Measures

We surveyed the women's chronic stress using Zung's Self-rating Depression Scale (SDS) and General Health Questionnaire-28 (GHQ28) at the second trimester and categorized the women into chronic high stress and normal groups. The SDS includes 20 questions that qualify the severity of depression symptoms. Each item ranges from 1 (none or a little of the time) to 4 (more or all of the time). The raw SDS score is the sum of all 20 items and ranges from 20 to 80.[13] This test is sensitive enough to detect depressive symptoms in normal populations. Its Japanese version has been ascertained to possess good internal consistency and test-retest reliability, and has been used in many research projects, such as population surveys.[14] The General Health Questionnaire (GHQ) is a 60-item self-administered screening tool designed to detect non-psychotic psychiatric illnesses. The GHQ28, a shorter version of the original GHQ, has been shown to be reliable and valid.[14] The GHQ28 was used to assess general health problems.[15] It is used in the general population and within community or non-psychiatric clinical settings, such as primary care or general medical outpatients.[16] The use of both the GHQ28 and SDS can enable the assessment of a variety of aspects of the mental health status in a normal population.[14] Using this questionnaire, we classified patients into chronic high stress and normal groups and made a comparison of the changes in stress markers between the two groups.

Determination of cortisol levels in the saliva

To collect a sufficient quantity of saliva, we used Salivette sampling devices (Sarstedt, Rommelsdorf, Germany). The Salivette includes a small cotton swab and stimulates saliva flow to a rate that enables a sufficient amount to be collected within 1 min. After centrifugation at 7000 *g* for 15 min, saliva was stored at –80°C until assay. Saliva cortisol levels were determined with a commercial enzyme immunoassay kit (CIRON, Tokyo, Japan). The intra-and interassay coefficient variabilities (CV) were <10%.[17]

Determination of CgA levels in the saliva

Salivary CgA levels were determined by ELISA using a previously described method.[18] The concentration of CgA in the saliva was determined using a YK070 Chromogranin A (Human) electro-immunoassay kit (Yanaihara Institute, Shizuoka, Japan) and the intra- and interassay CV were <5%.[19]

Statistical analysis

Data are given as mean ± SEM. The data were analyzed with StatView version 5.0 (SAS Institute, Cary, NC, USA). Statistical significance was assessed using Wilcoxon signed-rank test with a 5% significance level.

Results

The mean salivary cortisol levels (μg/dL ± SEM) were 0.282 ± 0.054 (first trimester), 0.693 ± 0.094 (second trimester), 0.330 ± 0.061 (early third trimester), 0.452 ± 0.069 (late third trimester), and 0.147 ± 0.028 (puerperal period). A repeat measure analysis of variance confirmed a significant effect of gestational age in salivary cortisol level ($P < 0.0001$). Cortisol levels in the saliva of pregnant women showed biphasic change during pregnancy. Mean salivary CgA/protein levels (pmol/mg ± SEM) were 1.919 ± 0.543 (first trimester), 4.203 ± 0.975 (second trimester), 3.617 ± 0.715 (early third trimester), 2.619 ± 0.481 (late third trimester), and 1.460 ± 0.262 (puerperal period). A repeat measure analysis of variance confirmed a significant effect of gestational age in salivary CgA/protein ($P = 0.0005$). CgA/protein levels in the saliva of pregnant women increased in the second and the early third trimesters and decreased to the puerperal period.

Figure 2 demonstrated that SDS score and general health questionnaire GHQ28 score were significantly correlated ($P < 0.005$). Patients in the chronic high stress

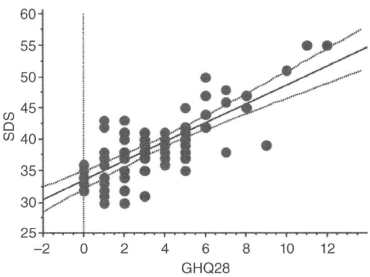

Figure 2 Correlation between the Zung self-rating depression scale (SDS) and General Health Questionnaire-28 (GHQ28) scores. SDS score and general health questionnaire GHQ28 score were significantly correlated. SDS = 33.527 + 1.516 × GHQ28; $r^2 = 0.58$ ($P < 0.005$).

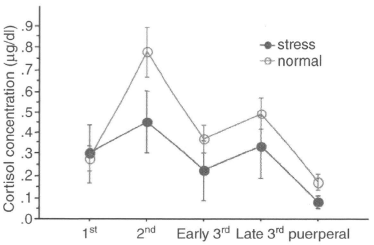

Figure 3 Difference in salivary cortisol concentrations between chronic high stress group (Zung self-rating depression scale [SDS] > 42) and normal group (SDS ≤ 42). Salivary cortisol concentrations of the chronic high stress group were significantly lower compared with those of the normal group at the second trimester, early third trimester, and late third trimester ($P < 0.01$).

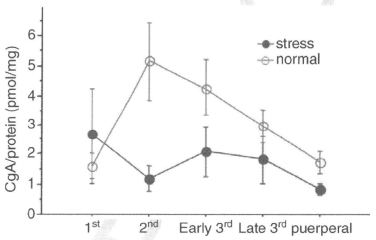

Figure 4 Difference in salivary chromogranin A (CgA) concentrations between the chronic high stress group (Zung self-rating depression scale [SDS] > 42) and normal group (SDS ≤ 42). Salivary CgA/protein concentrations of the chronic high stress group were significantly lower than those of the normal group ($P < 0.01$).

group (SDS > 42) were 17 of 69 pregnant women. Characteristics between the chronic high stress group and the normal group, such as maternal age, body weight, body mass index, smoking rate, parity, gravidity, delivery week, neonatal body weight and Apgar score were similar (data not shown). As shown in Figure 3, salivary cortisol concentrations of the chronic high stress group were significantly lower compared with those of the normal group at the second trimester, early third trimester, and late third trimester ($P < 0.01$). However, titers of salivary cortisol of the chronic high stress group were equal to those of the normal group at the first trimester and puerperal period. Salivary CgA/protein concentrations of the chronic high stress group were significantly lower than those of the normal group ($P < 0.01$) (Fig. 4). The elevation of CgA/protein in the saliva was suppressed in the chronic high stress group during pregnancy.

