High-Dose Vitamin C Injection to Cancer Patients May Promote Thrombosis Through Procoagulant Activation of Erythrocytes

Keunyoung Kim,*1 Ok-Nam Bae,†1 Sung-Hee Koh,*1 Seojin Kang,* Kyung-Min Lim,‡ Ji-Yoon Noh,* Sue Shin,§ Inho Kim,¶ and Jin-Ho Chung*,2

*College of Pharmacy, Seoul National University, Seoul 151-742, Korea; †College of Pharmacy, Hanyang University, Ansan 426-791, Korea; ‡College of Pharmacy, Ewha Woman's University, Seoul 120-750, Korea; §Department of Laboratory Medicine, Boramae Hospital, Seoul 156-707, Korea; and ¶College of Medicine, Seoul National University, Seoul 110-799, Korea

1These authors contributed equally to this study.
2To whom correspondence should be addressed at Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Korea. E-mail: jhc302@snu.ac.kr.

ABSTRACT

Potential risk of high-dose vitamin C consumption is often ignored. Recently, gram-dose vitamin C is being intravenously injected for the treatment of cancer, which can expose circulating blood cells to extremely high concentrations of vitamin C. As well as platelets, red blood cells (RBCs) can actively participate in thrombosis through procoagulant activation. Here, we examined the procoagulant and prothrombotic risks associated with the intravenous injection of gram-dose vitamin C. Vitamin C (0.5–5 mM) increased procoagulant activity of freshly isolated human RBCs via the externalization of phosphatidylserine (PS) to outer cellular membrane and the formation of PS-bearing microvesicles. PS exposure was induced by the dysregulation of key enzymes for the maintenance of membrane phospholipid asymmetry, which was from vitamin C-induced oxidative stress, and resultant disruption of calcium and thiol homeostasis. Indeed, the intravenous injection of vitamin C (0.5–1.0 g/kg) in rats in vivo significantly increased thrombosis. Notably, the prothrombotic effects of vitamin C were more prominent in RBCs isolated from cancer patients, who are at increased risks of thrombotic events. Vitamin C-induced procoagulant and prothrombotic activation of RBCs, and increased thrombosis in vivo. RBCs from cancer patients exhibited increased sensitivity to the prothrombotic effects of vitamin C, reflecting that intravenous gram-dose vitamin C therapy needs to be carefully revisited.

Key words: vitamin C; thrombotic risk; red blood cells; procoagulant activation; cancer patient

Most people believe that vitamins and dietary supplements are beneficial to health and large doses are frequently consumed with little concern for their potential toxicity. These days, however, the controversy is prevailing over the safety of high-dose vitamin consumption. Indeed, more than 60,000 cases of vitamin toxicity are reported annually in U.S. alone (Bronstein et al., 2010), reflecting its high prevalence and potential risks to public health. Vitamin C (ascorbic acid) is the single most popular vitamin (Padyatthy et al., 2004), which is widely consumed in high doses as a dietary supplement or adjuvant therapy to prevent or treat diverse diseases such as scurvy, common cold, and hepatitis (Levine et al., 2011; Naidu, 2003). Notably, recent studies revealed that excessive vitamin C consumption may aggravate cardiovascular diseases. Lee et al. (2004) reported that high vitamin C intake was strongly associated with the increased risk of cardiovascular disease mortality, coronary heart disease, and stroke in women with diabetes. Agarwal et al. (2012) also showed that vitamin C supplementation was positively...
correlated with the early atherosclerosis, reflecting that large dose of vitamin C may increase the risk of cardiovascular disease in susceptible populations.

Cancer patients, to whom extremely high doses of vitamin C are often administered for reducing pain or enhancing therapeutic activities of anticancer medications (Frei and Lawson, 2008; Hoffer et al., 2008; Ma et al., 2014; Ohno et al., 2009; Padayatty et al., 2010), may be highly vulnerable to vitamin C-associated cardiovascular toxicity. Elevated thrombotic risks in cancer patients have been well established (Prandoni et al., 2005; Young et al., 2012), and thrombosis is the second most common cause of death in patients with cancer (Khorana, 2009). Multiple factors are suggested to be involved that include clot-promoting effects of tumor cells, platelet-activating chemotherapy and increased coagulation activities (Sousou and Khorana, 2009; Young et al., 2012), suggesting that the potential risk of high-dose vitamin C to cancer patients needs to be thoroughly revisited.

