle diameters of the silica particles measured by Zetasizer were 33, 79, 326 and 945 nm, respectively (data not shown). The silica particles were confirmed to be well dispersed smooth-surfaced spheres by transmission electron microscopy (data not shown).

Initially, we attempted to identify protein biomarkers in mice by analyzing changes in the level of each plasma protein following treatment with silica nanoparticles using a proteomics approach. BALB/c mice were intravenously treated with nSP70 (0.8 mg/mouse) or saline and then plasma samples were collected 24 h later. Because albumin and immunoglobulins are known to account for the majority of plasma proteins, they were removed from the samples prior to analysis so that variation in the level of other proteins could be more closely monitored. The change of protein levels in plasma after treatment with nSP70 was assessed by SDS-PAGE analysis (Fig. 1). The intensity of a band of molecular mass 37 kDa was more intense in the plasma of nSP70 treated mice than that of saline treated control mice (Fig. 1). The band was excised and analyzed by LC/MS in order to identify the corresponding protein. This analysis identified the induced band after treatment with nSP70 as haptoglobin, one of the acute phase proteins.

3.2. The level of haptoglobin after treatment with silica particles

To assess the change of haptoglobin level in plasma after administration of silica particles, BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. We did not use nSP30 in the experiment, because nSP30 induced the toxic side effects after intravenous treatment at this dose. We confirmed that nSP70, nSP300 or mSP1000 at this dose did not induce any

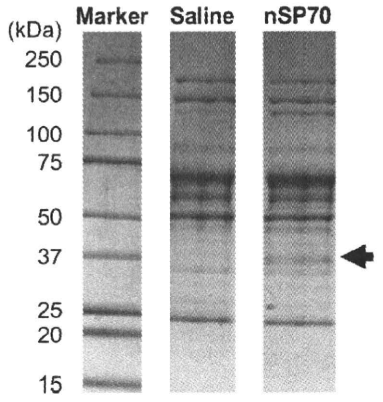

Fig. 1. SDS-PAGE analysis of plasma proteins. BALB/c mice were intravenously treated with nSP70 or saline at 0.8 mg/mouse. After 24 h, blood samples were collected. The change of protein levels in plasma after treatment of nSP70 was assessed by SDS-PAGE.

significant elevation of tissue injury and dysfunction markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) (data not shown). After 24 h, the level of haptoglobin in the plasma was analyzed by ELISA (Fig. 2A). The levels of haptoglobin in the plasma of nSP70 treated mice were significantly higher than those of saline treated control mice. In contrast, the levels of haptoglobin in the plasma of mSP1000 treated mice were almost the same as those of the saline treated control group. The haptoglobin levels of nSP300 treated mice were slightly higher than those of saline treated control mice. These results indicate that the levels of haptoglobin in the plasma of mice increase as the silica particle size decreases. Thus, haptoglobin appears to be a valuable biomarker for exposure to silica particles of nanometer size.

To assess the potential of haptoglobin as biomarker more precisely, we examined the sensitivity and time dependency of changes in haptoglobin level after treatment with silica particles. BALB/c mice were treated with nSP70, nSP300 or mSP1000 intravenously at 0.8 mg/mouse. After 6 h, 24 h, 3 day and 7 day, we examined the level of haptoglobin in the plasma by ELISA (Fig. 2B). No elevation of haptoglobin in the plasma of mSP1000 treated mice was observed. However, nSP70 and nSP300 treated mice showed a maximum level of haptoglobin 24 h after treatment. Furthermore, at 3 days after treatment, the level of haptoglobin in nSP70 treated mice was significantly higher than saline treated control mice. Next, BALB/c mice were treated with 0.2 and 0.05 mg/mouse nSP70 intravenously. After 24 h, we examined the level of haptoglobin in the plasma by ELISA (Fig. 2C). Mice treated with 0.2 and 0.05 mg/mouse nSP70 did not show any elevated level of haptoglobin. These results indicate that the level of haptoglobin is elevated as the particle size of silica particles decreases and that an increase of haptoglobin is dependent on the concentration of silica particles.