Discussion

Investigation of physiological stress markers in the saliva is a very useful method for objectively measuring stress. This method is non-invasive and non-stressful. Recent study demonstrated that music therapy during colonoscopy markedly reduces fear-related stress, as indicated by changes in salivary cortisol levels.[20] In the present study, the titration of salivary cortisol concentrations and CgA levels is a useful tool to determine maternal stress levels during pregnancy. Cortisol levels in the saliva of pregnant women showed biphasic change during pregnancy. Mean salivary cortisol levels increased from the first trimester to the second trimester. Those levels decreased from the second trimester to the early third trimester and increased at the late third trimester. Titers of salivary cortisol at the puerperal period returned to the levels at the first trimester. Serum concentration of circulating cortisol is increased during pregnancy.[21] The reason for the discrepancy between serum and saliva is unknown. The CgA/protein levels in the saliva of pregnant women increased in the second and the early third trimesters and decreased to the puerperal period. The mechanism of the change of stress marker levels during pregnancy is unknown. We speculated that the titers of those stress markers might be affected by hormonal changes, such as progesterone and immunological changes during pregnancy. Further investigations are necessary to determine the mechanism of the change of stress levels during pregnancy and the usefulness of cortisol and CgA as stress markers during pregnancy.

Psycho-mental health potentials were evaluated by both quality-of-life-related questionnaires and stress-related hormonal and cytokine levels, such as cortisol and interleukins. Self-reporting instruments represent an efficient and cost-effective way to identify individuals who should be evaluated additionally for the presence of a depressive disorder. The Zung

Self-rating Depression Scale (SDS) has 10 positively worded and 10 negatively worded items that cover affective, psychological, and somatic symptoms. The overall score represents the severity of the depressive symptoms.[22] GHQ28 represented the general health problems of pregnant women. To determine psychomental health potentials, several questionnaires have been used. Zung SDS and GHQ28 are useful tools because in Japan, the scale has been ascertained to have good internal consistency and test-retest reliability, and construct validity has been demonstrated in many research projects, including population surveys.[23]

In a population of Japanese women (mean age 46.3 years), mean SDS score was 37.8, 40.7, and 47.8 in the good lifestyle group, average lifestyle group, and poor lifestyle group, respectively,.[24] We noticed that there was a significant correlation between SDS and GHQ28. These results suggest that maternal depressive status represents general health during pregnancy. Several studies demonstrated maternal stress had adverse effects on pregnancy outcome.[2,5] The betterment of SDS and GHQ28 might affect the improvement of maternal and neonatal outcomes.

Maternal salivary cortisol and CgA of the chronic high stress group were significantly suppressed in comparison with those of the normal group. These results suggest that maternal chronic stress suppress the function or activity of the hypothalamic-pituitary-adrenal axis and sympatho-adrenomedullary system. Angelika *et al.* demonstrated that in children with allergic feature of atopic dermatitis under chronic psychosocial stress, the reaction of cortisol level to the stress test were suppressed.[25] Furthermore, Seng *et al.* found that for pregnant woman affected by posttraumatic stress disorder, the circadian rhythm of cortisol titer was suppressed and they had lower peak basal salivary cortisol concentrations.[26] Our results do not contradict those reports. The suppression of maternal response at the adrenal gland might affect the maternal immune defense system and endocrine function. This would be one possible reason why maternal stress has adverse effects on pregnancy outcome.

Acknowledgments

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Anti-β2 glycoprotein-I antibody increases the risk of pregnancy-induced hypertension: a case-controlled study

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ABSTRACT

The aim of this study was to evaluate whether anti-β2 glycoprotein-I antibody (anti-β2GPI) of the IgG or IgM classes is associated with the development of pregnancy-induced hypertension (PIH) or preeclampsia in the Japanese population. In a case-controlled cohort study, peripheral blood was obtained at 8–14 weeks of gestation from a consecutive series of 1155 women. The case group comprised 36 patients who later developed PIH during the pregnancy. Of the 36 PIH patients, 13 had severe PIH, 18 had preeclampsia and 11 had severe preeclampsia. One hundred and eleven age- and parity-matched women whose pregnancies ended in normal delivery without obstetric complications were selected as controls. We found that a titer of anti-β2GPI IgG ≥ 1.0 U/ml was a risk factor for severe PIH ($P = 0.023$, OR 5.7 95%CI 1.4–22.8). In addition, titers of anti-β2GPI IgM ≥ 1.2 U/ml was found to be a risk factor for PIH ($P = 0.001$, OR 8.8 95%CI 1.6–47.5). In women positive for anti-β2GPI but negative for lupus anticoagulant, anti-cardiolipin, phosphatidylserine-dependent anti-prothrombin, or kininogen-dependent anti-phosphatidylethanolamine antibodies, the presence of anti-β2GPI was not a significant risk factor for development of PIH or preeclampsia. In conclusion, the presence of anti-β2GPI antibody represents a risk factor for developing PIH and severe PIH. This finding supports the utility of anti-β2GPI determination as one of the laboratory criteria for anti-phospholipid syndrome classification. The usefulness of anti-β2GPI measurement among women without other anti-phospholipid antibodies requires further study.

1. Introduction

Anti-phospholipid antibodies (aPLs) are a heterogeneous group of autoantibodies directed against phospholipid-binding proteins. Anti-phospholipid syndrome (APS) refers to the association between aPLs and thrombosis or pregnancy morbidity. Criteria used widely as a consensus definition for APS were established by the Eighth International Symposium on Anti-phospholipid Antibodies Syndrome in Sapporo (Wilson et al., 1999). Obstetric complications included in this APS definition are recurrent pregnancy loss, unexplained fetal death, severe preeclampsia, intrauterine growth restriction, and premature delivery. Two types of aPLs were originally included in the laboratory criteria: anti-cardiolipin antibody (aCL) (either IgG or IgM), and lupus anticoagulant. In 2006, amendments to the Sapporo APS criteria were proposed at a workshop preceding the Eleventh International Congress on aPLs. Consequently, anti-β2 glycoprotein-I antibody (anti-β2GPI) of the IgG or IgM class was included as a

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laboratory criteria for the classification of definite APS (Miyakis et al., 2006).

Pregnancy-induced hypertension (PIH) and preeclampsia are amongst the major causes of mortality and morbidity during pregnancy and childbirth. The pathogenesis is multifactorial. PIH and preeclampsia can lead to multiple organ failure involving the cardiovascular and central nervous systems, the liver, and kidneys as well as cause coagulation breakdown. However, the association between aPLs and the risk of PIH and preeclampsia still remains controversial. Recently, we prospectively assessed aPLs including lupus anticoagulant, aCL, phosphatidylserine-dependent anti-prothrombin antibody, and kininogen-dependent anti-phosphatidylethanolamine antibody (aPE) in 1155 consecutive women during early pregnancy. We demonstrated that a positive test for aCL IgG or aPE IgG was associated with developing PIH during the third trimester in the SAPPORO study (Yamada et al., 2009). However, anti-β2GPI was not assessed in this study.

The present study was performed as a case-controlled study in a cohort of the SAPPORO study population to evaluate whether anti-β2GPI was associated with PIH or preeclampsia.