Therapeutic and nutritional benefit of vitamin C supplement stems from its antioxidant property. However, at millimolar concentrations (> 0.3 mM), prooxidant activity may prevail, which can inflict cytotoxicity through the alteration of cellular redox balance through generating ascorbyl radical and H₂O₂ (Chen et al., 2007, 2008; Kang et al., 2005). Indeed, the prooxidant property of vitamin C are utilized for anticancer activity, and large doses of intravenous vitamin C are being administered to the cancer patients as high as 70–80 g/m² or 1.5 g/kg to achieve the millimolar blood concentration (approximately 50 mM) of vitamin C (Hoffer et al., 2008; Stephenson et al., 2013). These prooxidant properties of high-dose vitamin C, however, can inflict oxidative damages and cytotoxicity against normal cells or tissues that include human lymphocytes, rat brain slices, and mouse bone marrow cells (Konopacka et al., 1998; Podmore et al., 1998; Song et al., 1999). Considering that blood cells would be directly exposed to high concentrations of vitamin C intravenously given, the oxidative damage of blood cells and subsequent alteration of hemodynamic homeostasis may be unavoidable.

Increasing evidences indicate that red blood cells (RBCs; erythrocytes) can represent an important target for drug- or chemical-induced toxicity. Various compounds including drugs (Zhou et al., 2010), environmental toxicants (Lim et al., 2010; Shin et al., 2007), and endogenous mediators (Chung et al., 2007; Noh et al., 2010) can increase procoagulant activity and thrombosis through phosphatidylserine (PS) externalization to outer membranes in RBCs (Daleke, 2008). Low-dose vitamin C (0.1 mM) was protective against cytotoxicity to RBC (Mahmud et al., 2010), but Ballin et al. (1988) demonstrated that high-dose vitamin C (1 mg/ml, approximately 5 mM) increased the formation of Heinz-body and echinocytes in RBCs from premature infants, reflecting that RBCs can be a target for vitamin C-induced toxicity. Yet, the mechanism underlying or the clinical implications of vitamin C-induced alteration of RBC remain to be elucidated.

In this study, we found that high concentration (0.5–5 mM) of vitamin C increases procoagulant activity in human RBCs. The underlying mechanisms and its implication in thrombosis was examined in vivo. Most importantly, we investigated whether the prothrombotic risks of vitamin C can be elevated in the cancer patients through examining the effects of vitamin C on the procoagulant activities of RBCs isolated from leukemia patients. We believe that our study may provide an important insight into the prothrombotic risks of high-dose vitamin C treatment.

