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Liver-specific microRNAs as biomarkers of nanomaterial-induced liver damage

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Abstract

Although nanomaterials are being used in various fields, their safety is not yet sufficiently understood. We have been attempting to establish a nanomaterials safety-assessment system by using biomarkers to predict nanomaterial-induced adverse biological effects. Here, we focused on microRNAs (miRNAs) because of their tissue-specific expression and high degree of stability in the blood. We previously showed that high intravenous doses of silica nanoparticles of 70 nm diameter (nSP70) induced liver damage in mice. In this study, we compared the effectiveness of serum levels of liver-specific or -enriched miRNAs (miR-122, miR-192, and miR-194) with that of conventional hepatic biomarkers (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) as biomarkers for nSP70. After mice had been treated with nSP70, their serum miRNAs levels were measured by using quantitative RT-PCR. Serum levels of miR-122 in nSP70-treated mice were the highest among the three miRNAs. The sensitivity of miR-122 for liver damage was at least as good as those of ALT and AST. Like ALT and AST, miR-122 may be a useful biomarker of nSP70. We believe that these findings will help in the establishment of a nanomaterials safety-assessment system.

1. Introduction

Nanomaterials are defined as substances that have at least one dimension less than 100 nm long. They are now widely used in cosmetics, foods, and medicines because they possess innovative functions such as high electrical conductivity, tensile strength, and tissue permeability [1, 2]. However, with the development of nanomaterials has come

concern that, unlike the case with micro-sized materials, their unique characteristics may induce unexpected biological responses [3–5]. For example, recent reports have shown that carbon nanotubes can induce mesothelioma-like lesions in mice, similar to those induced by asbestos [6]. In addition, our group has shown that silica nanoparticles with a diameter of 70 nm (nSP70) can induce severe liver damage and pregnancy complications in mice, and reactive oxygen species (ROS) generation and DNA damage *in vitro*, whereas micro-sized silica particles do not have these effects [7–10]. Nevertheless, because nanomaterials have the potential to improve our quality of life, it is important that we develop and promote the use of safe forms. Furthermore, the development of

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biomarkers of the biological effects of nanomaterials would be invaluable for establishing a nanomaterials safety-assessment system and strategies for the development, production, and use of safe forms of nanomaterials. We have already explored biomarkers, with a focus on proteins, and have shown that acute-phase proteins such as haptoglobin, serum amyloid A, and C-reactive protein could be useful biomarkers of the biological effects of silica or platinum nanoparticles [11–13]. However, the use of proteins alone is not sufficient for predicting the adverse biological effects of nanomaterials, as we need to evaluate biomarkers consisting of other biological molecules.

microRNAs (miRNAs) are highly conserved and small (18- to 25-nucleotide) RNAs that play pivotal roles in gene expression—specifically at the post-transcriptional level—in plants and animals. In humans, miRNAs are involved in the regulation of development, cell differentiation, proliferation, and apoptosis [14, 15], because they regulate as many as one-third of all messenger RNAs (mRNAs) [16, 17]. Furthermore, recent reports have demonstrated that miRNAs would serve as useful biomarkers for the non-invasive, tissue-specific evaluation of diseases and drug-induced tissue damage [18, 19]. This is because they show tissue-specific expression [20, 21] and exist in a stable form in the blood, saliva, and urine [22–24].

From this perspective, we focused on miRNAs as biomarkers for predicting the biological effects of nanomaterials. Although there have been some analyses of the changes in miRNA expression following exposure to nanomaterials [25, 26], there have been very few attempts to use miRNAs as biomarkers of nanomaterials. We therefore need to collect systematic information on miRNAs as biomarkers so as to predict nanomaterials-induced biological effects and thus establish a nanomaterials safety-assessment system.

In this study, as a first trial, we attempted to investigate whether liver-specific or -enriched miRNAs (miR-122, miR-192, and miR-194) could be used as reliable biomarkers for liver damage induced by nSP70.