3.3. Response of other acute phase proteins

Haptoglobin, CRP and SAA are typical acute phase proteins that are induced during infection and inflammation [19]. To assess the levels of CRP and SAA in plasma after administration of silica particles, BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 6 h, 24 h, 3 day and 7 day, we examined the level of CRP (Fig. 3A) and SAA (Fig. 3B) in the plasma of the mice by ELISA. At 6 h and 24 h, both the level of CRP and SAA in the plasma of mice treated with nSP70 was significantly higher than those of the saline treated control mice. Furthermore, the maximum level of CRP in nSP70 treated mice was observed at

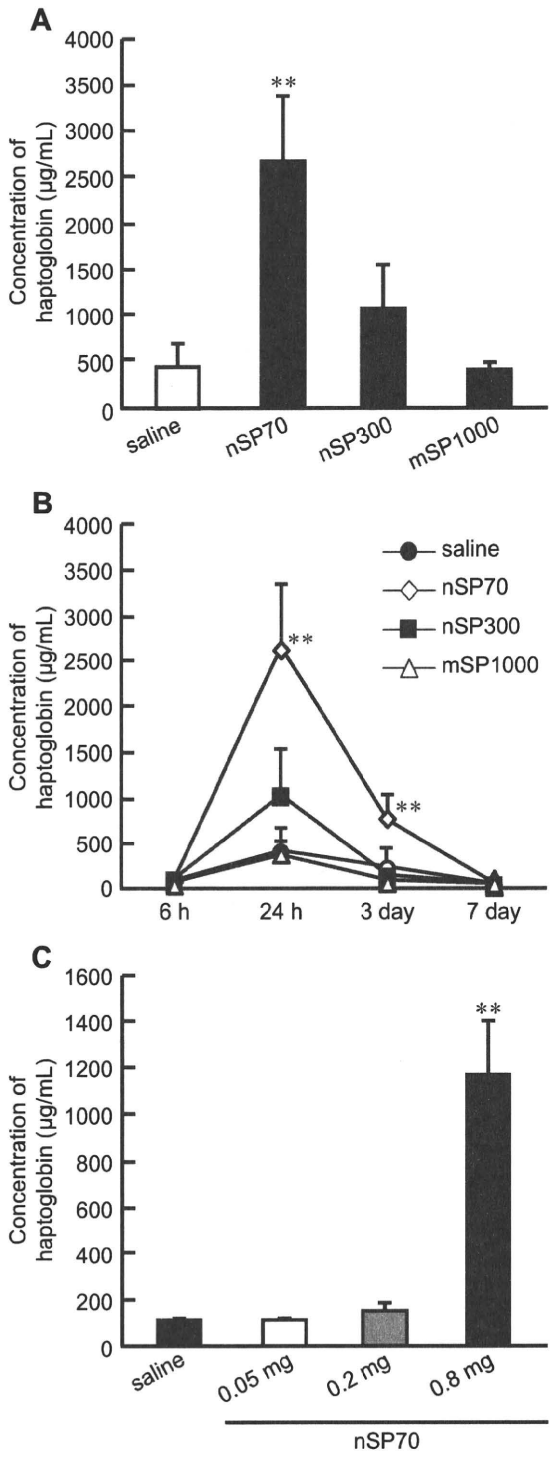

Fig. 2. The potential of haptoglobin as biomarker of nanomaterials. (A) The level of haptoglobin after treatment with silica particles. BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 24 h, the level of haptoglobin in the plasma of each mouse was examined by ELISA. (B) The time dependency of haptoglobin expression after treatment with silica particles. BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 6 h, 24 h, 3 day and 7 day, blood samples were collected. The level of haptoglobin in the plasma of the mice was determined by ELISA. (C) The sensitivity of haptoglobin after treatment of silica particles. BALB/c mice were intravenously treated with nSP70 at 0.8, 0.2 or 0.05 mg/mouse. After 24 h, blood samples were collected. The level of haptoglobin in the plasma of treated mice was determined by ELISA. Data are presented as mean ± SD ($n = 5–6$; $^{**}P < 0.01$ *versus* value for saline treated group by ANOVA).