### MATERIALS AND METHODS

**Materials.** Sodium ascorbate (vitamin C), CaCl₂, KH₂PO₄, NaCl, Na₂HPO₄·2H₂O, KCl, NaH₂PO₄, MgSO₄, NaHCO₃, MgCl₂, glucose, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), bovine serum albumin (BSA), N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES), Tris-HCl, Tris-base, Tris-acetate, sodium citrate, dimethyl sulfoxide, sodium dodecyl sulfate, ethanol, acetic acid, trichloroacetic acid, 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), perchloric acid, N-ethylmaleimide, catalase, DL-dithiothreitol (DTT), purified human thrombin, calcium ionophore A23187, mercury orange, glutaraldehyde solution, osmium tetroxide, and ATP bioluminescent assay kit were obtained from Sigma Chemical Co (St Louis, Missouri). 1-Palmitoyl-2-[6-[[7-(nitro-2,1,3-benzoxadiazole-4-yl)amino]caproyl]-sn-glyero-3-phosphatidylethanolamine (C₆-NBD-PS) and 1-oleoyl-2-[6-[[7-(nitro-2,1,3-benzoxadiazole-4-yl)amino]hexanoyl]-sn-glyero-3-phosphatidylcholine (C₆-NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Phycoerythrin-labeled monoclonal antibody against human glycoporphin A (anti-glycoporphin A-RPE) was from Dako (Glostrup, Denmark). Fluorescein-isothiocyanate (FITC)-labeled annexin V (annexin V-FITC) and phycocyanin-labeled antibody against rat CD61 (anti-CD61-PE) was from BD biosciences (San Diego, California). Purified human prothrombin, factor Xa and factor Va were from Hematologic Technologies, Inc (Essex Junction, Vermont) and the chromogenic substrate for thrombin (S2238) was purchased from Chromogenix (Milano, Italy). Fluo-4 acetoxyethyl ester (Fluo-4 AM) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were from Invitrogen (Eugene, Oregon). The hemoglobin assay kit was from BioAssay Systems (Hayward, California). Thromboplastin-D and bicinechonic acid (BCA) protein assay kit was from Thermo Scientific (Rockford, Illinois). Prothrombin time (PT) reagent and activated partial thromboplastin time (aPTT) reagent were from Instrumentation Laboratory (Lexington, Massachusetts).

**Preparation of RBCs.** With an approval from the Ethics Committee of Health Service Center at Seoul National University, human blood was obtained from healthy male donors (18–25 years old) using a vacutainer with acid citrate dextrose and a 21-gauge needle (Becton Dickinson, Franklin Lakes, New Jersey) on the day of each experiment. Platelet-rich plasma and buffy coat were removed by aspiration after centrifugation at 200 × g for 15 min. Packed RBCs were washed 3 times with phosphate buffered saline (PBS: 1 mM KH₂PO₄, 154 mM NaCl, 3 mM Na₂HPO₄, pH 7.4) and once with Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 32 mM HEPES, 5 mM glucose, pH 7.4). Washed RBCs were resuspended in Ringer solution to a cell concentration of 5 × 10⁷ cells/ml.

**Flow cytometric analysis of PS exposure and microvesicle generation.** After RBCs were treated with vitamin C for 24 h at 37°C, aliquots of blood samples were diluted with Annexin binding buffer (125 mM NaCl, 10 mM HEPES, pH 7.4) and further incubated with the following substances for 30 min at room temperature in dark. Annexin V-FITC was used as a marker for PS, while anti-glycoporphin A-RPE was used as an identifier of RBCs and RBC-derived microvesicles (MV). Negative controls for annexin V binding were stained with annexin V-FITC in the presence of EDTA instead of CaCl₂ adjusted to final 2.5 mM. The MV
from RBCs had diameters < 1 μm compared with the size of 1-μm-diameter standard beads in the histogram. Samples were analyzed on the flow cytometer FACS Calibur (Becton Dickinson, San Jose, California) equipped with argon-ion laser emitting at 488 nm. Data from 10,000 events were collected and analyzed using CellQuest Pro software (Becton Dickinson).

Microscopic observation using scanning electron microscopy. After vitamin C treatment, RBCs were fixed with 2% glutaraldehyde solution for 1 h at 4 °C and then centrifuged and washed with PBS. The postfixation with 1% osmium tetroxide was conducted for 30 min at room temperature. After washing with PBS, the samples were dehydrated serially with 50%, 75%, 90%, and 100% ethanol. After drying and coating with gold, the images were observed on scanning electron microscope (JEOL, Japan).

Measurement of hemolysis, reactive oxygen species generation and intracellular level of glutathione. After vitamin C treatment, RBCs were centrifuged at 12,000 × g for 1 min and the supernatant was obtained for measurement of hemolysis. The extent of hemolysis was determined spectrophotometrically at 540 nm.

To detect reactive oxygen species (ROS) generation, RBCs were preincubated with 5 nM MDCFH-DA for 30 min at 37 °C. After washing with Ringer solution containing 1 mM CaCl2 to remove excess DCFH-DA, RBCs were incubated with vitamin C. The aliquots were diluted and the fluorescence of intracellular DCF was measured using flow cytometry. For the measurement of intracellular glutathione (GSH) level, RBCs were treated with 2% glutaraldehyde for 24 h at 37 °C, and incubated with 40 μM of mercuric orange for 3 min at 37 °C. Cells were diluted in Ringer solution and analyzed by flow cytometry.