2. Materials and methods

2.1. Materials

Silica particles were purchased from micromod Partikeltechnologie (Rostock/Warnemünde, Germany). Silica particles with diameters of 70, 300, and 1000 nm (nSP70, nSP300, and mSP1000, respectively) were used. In addition, we used nSP70 modified with surface functional groups—a carboxyl group (nSP70-C) or an amino group (nSP70-N). The particles were sonicated for 5 min and then vortexed for 1 min before use. Lipopolysaccharide (LPS) and D-galactosamine hydrochloride (D-GalN) were purchased from Sigma-Aldrich (Tokyo, Japan).

2.2. Animals

Female BALB/c mice were purchased from Nippon SLC, Inc. (Shizuoka, Japan) and used at 6–7 weeks of age. All of the animal experimental procedures were performed in

accordance with the Osaka University and National Institute of Biomedical Innovation guidelines for the welfare of animals.

2.3. Blood sample collection

BALB/c mice ($n = 5$ or 6 per group) were treated in various experiments with nSP70 at 10, 20, or 40 mg kg^{−1} or with nSP70-C, nSP70-N, nSP300, or mSP1000 at 40 mg kg^{−1}, via injection into the tail vein. As positive control of induction of liver damage, BALB/c mice ($n = 5$ or 6 per group) were intraperitoneally treated with LPS at 10 μg kg^{−1} and D-GalN at 700 mg kg^{−1}. Blood samples were collected at varying times (4, 8, 24, or 72 h after treatment) in several experiments. Sera were harvested by blood centrifugation at 8000g for 15 min.

2.4. Biochemical analysis

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with a DRI-CHEM 7000 biochemical analyzer (Fujifilm Corp., Tokyo, Japan).

2.5. Isolation of total RNA from serum

We extracted total RNA, including small RNA, from serum samples by using a miRNeasy Mini Kit (QIAGEN, Tokyo, Japan) in accordance with the manufacturer's instructions. In brief, 250 μl of QIAzol lysis reagent was added to 50 μl of serum. After the addition of 50 μl of chloroform, the samples were mixed completely and centrifuged. Then, 100 μl of supernatant was transferred to a new tube and mixed with 150 μl of 100% ethanol. The sample was then applied directly to a RNeasy Mini spin column (QIAGEN) and the combined RNA to column was cleaned with wash buffers to remove impurities. Total RNA was eluted in 30 μl of RNase-free water.

2.6. Real-time quantitative reverse-transcription PCR (qRT-PCR)

Serum levels of miR-122, miR-192, and miR-194 were measured by using TaqMan RT-PCR. Each miRNA in 5 μl of purified total RNA was reverse transcribed with a miRNA-specific stem-loop reverse transcriptase primer (Life Technologies, Tokyo, Japan). TaqMan RT-PCR was performed on a StepOne Plus real-time PCR system (Life Technologies, Tokyo, Japan). Generally, typical internal controls for miRNA expression, such as U6 RNA and 5S rRNA, are degraded in serum samples [27]. Therefore, we equalized all of the conditions and then normalized the levels of each miRNA by serum volume. The expression level of each miRNA is shown as a relative fold-change. Relative expression was compared between the silica-particle-treated group and the untreated group.

2.7. Statistical analyses

All results are expressed as means ± SEM differences were compared by using the Student's *t*-test or Bonferroni test after ANOVA (analysis of variance).

