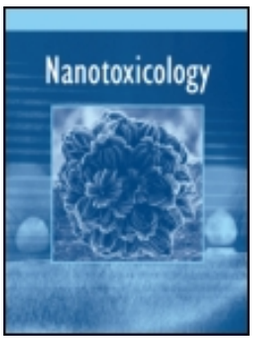

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Silver nanoparticles enhance thrombus formation through increased platelet aggregation and procoagulant activity

EUN-AH JUN[1]*, KYUNG-MIN LIM[2]*, KEUNYOUNG KIM[1], OK-NAM BAE[1], JI-YOON NOH[1], KYU-HYUCK CHUNG[3], & JIN-HO CHUNG[1]

[1]*College of Pharmacy, Seoul National University, Seoul,* [2]*Amore-Pacific Co., R&D Center, Gyeonggi-do, and* [3]*College of Pharmacy, Sungkyunkwan University, Gyeonggi-do, Korea*

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Abstract

Despite the wide use of silver nanoparticles (nano Ag), its toxicity still remains poorly understood. In this report, nano Ag induced an increase in platelet aggregation and procoagulant activation which are the key contributors to thrombotic diseases. In freshly isolated human platelets, nano Ag induced platelet aggregation and procoagulant activation evident by increased phosphatidylserine exposure and thrombin generation. Interestingly, the sub-threshold level of thrombin enhanced nano Ag-induced platelet activation significantly indicating that the prothrombotic effects of nano Ag might be further potentiated in activated platelets. An increase in intracellular calcium mediated nano Ag induced platelet activation and P-selectin expression, and serotonin release was also enhanced by nano Ag. Consistent with the *in vitro* results, exposure to nano Ag (0.05–0.1 mg/kg i.v. or 5–10 mg/kg intratracheal instillation) *in vivo* enhanced venous thrombus formation, platelet aggregation, and phosphatidylserine externalization *ex vivo* in rats suggesting that nano Ag, indeed, does enhance thrombus formation through platelet activation.

Keywords: *Silver nanoparticles, platelet aggregation, platelet procoagulant activity, thrombus formation, cardiovascular toxicity*

Abbreviations: ACD, acid citrate dextrose; CVD, cardiovascular disease; FITC, Fluorescein-isothiocyanate; LDH, lactate dehydogenase; Nano Ag, silver nanoparticles; PE, phycoerythrine; PPP, platelet poor plasma; PRP, platelet rich plasma; PS, phosphatidylserine; PGE_1, prostaglandin E_1; WP, washed platelets

Introduction

Recent advances in nanotechnology have resulted in greatly increased uses of nanomaterials in all aspects of human life (Colvin 2003; Oberdörster et al. 2005). Among these, silver nanoparticles (nano Ag) have gained a huge popularity due to its antimicrobial effect (Blaser et al. 2008; Lu et al. 2008) and are being widely introduced into medical appliances. Nano Ag is also being used in various consumer products including disinfectants, deodorants, antimicrobial sprays, laundry devices and garments (Chen and Schluesener 2008) raising concerns over its inevitable side effects. Some studies, indeed, have shown the potential toxicity of nano Ag. Nano Ag is known to induce a strong cytotoxicity against mammalian cell lines (Hussain et al. 2005) and interferes with DNA replication (Yang et al. 2009). Subchronic inhalation of nano Ag can cause alveolar inflammation and small granulomatous lesions in the lungs and bile duct hyperplasia in the liver (Sung et al. 2009). However, little is known about the effects of nano Ag on thrombosis to our best knowledge.

In the development of thrombotic diseases, platelets play a predominant role by forming a thrombus and activating a coagulation cascade. They are highly reactive to shear stress, endogenous activators and blood-borne chemicals with prothrombotic potential (Lee et al. 2002; Bae et al. 2007). Following exposure to these prothrombotic stimuli, platelets undergo aggregation (Ruggeri 2002; Gawaz 2004), P-selectin expression, serotonin release, and procoagulant activation by phosphatidylserine (PS) exposure and thrombin generation (Lentz 2003). Recently, it has

Correspondence: Dr Jin-Ho Chung, Shinrim-dong San 56-1, Gwanak-ku, College of Pharmacy, Seoul National University, Seoul 151-742, Korea. Tel: +822 880 7856. Fax: +822 885 4157. E-mail: jhc302@snu.ac.kr
*These authors equally contributed to this work.

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been reported that carbon nanotubes can have proaggregatory effects mediated by calcium-dependent glycoprotein IIb/IIIa activation and P-selectin expression (Radomski et al. 2005; Nemmar et al. 2007b) indicating the prothrombotic potential of nanoparticles.

Due to the reduced particle size and increased surface area, nanoparticles can translocate into systemic blood flow through oral (Kim et al. 2008) or inhalation routes (Monroe et al. 2002; Oberdörster et al. 2002; Pery et al. 2009). Systemic translocation of nano Ag through inhalation has also been reported (Sung et al. 2009) suggesting that an interaction between nano Ag and platelets might occur. In the present study, our aim was to investigate the effects of nano Ag on platelets in an effort to elucidate the prothrombotic potential of nano Ag. In addition, we elucidated the mechanism underlying the nano Ag-induced platelet activation and examined the clinical relevance of these events using *in vivo* rat models.

Materials and methods

Materials

Nano Ag powder (<100 nm, catalog no. Aldrich 576832), micro Ag powder (5,000–8,000 nm, catalog no. Aldrich 327093), thrombin, citric acid, trisodium citrate and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Phycoerythrin (PE)-labeled monoclonal antibody against human glycoprotein Ib (anti-GP Ib-PE Ab), fluorescein-isothiocyanate (FITC)-labeled monoclonal antibody against rat glycoprotein IIIa (anti-GP IIIa-FITC Ab), FITC- or PE-labeled annexin V (annexin V-FITC, annexin-PE) and FITC-labeled anti-CD62P were purchased from BD Biosciences (San Diego, CA, USA). Fluo-3 acetoxymethylester (Fluo-3 AM) was obtained from Molecular Probes (Eugene, OR, USA), and ^{14}C-serotoin (55 mCi/mmol) from GE Healthcare (Buckinghamshire, UK). Purified human prothrombin (factor II), factor Xa and factor Va were purchased from Hematologic Technologies, Inc. (Essex Junction, VT, USA). Thromboplastin was obtained from Organon Teknika BRI (Rockville, MD), and all other reagents used were of the highest purity available.

