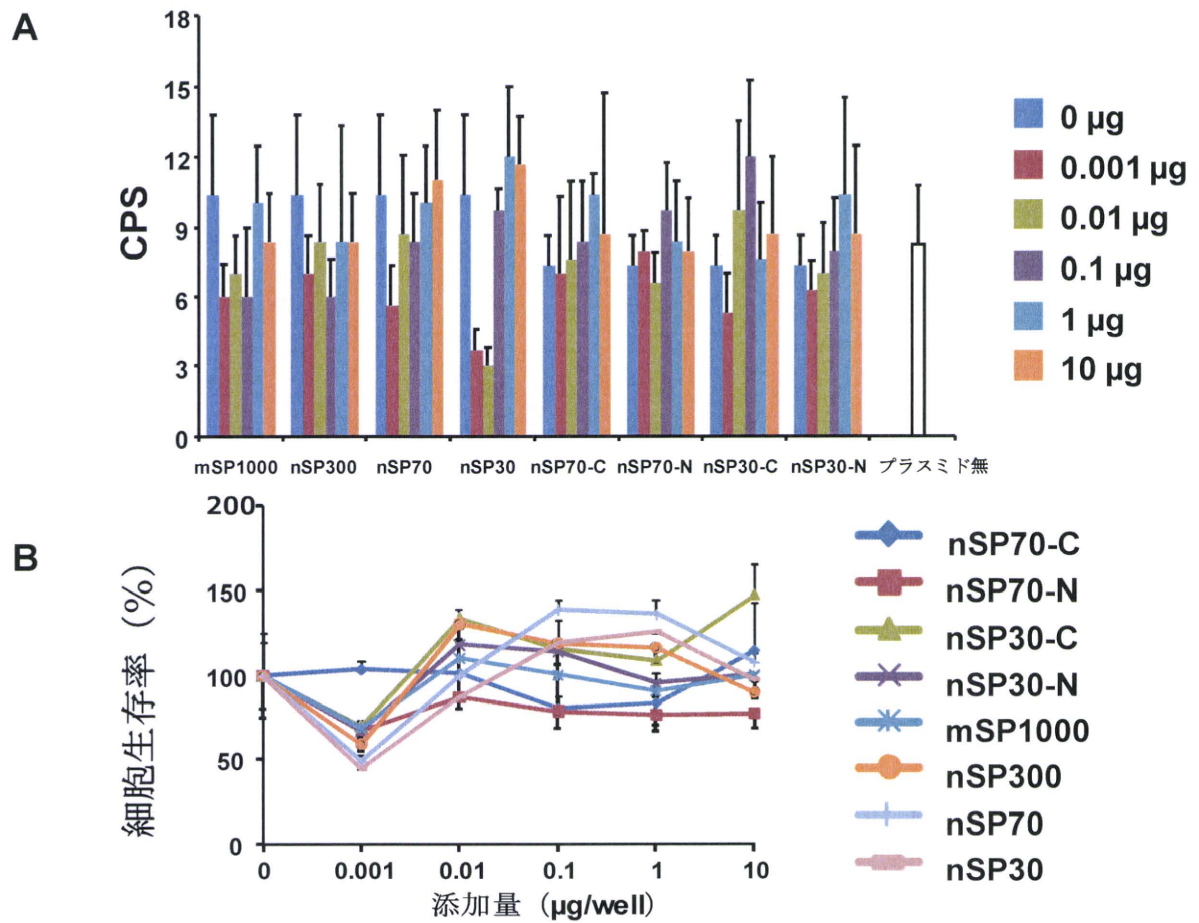**Figure 1.** シリカ粒子の **siRNA** キャリアとしての有用性評価

ルシフェラーゼ遺伝子に対する **siRNA** と、sigma-mSP、sigma-nSP、sigma-mesoSP との混合液をルシフェラーゼ発現肝細胞に作用させ、48 時間後のルシフェラーゼ活性（A）と細胞傷害性（B）を評価した。

Figure 2. シリカ粒子のプラスミドキャリアとしての有用性評価

Luciferase 遺伝子を導入するプラスミドと、各シリカ粒子との混合液を HepG2 細胞に作用させ、24 時間後のルシフェラーゼ活性（A）と細胞傷害性（B）を評価した。