3. Results

3.1. Physicochemical properties of silica nanoparticles

Silica nanoparticles are common nanomaterials that are being used in cosmetics, foods, and medicines [28, 29]. Considering that further use of silica nanoparticles is expected, it is important to assess their safety. We used silica particles with diameters of 70 nm (nSP70), 300 nm (nSP300), and 1000 nm (mSP1000), as well as nSP70 with carboxyl (nSP70-C) or amino (nSP70-N) surface functional groups. In our previous study, we confirmed by transmission electron microscopy that all silica particles of the above-listed sizes and types were smooth-surfaced spheres [7–10]. We have already shown that the hydrodynamic diameters of nSP70, nSP70-C, nSP70-N, nSP300, and mSP1000 are 64.6, 69.6, 71.8, 322, and 1140 nm, respectively, and their zeta potentials (overall surface potentials) are -52.7 , -76.3 , -29.0 , -62.1 , and -67.0 mV, respectively [7–10]. The carboxyl and amino surface modifications therefore altered the surface charge of the nSP70 particles. In addition, we have already shown that the size-distribution spectrum of each set of silica particles had a single peak, and the measured hydrodynamic diameter corresponded almost precisely to the primary particle size of each set of silica particles [7–10]. These results indicated that the silica particles used in this study were well dispersed in solution.

3.2. Liver-specific or -enriched miRNAs as biomarkers of silica nanoparticles

To evaluate the usefulness of microRNAs (miRNAs) as biomarkers for nanomaterials, we focused on a model of liver damage induced by nSP70. We have previously shown that treatment of mice with high-dose nSP70 intravenously via the tail vein induced lethal toxicity and severe liver damage. Therefore, we assessed the usefulness of the liver-specific or -enriched miRNAs miR-122, miR-192, and miR-194 as biomarkers of liver damage. miR-122 and miR-192 are the two miRNAs expressed most abundantly in the human liver [30], and hepatic miR-194 is highly expressed in hepatic epithelial cells, including hepatocytes, which are parenchymal cells and account for more than 80% of liver cells [31]. Therefore, to collect systematic information on whether miRNAs have the potential to be biomarkers of nanomaterials, we selected those three major miRNAs in liver. Mice were given nSP70, nSP300, or mSP1000 via the tail vein. A dose rate of 40 mg kg^{-1} was chosen, because in the case of nSP70 this dose rate significantly increases the levels of ALT and AST [10]. As positive control, mice were intraperitoneally treated with LPS and D-GalN. At 8 h after treatment, we evaluated the serum level of each miRNA by using qRT-PCR and those of ALT and AST (i.e. the conventional hepatic biomarkers) by using biochemical analysis. The levels of miR-122 (figure 1(A)), ALT (figure 1(B)), and AST (figure 1(C)) in LPS/D-GalN-treated mice were significantly increased compared to those of vesicle-treated mice. The levels of miR-122 (figure 1(A)) and miR-192 (figure 1(D))

in nSP70-treated mice were significantly greater than those in saline-, nSP300- or mSP1000-treated mice and untreated mice. In contrast, the serum levels of miR-194 did not differ significantly among any groups (figure 1(E)). In addition, the levels of ALT (figure 1(B)) and AST (figure 1(C)) in nSP70-treated mice were significantly greater than those in saline-, nSP300- or mSP1000-treated mice and untreated mice. Thus, miR-122 and miR-192 may be useful as biomarkers for evaluating liver damage induced by nSP70. We focused on miR-122 in the following assessment, because it had the highest rate of expression among the three miRNAs.

3.3. Sensitivity and time-dependency of miR-122 expression

To more precisely assess the serum levels of miR-122 as a biomarker, we examined the sensitivity and time-dependency of miR-122 expression after treatment with nSP70. Mice were intravenously treated with nSP70 at 10, 20, or 40 mg kg^{-1} . Blood samples were collected 8 h after treatment and the levels of miR-122, ALT, and AST were measured. The serum levels of miR-122 (figure 2(A)), ALT (figure 2(B)), and AST (figure 2(C)) showed dose-dependent patterns of expression. In mice treated with 40 mg kg^{-1} nSP70 and 20 mg kg^{-1} in the case of AST, the levels of all three markers were significantly greater than in untreated or saline-treated mice. Thus, as was the case with ALT and AST, the increase in serum miR-122 levels was dependent on the dose of nSP70. Next, to assess the time-dependency of miR-122 expression, we examined the serum levels of miR-122 (figure 2(D)), ALT (figure 2(E)), and AST (figure 2(F)) at 4, 8, 24, and 72 h after intravenous injection of nSP70 at 40 mg kg^{-1} . The serum levels of miR-122 in nSP70-treated mice were significantly higher than those in saline-treated mice at every time point, as was the case with ALT and AST. These results suggested that the pattern of release of miR-122 into the blood was the same as those of ALT and AST.