Preparation and characterization of Ag particle suspension

Preparation of Ag suspension was carried out according to the methods previously described (Radomski et al. 2005; Yang et al. 2009) with a slight modification. Nano Ag was selected based on the study of Yang et al. (2009). Nano Ag or micro Ag powder was dispersed in distilled water as 100X stock solution (1–25 mg/ml) and sonicated with a probe-type sonicator with a maximum output power, 200 Watts (Branson Sonifier, Danbury, CT) for 2 min to prevent particle self-assembly (agglomeration). In addition, prior to every experiment, nano Ag suspensions were vigorously vortexed for 20 sec. For the characterization of nano Ag after spiking in platelet suspension, an aliquot of nano Ag-spiked incubation buffer (Tyrode buffer; 134 mM NaCl, 2.9 mM KCl, 1.0 mM $MgCl_2$, 10.0 mM HEPES, 5.0 mM glucose, 12.0 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, and 0.3% BSA, pH 7.4) was analyzed with zeta-size meter (Malvern instruments, UK) or was dried and observed with TEM to examine the size distribution and the shape of nano Ag.

Preparation of human washed platelet

With an approval from the Ethics Committee of the Health Service Center at Seoul National University, human blood was collected from healthy male donors (18–25 years old) through venipuncture on the day of experiments using acid-citrate-dextrose (ACD, 10%) and prostaglandin E_1 (PGE_1; 1 μM). Washed platelets (WP) were prepared by differential centrifugation as previously described (Lee et al. 2002). Briefly, after isolation of platelet rich plasma (PRP) by centrifugation at 150 *g* for 15 min, platelets were pelleted by a stronger centrifugation at 500 *g* for 10 min. Pellet was resuspended with Tyrode buffer containing 1 μM PGE_1 and 10% ACD. After centrifugation at 400 *g* for 10 min, platelets were resuspended in Tyrode buffer to a cell concentration of 3×10^8 cells/ml and final $CaCl_2$ concentration was adjusted to 2 mM prior to use.

Measurement of platelet aggregation using single cell counts

To overcome the interference of light transmission detection of lumi-aggregometer by nanoparticles, the extent of aggregation was determined by single cell counting method (Chung et al. 2006). Briefly, WP was incubated with nano Ag and/or sub-threshold level of thrombin (0.01–0.02 U/ml for WP) at 37°C for 5 min in aggregometer (Chrono-log Corp., USA) and the resulting platelet suspension was fixed with 0.5% glutaraldehyde. Single cells were counted under light microscopy using hemacytometer.

Aggregation % was calculated according to the following equation:

Aggregation (%) = (1 − single cell count after incubation/initial cell count before incubation) × 100

Transmission electron microscopic (TEM) observation of platelet aggregation

WP was treated with nano Ag and/or sub-threshold concentration of thrombin at 37°C for 5 min and fixed with same volume of 2% glutaraldehyde. After centrifugation at 400 *g* for 10 min, the pellet was resuspended and washed once with 2% glutaraldehyde, twice with PBS, once with OsO_4 for 1 h and then undergone serial dehydration with ethanol (once with 10, 30, 50, 70, 90% and three times with 100% ethanol). The dehydrated pellet was substituted with propylene oxide twice, then embedded in spur four times, and dried at 70°C for 8 h. The embedded sample was observed with TEM (JEM 1010, JOEL, Japan).

Flow cytometric analysis of phosphatidylserine exposure

Annexin V-FITC was used as a marker for phosphatidylserine (PS) exposure and anti-GPIb(CD42b)-PE for platelet identifier. Negative controls for annexin V binding were prepared in the presence of 4 mM EDTA. Platelets were incubated with annexin V-FITC and anti-CD42b-PE for 20 min and analyzed on the flow cytometer, BD FACS Calibur (BD Biosciences) equipped with argon lasor (λ_{ex} 488 nm). CD42b-positive platelets were determined by side and forward scatters and PS-exposed platelets were identified with annexin V-FITC positivity among CD42b-positive population. Data from 5,000 events were collected and analyzed using CellQuest Pro software.

Measurement of platelet procoagulant activity

WP was diluted to 1×10^7 cells/ml and then incubated with nano Ag and/or a sub-threshold concentration of thrombin (0.05 U/ml) or saline for 5 min at 37°C. An aliquot of WP suspension was incubated with 5 nM factor Xa and 10 nM factor Va in Tyrode buffer containing 2 mM $CaCl_2$ for 3 min at 37°C. Thrombin formation was initiated by addition of 2 μM prothrombin. Exactly 3 min after the addition of prothrombin, an aliquot of the suspension was transferred to a tube containing EDTA stop buffer (50 mM Tris-HCl, 120 mM NaCl, and 2 mM EDTA, pH 7.4). Thrombin activity was determined by using the chromogenic substrate S2238, a synthetic tripeptide (H-D-phenylalanyl-L-pipecolyl-arginine-nitroanilide) substrate for thrombin (Chromogenix, Milano, Italy). The rate of thrombin formation was calculated from the change in absorbance at 405 nm with a standard calibration curve.