最適な核酸医薬の設計と治療効果の検討

研究分担者　形山　和史　　大阪大学大学院薬学研究科　分子生物学分野　助教

研究要旨

HCV に対する核酸医薬は次世代の C 型肝炎治療薬として期待されているものの、静脈内投与後の体内安定性・肝臓集積率・細胞内移行率の低さなどから、その開発は遅々として進展していない。本研究では、ナノキャリアの優れた肝移行能を核酸医薬の標的化に応用し、HCV 感染・複製阻害を達成する新しいタイプの HCV 治療薬開発を目指す。本年度は、本研究の基盤となるナノマテリアルの核酸医薬送達能ならびに肝細胞適合性を簡便に定量する為の細胞評価系を構築した。

A. 研究目的

　HCV に対する核酸医薬は次世代の C 型肝炎治療薬として期待されているものの、静脈内投与後の体内安定性・肝臓集積率・細胞内移行率の低さなどから、その開発は遅々として進展していない。本研究では、ナノキャリアの優れた肝移行能を核酸医薬の標的化に応用し、HCV 感染・複製阻害を達成する新しいタイプの HCV 治療薬開発を目指す。本年度は、ナノマテリアルによる核酸医薬送達法のスクリーニング及び最適化を行うために、small interfering RNA (siRNA)の細胞内送達能とその効果発現を簡便に検出可能な評価系を構築した。具体的には、遺伝子の量的変化を簡便かつ鋭敏に評価できる *Firefly* luciferase と、遺伝子発現の量的変化を可視化することが可能な緑色蛍光タンパク Venus を応用し、siRNA 効果判定に適した安定発現細胞株を作製した。細胞は SK HEP-1（ヒト肝癌由来細胞株）および様々な研究で利用されている HeLa 細胞（ヒト子宮頸癌細胞株）を用い、遺伝子の安定導入にはレンチウイルスベクターシステムを利用した。

B. 研究方法

プラスミド構築

様々な細胞で外来遺伝子を安定的に発現可能なヒト EF-1αプロモーターを有するレンチウイルスベクタープラスミドを選択した。使用する各遺伝子が含まれるプラスミドを鋳型に目的の配列を PCR で増幅した後、レンチウイルスベクタープラスミドの適切な位置にクローニングし、そのシークエンスを確認した。IRES（internal ribosome entry site）を用いて１つのベクターから２つのタンパクを産生させるレンチウイルスベクター（Luc-IRES-Venus）は、CSII-EF-MCS-IRES-Venus のマルチクローニングサイトに Firefly luciferase 遺伝子を挿入することで作製した。自己開裂ペプチド（T2A ペプチド配列）を用いて１つのベクターから２つのタンパクを同時に発現するタイプのレンチウイルスベクター（Venus-T2A-Luc）は、2A ペプチドをコードする配列を含む合成オリゴプライマーを使用した Overlapping PCR 法により作製した。なお、レンチウイルスベクターは三好浩之先生（理研バイオリソースセンター）よりご供与頂いた。

レンチウイルスベクターの作製

293T 細胞を poly-L-lysine でコートした 100 mm dish へ 5 x 10^6 cells ずつ播種した。一晩培養後、

各遺伝子を搭載したレンチウイルスベクタープラスミドを HIVgp 発現プラスミド、VSV-G/Rev 発現プラスミドと共に Lipofectamine 2000 を用いてトランスフェクションした。翌日、Forskolin を含む培地に交換し、トランスフェクションから 2〜3 日培養後上清を回収し、0.45 μm のフィルターで濾過してレンチウイルス溶液とした。レンチウイルス溶液は、-80℃で保存した。本研究で用いたレンチウイルスベクターは緑色蛍光タンパク Venus を発現するため、その発現を指標に感染力価（TU/ml）を算出した。

細胞の培養
HeLa 細胞と 293T 細胞は 10% FCS と L-glutamine を添加した High Glucose DMEM 培地を用いて 37℃、飽和蒸気圧、5%CO_2 存在下で培養した。

導入遺伝子発現量の評価
ウイルスベクターを作用させた細胞を 2 分割し、片方を Venus 遺伝子の発現測定に、もう片方を Firefly luciferase 遺伝子の発現測定に供した。Venus 遺伝子の測定：ウイルスベクター作用 48 時間後、細胞を回収し、フローサイトメーター（LSR II）を用いて蛍光を測定した。Firefly Luciferase 遺伝子の測定：ウイルスベクター作用 48 時間後、Pica Gene LT 2.0 を用いてルシフェラーゼ活性を測定した。