3.4. miR-122 response after treatment of mice with surface-modified nSP70

Our group has previously demonstrated that modification of nSP70 with surface functional groups such as a carboxyl group (nSP70-C) or amino group (nSP70-N) can reduce the toxic effects of nSP70 (e.g., pregnancy complications in mice and ROS generation and DNA damage *in vitro*) [8, 9]. Here, we tested the use of miR-122 as a biomarker to determine whether the safety of nSP70 could be improved by adding functional groups. BALB/c mice were treated intravenously with nSP70, nSP70-C, or nSP70-N at 40 mg kg^{-1} . At 8 h after treatment, we measured the levels of miR-122 (figure 3(A)), ALT (figure 3(B)), and AST (figure 3(C)). The serum levels of miR-122 in nSP70-C- or nSP70-N-treated mice were significantly lower than that in nSP70-treated mice and were almost the same as those in untreated or saline-treated mice. The serum levels of ALT after treatment with nSP70-C or nSP70-N showed a trend similar to those of miR-122. In contrast, although the level of AST in nSP70-N-treated mice was significantly lower than that in nSP70-treated mice, the

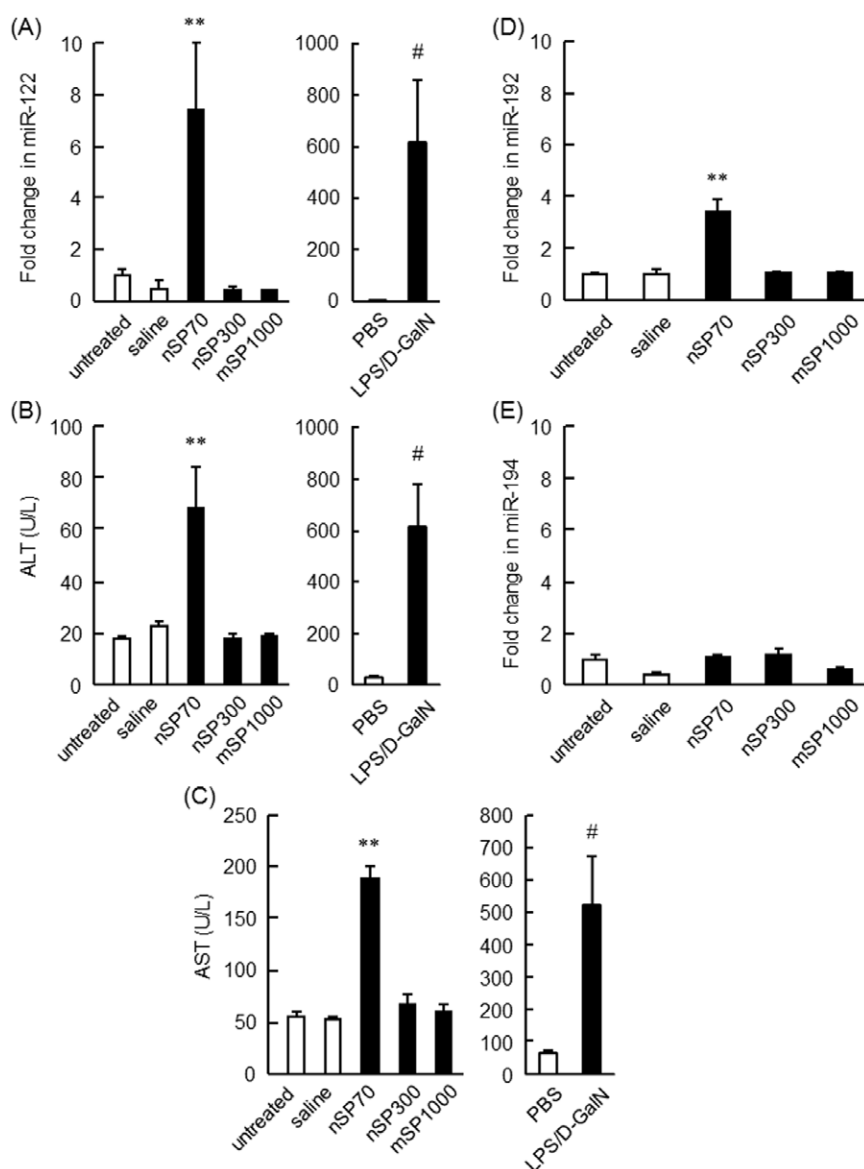


Figure 1. Usefulness of liver-specific microRNAs (miR-122, miR-192, and miR-194) as biomarkers for the development of safe silica nanoparticles. BALB/c mice were intravenously treated with nSP70, nSP300, or mSP1000 at 40 mg kg⁻¹. In addition, as positive control, mice were intraperitoneally treated with LPS and D-GalN. After 8 h, the serum levels of each microRNA, (A) miR-122, (D) miR-192, and (E) miR-194, were examined by real-time quantitative reverse-transcription PCR. Serum levels of (B) alanine