Measurement of serotonin secretion

Platelet rich plasma (PRP) was pre-incubated with 0.5 μCi/ml ^{14}C-serotonin (55 mCi/mmol) for 45 min at 37°C to load ^{14}C-serotonin and then ^{14}C-serotonin-loaded washed platelets were prepared as described above. After incubation with nano Ag for 30 min at 37°C, subthreshold concentration of thrombin or saline (vehicle) was added to platelets. The reaction was terminated by the addition of EDTA (final 5 mM). The resultant platelet suspensions were centrifuged at 12,000 *g* for 1 min, and the supernatant was obtained to determine ^{14}C-serotonin secretion. Radioactivity in each sample was measured in Wallac 1409 liquid scintillation counter (Perkin Elmer, Boston, MA, USA), after mixing with ACSII scintillation cocktail (GE Healthcare). Serotonin secretion was expressed as the percentage of the total serotonin content as measured in the supernatant from the cell lyzed with 0.3% Triton X-100.

Measurement of P-selectin expression

After the incubation with nano Ag at 37°C for 10 min, subthreshold concentration of thrombin (0.05 U/ml) was added to platelets. Anti-CD62P-FITC was used as a marker for P-selectin expression, while platelets were identified by anti-CD42b-PE. Platelets were incubated with anti-CD62P-FITC and anti-CD42b-PE for 20 min and analyzed on the flow cytometer BD FACS Calibur equipped with argon lasor (λ_{ex} 488 nm). Data from 5,000 events were collected and analyzed using CellQuest Pro software.

Determination of intracellular calcium levels

Intracellular calcium change was determined using Fluo-3 AM with flow cytometry according to the previously described method (do Ceu Monteiro et al. 1999). Fluo-3 AM (5 μM) was loaded to platelets in the presence of pluronic F-127 (0.2%) for 45 min at 37°C. Then platelets were spun-down by centrifugation at 300 *g* for 10 min, and resuspended with Tyrode buffer. Dye-loaded

platelets were incubated with nano Ag (250 μg/ml) and/or sub-threshold concentration of thrombin (0.05 U/ml). The platelet identifier, anti-CD42b-PE was added to the WP and incubated for 20 min and analyzed on the flow cytometer, BD FACS Calibur equipped with argon lasor (λ_{ex} 488 nm) to measure intracellular calcium increase. Data from 5,000 events were collected and analyzed using CellQuest Pro software.

Measurement of platelet aggregation in rat whole blood and in vivo rat venous thrombosis model

Male Sprague-Dawley rats weighing 250–350 g were used in all experiments. Before the experiments, animals were acclimated for one week. Food and water were provided *ad libitum*. All the protocols were approved by the Ethics Committee of Animal Service Center at Seoul National University. For the assessment of nano Ag-induced aggregation in whole blood, we used an impedence aggregometer, Multiplate® platelet function analyzer (Dynabyte, Munich, Germany), according to the manufacturer's instruction. Briefly, citrated whole blood (3.8% sodium citrate) was withdrawn from abdominal aorta and incubated with an equal volume of saline supplemented with $CaCl_2$ (4 mM) for 3 min at 37°C. Nano Ag with or without subthreshold thrombin was then added and aggregation was monitored for 30 min.

In vivo venous thrombosis was induced by stasis and hypercoagulability as described previously (Shin et al. 2007). Briefly, after rats were anesthetized with urethane (1.25 g/kg, i.p.), the abdomen was surgically opened to expose vena cava carefully. Two loose cotton-thread loops were placed 16 mm apart around the vena cava and all side branches were ligated tightly with cotton-threads. Thirty min after intravenous injection of saline (as vehicle) or nano Ag suspension into a left femoral vein, 1,000-fold diluted thromboplastin was infused for 2 min to induce thrombus formation. Stasis was initiated by tightening the two threads, first, the proximal and the distal thereafter. The abdominal cavity was provisionally closed and blood stasis was maintained for 15 min. After the abdomen was re-opened, the ligated venous segment was excised and opened longitudinally to collect the thrombus. Isolated thrombus was blotted off excess blood and immediately weighed using a bench balance.

Intratracheal instillation of nano Ag

The effects of nano Ag intratracheal instillation on *ex vivo* platelet aggregation and procoagulant activity were measured according to the methods previously described (Nemmar et al. 2007b). Male Sprague-Dawley rats weighing 250–350 g were used in experiments. After rats were anesthetized with urethane (1.25 g/kg, i.p.) and placed on supine position, a tube was inserted into the trachea and nano Ag suspension or saline (as vehicle) was instilled (0.5 ml/kg), followed by an air bolus (1 ml/kg). Six hours after intratracheal instillation, citrated blood (3.8% sodium citrate) was withdrawn from abdominal aorta, and PRP (3×10^8 cells/ml) was isolated. For platelet aggregation, PRP (3×10^8 cells/ml) was incubated with subthreshold concentration of thrombin (0.02 U/ml) at 37°C for 5 min and fixed in 0.5% glutaraldehyde for single cell count as described above. For PS exposure examination, an aliquot of blood sample was diluted 200-fold with the tyrode buffer containing 2 mM $CaCl_2$, and was stained with annexin V-FITC and anti-CD61-PE for 15 min in the dark. PS exposure was measured with a flow cytometer, and PS-exposed platelets were identified with annexin V-FITC positivity among CD61-positive population. Data from 50,000 events were collected and analyzed using CellQuest Pro software.

Statistical analysis

Values are shown as means ±SEM for all treatment groups. After normality test, the data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test or Student's *t*-test to determine the statistical difference from the corresponding vehicle or thrombin control. Statistical analysis was performed using SPSS software (Chicago, IL, USA). In all cases, a p value of <0.05 was used to determine significance.