Determination of protein thiol levels. One milliliter of vitamin C-treated RBCs was centrifuged at 7000 × g for 1 min, and pellet was resuspended with lysis buffer (5 mM sodium phosphate, pH 8) and incubated on ice for 30 min. Total lysate was resuspended with 5% perchloric acid on the ratio of 2:5, and then centrifuged at 7000 × g for 2 min. The pellet was solubilized in 1 ml of Tris-EDTA buffer (0.5 mM Tris-HCl, 5 mM EDTA, pH 7.6) containing 1% sodium dodecyl sulfate. DTNB (250 μM) was added to the samples, and the change of the absorbance was measured at 412 nm. The content of protein thiol was calculated on the basis of a glutathione calibration curve and divided by the protein content, which was measured by BCA protein assay kit.

Measurement of intracellular ATP and calcium levels. After incubation with vitamin C, RBCs were washed and resuspended in Tris buffer containing 1 mM CaCl2. The aliquot was mixed vigorously with TAE buffer (100 mM Tris-acetate, 2 mM EDTA, pH 7.8) containing 10% trichloracetic acid, and then cooled in ice for 20 min. The sample was centrifuged and the aliquot of resultant supernatant was mixed with cold TAE buffer. The intracellular ATP level was measured by luciferin/luciferase assay in Luminoskan (Labsystems, Franklin, Massachusetts) using an ATP assay kit and divided by the hemoglobin content, which was measured by Hemoglobin assay kit. To detect intracellular calcium increase, RBCs were loaded with 3 μM Fluo-4 AM for 1 h at 37 °C in the dark. After excess Fluo-4 AM was removed, vitamin C was treated to RBCs. The aliquots were diluted and analyzed in flow cytometer.

Determination of phospholipid translocation. RBCs were incubated with vitamin C and then loaded with 0.5 μM C6-NBD-PS or C8-NBD-PC. Aliquots from the cell suspension were taken at the indicated time intervals and incubated with cold Ringer solution for 10 min in the presence or absence of 1% BSA, respectively. Samples were analyzed on the flow cytometer. The amount of internalized probe was determined by comparing the fluorescence intensity associated with the cells before and after back extraction.

Prothrombinase assay. After treatment with vitamin C, RBCs were incubated with 5 nM factor Xa and 10 nM factor Va in Tyrode buffer (134 mM NaCl, 10 mM HEPES, 5 mM glucose, 2.9 mM KCl, 1 mM MgCl2, 12 mM NaHCO3, 0.34 mM Na2HPO4, 0.3% BSA, 2 mM CaCl2, pH 7.4) for 3 min at 37 °C. Thrombin formation was initiated by addition of 2 μM prothrombin. Exactly 3 min after addition of prothrombin, an aliquot of the suspension was transferred to a tube containing stop buffer (50 mM Tris-HCl, 120 mM NaCl, 2 mM EDTA, pH 7.9). Thrombin activity was determined using chromogenic substrate S2238. The rate of thrombin formation was calculated from the change in absorbance at 405 nm, using a calibration curve generated with active site-titrated thrombin.

Adherence of RBCs to HUVECs. The HUVEC (3 passages) were maintained in the endothelial cell growth media kit at 37 °C in a 95% air/5% CO2 incubator. Endothelial cells (1 × 105 cells) were seeded into grid dishes (μ-dish 35 mm, 0.5 mm grid; ibidi, Germany) and grown for 5 days. Vitamin C-treated RBCs were washed twice and resuspended in EB2-2 to a cell concentration of 5 × 105 cells/ml. After HUVEC was washed twice with EBM-2, vitamin C-exposed RBCs were layered onto confluent HUVEC monolayer and incubated for 45 min at 37 °C. After the incubation, the grid dish was rinsed 3 times with EB2-2 to remove nonadherent RBCs. The number of adherent RBC was counted on light microscope. The experiments were performed in triplicate and 25 fields were selected randomly to count RBC number.