倫理面への配慮
本研究は、国立大学法人大阪大学の組換え DNA 実験委員会の承認を得た後に行った。なお本研究においては、公知の細胞株以外のヒト由来試料は使用していない。

C. 研究結果
　遺伝子の量的変化を簡便かつ鋭敏に評価できる Firefly luciferase と、可視化することが簡便な緑色蛍光タンパク Venus をモデル標的遺伝子とすることで、siRNA の細胞内送達能とその効果発現のスクリーニングに適した評価細胞を作製した。2 つの遺伝子を同時に発現させる際には IRES 配列が利用される場合が多い。しかしながら IRES 配列の前後に挿入する遺伝子の種類や組み合わせなどにより、それぞれの発現量が大きく変動してしまい、理想とする遺伝子発現が観察できない場合もある。従って本研究では、IRES 配列を利用する場合（Luc-IRES-Venus）に加え、自己開裂ペプチドを用いて 1 つのプロモーターで 2 つのタンパクを同時に発現するタイプ（Venus-T2A-Luc）のレンチウイルスベクターを作製した。

　様々な細胞種で安定的な遺伝子発現を達成する為にヒト EF-1αプロモーターを選択し、その下流に自己開裂ペプチドをコードする遺伝子配列（T2A）あるいは IRES 配列で連結したモデル標的遺伝子を挿入したレンチウイルスベクタープラスミドを構築した（Fig. 1A）。これらを HIVgp 発現プラスミド、VSV-G/Rev 発現プラスミドと共に 293T 細胞に共導入し、目的とするレンチウイルスベクターを作製した。

　自己開裂ペプチドで連結した場合（Venus-T2A-Luc）には、レンチウイルスベクターの感染量、Venue 発現および luciferase 発現量に正の相関が観察された（Fig. 1B, C）。一方、IRES 配列を用いたベクター感染細胞でも両遺伝子の発現が確認できたが、その発現量に相関が認められなかった。また、Venus-T2A-Luc を感染させたヒト肝癌由来細胞株 SK HEP-1 でも同様に、Venus と luciferase を安定的に発現させることに成功した（data not shown）。

D. 考察
　本研究では、Venus 及び Firefly luciferase 遺伝子を同時に発現するレンチウイルスベクターを構築した。また、IRES 配列を用いる場合に比べ、

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2A ペプチドを利用する方が本研究に適した遺伝子発現パターンを示す事を確認した。本ベクターは種々の細胞に効率良く感染することが可能である。また、ゲノムにインテグレーションされるタイプのベクターである為、安定発現細胞株の作製に適している。モデル標的遺伝子として選択した Venus は緑色蛍光の強度から発現量評価に利用することも可能であるが、遺伝子導入された細胞をセルソーターで単離する場合のマーカーとしても有用であり、目的とする細胞株を容易に作製することが可能となる。本年度は HeLa 細胞と SK HEP-1 細胞を利用して安定発現細胞株を作製したが、次年度以降、HCV に対する核酸医薬の評価に適した他の細胞株（HepG2 や Huh-7 等）にも応用し、ナノマテリアルによる核酸医薬送達法のスクリーニングに応用する予定である。

E. 結論

　ナノマテリアルによる核酸医薬送達法のスクリーニング及び最適化を行うための細胞株を作製することに成功した。本細胞株を利用することで、ナノマテリアルによる siRNA の細胞内送達能とその効果を簡便に評価することが可能となる。