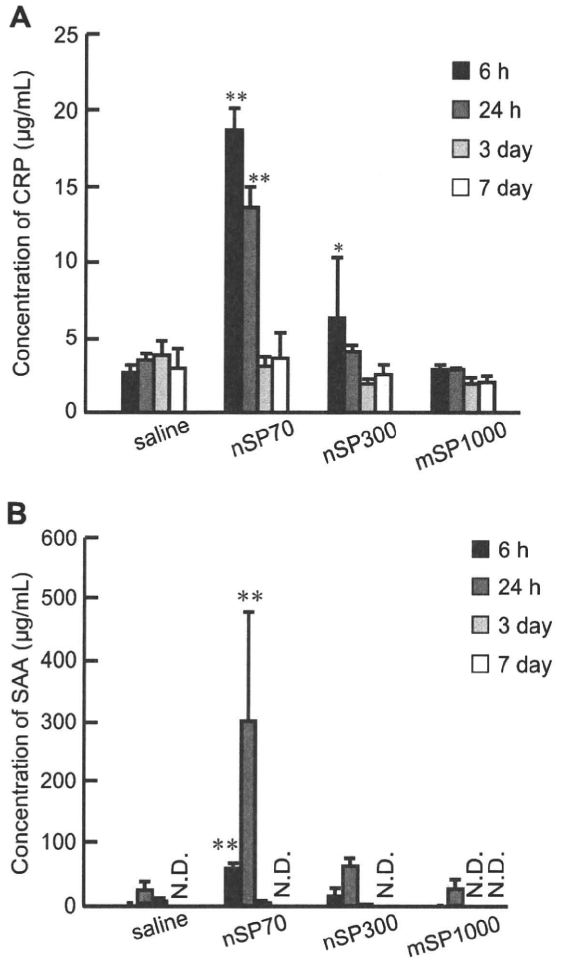

Fig. 3. Response of other acute phase proteins. BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 6 h, 24 h, 3 day and 7 day, blood samples were collected. The levels of (A) CRP and (B) SAA in the plasma of treated mice were examined by ELISA. Data are presented as mean ± SD ($n = 5–6$; $*P < 0.05$, $**P < 0.01$ *versus* value for saline treated group by ANOVA; N.D., not detected).

6 h after treatment, whereas that of haptoglobin and SAA was observed at 24 h. In contrast, the level of CRP and SAA in plasma of mSP1000 treated mice were almost the same as that of the saline treated control mice at all time points. The level of CRP in the plasma of nSP300 treated mice was slightly higher than that of saline treated control mice at 6 h. Our results suggest that both SAA and CRP may be useful biomarkers for predicting the risk from exposure to silica nanoparticles as well as haptoglobin. Indeed, these biomarkers could give even better response and sensitivity when used in combination.

3.4. The level of acute phase proteins through various routes

Exposure to nanomaterials in our daily lives can occur through various different routes. For example, nanomaterials contained in foods and drug medicines are taken up orally, whereas nanomaterials spread in the environment generally enter the body intranasally. Therefore, there is a need to evaluate suitable biomarkers for the exposure of nanomaterials through various routes. To assess the response of acute phase proteins to