Results

The effects of nano Ag on platelet aggregation

To determine the effects of silver nanoparticles (nano Ag) on platelet aggregation, human washed platelets (WP) were treated with various concentrations of nano Ag for 5 min, and the extent of platelet aggregation was measured by single cell count as described in the *Methods* section. As seen in Figure 1A, nano Ag induced platelet aggregation in a concentration-dependent manner starting from 100 μg/ml (28.2 ± 5.9% at 100 μg/ml and 54.1 ± 11.6% at 250 μg/ml vs. 5.4 ± 1.4% for the control group). Remarkably, with the co-treatment of sub-threshold thrombin (0.01–0.02 U/ml, the maximum concentration of thrombin inducing less than 20% aggregation), the

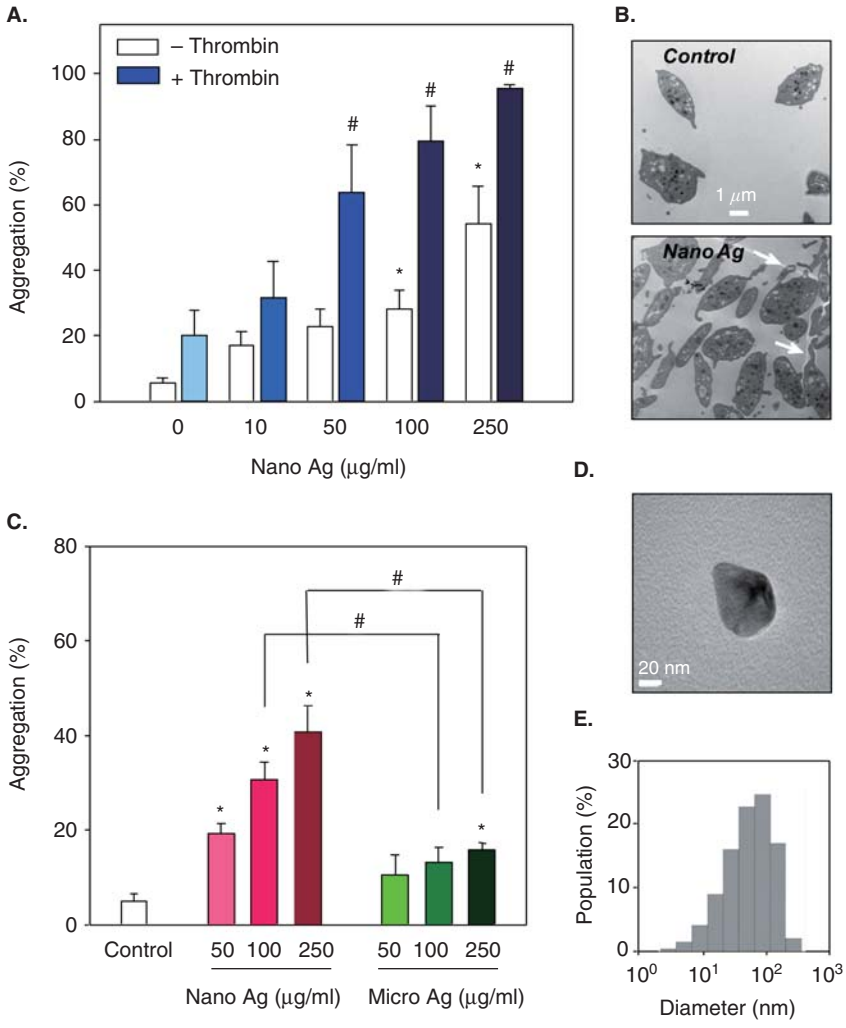

Figure 1. Effects of nano Ag on platelet aggregation and characterization of nano Ag (A) Freshly isolated human washed platelets were treated with nano Ag alone or co-treated with sub-threshold levels of thrombin for 5 min, and the extent of aggregation was estimated by single cell counting. Values are the mean ± SEM from 3–4 independent experiments. *represents a significant difference compared to the thrombin(–) control ($p < 0.05$, one-way ANOVA). #represents a significant difference compared to the thrombin(+) control ($p < 0.05$, one-way ANOVA). (B) Nano Ag-treated platelets (250 μg/ml) were observed by TEM. The arrow indicates the pseudopods extending from the activated platelets. (C) Comparison of the pro-aggregatory effects of nano Ag and micro-sized Ag. Values are the mean ± SEM from four independent experiments. *represents a significant difference compared to the corresponding control ($p < 0.05$, one-way ANOVA). #represents a significant difference between nano Ag and micro Ag ($p < 0.05$, Student's *t*-test). (D) TEM image of nano Ag. (E) Size distribution of nano Ag in Tyrode buffer solution measured by zetasizer.

pro-aggregatory effect of nano Ag was potentiated (63.7 ± 14.7% at 50 μg/ml, 79.3 ± 10.7% at 100 μg/ml, and 95.5 ± 1.2% at 250 μg/ml vs. 19.9 ± 7.7% for the control group). Under transmission electron microscopy (TEM, Figure 1B), nano Ag treatment induced typical pseudopod development from platelets confirming nano Ag-induced platelet activation. In contrast to nano Ag, silver microparticles (5–8 μm) induced aggregation was substantially weaker (Figure 1C). In addition, the removal of nano Ag by filtration abolished the pro-aggregatory responses, and no LDH leakage was measured after nano Ag incubation (data not shown). The nano Ag used in this study had a size distribution of approximately 10–100 nm with a spherical appearance (Figure 1D, 1E) which agreed well with the specification provided by the supplier.

The effects of nano Ag on platelet procoagulant activity

Platelet procoagulant activities, another important contributor to thrombus formation (Heemskerk et al. 2002), can also be enhanced by prothrombotic stimuli. To examine the effects of nano Ag on platelet procoagulant activities, phosphatidylserine

(PS) exposure, a representative marker for procoagulant activation, was examined after incubation with nano Ag using flow cytometry with FITC-tagged annexin V (PS-positivity marker). Incubation with nano Ag substantially increased PS-exposing platelets in a concentration-dependent manner and in a similar pattern to the nano Ag-induced aggregation of platelets. The addition of sub-threshold level thrombin significantly amplified these responses (Figure 2A). PS externalized on the outer cellular membrane provides a site for the assembly of coagulant enzymes facilitating rapid thrombin generation and coagulation cascades. To investigate whether PS exposure in platelets by nano Ag treatment can induce thrombin generation, aliquots of nano Ag-exposed platelets were used for a prothrombinase assay to measure thrombin generation. The assay showed that nano Ag-exposed platelets had significantly enhanced thrombin generation (Figure 2B).