aminotransferase (ALT) and (C) aspartate aminotransferase (AST) were measured by biochemical analysis. Data are presented as means \pm SEM ($n = 6$; ** $P < 0.01$ versus value for saline-, nSP300-, mSP1000-treated group and untreated group by ANOVA; # $P < 0.05$ versus value for PBS-treated group by Student's t -test).

AST level in nSP70-C-treated mice was significantly greater than that in untreated or saline-treated mice. These results suggest that serum miR-122 may be useful as a biomarker for assessing improvement of the safety of nSP70 through surface modification.

4. Discussion

Our group showed previously that nSP70 can induce severe liver damage in mice [10]. Here, we examined whether liver-specific or -enriched miRNAs (miR-122, miR-192, and miR-194) were potentially useful as biomarkers of the liver damage induced by nSP70. Although miR-192 and miR-194

are thought to be involved in controlling proliferation and metastasis in liver cancer, the details of their functions are less well understood than in the case of miR-122 [31]. miR-122 is a key regulator of cholesterol and fatty-acid metabolism in the liver [32]. Therefore, it is likely that changes in the levels of miR-122 will predict not only liver damage but also the biological effects of that damage, such as abnormalities in fat metabolism. In addition, miR-122 has also been shown to be biomarkers for drug-induced liver injury in mice, rats, and human [33–35].

First, we showed that serum levels of miR-122 and miR-192 in nSP70-treated mice were elevated 8 h after treatment (figures 1(A) and (D)), as were those of ALT and AST (figures 1(B) and (C)), and that the levels of miR-122