In vivo experiments. All the protocols used in vivo experiments were approved by the Ethics Committee of Animal Service Center at Seoul National University. Male Sprague Dawley rats (SamTako, Osan, Korea) weighing 200–250 g were used for animal studies. Before the experiments, animals were acclimated for 1 week, and food and water were provided ad libitum.

Thrombus formation was induced by stasis combined with hypocoagulability. After anesthesia with urethane (1.25 g/kg, ip), the abdomen was surgically opened and the vena cava was exposed after careful dissection. Two loose cotton threads were prepared 16 mm apart around the vena cava. All side branches were ligated tightly with cotton threads. One hour after an intravenous injection of vitamin C into a left femoral vein, thromboplastin-D was infused (500 μl/kg/min) to induce thrombus formation. Stasis was initiated by tightening the proximal thread first, and after 30 s the distal thread. The abdominal cavity was provisionally closed, and blood stasis was maintained for 15 min. After the abdomen was reopened, the ligated venous segment was excised and opened longitudinally to remove the thrombus. The isolated thrombus was blotted of excess blood and immediately weighed. The thrombus was embedded in optimal cutting temperature compound (Tissue-Tek Products, Torrance, California) and cross sectioned into 10-μm thickness in a cryostat (CM1850, Leica, Germany) for hematoxylin and eosin staining.

To measure PS exposure on blood cells and coagulation time (PT and aPTT), blood was collected from the abdominal aorta using 3.8% trisodium citrate as anticoagulant at 1 h after an intravenous injection of vitamin C. An aliquot of the blood
The sample was diluted 200-fold with the following buffer (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2.0 mM MgCl$_2$, 1.0 mM Na$_2$HPO$_4$, 5.0 mM glucose, 5 mg/ml BSA, 2.5 mM CaCl$_2$, pH 7.4) and was stained with annexin V-FITC for 15 min in the dark. Anti-CD61-PE was added to identify platelets. Samples were analyzed using flow cytometry. For PT and aPTT assay, plasma was obtained from citrated-whole blood by centrifugation at 1500 × g for 20 min. PT and aPTT were measured in BBL Fibrometer (Becton Dickinson, Cockeysville, Maryland), according to the procedures in PT and aPTT reagent kit, respectively.

Experiments using RBCs from cancer patients. With an approval from the Ethics Committee of Health Service Center at Seoul National University Hospital, human blood was obtained from cancer patient male donors (N = 10; 32–73 years old; mean ± SD, 55.3 ± 4.4) with leukemia and healthy control male donors (N = 7; 42–60 years old; mean ± SD, 50.3 ± 3.1) using a vacutainer with acid citrate dextrose and a 21-gauge needle on the day of each experiment. RBC preparation, measurement of PS exposure, MV generation and ROS generation, and prothrombinase assay were done as described earlier.

Statistical analysis. The means and standard errors of means were calculated for all treatment groups. The data were subjected to 1-way analysis of variance followed by Duncan’s multiple range test or student t test to determine which means were significantly different from the control. In all cases, a P value of < .05 was used to determine significant differences.

RESULTS
Blood cells can be directly exposed to extremely high concentration of vitamin C administered through intravenously injection. RBCs, a major component of blood cells, can actively contribute to blood clotting through PS externalization and PS-bearing MV formation (Daleke, 2008; Shantsila et al., 2010). To study if vitamin C may activate thrombotic activities of RBCs, we examined PS externalization in outer membrane of human RBCs. Vitamin C (0.5–5 mM) induced PS exposure in a concentration- and time-dependent manner (Fig. 1A and 1B) as determined by the extent of annexin V binding. In addition to PS exposure, MV generation from RBCs was increased by vitamin C (Fig. 1C and 1D). The morphological change of RBCs from normal discoid to echinocyte was also observed (Fig. 1E), confirming the shedding of MV from spiculated RBCs. High-dose vitamin C did not induce hemolysis up to 5 mM, indicating that PS externalization and MV generation were not from nonspecific cytotoxicity.