F. 健康危険情報
該当事項無し
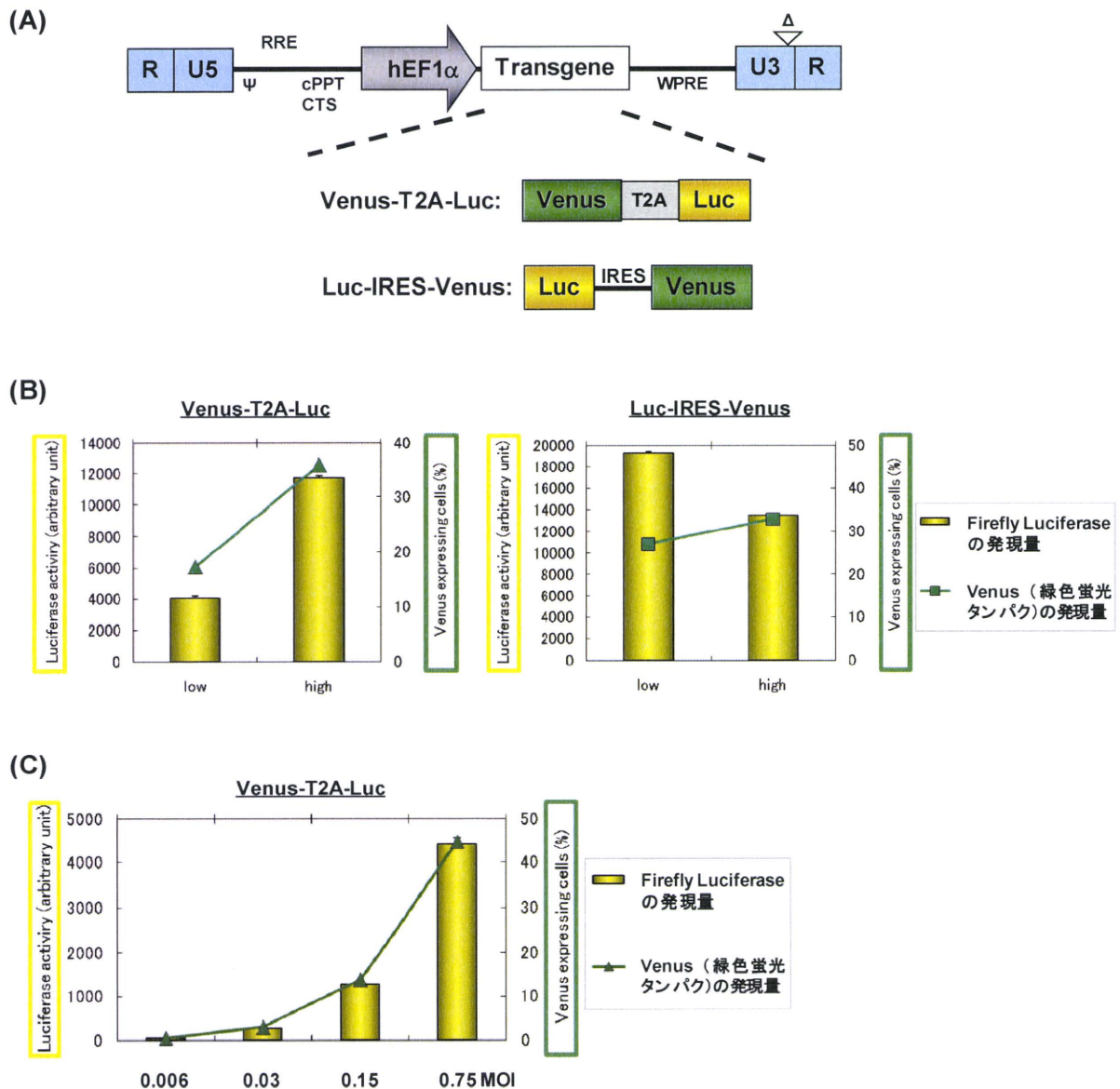
G. 研究発表
該当事項無し

H. 知的財産権の出願・登録状況
①特許取得
該当事項無し

②実用新案登録
該当事項無し

③その他
該当事項無し

I. 研究協力者
該当事項無し

Figure 1 . Construction and characterization of lentivirus vectors carrying a 2A-peptide or IRES linked bicistronic expression cassette.

(A) Transgenes, linked by T2A or IRES sequence, were expressed under the control of the EF1a promoter. Abbreviations: CMV, Human cytomegalovirus immediate early promoter; Ψ, Packaging signal; RRE, Rev responsive element; cPPT, Central polypurine tract; CTS, Central termination sequence ; WPRE, Woodchuck hepatitis virus posttranscriptional regulatory element; T2A, 2A peptide sequence from Thosea asigna virus.

(B, C)   Hela cells were transduced with the indicated dose of the lentivirus vectors. After 2 days, the cells were harvested and analyzed for the levels of transgene expression. Venus expression was examined by flowcytometry and luciferase expression was determined by a standard luciferase assay.