The effects of nano Ag on intracellular calcium, P-selectin expression and serotonin release

An increase in intracellular calcium in platelets is a key event for aggregation and procoagulant activation (Solum 1999). The role of the increase in intracellular calcium in nano Ag-induced prothrombotic events was examined using Fluo-3 AM with flow cytometry. As seen in Figure 3A, treatment with nano Ag

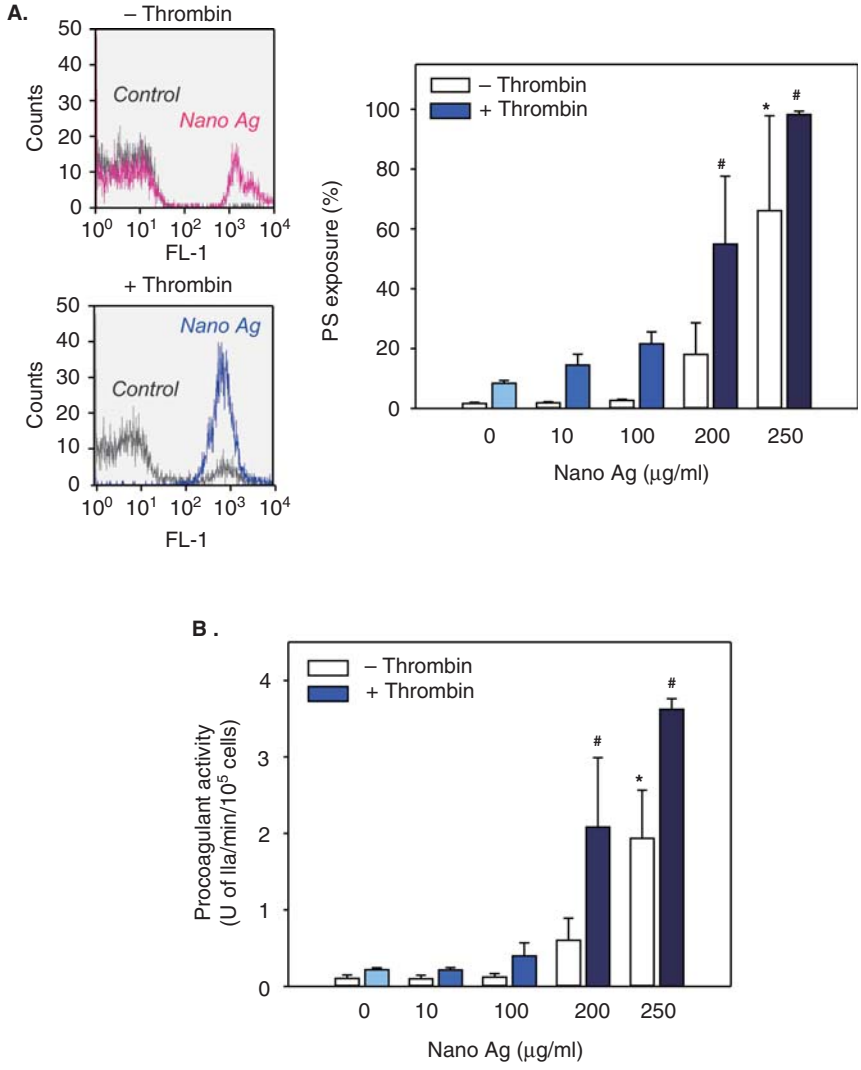

Figure 2. Effects of nano Ag on phosphatidylserine (PS) exposure and procoagulant activities. After platelets were treated with nano Ag (250 μg/ml) alone or co-treated with thrombin for 5 min, PS exposed platelets were identified with annexin V in a flow cytometer. (A) A representative histogram (left) and chart (right). (B) The effect of nano Ag on thrombin generation in platelets. Values are the mean ± SEM from three independent experiments. *represents a significant difference compared to the thrombin(−) control ($p < 0.05$, one-way ANOVA). #represents a significant difference compared to the thrombin(+) control ($p < 0.05$, one-way ANOVA).

increased significantly the intracellular calcium levels. Consistent with platelet aggregation and procoagulant activities, the sub-threshold level of thrombin significantly amplified this nano Ag-mediated intracellular calcium increase. To verify the role increased intracellular calcium plays in nano Ag-induced platelet activation, the platelets were pretreated with a calcium chelator, EGTA. 5 mM of EGTA significantly attenuated the effect of nano Ag on the induction of platelet aggregation (Figure 3B). P-selectin expression and serotonin release are secondary events caused by increased intracellular calcium in platelets, which have important roles in the interaction of platelets with leukocytes or vascular tissues in inflammation (Nemmar et al. 2007b). Along with aggregation and procoagulant activation, P-selectin expression and serotonin release were also increased by treatment with nano Ag (Figure 3C, 3D).

Prothrombotic effects of nano Ag in vivo

The pro-aggregatory effects of nano Ag were examined in rat platelets to evaluate the cross species difference prior to the experiments in the rat *in vivo*. Nano Ag enhanced rat platelet aggregation as well as PS exposure with a similar pattern to those observed for human platelets (Figure 4A, 4B). Measurement of platelet