To identify the mechanisms underlying PS-exposure by vitamin C, we investigated the alteration of intracellular redox state in RBCs. High concentration of vitamin C induced oxidative stress in RBCs as determined by DCF DA-enhanced fluorescence (Fig. 2A). The levels of GSH and protein thiol, which represent the intracellular antioxidant buffer in RBCs, were significantly decreased by vitamin C treatment (Fig. 2B and 2C). The intracellular level of ATP and calcium, the important factors to maintain cellular homeostasis, was significantly altered by vitamin C (Fig. 2D and 2E). Perturbation of thiol, ATP, and calcium levels can affect activities of intracellular enzymes including scramblase and flippase, which regulate transmembrane lipid asymmetry. Scramblase, which transports phospholipid bidirectionally, is activated by increased calcium level resulting in PS exposure. On the other hand, flippase is responsible for recovering the exposed PS to inner leaflet, and its activity is dependent on intracellular ATP and thiol level (de Jong and Kuypers, 2006; Lim et al., 2010). Consistent with the effects of vitamin C on intracellular calcium, ATP, and thiol, the activities of these lipid transporting enzymes were significantly affected by vitamin C; vitamin C activated scramblase, while flippase was inhibited (Fig. 2F and 2G). Pretreatment of EGTA, catalase, and DTT that can alleviate the alteration of intracellular calcium, ROS, and thiol, respectively, significantly inhibited vitamin C-induced PS exposure in RBCs (Fig. 2H), demonstrating that oxidative stress, calcium increase, and thiol depletion may underlie the prothrombotic effects of vitamin C on RBCs.

Next, we examined the pathological implication of RBC alteration by vitamin C. PS externalized in outer membrane of RBCs enhances thrombogenic activity of RBCs (Chung et al., 2007). Exposed PS accelerates blood coagulation by providing a site for the activation of coagulation factors. To examine if vitamin C
may increase procoagulant activities, we measured the conversion of prothrombin to thrombin in RBCs following vitamin C treatment. Vitamin C significantly increased thrombin generation in RBCs in a concentration-dependent manner (Fig. 3A). Vitamin C-enhanced thrombin generation was significantly reversed by the pretreatment of EGTA, catalase, and DTT, consistently with the reversal of vitamin C-induced PS externalization in RBCs by these modulators (Fig. 3B). In addition to promoting blood clotting, PS exposure facilitates RBC adhesion to endothelial cells (Closse et al., 1999; Wautier et al., 2011). As shown in Fig. 3C, the adherence of RBCs to human endothelial cells was increased by high concentration of vitamin C.

To prove the thrombogenic effects of vitamin C on RBCs in vivo, we employed in vivo venous thrombosis animal model in rats. Intravenous administration of vitamin C (0.5 or 1 g/kg) significantly increased thrombus formation in rats (Fig. 4A and 4B). Formation of thrombus is the result of a coordinated interplay between blood coagulation system and blood cells that include RBCs and platelets. To identify whether RBCs are the main mediators for thrombogenic activity of vitamin C, the alteration of platelets, coagulation factors, and RBCs were analyzed following in vivo vitamin C administration. As shown in Figure 4C and 4D, PS externalization in platelets was not induced and coagulation clotting times were not affected by intravenous administration of high-dose vitamin C. On the contrary, significant increase in PS-exposed RBCs could be observed (Fig. 4E), demonstrating that vitamin C-enhanced in vivo thrombogenic activity through the procoagulant activity of RBCs.