2. Materials and methods

2.1. Subjects

A previous study, designated Sapporo Multiple Anti-phospholipid Testing for the Prediction of Obstetric Outcome study (the SAPPORO study) was performed in the city of Sapporo, Japan, and conducted with informed consent from all of the subjects (Yamada et al., 2009). The study was approved by the institutional ethics board of Hokkaido University Graduate School of Medicine. Peripheral blood was obtained at 8–14 weeks of gestation from a consecutive series of 1155 women with living fetuses who visited the Hokkaido University Hospital or an affiliate hospital. The sera were collected and stored at −80 °C.

The present study was performed as a case-controlled study in a cohort from the SAPPORO study population, and comprised 36 cases that developed PIH during pregnancy. The 36 PIH cases (age range 22–41, mean ± SD 32.1 ± 4.6 years old, 25 (69%) nulliparity) included 13 severe PIH, 18 preeclampsia and 11 severe preeclampsia cases. A group of 111 age and parity-matched women whose pregnancies ended in normal delivery without obstetric complications were randomly selected as controls (age range 22–41, mean ± SD 32.1 ± 4.6 years old, 76 (69%) nulliparity). This random selection was performed by one scientist (I.F.) who is not a medical doctor and did not have the knowledge of results of other aPLs.

In this study, we used PIH and preeclampsia criteria defined by the Japan Society of Obstetrics and Gynecology. PIH was defined as hypertension (systolic blood pressure > 140 mm Hg or diastolic blood pressure > 90 mm Hg) detected after 20 weeks gestation. Severe PIH was defined when at least one of the following criteria was met: (1) blood pressure ≥ 160/110 mm Hg after 20 weeks gestation, regardless of the complication of proteinuria, or (2) blood pressure ≥140/90 mm Hg after 20 weeks gestation complicated by proteinuria ≥2.0 g/day. Preeclampsia was defined as hypertension (≥140/90 mm Hg) and proteinuria (≥300 mg/day) detected after 20 weeks gestation. Severe preeclampsia was defined when at least one of the following criteria was met: (1) blood pressure ≥160/110 mm Hg after 20 weeks gestation complicated by proteinuria ≥300 mg/day, or (2) blood pressure ≥140/90 mm Hg after 20 weeks gestation complicated by proteinuria ≥2.0 g/day. Blood pressures were measured repeatedly.

2.2. Anti-phospholipid antibody measurement

Anti-β2GPI antibodies were measured by ELISA in the stored sera using a protocol reported previously (Amengual et al., 1996). Purified human β2GPI was purchased from Yamasa Corp. Tokyo, Japan. Irradiated microtitre plates (Maxisorp, Nunc, Denmark) were coated with 4 μg/ml of purified β2GPI in phosphate-buffered saline (PBS) at 4 °C and washed twice with PBS. To avoid non-specific binding of proteins, wells were blocked with 150 μl of 3% gelatin (BDH Chemicals Ltd., Poole). After three washes with PBS containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO, USA) (PBS–Tween), 50 μl of serum sample diluted 1:50 with PBS containing 1% bovine serum albumin (Sigma) (PBS–1% BSA) was added to duplicate wells. Plates were incubated for 1 h at room temperature and washed three times with PBS–Tween. Fifty microlitres per well of the appropriate dilution of alkaline phosphatase-conjugated goat anti-human IgG and IgM (Sigma) in PBS–1%BSA was added. After 1 h of incubation at room temperature and after four washes in PBS–Tween, 100 μl/well of 1 mg/ml p-nitrophenylphosphate disodium (Sigma) in 1 M diethanolamine buffer (pH 9.8) was added. Following colour development, optical density at 405 nm was measured by a Multiskan ascent plate reader (Thermo electron corporation, Waltham MA, USA).

One of the serum samples that had showed high binding to β2GPI coated onto the irradiated plates was used as a positive control. Normal ranges of anti-β2GPI IgG (<2.2 U/ml) and IgM (<6.0 U/ml) with cut-off values at the 99th percentile were established in preliminary experiments using non-pregnant 132 healthy controls. Cut-off values of anti-β2GPI IgG (normal < 1.0 U/ml) and anti-β2GPI IgM (normal < 1.2 U/ml) were established as the most appropriate values dividing the subjects in this study. To define the intra-assay precision for anti-β2GPI IgG and IgM, 3 patient samples with high, medium and low titers of anti-β2GPI IgG or IgM were replicated 12 times on the same 96-well ELISA plate, and coefficients of variation were calculated from the optical density results.

2.3. Statistical analysis

Statistical differences were analyzed by the chi-square test (df = 1). Fisher's exact test was used when the number of observations was ≤5. A $P < 0.05$ was considered statistically significant. Odds ratios (OR) and 95% confidence intervals (95%CI) were calculated to evaluate the association between anti-β2GPI and PIH or preeclampsia. All statistical analyses were conducted with a statistical anal-

Table 1
IgG anti-β2 glycoprotein-I as a risk factor for pregnancy-induced hypertension and preeclampsia with a cut-off value or 2.2 U/ml.

Outcome	Positive frequency of IgG anti-β2GPI						*P* value	Odds ratio	95%* confidence intervals
	Women with positive other aPL[a]		Women with negative other aPL[a]		Total				
Normal (controls)	1/12	8.3%	5/99	5.1%	6/111	5.4%			
PIH	2/8	25.0%	2/28	7.1%	4/36	11.1%	0.26	2.2	0.6–8.3
Severe PIH	2/5	40.0%	1/8	12.5%	3/13	23.1%	0.053	5.3	1.1–24.3
Preeclampsia	1/4	25.0%	1/14	7.1%	2/18	11.1%	0.31	2.2	0.4–11.8
Severe preeclampsia	1/3	33.3%	1/8	12.5%	2/11	18.2%	0.15	3.9	0.7–22.1

PIH, pregnancy-induced hypertension; anti-β2GPI, anti-β2 glycoprotein-I antibody; aPL, anti-phospholipid antibody.
[a] Other aPLs include IgG/IgM anti-cardiolipin antibody, lupus anticoagulant, IgG/IgM phosphatidylserine-dependent anti-prothrombin antibody, and IgG kininogen-dependent anti-phosphatidylethanolamine.

ysis system package (SAS version 9.1, SAS Institute Japan Ltd., Tokyo, Japan).

3. Results

The numbers of women with a positive anti-β2GPI IgG test of ≥2.2 U/ml (or ≥1.0 U/ml) were as follows: 6 (8) in 111 controls, 4 (6) in 36 PIH, 3 (4) in 13 severe PIH, 2 (4) in 18 preeclampsia, and 2 (3) in 11 severe preeclampsia. We found no women tested positive for ≥6.0 U/ml of anti-β2GPI IgM. The numbers of women with a positive anti-β2GPI IgM test of ≥1.2 U/ml were as follows: 2 in controls, 5 in PIH, 2 in severe PIH, 2 in preeclampsia, and 1 in severe preeclampsia. The anti-β2GPI intra-assay coefficients of variation for the high, medium and low positive samples were 4.4%, 4.1% and 8%, respectively for the IgG assay, and 4.6%, 5.4% and 8.8% for the IgM assay, representing good assay precision.