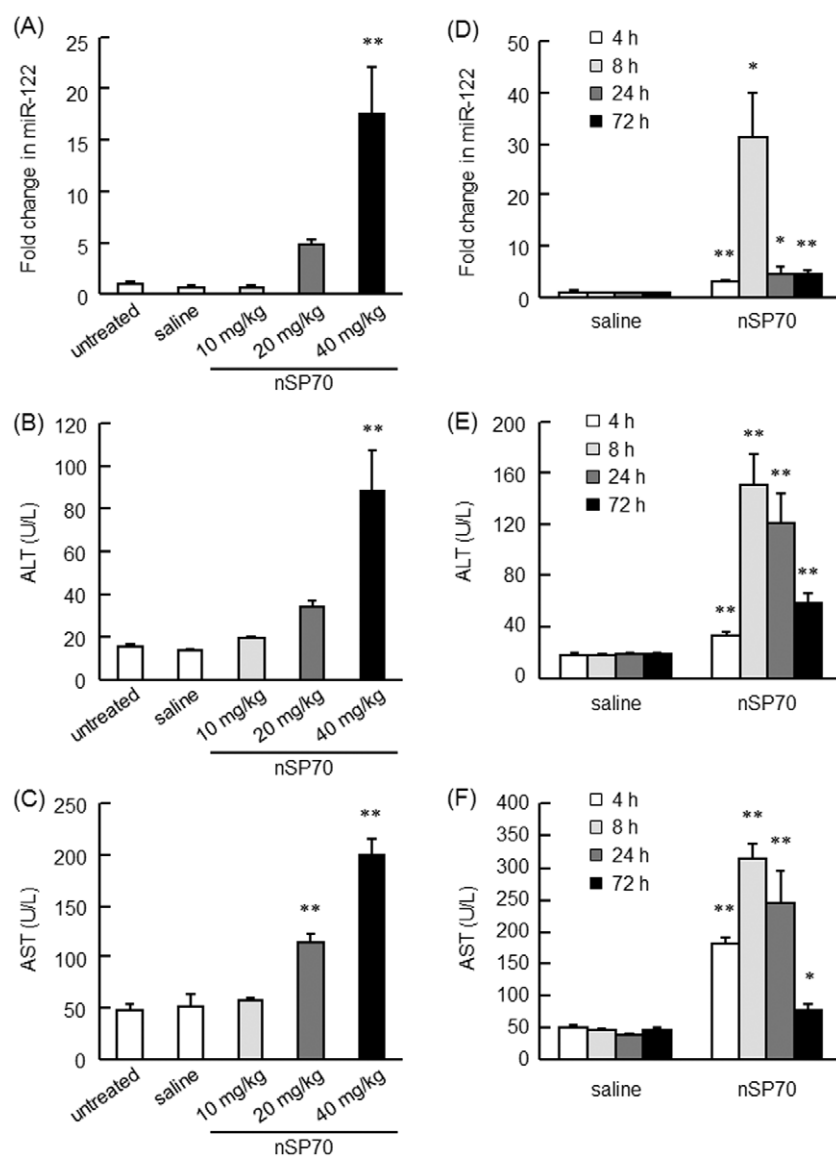


Figure 2. Serum levels of miR-122 after treatment with nSP70. BALB/c mice were intravenously treated with nSP70 at 10, 20, or 40 mg kg⁻¹. At 8 h after treatment with nSP70, the levels of (A) miR-122, (B) alanine aminotransferase (ALT), and (C) aspartate aminotransferase (AST) were analyzed. In addition, at 4, 8, 24, and 72 h after treatment with nSP70 at 40 mg kg⁻¹, the levels of (D) miR-122, (E) ALT, and (F) AST were examined. Data are presented as means \pm SEM ($n = 5$ or 6 ; * $P < 0.05$, ** $P < 0.01$ versus value for saline-treated group and untreated group by ANOVA).

were much higher than those of miR-192. We considered that this was because miR-122 accounts for 70% of all liver miRNA. By contrast, the serum levels of miR-194 in nSP70-treated mice did not differ significantly from those in untreated or saline-treated mice (figure 1(E)). The expression of miR-194 may change markedly with time; because we analyzed this expression only at 8 h after treatment, further time-course studies are needed before this miRNA can be discarded as a biomarker.