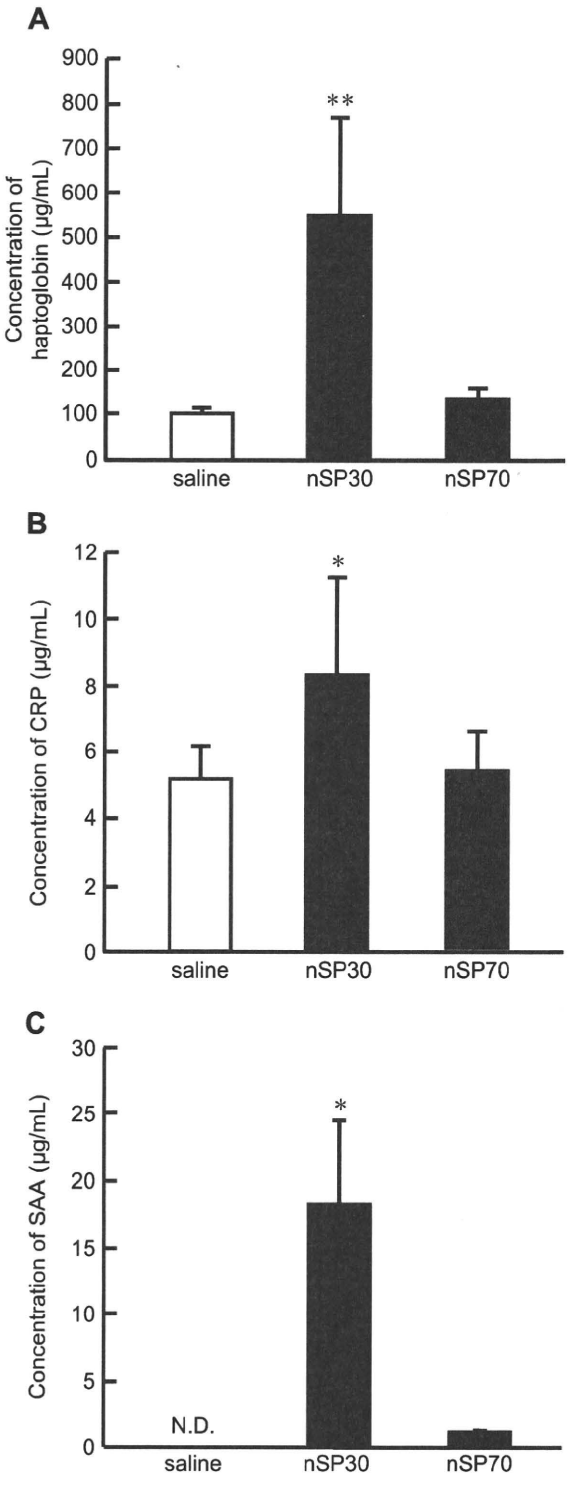

Fig. 4. Application of acute phase proteins to assess exposure of nanomaterials through various routes. To assess the administration of silica nanoparticles through an intranasal route, BALB/c mice were treated with nSP30, nSP70 or saline intranasally at 0.5 mg/mouse. Blood samples were collected 24 h after treatment. The level of (A) haptoglobin, (B) CRP and (C) SAA in the plasma were examined by ELISA. Data are presented as mean ± SD ($n = 5–6$; $*P < 0.05$, $**P < 0.01$ *versus* value for saline treated group by ANOVA; N.D., not detected).