Next, we explored the clinical relevance of our findings. To simulate the clinical settings where gram dose of vitamin C is intravenously given for the cancer patients, we examined the effects of vitamin C on RBCs isolated from leukemia patients. In RBCs isolated from leukemia patients, vitamin C treatment significantly increased PS externalization and thrombogenic activity, in a similar pattern to the alterations of RBCs from normal volunteers (Fig. 5A and 5B). Of note, RBCs isolated from the

FIG. 2. Effects of vitamin C on phospholipid translocation. After RBCs were incubated with several concentrations of vitamin C for 24 h, reactive oxygen species (ROS) generation (A), intracellular GSH level (B), protein thiol content (C), ATP level (D), and intracellular calcium level (E) were determined as described in Materials and Methods section. F and G, After RBCs were incubated with distilled water (DW, control) or vitamin C for 4 h, C6-NBD PC translocation by scramblase (F) or C6-NBD PS translocation by flippase (G) were examined. H, RBCs were preincubated with EGTA (5 mM), catalase (100 μg/ml) or dithiothreitol (DTT) (2 mM) for 15 min followed by vitamin C treatment for 24 h. PS exposure was determined in flow cytometry. Values are mean ± SEM of 3–4 independent experiments. * Significant differences from control group (P < .05); # significant differences from vitamin C alone group (P < .05).
leukemia patients showed significantly enhanced susceptibility to the prothrombotic activation by vitamin C, as evidenced by the increased RBC alteration by vitamin C in the aspect of concentrations and sizes of effect. Susceptibility of RBCs from leukemia patients to the prothrombogenic effects of vitamin C could also be observed in MV generation and intracellular ROS generation (Fig. 5C and 5D).

DISCUSSION

Here, we demonstrated that high concentration (0.5–5 mM) of vitamin C may escalate thrombotic risk through the procoagulant activation of RBCs. High-dose vitamin C induced oxidative stress, collapsed cellular redox homeostasis, and depleted ATP, leading to the dysregulation of enzymes maintaining membrane phospholipid asymmetry in human RBCs. PS externalization and MV generation accomplished the procoagulant activation of RBCs by vitamin C. In vivo relevancy was also verified in rat venous thrombosis models, where the administration of vitamin C significantly increased thrombus formation. Of note, RBCs isolated from leukemia patients showed higher susceptibility to procoagulant activation and thrombosis induced by vitamin C, suggesting that cancer patients may be more vulnerable to the thrombotic risk of high-dose vitamin C.

Gram doses of vitamin C, often in parenteral dosage forms, are being recommended for the cancer patients to maximize the anticancer effects of vitamin C without any extra precaution. Here, we showed that RBCs from leukemia patients are susceptible to vitamin C-induced procoagulant activation, suggesting that high-dose vitamin C may increase the risk of thrombosis in cancer patients through the procoagulant activation of RBCs at least in part. Excessive procoagulant activities of blood cells accelerate blood clotting and augment thrombosis (Chung et al., 2007; Du et al., 2014; Mallat et al., 2000). Moreover, PS exposure on RBCs results in increased adhesion of blood cells to damaged endothelium or denuded blood vessels that is associated to a higher risk of thrombosis and cardiovascular complications (Borst et al., 2012; Wautier et al., 2011). Notably, cancer patients are at the higher risks of cardiovascular diseases and thrombosis, as found in epidemiological evidence where deep vein thrombosis, pulmonary embolism, and venous thromboembolism are frequently observed in the patients with pancreas, ovary, and brain cancer (Khorana, 2009; Lee and Levine, 2003; Young et al., 2012). Considering the close association between cancer and thrombosis, procoagulant alteration of RBCs following high-dose vitamin C should be examined in more detail in the future. Further studies with RBCs from the patients with other cancer types will be instrumental to prove the risk of high-dose vitamin C in cancer patients.