The serum levels of ALT and AST in mice treated with nSP70 at 40 mg kg⁻¹ were beyond the physiological range, at about 4 times the values in untreated or saline-treated mice. In contrast, the serum level of miR-122 in nSP70-treated mice was about 15 times that in untreated or saline-treated mice (figures 2(A)–(C)). This suggests that miR-122 may be a more sensitive biomarker than the currently used ALT and AST

in detecting liver damage induced by nSP70. In addition, as shown in figures 2(A)–(C), we evaluated the dose-dependent expression of miR-122 only at 8 h after treatment with nSP70, and we showed that the pattern of expression of miR-122 was almost the same as that of ALT and AST. In another study, injection of acetaminophen did not cause serum ALT elevation at 1 h, although obvious increases in miR-122 and miR-192 levels were already observable [36]. Therefore, evaluation at time points earlier than 8 h after nSP70 injection with lower doses could reveal the possibility that miR-122 is a more sensitive predictor of nSP70-induced liver damage than ALT and AST.

As shown in figures 2(D)–(F), the serum levels of ALT and AST in mice treated with nSP70 at 24 h showed significantly greater than those in saline-treated mice, while for miRNA-122, it dropped at 24 h to the same level as

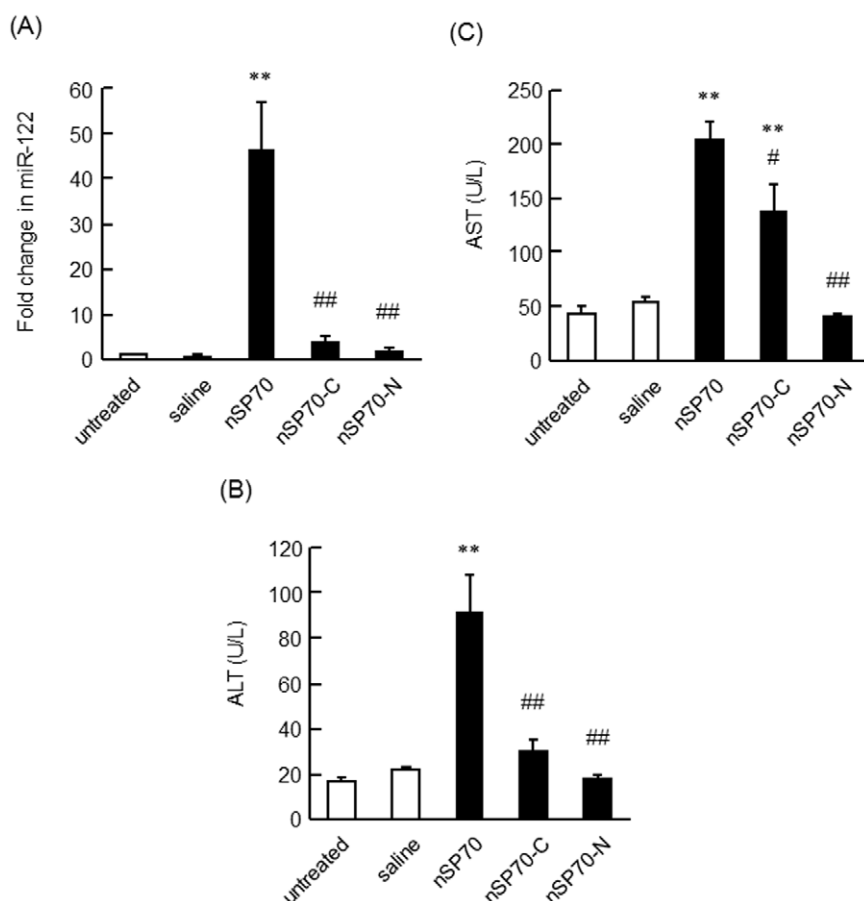


Figure 3. Responses of serum levels of miR-122 to treatment with surface-modified nSP70. BALB/c mice were intravenously treated with nSP70, nSP70-C, or nSP70-N at 40 mg kg^{-1} . Serum levels of (A) miR-122, (B) alanine aminotransferase (ALT), and (C) aspartate aminotransferase (AST) were measured 8 h after treatment. Data are presented as means \pm SEM ($n = 5$ or 6 ; ** $P < 0.01$ versus value for saline-treated group and untreated group; # $P < 0.05$, ## $P < 0.01$ versus value for nSP70-treated group by ANOVA).