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Higashisaka K, Yoshioka Y, Yamashita K, Morishita Y, Fujimura M, Nabeshi H, Nagano K, Abe Y, Kamada H, Tsunoda S, Yoshikawa T, Itoh N, Tsutsumi Y.	Acute phase proteins as biomarkers for predicting the exposure and toxicity of nanomaterials	Biomaterials	32(1)	3-9	2011

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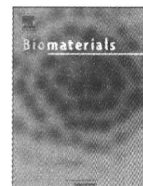
Leading Opinion

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

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ARTICLE INFO

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.

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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

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$_4$HCO$_3$ 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$_4$HCO$_3$ 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
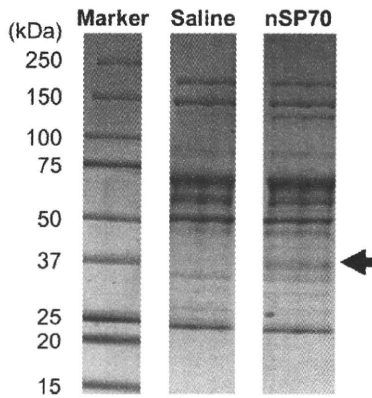
Fig. 1. SDS-PAGE analysis of plasma proteins. BALB/c mice were intravenously treated with nSP70 or saline at 0.8 mg/mouse. After 24 h, blood samples were collected. The change of protein levels in plasma after treatment of nSP70 was assessed by SDS-PAGE.

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
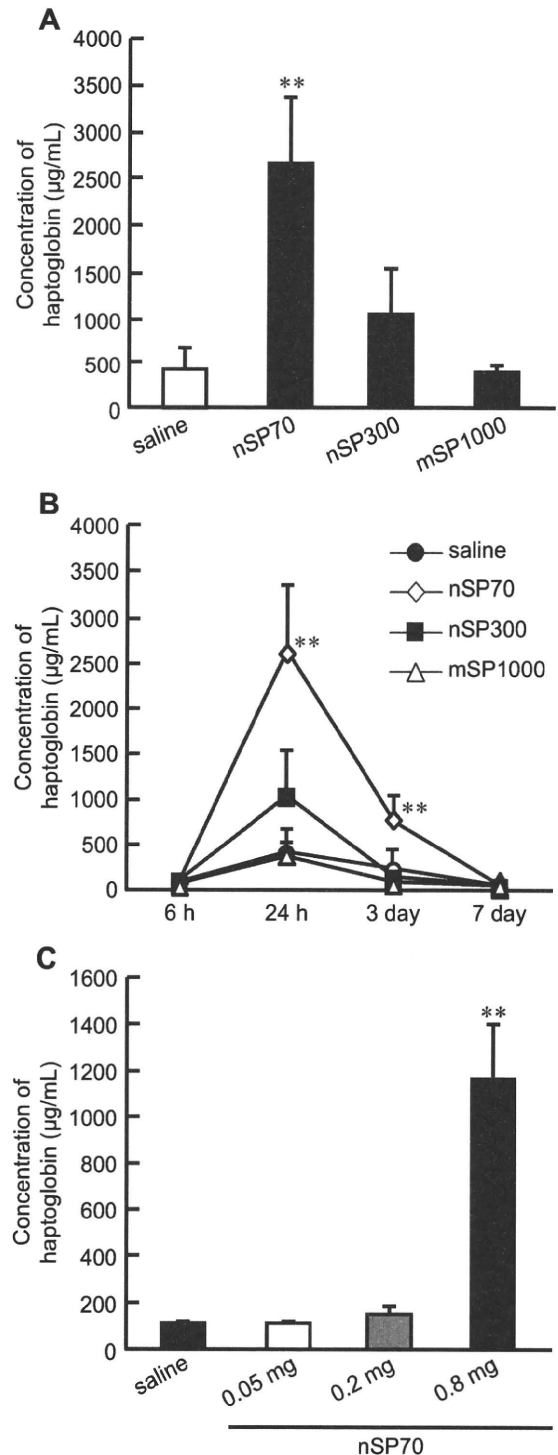
Fig. 2. The potential of haptoglobin as biomarker of nanomaterials. (A) The level of haptoglobin after treatment with silica particles. BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 24 h, the level of haptoglobin in the plasma of each mouse was examined by 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).