Vitamin C has dual biological effects depending on the applied concentrations: it reduces oxidative damage in low-concentration range (up to 0.1 mM) but in higher concentration (0.3–20 mM), it acts as a prodrug of H₂O₂ (Levine et al., 2011). The generation of cytotoxic H₂O₂ by high-dose vitamin C was
mediated by transition metals such as iron. The prooxidant activity of vitamin C has been suggested to be useful for the treatment of cancer as a stand-alone or an adjuvant for chemotherapy. Chen et al. (2008) reported that intraperitoneal administration of 4 g/kg vitamin C in rats significantly reduced the size of transplanted tumors of ovarian, pancreatic, and glioblastoma origins through the generation of ascorbyl radical and H$_2$O$_2$. Apoptosis of melanoma cells and neuroblastoma cells were induced by the treatment of 3–10 mM vitamin C in vitro (Carosio et al., 2007; Kang et al., 2005). However, it is highly controversial whether the prooxidant vitamin C selectively affects cancer cells (Frei and Lawson, 2008). There are several lines of evidence for potential adverse effect of high-dose vitamin C against normal cells as well. In normal bovine lung microvascular endothelial cells, treatment of vitamin C up to 10 mM induced free radical formation, H$_2$O$_2$ generation, GSH depletion, and alteration of cell morphology (Varadharaj et al., 2005). It is also observed that the functional thiol was decreased in hepatic endoplasmic reticulum following vitamin C treatment (Csala et al., 1999). In addition, prooxidant effects of high-dose vitamin C (5–7 mM) can activate phospholipase A$_2$, cyclooxygenase, lipooxygenase, and phospholipase D in endothelial cells (Steinhour et al. 2008; Varadharaj et al., 2006), stimulating the secretion of arachidonic acid and the alteration of cellular membranes, which can promote further prothrombotic activation and adhesion of RBCs (Chung et al., 2005; Lang et al., 2005).

The upper limit dose of vitamin C in the Dietary Reference Intakes guidelines is 2 g, and the plasma concentration following oral consumption of vitamin C (up to 2.5 g/day) did not exceed 0.25 mM (Levine et al., 1996; Padayatty et al., 2004), due to the tight regulation of oral vitamin C kinetics, such as limited bioavailability, tissue accumulation, and renal secretion (Levine et al., 2011). Although the plasma concentration of vitamin C is in limited range following dietary intake, the plasma vitamin C could be rapidly surged up to 50 mM by intravenous treatment (Padayatty et al., 2004). Intravenous administration of 1.5 g/kg vitamin C (0.5–1.0 g/min) can accomplish the plasma concentration of 30 mM in humans (Hoffer et al., 2008). In the phase I clinical trial in cancer patients, 70 g/m$^2$ dose of vitamin C produced a $C_{\text{max}}$ of 49 mM (Stephenson et al., 2013) suggesting that millimolar vitamin C can be easily achieved following intravenous administration. Here, we used vitamin C at in vitro concentration of 0.5–5 mM and in vivo dose of 0.5–1.0 g/kg, which are in the concentration range that may be achieved after intravenous administration in cancer patients (Du et al., 2010; Ohno et al., 2009; Padayatty et al., 2006). Furthermore, prothrombotic activation occurred at lower concentration range of vitamin C in RBCs from cancer patients, while its extent was higher than normal patients (Fig. 5), suggesting that leukemia patients may be at higher risk of vitamin C-associated prothrombotic risks.

In conclusion, we demonstrated that high dose of vitamin C may cause thrombogenic PS exposure in RBCs, leading to increased venous thrombosis in vivo. RBCs act as a primary buffer for vitamin C-generated oxidative stress in blood through quenching H$_2$O$_2$ by the action of catalase and peroxidase (Chen et al., 2005, 2007). Consequently, RBCs might be an early and major target of vitamin C-induced toxicity in blood as shown in our study where vitamin C disrupted cellular homeostasis such as calcium, GSH, protein thiol, and ATP. The potential mechanisms for vitamin C-induced prothrombotic activation of RBCs can be summarized in Fig. 6. Clinical importance was explored by the higher susceptibility to vitamin C in RBCs isolated from leukemia patients. Considering that the use of high dose of
vitamin C is now expanding in cancer patients who are at the higher risk of thrombosis, we believe that it is necessary to verify the safety of high-dose vitamin C and its clinical implications.

FUNDING
This work was supported by the National Research Foundation of Korea (NRF), grants funded by the Korean government (No. 2007-0056817 from the Ministry of Science, ICT and Future Planning and No. NRF-2014R1A1A2058459 from the Ministry of Education).

REFERENCES


Sodium ascorbate induces apoptosis in neuroblastoma cell lines by interfering with iron uptake. Mol. Cancer 6, 55.