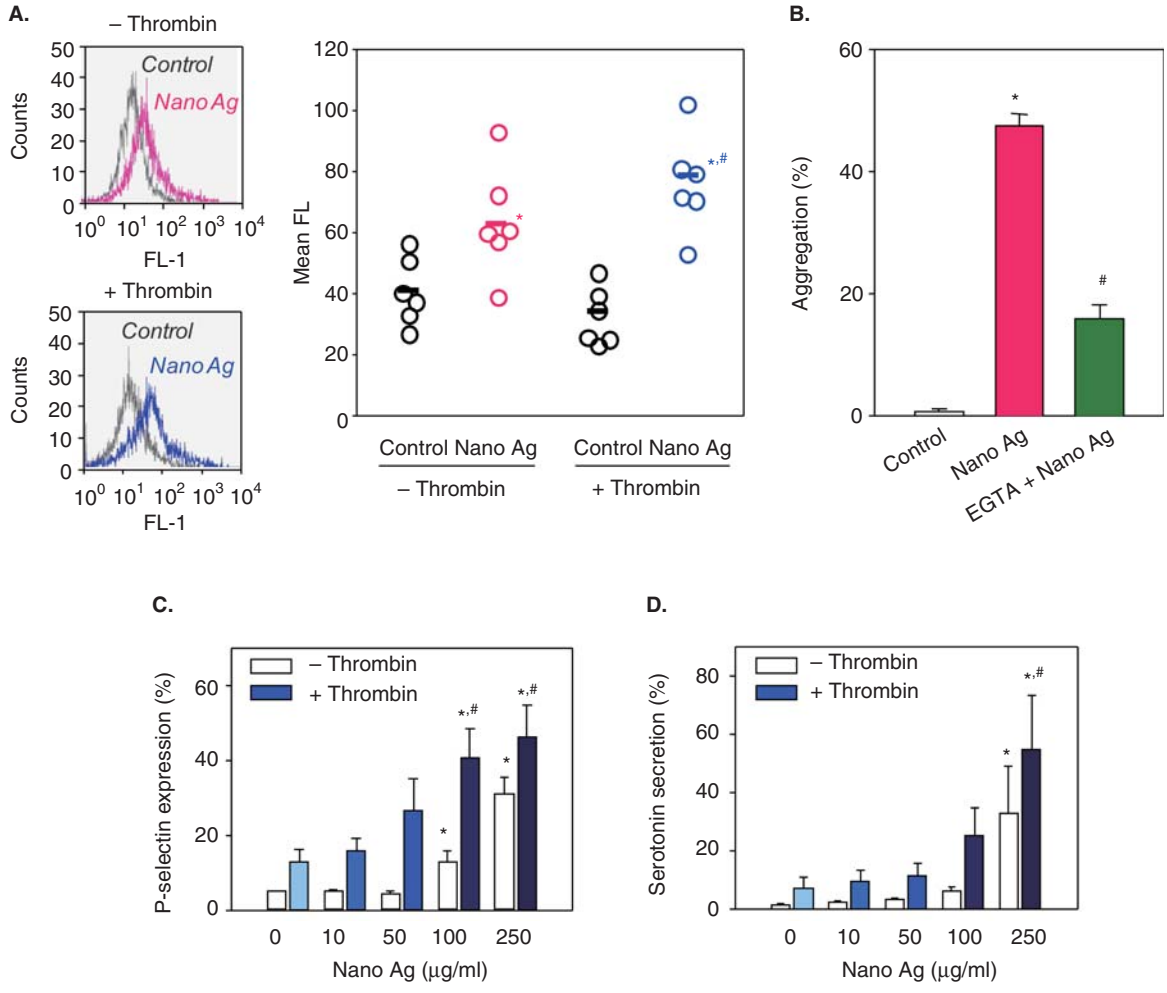

Figure 3. Effects of nano Ag on intracellular calcium levels, serotonin release and P-selectin expression in human washed platelets. (A) Fluo-3 AM loaded platelets were treated with nano Ag (250 μg/ml) alone or co-treated with thrombin and intracellular calcium levels was recorded using flow cytometry. Values are the mean ± SEM from six independent experiments. *represents a significant difference compared to the thrombin(−) control ($p < 0.05$, one-way ANOVA). #represents a significant difference compared to the thrombin(+) control ($p < 0.05$, Student's *t*-test). (B) Platelets were pre-incubated with or without EGTA (5 mM) and nano Ag (250 μg/ml) was added to initiate aggregation. Values are the mean ± SEM from three independent experiments. *represents a significant difference compared to the control ($p < 0.05$, Student's *t*-test). #represents a significant difference compared to nano Ag ($p < 0.05$, Student's *t*-test). (C) P-selectin expression and (D) serotonin release were measured after incubation of nano Ag with or without thrombin. Values are the mean ±SEM from 3–5 independent experiments. *represents a significant difference compared to the thrombin(−) control ($p < 0.05$, one-way ANOVA). #represents a significant difference compared to the thrombin(+) control ($p < 0.05$, one-way ANOVA).

aggregation with an impedance aggregometer for rat whole blood also confirmed the concentration-dependent pro-aggregatory effects of nano Ag and the synergy by co-treatment with thrombin (Figure 4C). To investigate the *in vivo* relevance of these *in vitro* findings, rat thrombosis models were conducted after the administration of nano Ag. In a rat venous thrombosis model where thrombus was induced by stasis and hypercoagulability through coagulation and platelet activation (Herbert et al. 1992), thrombus formation increased significantly by exposure to nano Ag (0.1 mg/kg, 25 ~ 30 μg/rat, i.v. bolus) as measured by increased thrombus weight (Figure 4D; 4.7 ± 1.6 mg at 0.05 mg/kg and 9.7 ± 2.2 mg at 0.1 mg/kg vs. 1.5 ± 1.2 mg for the control group). In addition, after intratracheal instillation of nano Ag (5–10 mg/kg, 1 ~ 4 mg/rat), platelet aggregation response (13.3 ± 2.4% at 10 mg/kg vs. 5.0 ± 0.9% for the control group) and PS exposure (2.0 ± 0.2% at 10 mg/kg vs. 1.1 ± 0.1% for the control group) *ex vivo* significantly increased (Figure 5A, 5B) confirming that nano Ag could indeed induce platelet activation.