The results of statistical analyses are shown in Tables 1–3. A titer ≥1.0 U/ml of anti-β2GPI IgG was found to be a significant risk factor for severe PIH (P = 0.023, OR 5.7 95%CI 1.4–22.8) (Table 2). A titer ≥1.2 U/ml of anti-β2GPI IgM was a significant risk factor for PIH (P = 0.001, OR 8.8 95%CI 1.6–47.5) (Table 3). We also found a possible association between titers ≥1.0 U/ml of anti-β2GPI IgG and severe preeclampsia (P = 0.061, OR 4.8 95%CI 1.1–21.8) (Table 2), between titers ≥2.2 U/ml of anti-β2GPI IgG and severe PIH (P = 0.053, OR 5.3 95%CI 1.1–24.3) (Table 1), and between titers ≥1.2 U/ml of anti-β2GPI IgM and severe PIH (P = 0.054, OR 9.9 95%CI 1.3–77.4) (Table 3). However, these associations did not reach statistical significance.

Of the 6 PIH women with titers ≥1.0 U/ml of anti-β2GPI IgG, one had aPE, another had aCL plus lupus anticoagulant, and the other 4 women had neither lupus anticoagulant, aCL, phosphatidylserine-dependent anti-prothrombin antibody, nor aPE. Among the 30 PIH women with a negative test of anti-β2GPI IgG, five had aPE IgG, one had aCL plus phosphatidylserine-dependent anti-prothrombin antibody, and the other 24 women had no aPLs. Of the 8 control women with titers ≥1.0 U/ml of anti-β2GPI IgG, two had phosphatidylserine-dependent anti-prothrombin antibody, and the other 6 women had no aPLs. Of the 103 control women testing negative for anti-β2GPI IgG, eight had aPE IgG, two had phosphatidylserine-dependent anti-prothrombin antibody, one had aCL, one had lupus anticoagulant, and the other 91 women had no aPLs. These aPL characterisics were quoted from known data in the SAPPORO study (Yamada et al., 2009).

Similarly, of the 5 PIH women with titers ≥1.2 U/ml of anti-β2GPI IgM, one had aCL plus lupus anticoagulant, another had aCL plus phosphatidylserine-dependent anti-prothrombin antibody and the other 3 women had neither lupus anticoagulant, aCL, phosphatidylserine-dependent anti-prothrombin antibody, nor aPE. Among the 31 PIH women testing negative for anti-β2GPI IgM, six had aPE IgG, and the other 25 women had no aPLs. Of the 2 control women with titers ≥1.2 U/ml of anti-β2GPI

Table 2
IgG anti-β2 glycoprotein-I as a risk factor for pregnancy-induced hypertension and preeclampsia with a cut-off value of 1.0 U/ml.

Outcome	Positive frequency of IgG anti-β2GPI						*P* value	Odds ratio	95% confidence intervals
	Women with positive other aPL[a]		Women with negative other aPL[a]		Total				
Normal (controls)	2/12	16.7%	6/99	6.1%☆	8/111	7.2%*			
PIH	2/8	25.0%	4/28	14.3%☆	6/36	16.7%	0.065	2.6	0.8–8.8
Severe PIH	2/5	40.0%	2/8	25.0%☆	4/13	30.8%*	0.023	5.7	1.4–22.8
Preeclampsia	1/4	25.0%	3/14	21.5%☆	4/18	22.2%	0.065	3.2	0.9–11.9
Severe preeclampsia	1/3	33.3%	2/8	25.0%☆	3/11	27.3%	0.061	4.8	1.1–21.8

PIH, pregnancy-induced hypertension; anti-β2GPI, anti-β2 glycoprotein-I antibody; aPL, antiphospholipd antibody.
[a] Other aPLs include IgG/IgM anti-cardiolipin antibody, lupus anticoagulant, IgG/IgM phosphatidylserine-dependent anti-prothrombin antibody, and IgG kininogen-dependent anti-phosphatidylethanolamine.
* Statistically significant (P = 0.023).
☆ Not significant (P > 0.05).

Table 3
IgM anti-β2 glycoprotein-I as a risk factor for pregnancy-induced hypertension and preeclampsia with a cut-off value of 1.2 U/ml.

Outcome	Positive frequency of IgM anti-β2GPI						*P* value	Odds ratio	95% confidence intervals
	Women with positive other aPL[a]		Women with negative other aPL[a]		Total				
Normal (controls)	0/12	0%	2/99	2.0%☆	2/111	1.8%*			
PIH	2/8	25.0%	3/28	10.7%☆	5/36	13.9%*	0.001	8.8	1.6–47.5
Severe PIH	1/5	20.0%	1/8	12.5%☆	2/13	15.4%	0.054	9.9	1.3–77.4
Preeclampsia	0/4	0%	2/14	14.3%☆	2/18	11.1%	0.093	6.8	0.9–51.8
Severe preeclampsia	0/3	0%	1/8	12.5%☆	1/11	9.1%	0.249	5.5	0.5–65.5

PIH, pregnancy-induced hypertension; anti-β2GPI, anti-β2 glycoprotein-I antibody; aPL, anti-phospholipid antibody.
[a] Other aPLs include IgG/IgM anti-cardiolipin antibody, lupus anticoagulant, IgG/IgM phosphatidylserine-dependent anti-prothrombin antibody, and IgG kininogen-dependent anti-phosphatidylethanolamine.
* Statistically significant ($P = 0.001$).
☆ Not significant ($P > 0.05$).

IgM, none had aPLs. Of the 109 control women testing negative for anti-β2GPI IgM, eight had aPE IgG, two had phosphatidylserine-dependent anti-prothrombin antibody, one had aCL, one had lupus anticoagulant, and the other 97 women had no aPLs.