72 h. In general, miRNAs are considered to be stable in the blood [37]. However, Yamaura *et al* showed that although miR-122 was stable in human plasma, it was unstable in rat plasma [38]. Therefore, although detailed information on the stability of mouse miR-122 is unavailable, it is possible that RNase action in the blood leads to a rapid decrease in miR-122 levels. Currently, there are two hypothetical pathways by which miRNAs can enter the circulation [39]. One is by direct leakage from cells, and the other is by release from cells via microvesicles. It is thought that direct secretion of miRNAs occurs in tissue damage or cell apoptosis [39]. We previously demonstrated that nSP70 administered via the tail vein was distributed mainly to the liver and induced cytotoxicity in primary hepatocytes isolated from nSP70-treated mice [7]. Taking these results together, it is conceivable that most miR-122 is leaked into the blood and degraded by RNase between 8 and 24 h after nSP70 treatment. On the other hand, the level of miR-122 in nSP70-treated mice showed significant increments compared to that of saline-treated mice over the time course of the experiment. Therefore, its change in expression over the full time course of the experiment was still useful for evaluating nSP70-induced liver damage. For these reasons, we consider that miR-122 is as useful as ALT and AST in evaluating nSP70-induced liver damage.

However, because we cannot explain this in detail, we are now trying to investigate the mechanism involved.

Here, we evaluated the time course of miR-122 expression over only a short period of time after nSP70 treatment. However, considering that we are exposed to nanomaterials on a daily basis, there is a need to investigate the biological effects of long-term exposure to them. Recent reports have shown that long-term exposure to titanium dioxide nanomaterials in mice induces ROS production in the lung [40]. A nanomaterials safety-assessment system is required for the evaluation of not only acute effects but also chronic effects. Therefore, we intend to analyze nanomaterial-induced chronic biological effects by evaluating the long-term course of miRNA expression.

Although the levels of miR-122, ALT, and AST were lower in mice treated with modified nSP70 than in those treated with unmodified nSP70 (figure 3), the AST level in nSP70-C-treated mice was higher than that in untreated mice. It is possible that nSP70-C and nSP70-N is safer than nSP70, because we previously demonstrated that differences in the surface charge state or kind of functional group of nanomaterials lead to different biological effects and cell responses [41–43]. On the other hand, AST is abundant not only in the liver but also in the skeletal muscle, heart, and kidney. Taking these results together, we consider that the

increase in AST in nSP70-C-treated mice was attributable to damage to tissues other than the liver. We are now analyzing in detail the effects of nanomaterials by focusing on other tissues.

This study is just the first step in the development of a miRNA-based safety-assessment system for nanomaterials. We have shown here that miR-122 is almost as useful as ALT and AST as a biomarker for nanomaterials. Recent reports have shown that many miRNAs exist not only in blood but also in other body fluids such as urine or saliva [22, 23]. In light of these reports, we consider that miR-122 might have the potential to predict nSP70-induced liver damage non-invasively from analyses of urine or saliva. In addition, many tissue-specific miRNAs other than miR-122 have been reported [44]. Therefore, to develop more specific or more sensitive biomarkers than the established system, there is a strong need for comprehensive analyses of the miRNAs associated with various tissues, such as brain and pancreas, for which we do not have enough useful biomarkers. Considering that miRNAs regulate the expression of the mRNAs involved in protein translation, identifying the changes that occur in miRNA expression upon exposure to nanomaterials will enable us to predict the biological effects of these materials. We are now trying comprehensively to explore those miRNAs that show changes in expression levels upon exposure to nanomaterials. We hope that these studies will help to establish a system for evaluating the safety and usefulness of nanomaterials.

5. Conclusions

We have revealed here that miR-122 and miR-192 may be useful biomarkers of liver damage induced by nSP70. miR-122, in particular, may be comparable to ALT and AST as a biomarker for this purpose.

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