Discussion and conclusion

In this study, we have demonstrated that nano Ag enhanced platelet activation determined by an

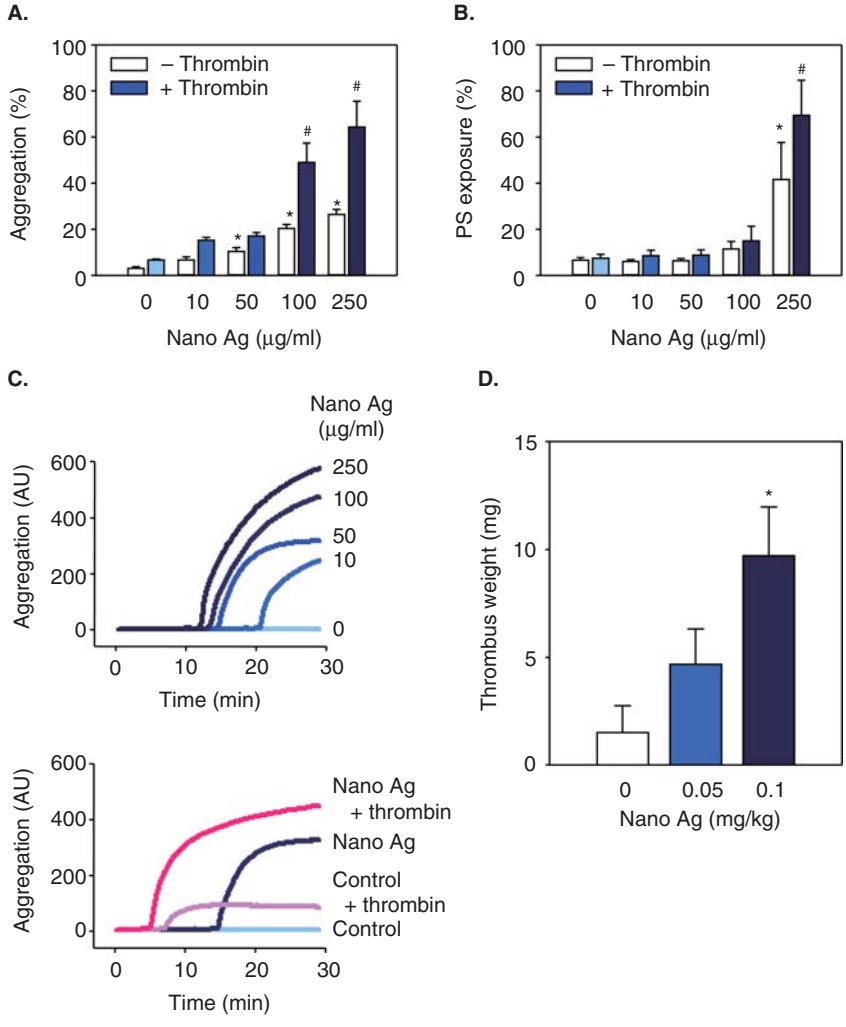

Figure 4. Effects of nano Ag on rat platelets and thrombus formation in rats *in vivo.* Rat washed platelets were treated with nano Ag with or without thrombin (A) the extent of platelet aggregation and (B) PS exposure were determined. Values are the mean ±SEM from three independent experiments. *represents a significant difference compared to the thrombin(−) control ($p < 0.05$, one-way ANOVA). #represents a significant difference compared to the thrombin(−) control ($p < 0.05$, one-way ANOVA). (C) Nano Ag-induced platelet aggregation (upper) and synergy with thrombin (at 50 μg/ml nano Ag, lower) was measured for 30 min in rat whole blood by an impedance aggregometer. The values are representative of more than three independent experiments. (D) After *i.v.* bolus of nano Ag (in 0.3 ml/kg saline), thromboplastin was infused to initiate thrombus formation as described in the *Methods* section. Values are the mean ± SEM from 4 animals. *represents a significant difference compared to the control ($p < 0.05$, one-way ANOVA).

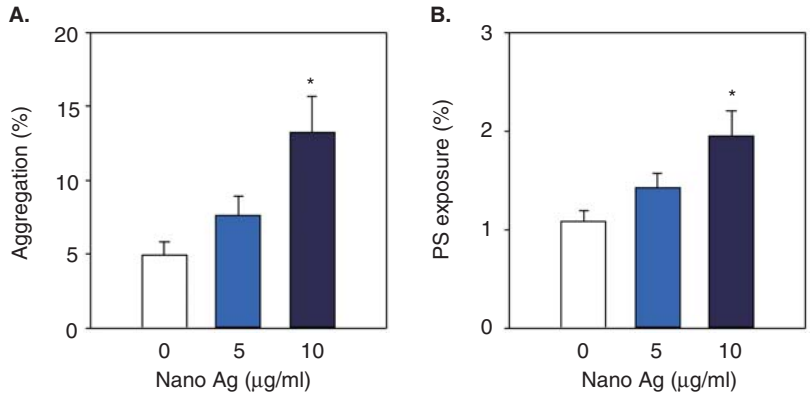

Figure 5. Effects of intratracheal instillation of nano Ag on *ex vivo* platelet aggregation and phosphatidylserine exposure in rats. Six hours after the animals were intratracheally instilled with nano Ag, the *ex vivo* platelet aggregation response and phosphatidylserine exposure was measured using the sub-threshold level of thrombin. Values are the mean ± SEM from 4–6 animals. *represents a significant difference compared to the corresponding control ($p < 0.05$, one-way ANOVA).

increase in platelet aggregation and procoagulant activity in freshly isolated human platelets. The co-treatment with the sub-threshold level of thrombin, an activating agonist, amplified these prothrombotic effects suggesting that co-existing thrombotic risk could act synergistically with exposure to nano Ag. Increased intracellular calcium appeared to mediate these nano Ag-mediated prothrombotic effects. P-selectin expression and serotonin secretion which are secondary but yet important events related to the increase in calcium were also enhanced by nano Ag. Especially, the prothrombotic effects of nano Ag can be seen *in vivo* in a rat venous thrombosis model and in an intratracheal instillation test where nano Ag enhanced platelet aggregation, procoagulant activity and thrombus formation indicating that exposure to nano Ag could indeed induce prothrombotic effects through platelet activation.