Among women negative for other aPL, the incidence of positivity for anti-β2GPI IgG (≥2.2 U/ml) in women with PIH ($P = 0.96$, OR 1.5 95%CI 0.3–7.9), severe PIH ($P = 0.93$, OR 2.7 95%CI 0.3–26.3), preeclampsia ($P = 0.75$, OR 1.4 95%CI 0.16–13.4) or severe preeclampsia ($P = 0.93$, OR 2.7 95%CI 0.3–26.3), was not significantly different from those in controls (Table 1). Similarly, among women negative for other aPL, the incidence of positivity for anti-β2GPI IgG (≥1.0 U/ml) in women with PIH ($P = 0.30$, OR 2.6 95%CI 0.7–9.9), severe PIH ($P = 0.21$, OR 5.2 95%CI 0.9–31.3), preeclampsia ($P = 0.14$, OR 4.2 95%CI 0.9–19.3) or severe preeclampsia ($P = 0.21$, OR 5.2 95%CI 0.9–31.3), was not significantly higher than in controls (Table 2). Among women negative for other aPL, the incidence of positivity for anti-β2GPI IgM (≥1.2 U/ml) in groups with PIH ($P = 0.12$, OR 5.8 95%CI 0.9–36.7), severe PIH ($P = 0.54$, OR 6.9 95%CI 0.6–86.1), preeclampsia ($P = 0.12$, OR 8.1 95%CI 1.0–62.7) or severe preeclampsia ($P = 0.54$, OR 6.9 95%CI 0.6–86.1) was not significantly higher than in controls (Table 3).

4. Discussion

It has been reported that anti-β2GPI is associated with increased risk of recurrent spontaneous abortion and pregnancy loss among women with aPLs such as lupus anticoagulant (Falcón et al., 1997; Forastiero et al., 1997; Lee et al., 1999; Sailer et al., 2006), and among women without lupus anticoagulant (Stern et al., 1998). Conversely, other studies did not find an association (Ailus et al., 1996; Arnold et al., 2001). Previous prospective studies assessing associations between anti-β2GPI and PIH or preeclampsia also found conflicting results. One report noted that preeclampsia and eclampsia were related to the presence of anti-β2GPI in the maternal blood (Faden et al., 1997). However, other studies reported no association between anti-β2GPI and PIH (Lynch et al., 1999), or preeclampsia and HELLP syndrome (Lee et al., 2003). In the present study, we demonstrated that a positive test of anti-β2GPI IgG in early pregnancy was a significant risk factor for later developing severe PIH; and that a positive test of anti-β2GPI IgM was a significant risk factor for later developing PIH. It seems that the rate of positive anti-β2GPI test in a subset of severe cases is higher than those in the total PIH group. Of the PIH women with anti-β2GPI IgG or IgM, only one patient had lupus anticoagulant, suggesting that our study population was little affected by other aPLs. However, our results contrast with the lack of association observed in the abovementioned two cohort studies (Lynch et al., 1999; Lee et al., 2003). Most subjects of the former study included mild, but not severe PIH (Lynch et al., 1999). The latter study showed low frequencies of positive anti-β2GPI IgG test in controls (2%) and severe PIH (2%) using their cut-off values (Lee et al., 2003). The discrepancy may be explained by the difference in populations included in the studies, or related to the definition of the cut-off levels. Anti-β2GPI assays are not universally standardized, which leads to inter-laboratory variation.

There is a large body of evidence for an involvement of anti-β2GPI in hypercoagulation status and thrombosis (Martinuzzo et al., 1995; Amengual et al., 1996; Zanon et al., 1999; Zoghlami-Rintelen et al., 2005; Pengo et al., 2005; de Laat et al., 2004). A multivariate analysis in a multicenter study has demonstrated that anti-β2GPI and aPE, but not lupus anticoagulant or aCL, were significantly associated with thrombosis (Sanmarco et al., 2007). Anti-β2GPI induces activation of endothelial cells, resulting in a proinflammatory state which favours prothrombotic diathesis (D'Ippolito et al., 2007). Recently, a study has demonstrated β2GPI can naturally inhibit von Willebrand factor (VWF)-dependent platelet adhesion and aggregation. Anti-β2GPI of APS patients neutralized the β2GPI–VWF interactions, contributing to hypercoagulation status in these patients (Hulstein et al., 2007). It is likely that the thrombotic insult of anti-β2GPI to placental angiogenesis or the circulation is causally associated with PIH. Additionally, β2GPI binds to trophoblast cells (Di Simone et al., 2007). Antibody binding to β2GPI downregulates trophoblast chorionic gonadotropin synthesis and secretion (Di Simone et al., 2005). Such a direct effect on trophoblast may contribute to inhibition of trophoblast invasiveness and lead to defective placentation (Di Simone et al., 2007), and may be causally associated with PIH.

In conclusion, anti-β2GPI was found to represent a risk factor for developing PIH in this case-controlled cohort study, providing evidence to support the utility of anti-β2GPI determination as one of the laboratory criteria for APS classification. In the previous SAPPORO prospective study, aPLs were measured during pregnancy and women with a history of recurrent spontaneous abortion or thrombosis who tested positive for lupus anticoagulant or aCL underwent low dose aspirin therapy. The knowledge of the presence of these aPLs could potentially influence the physician in favour of an early pregnancy termination. These possible biases can be excluded in the present case-controlled study. However, the subject number in our study is relatively small and furthermore we did not measure anti-β2GPI repeatedly 12 weeks apart, as required by the updated SAPPORO criteria (Miyakis et al., 2006). It is well known that aPLs share antigen epitopes and presence of one aPL increases the chance of the presence of the other aPLs. In women positive for anti-β2GPI but negative for lupus anticoagulant, aCL, phosphatidylserine-dependent anti-prothrombin antibody, or aPE, the presence of anti-β2GPI was not a significant risk factor for development of PIH or preeclampsia. This may partly be due to small numbers but it is also possible that an anti-β2GPI is only a marker for the presence of other more important aPLs. Larger studies designed to include appropriate adjustments for the presence of several aPLs should be undertaken to clarify this.

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CASE REPORT

Intravenous immunoglobulin therapy for aspirin-heparinoid-resistant antiphospholipid syndrome

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Abstract We encountered a woman who had a history of repeated fetal losses and positive tests for lupus anticoagulant, phosphatidylserine-dependent antiprothrombin (aPS/PT) IgG, IgM and kininogen-dependent antiphosphatidylethanolamine (aPE) IgG, IgM. Her previous pregnancy had ended in intrauterine fetal death at 24 weeks of gestation despite a therapy of low-dose aspirin, prednisolone and danaparoid. During the present pregnancy, she was treated with repeated intravenous infusions of immunoglobulin (IVIg) together with low-dose aspirin, prednisolone and heparin. When thrombocytopenia developed, she delivered a female baby weighing 2,152 g at 34 weeks of gestation by cesarean section. Titers of aPS/PT IgM and aPE IgM were reduced or maintained at low levels by repeated IVIg therapies. The IVIg therapy might be effective for aspirin-heparinoid-resistant antiphospholipid syndrome.