A

B
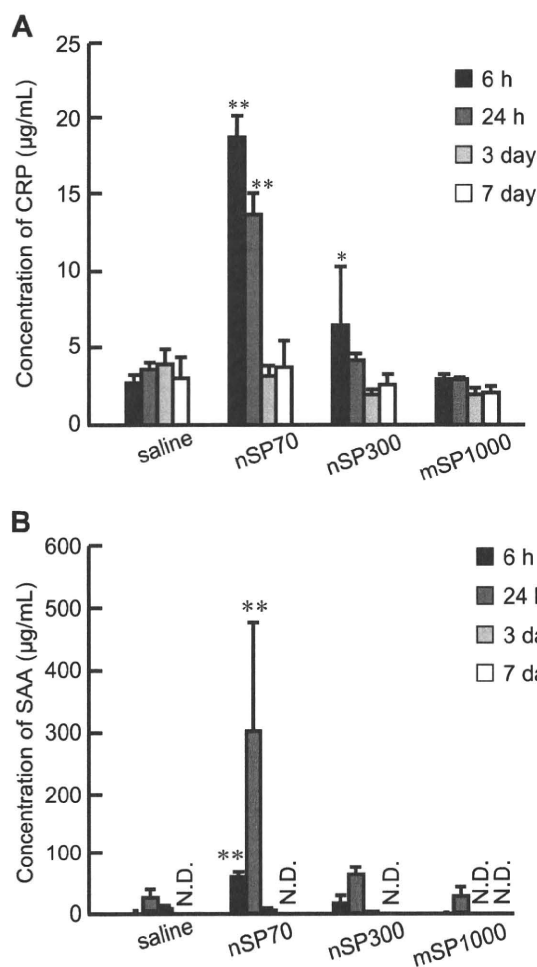
Fig. 3. Response of other acute phase proteins. BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 6 h, 24 h, 3 day and 7 day, blood samples were collected. The levels of (A) CRP and (B) SAA in the plasma of treated mice were examined by ELISA. Data are presented 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