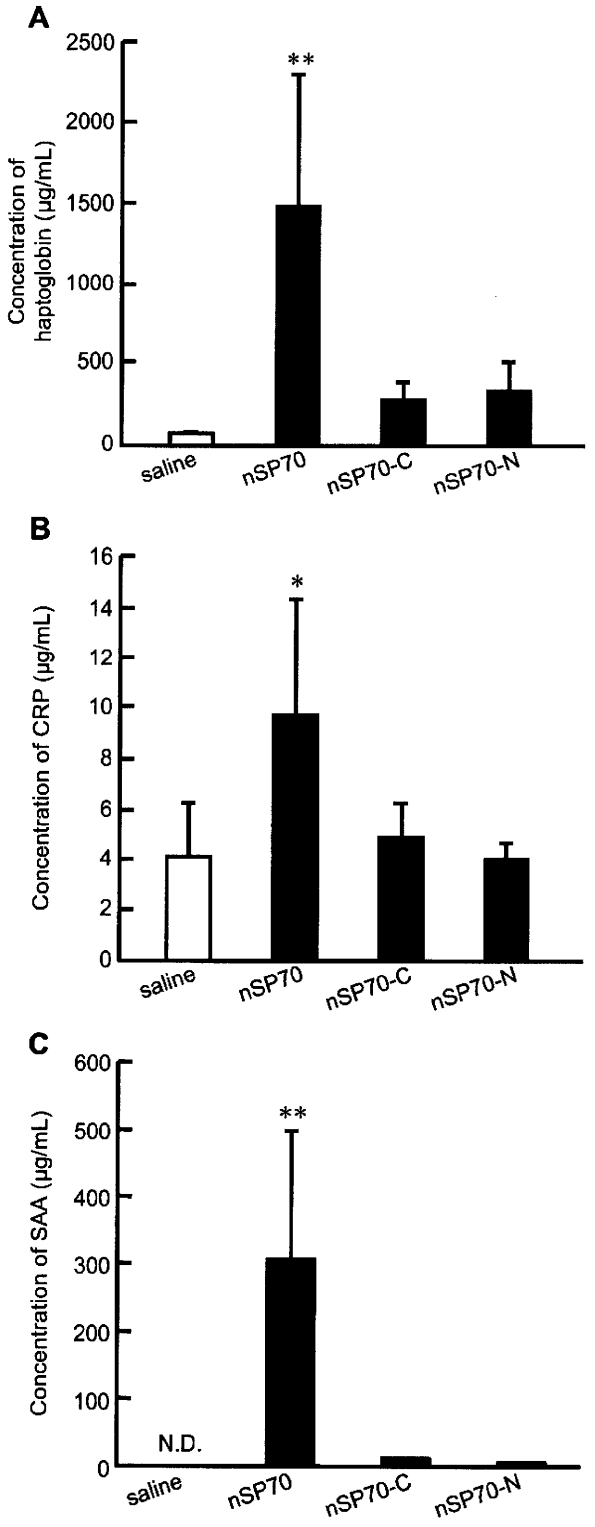

Fig. 5. Responses of acute phase proteins by the exposure to surface modified nSP70. BALB/c mice were intravenously treated with nSP70 modified with amino or carboxyl groups at 0.8 mg/mouse. After 24 h, the level of (A) haptoglobin, (B) CRP and (C) SAA in the plasma of treated mice were examined by ELISA. Data are presented as mean ± SD ($n = 5–6$; $^*P < 0.05$, $^{**}P < 0.01$ *versus* value for saline treated group by ANOVA; N.D., not detected).

silica particles introduced *via* different routes, we examined the level of haptoglobin, CRP and SAA in plasma after treatment of silica particles intranasally (Fig. 4). In this experiment, we used nSP30 and nSP70. For the administration of silica nanoparticles through an intranasal route, BALB/c mice were treated with nSP30, nSP70 or saline intranasally at 0.5 mg/mouse. After 24 h, we examined the level of haptoglobin (Fig. 4A), CRP (Fig. 4B) and SAA (Fig. 4C) in the plasma of the mice by ELISA. We showed that the level of haptoglobin, CRP and SAA in the plasma of mice treated with nSP30 intranasally was significantly higher than those of the saline treated control mice, although intranasal administration of nSP70 did not cause elevation in the plasma level of each acute phase protein in the treated mice. These results suggest that acute phase proteins could be useful biomarkers for predicting the risk arising from exposure to silica nanoparticles through various routes.

3.5. The level of acute phase proteins after treatment with surface modified silica nanoparticles

It has recently become evident that particle characteristics, including particle size and surface properties, are important factors in pathologic alterations and cellular responses [8,20–22]. Previously, our group also showed that surface modification of silica particles with functional groups such as amino or carboxyl group suppressed toxic biological effects of silica particles such as inflammatory responses [23]. To assess whether acute phase proteins could be useful biomarkers to predict risk factors associated with exposure to silica particles, we examined the level of haptoglobin (Fig. 5A), CRP (Fig. 5B) and SAA (Fig. 5C) in the plasma of mice after administration of nSP70 with amino or carboxyl group surface modifications. BALB/c mice were treated with 0.8 mg/mouse of these silica particles intravenously. After 24 h, we examined the level of haptoglobin, CRP and SAA in the plasma of the treated mice by ELISA. Our results showed that the level of these acute phase proteins in the plasma of nSP70 with amino or carboxyl group treated mice were significantly low compared with nSP70 treated mice.

4. Discussion

Our goal was to identify the biomarkers of nanomaterials for predicting their potential toxicity and to provide basic information for the creation of safe nanomaterials. To achieve these purposes, we tried to identify biomarkers in blood using a proteomics analysis. At first, we showed that the silica nanoparticles with small particle sizes (diameter <100 nm) induced a higher level of acute phase proteins such as haptoglobin, CRP and SAA than larger silica particles (diameter >100 nm) after intravenously treatment (Figs. 2 and 3). Previously, our group has shown that silica nanoparticles with relatively small particle size such as nSP70 induce a greater level of toxicity, including liver injury, compared to those of larger particle size [10,11]. Thus, there is a correlation between toxicity induced by the silica nanoparticles and the level of each potential plasma biomarker. Therefore, these acute phase proteins appear to be good biomarkers for predicting the strength of toxicity induced by silica nanoparticles.