Keywords Antiphospholipid syndrome · Aspirin · Heparin · Heparinoid · Immunoglobulin

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Introduction

Women with antiphospholipid syndrome (APS) have an increased risk of pregnancy loss and obstetrical complications. Detrimental effects of antiphospholipid antibody (aPL) are attributed to pathological mechanisms including thrombotic changes, suppression of hCG release [1], induction of complement activation and placental injury [2], and a direct effect on trophoblast cells [3]. Although the management of pregnancy in women with APS has been a subject of much debate, antiplatelet and anticoagulation therapies are usually recommended [4]. A randomized controlled study demonstrated a high live birth rate (71%) with low-dose aspirin (LDA) plus heparin as compared with 42% with LDA alone in APS women [5]. The women treated with LDA plus heparin had fewer maternal adverse effects, and this treatment was found to be superior to LDA plus steroids [6].

However, we encounter APS women who undergo LDA plus heparin/heparinoid with or without steroids and fail to have a healthy infant. Such patients can be designated as having aspirin-heparin/heparinoid-resistant APS. In the present report, a case of aspirin-heparinoid-resistant APS was treated successfully with repeated intravenous infusions of immunoglobulin (IVIg), LDA, heparin and prednisolone. We assessed changes in levels of serum complements and antiphospholipid antibodies (aPLs) through the course of pregnancy.

Case report

A 38-year-old non-pregnant woman with a history of repeated fetal losses and APS was referred to the infertility clinic of The Hokkaido University Hospital for

consultations. Her first pregnancy had ended in spontaneous abortion at 7 weeks of gestation (GW). Only the gestational sac was detected without fetal heart movement in this missed abortion. She was treated with LDA (81 mg/day), danaparoid sodium (Orgaran™, Schering-Plough Co., Ltd., Tokyo, Japan; 2,500 unit/day) and prednisolone (PSL 10 mg/day) during the second pregnancy. However, growth restriction and mild preeclampsia developed at 22 GW, and this pregnancy resulted in intrauterine death of a female fetus weighing 344 g at 24 GW. Pathological examination of the placenta revealed ischemic changes with relatively small villi, abundant syncytial nodes, fibrin depositions and necrosis.

Laboratory data obtained in our infertility clinic after her first visit were as follows: white blood cells 6,400/μl, red blood cells 336 × 10^4/μl, hemoglobin 11.9 g/dl, hematocrit 34.4%, platelet 29.4 × 10^4/μl, fibrinogen 255 mg/dl, fibrinogen degradation product <2.6 mg/ml, D-dimer 0.58 mg/ml (normal < 1.0), prothrombin time 11.6 s, APTT 44.2 s (normal 26.1–36.5), coagulation factor XII 30.5% (normal > 50.0), C-reactive protein 0.03 mg/dl, C3 55 mg/dl (normal 86–160), C4 8 mg/dl (normal 17-45), CH50 20.3 U/ml (normal 31.5–48.4), antinuclear antibody (ANA) 1:640, anti-DNA antibody 0.0 IU/ml, anti-SSA antibody 122.3 index (normal < 10.0), anticardiolipin (aCL) IgG < 8 U/ml, aCL IgM < 5 U/ml, β2 glycoprotein I-dependent anticardiolipin (aCLβ2GPI) IgG < 1.3 U/ml, phosphatidylserine dependent anti-prothrombin (aPS/PT) IgG 235 U/ml (normal < 2.0), aPS/PT IgM 37.5 U/ml (normal < 9.2), lupus anticoagulant (LA) 2.01 (normal < 1.3; DRVVT method), PAIgG 33.5 ng/10^7 cells (normal 9.0–25.0), and negative tests for anti-platelet antibody or proteinurea. Thus, decreases in levels of serum C3, C4, CH50 concentrations and factor XII activity, and positive tests for ANA, anti-SSA antibody, aPS/PT IgG, aPS/PT IgM, LA and PAIgG were detected prior to her third pregnancy. On that occasion, kininogen-dependent anti-phosphatidylethanolamine (aPE) IgG or IgM was not measured.

A combined therapy of LDA (81 mg/day, orally), PSL (10 mg/day, orally), unfractionated heparin (5,000 unit × 2 sc/day) and intravenous infusions of intact type immunoglobulin (IVIg) for the third pregnancy was planned with informed consent. LDA was commenced before conception. Immediately after a positive pregnancy test was obtained, she was hospitalized, and received PSL and unfractionated heparin. LDA was maintained until 31 GW. PSL (10 mg/day) was increased to 15 mg/day at 13 GW, because serum levels of complements (C3, C4, CH50) decreased; PSL was increased to 30 mg/day for 2 weeks at 26 GW, because serum levels of C4 and CH50 tended to decrease further. Unfractionated heparin (10,000 unit/day) was increased to 12,000 units/day at 24 GW (Fig. 1).

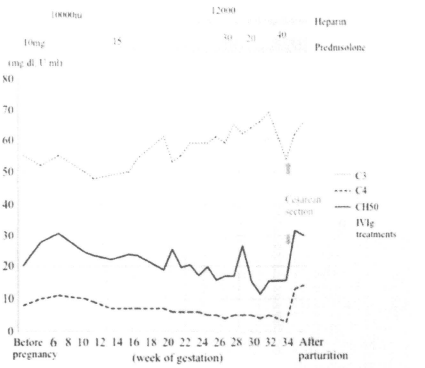

Fig. 1 Changes in serum complement levels

At 12 GW, the first course of IVIg therapy (20 g/day, 5 consecutive days, total 100 g) was employed using Kenketsu Glovenin-I™ (Nihon Pharmaceutical Co., Ltd., Tokyo) or Kenketsu Venilon-I™ (Teijin Co., Ltd., Tokyo, Japan) followed by 40 g (20 g/day, 2 consecutive days) of IVIg at 16 GW. After IVIg (20 g/day, 1 day) at 20 GW skin eruptions developed. Therefore, additional IVIg (20 g/day, 1 day) was applied at 22 GW. At 24 and 28 GW, administration of 40 g of IVIg was completed without adverse effects (Fig. 1).

The fetal growth was appropriate for the gestational age, and no sign of pregnancy-induced hypertension or abnormal maternal blood tests was detected until 32 GW. Then, platelet counts decreased from 14.5 × 10^4/μl to 6.9 × 10^4/μl. PSL was increased to 40 mg/day, and IVIg therapy (20 g/day, 5 consecutive days, total 100 g) was performed with informed consent. However, platelet counts minimally increased to 8.3 × 10^4/μl. Therefore, we performed cesarean section at 34 GW, and she delivered a female baby weighing 2,152 g without thrombocytopenia or neonatal lupus erythematosus. Before cesarean section, unfractionated heparin was changed to 5,000 unit/day of low molecular weight heparin and dalteparin sodium (Fragmin™, Eisai Co., Ltd., Tokyo, Japan). PSL was tapered off after the operation. The patient, with a platelet count of 15.6 × 10^4/ml, was discharged 11 days after delivery. Placental pathological findings indicated small infarctions and calcification.