A

B

C
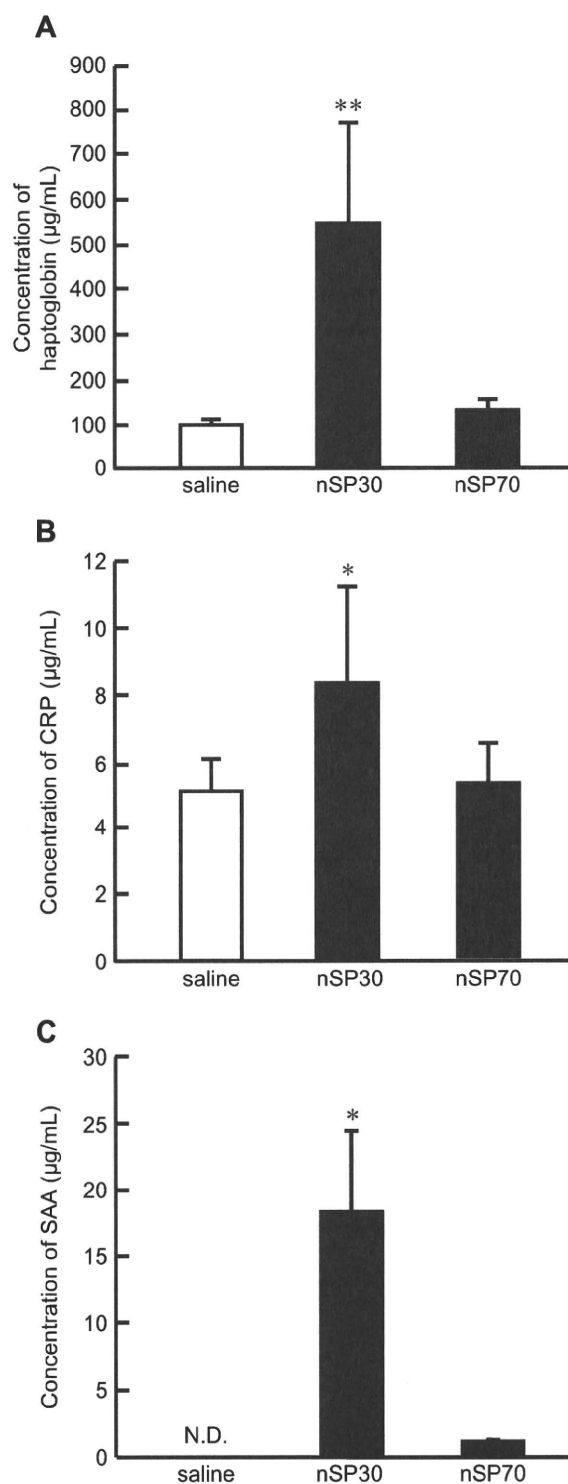
Fig. 4. Application of acute phase proteins to assess exposure of nanomaterials through various routes. To assess the administration of silica nanoparticles through an intranasal route, BALB/c mice were treated with nSP30, nSP70 or saline intranasally at 0.5 mg/mouse. Blood samples were collected 24 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).
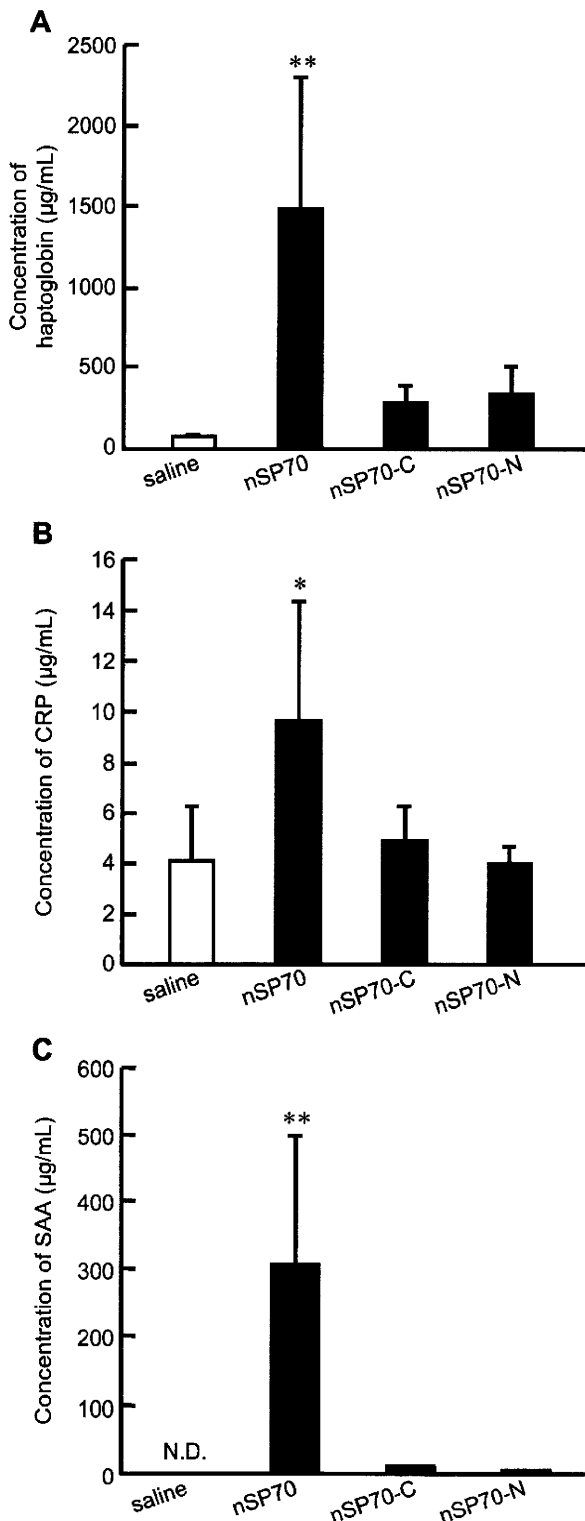
Fig. 5. Responses of acute phase proteins by the exposure to surface modified nSP70. BALB/c mice were intravenously treated with nSP70 modified with amino or carboxyl groups at 0.8 mg/mouse. After 24 h, the level of (A) haptoglobin, (B) CRP and (C) SAA in the plasma of treated mice were examined by ELISA. 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 nano-materials 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 asso-ciated 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 addi-tion, 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 pre-dicting 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 struc-tures 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 nano-particles 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.

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