The acute phase response is the nonspecific early response of an organism to infection and inflammation[24]. It comprises a whole array of systemic reactions and induction of a group of serum proteins called the acute phase proteins [25]. Monitoring the progression of infection and cancer by acute phase protein measurements in blood samples is used extensively in human patients. For example, haptoglobin is a biomarker of pancreatic cancer [26]. CRP is used as an index for the development of atrial fibrillation and maintenance [27], although mouse CRP is

synthesized only in trace amounts unlike its human counterpart [28]. In addition, both SAA and CRP are used as an index for adverse prognosis of breast cancer [29]. Therefore, we believe that these diagnostic systems using acute phase proteins for human health would be useful for predicting the risk of exposure to nanomaterials as well as their likely toxicities. In addition, we showed that the induction time for the maximum level of haptoglobin, SAA and CRP are different after treatment with the silica nanoparticles (Figs. 2 and 3). Therefore, the predictive quality of these biomarkers is improved when they are used in combination.

Epidemiological studies have suggested that increased levels of ambient particle including particle with nanometer size are associated with adverse effects in the respiratory and cardiovascular systems [30]. Some reports have shown that humans exposed to ambient particle have increased blood levels of CRP [31]. In addition, epidemiological studies have shown associations between increased concentrations of SAA and CRP in plasma, and increased risk of cardiovascular diseases [32] and cancer [33]. Therefore we consider that acute phase proteins would be biomarkers for predicting the risk of inflammatory disease, cardiovascular diseases and cancer after exposure by nanomaterials.

In recent years there has been increasing use of nanomaterials in cosmetics, due to their light-diffusing properties and absorbencies, as well as in foodstuffs, such as additives in powdered foods. In particular, silica particles have been extensively used in many consumer products. For example, in the US, the use of amorphous silica is limited to less than 2.0% by weight of common salt. Other limits are defined for finished foods (<1%) and dried egg products (<5%). We cannot avoid exposure to nanomaterials, either from the unintentional release of waste products into the environment or by exposure to medicines, cosmetics and foodstuffs. Thus, it is important to carry out a safety analysis of nanomaterials after exposure *via* various routes. In this study, we showed that the level of acute phase proteins in the plasma of mice treated with nSP30 intranasally was elevated, although nSP70 did not induce elevation of each acute phase protein (Fig. 4). Therefore we consider that nSP30 would induce any toxic biological effects after intranasally treatment. Now we are trying to examine the pharmacokinetics and biological effects of nSP30 after intranasally treatment.

We then examined the effects of surface modification of silica nanoparticles on the production of acute phase proteins, because it has become evident that surface properties are important factors in the biological effects of particles. We showed that nSP70 with amino or carboxyl group surface modifications did not induce the production of each acute phase proteins (Fig. 5). Previously, we showed that surface modification of silica particles with functional groups such as amino or carboxyl group suppressed toxic biological effects of silica particles such as inflammatory responses [23]. These results also suggest that acute phase proteins could be a promising candidate biomarker for predicting the strength of toxicity induced by silica nanoparticles, although it is need to examine the toxic biological effects of silica nanoparticles with functional groups. Over recent years, nanomaterials have been introduced into our everyday lives. For example, silica particles, titanium dioxide and fullerenes of various crystallographic structures and surface functional groups are used in a range of different consumer products. Therefore, we are now trying to evaluate the response of acute phase proteins to exposure from various nanomaterials.

In general, acute phase proteins are known to be released from the liver mainly as a result of inflammatory cytokines such as interleukin (IL)-6 [19]. However, we confirmed that the levels of IL-6 were not elevated in the plasma of mice treated with silica particles at 24 h after treatment (data not shown). Therefore it is unclear why nanomaterials induce the production of acute phase proteins. We already showed that although silica particles with micrometer size tend to be taken up by Kupffer cells, silica nanoparticles with small particle sizes distribute around hepatic parenchymal cells (unpublished data). It is conceivable that instead of inflammatory cytokines, small silica particles act directly on the liver to induce the release of acute phase proteins. We are currently analyzing the detailed mechanism by which silica particles induce acute phase proteins in order to identify additional protein biomarkers.

5. Conclusions

We show here that acute phase proteins such as haptoglobin, CRP and SAA can act as useful biomarkers for analyzing the risk of exposure to nanomaterials and their associated toxicity. We believe that such information would be vital for the future development of predictive tests for estimation of the potential toxicity of new nanomaterials based on their physiochemical characteristics.

Acknowledgements

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