We repeatedly measured aPLs during the pregnancy. Serum levels of aCL IgG, aCL IgM or aCLβ2GPI IgG were all negative through the course of pregnancy. LA tests were constantly positive at similar levels (1.7–1.9) during pregnancy. We measured aPE IgG and IgM from 13 GW

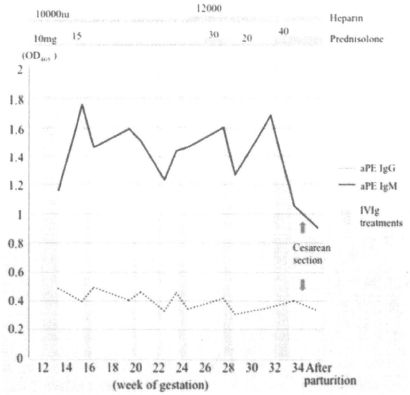

Fig. 2 Changes in titers of kininogen-dependent antiphosphatidylethanolamine antibodies. aPE, kininogen-dependent antiphosphatidylethanolamine

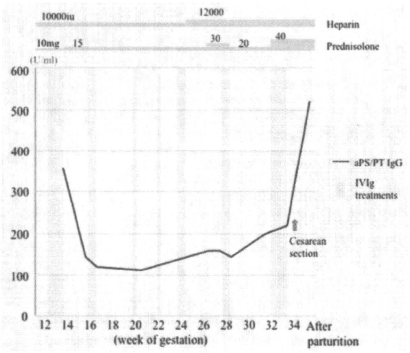

Fig. 3 Changes in titers of phosphatidylserin-dependent antiprothrombin IgG. aPS/PT, phosphatidylserin-dependent antiprothrombin

of the index pregnancy. Figure 2 shows the changes of serum levels of aPE IgG (normal range < 0.300) and IgM (normal range < 0.450). The level of aPE IgG tended to decrease during pregnancy. The level of aPE IgM increased from 1.16 at 13 GW to 1.75 at 15 GW, and significantly ($P = 0.028$ by paired t test) decreased after each course of IVIg therapy. The levels of aPE IgM (mean + SD 1.60 + 0.10, range 1.48–1.75) 4 days before commencement of IVIg decreased to 1.33 + 0.18 (range 1.06–1.51) 2

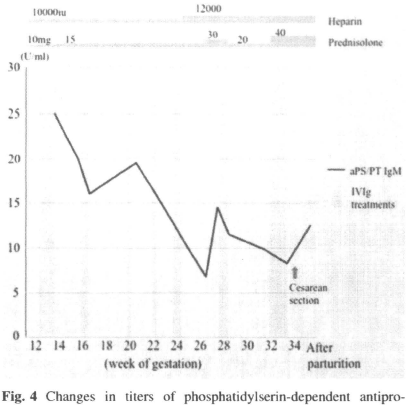

Fig. 4 Changes in titers of phosphatidylserin-dependent antiprothrombin IgM. aPS/PT, phosphatidylserin-dependent antiprothrombin

or 3 days after completion of IVIg. Titers of aPS/PT IgM were reduced; titers of aPS/PT IgG at 13 GW were higher than those of nonpregnant status, but were maintained at low levels during repeated IVIg therapies (Figs. 3, 4). The measurement methods of all of the abovementioned aPLs and the normal ranges were shown elsewhere [7, 8].

Discussion

This case of aspirin-heparinoid-resistant APS was treated successfully by repeated IVIg together with LDA, heparin and steroids. Titers of aPS/PT IgG, IgM and aPE IgG, IgM were reduced or maintained at low levels. The level of aPE IgM significantly decreased after each IVIg treatment. It was known that heparin had a function of suppressing the complement activity and protected mice from pregnancy complications induced by aPL [9]. Other investigators reported the function of heparin to inactivate complements in various diseases [10]. Therefore, we increased a dose of heparin at 24 GW when serum levels of C4, CH50 decreased. However, these complement levels were not restored, so we increased the dose of prednisolone at 26 GW.

Carreras et al. [11] first reported successful IVIg therapy in a pregnant woman with LA and a history of nine recurrent pregnancy losses (RPL). A randomized controlled trial comparing LDA plus heparin plus IVIg with LDA plus heparin therapies in 16 APS patients failed to show differences in the efficacy [12]. Triolo et al. [13] reported that administration of LDA plus low molecular weight heparin resulted in a higher birth rate (84%) than

IVIg alone (57%) in RPL women with aCL/β2GPI. But later, they also reported successful IVIg therapy in eight of ten APS women previously unresponsive to LDA plus heparin [14]. Therefore, a certain subgroup of APS women who are resistant to aspirin-heparin therapy as presented in the present report might benefit from the possible advantages of IVIg therapy.

The optimal dosage of IVIg in APS women during pregnancy was not determined and still needs to be debated. Yamada et al. first performed high-dose IVIg therapy (20 g/day, 5 consecutive days, total 100 g) in early pregnancies of women with unexplained severe RPL, demonstrating a high live birth rate [15–17]. Carreras et al. [11] performed IVIg therapy (400 mg/kg day, 5 consecutive days at 17 GW; and 2 days at 22, 27 GW) in APS women. Others reported monthly 1 g/kg IVIg therapies [14]. The present patient had a history of intrauterine fetal death at 24 GW, so we planned high-dose IVIg therapy at 12 GW followed by cyclic courses of 40 g IVIg every 4 weeks from 12 to 32 GW.

The mechanisms of IVIg efficacy for pregnant women with APS have not been fully assessed. The following possible mechanisms explain its broad activity: (1) provision of anti-idiotypic antibodies and the function as an immunomodulator, (2) interference with the complement activation and the cytokine network, (3) modulation of the expression and function of Fc receptors, and (4) differentiation and effector functions of T and B cells [18, 19]. As for the anti-idiotypic antibody function, inhibitory effects of IVIg on aCL and LA were reported [20–22]. Caccavo et al. [20] demonstrated that aCL binding to cardiolipin was suppressed by $F(ab')_2$ fragments derived from IVIg in a dose-dependent manner. Galli et al. [21] also demonstrated dose-dependent suppression of LA activity in patients, using either IVIg or $F(ab)_2$ fragments. IVIg may induce a long-term decrease in autoantibody production by acquiring the inactivation of idiotype-bearing B cell clones [23]. We for the first time found that repeated IVIg reduced serum levels of aPS/PT and aPE in the present patient with aspirin-heparinoid-resistant APS, and IVIg might have anti-idiotypic antibody effects against these aPLs.