Nano Ag enhanced the procoagulant activity of platelets as well as aggregation concomitantly. Platelet procoagulant activation is an important event distinct from typical platelet aggregation in several aspects such as differences in the time course and the extent of responses to endogenous agonists. Procoagulant activation is accomplished by PS externalization, accelerating thrombin generation through increased assembly of prothrombinase and tenase on platelet membranes (Zwaal et al. 2005) while platelet aggregation is achieved by a series of events which culminate in GPIIb/IIIa activation and fibrin binding. The procoagulant activity of platelets is important in haemostasis and thrombosis (Solum 1999) by working as a positive feedback mechanism for the activation of platelets and by enhancing the formation of coagulation and thrombotic products (Zwaal and Schroit 1997; Monroe et al. 2002). These procoagulant and pro-aggregatory activities of nano Ag were similar to those of arsenic (Bae et al. 2007), a major thrombotic risk factor with many lines of epidemiological evidence. In this regard, we believe that the prothrombotic activities of nano Ag should be considered more seriously, and the potential thrombotic risk associated with exposure to nano Ag should be addressed in more detail.

Increase of intracellular calcium levels was shown to be the key mechanism underlying nano Ag-induced platelet activation (Figure 3A). In our results, nano Ag potentiated the thrombin-induced intracellular calcium increase by more than two-fold, and pretreatment with a calcium chelator attenuated nano Ag-induced platelet aggregation. This is in good agreement with the previous report on the ultrafine carbon black-induced intracellular calcium increase in monocytic cell lines (Stone et al. 2000). Calcium increase is directly related to GPIIb/IIIa activation in platelets, and it prompts a series of platelet activation responses such as P-selectin expression, serotonin secretion and procoagulant activity through PS-exposure by the activation of scramblase. Similar responses have been observed with thapsigargin, an inhibitor of calcium-ATPase, which potentiates the thrombin-induced procoagulant activity by increasing the intracellular calcium levels (Smeets et al. 1993). In addition, our data can explain well the previous observation of carbon nanoparticle-induced P-selectin and GPIIb/IIIa activation by Radomski et al. (2005) although a further confirmatory study is necessary to elucidate the upstream events leading to nano particle-induced intracellular calcium increases.

Due to the interference of nanoparticles in turbidometric measurements, single cell count method was adopted in the current study, which is a notable difference from the previous study by Radomski

et al. (2005). We found that the cloudy feature of nano Ag interferes substantially with the light transmission, especially at high concentrations. However, we could obtain tracing data and the time course of nano Ag-induced platelet aggregation using an impedance aggregometer (Figure 4C) which determines platelet aggregation by directly measuring platelet adhesion to the electrodes (Seyfert et al. 2007). This result suggests that the impedance aggregometer could be a good alternative for the study of platelet response to various nanoparticles.

Despite the risk of direct exposure of platelets to nano Ag through the use of nano Ag incorporated medical appliances, there has been no research on the prothrombotic effects of nano Ag. Concern over the imprudent use of nano Ag and urgent need for this kind of research has been raised by the recent review by Chen and Schluesener (2008). As shown Figure 1A and Figure 4D, significant prothrombotic effects of nano Ag were observed from 50 μg/ml *in vitro* which is in the proximate range to the antibacterial concentrations of nano Ag (Shrivastava et al. 2007) suggesting that there is not much safety margin for the antibacterial effects. Interestingly, the prothrombotic dose levels of nano Ag in our study match well with those of carbon nanomaterials or carbon ultrafine particles reported by Radomski et al. (2005) and by Nemmar et al. (2007a, 2007b) where prothrombotic effects could be observed *in vitro* at 25 ~ 50 μg/ml and *in vivo* at 20 ~ 100 μg/kg i.v. and 10 ~ 20 mg/kg intratracheal instillation. Considering that the epidemiological evidence supports the prothrombotic and cardiovascular risk of ultrafine carbon particles (Seaton et al. 1999; Pope et al. 2004), our results suggest that the potential prothrombotic risk of nano Ag can not be excluded.

Currently, there are no guidelines for nano Ag exposure levels to the best of our knowledge, and it is difficult to assess the relevance of the nano Ag dose we used in this study to real human exposure. The only available information is from the American Conference of Governmental Industrial Hygienists (ACGIH) guideline recommending a silver dust threshold limit volume for an 8 h daily to be below 0.1 mg/m^3. This level might be estimated to be 0.5–1.5 mg/adult when the breathing volume is assumed to be 10–30 l/min/adult indicating a large difference from the *in vivo* dose used in this study. However, when considering that platelet activation and thrombosis were observed after a single exposure to a dose of nano Ag, the prothrombotic effects of nano Ag cannot be completely ruled out although further studies involving chronic and wider dose ranges for exposure in the real world are necessary.

Conspicuously, the platelet activating effects of nano Ag was substantially amplified by the subthreshold level of thrombin suggesting a higher risk of nano Ag-induced prothrombotic effects in patients with compounding thrombotic complications. Thrombin also potentiated nano Ag-induced P-selectin expression and serotonin release, important mediators which enable platelets to participate in inflammation and vasoconstriction through interaction with leukocytes (Vandendries et al. 2004; Nemmar et al. 2007b) and blood vessels (Lee et al. 1998). In this regard, nano Ag-induced platelet activation might become more pronounced in a susceptible population with compounding thrombotic risk factors such as diabetes, hypertension, atherosclerosis, and thrombotic disease.

In conclusion, we showed that nano Ag enhances platelet aggregation and procoagulant activity through an increase in intracellular calcium, and these effects are substantially amplified in the presence of subthreshold levels of thrombin suggesting that nano Ag increases the prothrombotic risk in susceptible patients with compounding cardiovascular diseases. We believe this study provided important evidence in the understanding of prothrombotic risks for nanomaterials.

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NOTICE OF CORRECTION

The Early Online version of this article published online ahead of print on 08 September 2010 contained an error in Figures 4D and 5. The unit of X-axis μg/ml should have read mg/kg. This has been corrected for the current version.