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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

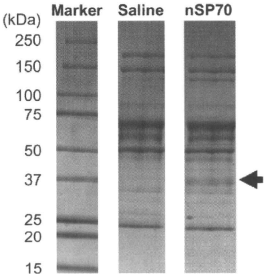

Fig. 1. SDS-PAGE analysis of plasma proteins. BALB/c mice were intravenously treated with nSP70 or saline at 0.8 mg/mouse. After 24 h, blood samples were collected. The change of protein levels in plasma after treatment of nSP70 was assessed by SDS-PAGE.

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

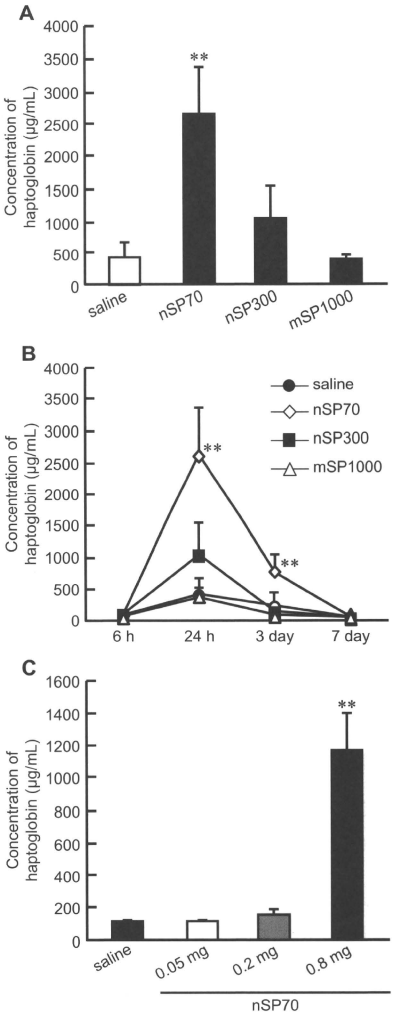

Fig. 2. The potential of haptoglobin as biomarker of nanomaterials. (A) The level of haptoglobin after treatment with silica particles. BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 24 h, the level of haptoglobin in the plasma of each mouse was examined by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

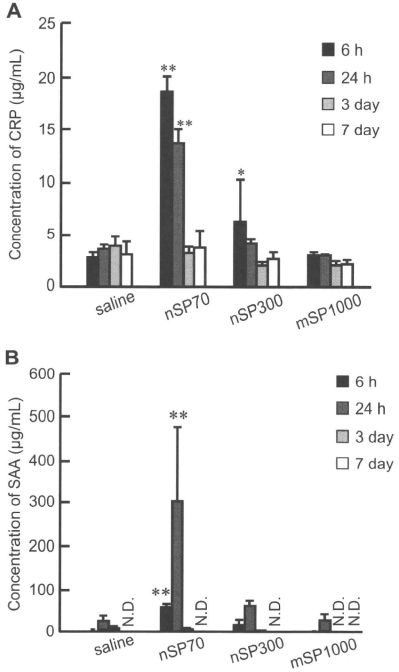

Fig. 3. Response of other acute phase proteins. BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 6 h, 24 h, 3 day and 7 day, blood samples were collected. The levels of (A) CRP and (B) SAA in the plasma of treated mice were examined by ELISA. Data are presented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

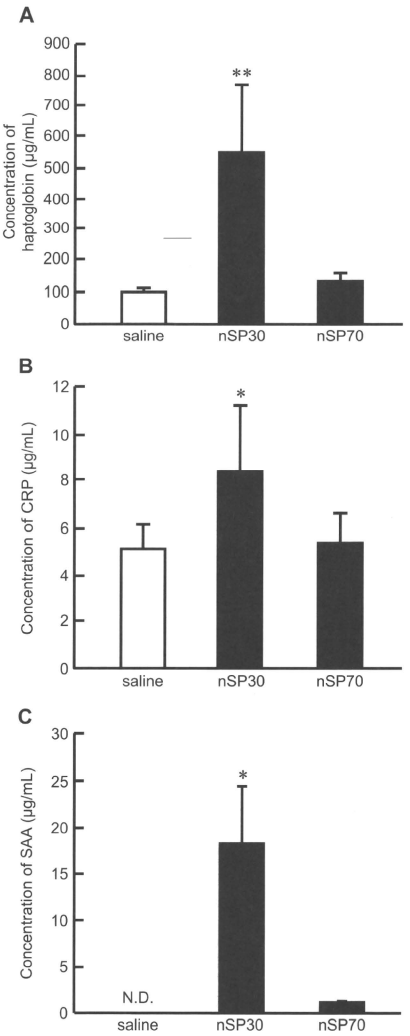

Fig. 4. Application of acute phase proteins to assess exposure of nanomaterials through various routes. To assess the administration of silica nanoparticles through an intranasal route, BALB/c mice were treated with nSP30, nSP70 or saline intranasally at 0.5 mg/mouse. Blood samples were collected 24 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).

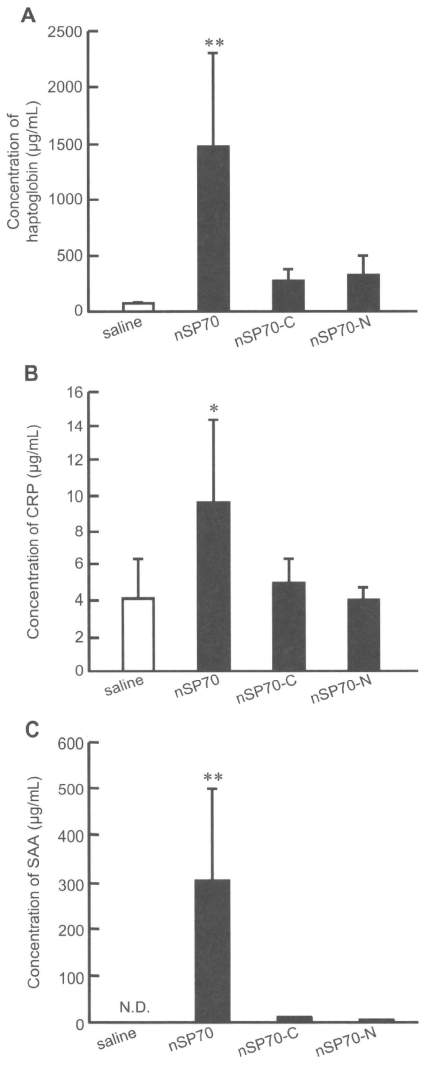

Fig. 5. Responses of acute phase proteins by the exposure to surface modified nSP70. BALB/c mice were intravenously treated with nSP70 modified with amino or carboxyl groups at 0.8 mg/mouse. After 24 h, the level of (A) haptoglobin, (B) CRP and (C) SAA in the plasma of treated mice were examined by ELISA. 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.

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