Doxorubicin-Induced Platelet Procoagulant Activities: An Important Clue for Chemotherapy-Associated Thrombosis

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Thrombotic risk associated with chemotherapy including doxorubicin (DOX) has been frequently reported; yet, the exact mechanism is not fully understood. Here, we report that DOX can induce procoagulant activity in platelets, an important contributor to thrombus formation. In human platelets, DOX increased phosphatidyserine (PS) exposure and PS-bearing microparticle (MP) generation. Consistently, DOX-treated platelets and generated MPs induced thrombin generation, a representative marker for procoagulant activity. DOX-induced PS exposure appeared to be from intracellular Ca²⁺ increase and ATP depletion, which resulted in the activation of scramble and inhibition of flipase. Along with this, apoptosis was induced by DOX as determined by the dissipation of mitochondrial membrane potential (ΔΨm), cytochrome c release, Bax translocation, and caspase-3 activation. A Ca²⁺ chelator ethylene glycol tetraacetic acid, caspase inhibitor Q-VD-OPh, and antioxidants (vitamin C and trolox) can attenuate DOX-induced PS exposure and procoagulant activity significantly, suggesting that Ca²⁺, apoptosis, and reactive oxygen species (ROS) were involved in DOX-enhanced procoagulant activity. Importantly, rat in vivo thrombosis model demonstrated that DOX could manifest prothrombotic effects through the mediation of platelet procoagulant activity, which was accompanied by increased PS exposure and ΔΨm dissipation in platelets.

Key Words: doxorubicin; platelets; thrombosis; PS exposure.

Doxorubicin (DOX) is an anthracycline anticancer drug widely prescribed for the treatment of solid tumors and hematologic malignancy including breast, ovarian and bladder cancer, osteosarcoma, and non-Hodgkin’s lymphoma. Although DOX is effective against diverse types of cancers, the active and combinatorial use with other anticancer agents for the maximization of its anticancer effects is being seriously impeded by adverse effects, such as cardiomyopathy and thrombus formation (Singal and Iliskovic, 1998; Zangari et al., 2002). Especially, in many clinical trials, for new DOX formulations or combinatorial therapy with other chemotherapeutic agents, increased thrombus formation has been frequently encountered, of which incidence almost reached up to 20–23% of DOX-treated patients (Lokich et al., 1986; Zangari et al., 2002).

The elevated thrombotic risks in cancer patients have been well documented (Prandoni et al., 2005) and multiple factors are suggested to be involved in it, including clot-promoting effects of tumor cells and increased coagulation activities (Sousou and Khorana, 2009). Of these, chemotherapy is being considered as a major culprit for the increased thrombosis in cancer patients. Supporting this, a clinical trial with 205 breast cancer patients revealed that higher incidences of thrombosis occurred during chemotherapy (14 cases [6.8%] vs. none during the period without chemotherapy) (Levine et al., 1988). In another study with breast cancer patients, the occurrence of thrombosis almost elevated up by 17.6% in the patients under chemotherapy (Goodnough et al., 1984), and this trend has been confirmed unequivocally again in multiple myeloma patients (Libourel et al., 2010), demonstrating a strong association between chemotherapy and thrombosis.

In order to prevent the chemotherapy-associated thrombosis, many efforts have been made to uncover the mechanism underlying the thrombogenic activities of chemotherapy. Diverse cells, plasma proteins, or tissues involved in thrombosis, such as monocytes (Paredes et al., 2003; Walsh et al., 1992), coagulation factors (Rogers et al., 1988), and vascular endothelium (Cwikel et al., 1996), have been examined for their roles in the chemotherapy-associated thrombosis. Of these, platelets are considered to play a critical role in increased thrombotic risks in the patients under chemotherapy (Bernat and Herbert, 1994; Togna et al., 2000), but the exact mechanism has not been fully clarified yet. Following the exposure to prothrombotic stimuli, including shear stress, endogenous activators, and blood-borne chemicals (Bae et al., 2007; Lee et al., 2002), platelets undergo
aggregation (Gawaz, 2004; Ruggeri, 2002), express procoagulant activity through phosphatidylserine (PS) exposure and thrombin generation (Lentz, 2003), and release vasoactive mediators like serotonin and thromboxane (Lee et al., 1998). In particular, it is recently demonstrated that the procoagulant activity plays a key role in the thrombosis observed in many cardiovascular diseases (CVDs) (Bonomini et al., 2004; Heemskerk et al., 2002; Lentz, 2003; Zwaal et al., 2005).

Procoagulant activity is accomplished by the externalization of PS on outer cellular membrane and the generation of PS-bearing microparticle (MP) that provide sites for the assembly of coagulant enzymes, facilitating a rapid thrombin generation and efficient blood clotting (Lentz, 2003; Zwaal et al., 2005).

Here, we aimed to investigate the effect of DOX on platelet activation using freshly isolated human platelets in an effort to elucidate the mechanism underlying the prothrombotic effects of DOX. Interestingly, although DOX did not affect platelet aggregation response, it enhanced the platelet procoagulant activity potently as measured by increased PS exposure, MP, and thrombin generation. We tried to elucidate the mechanism underlying and examined the clinical significance through conducting an in vivo rat venous thrombosis model.

MATERIALS AND METHODS

Materials. DOX, purified human thrombin, apyrase, citric acid, trisodium citrate, fibrinogen, calcium ionophore A23187, bovine serum albumin (BSA), N-ethylmaleimide, ATP bioluminescent assay kit, and Triton X-100 were obtained from Sigma Chemical Co. (St Louis, MO). Fluoro-3-acetoxyethyl ester (Fluo-3 AM) and 2,7'-dichlorodihydrofluorescein diacetate (DCF) were from Molecular Probes (Eugene, OR) and 1-palmitoyl-2-[6-(7-nitro-2,3-benzoxadiazol-4-yl)amino-caproyl]-sn-glycero-3-phosphoserine (C6-NBD-PS) and 1-oleoyl-2-[6-(7-nitro-2,3-benzoxadiazol-4-yl)amino-6-oxo-caproyl]-sn-glycero-3-phosphocholine (C6-NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Laemli sample buffer, 30% acrylamide/Bis solution, ammonium persulfate, SDS, glycine, Tween 20, and Immune Blot polyvidnylidene fluoride (PVDF) membrane were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Horseradish peroxidase (HRP)-linked anti-mouse and anti-rabbit IgG antibody were from Cell Signaling Technology, Inc. (Danvers, MA). ECL detection reagent was from Amersham Biosciences (Piscataway, NJ). The chromogenic substrate for thrombin (S2238) was purchased from Chromogenix (Milano, Italy). Purified human prothrombin, factor Xa, and factor Va were from Hematologic Technologies, Inc. (Essex Junction, VT). Fluorescein-isothiocyanate (FITC)-labeled annexin V (annexin V-FITC) and phycoerythrin (PE)-labeled glycophorin Ia antibody (anti-GP Ib-PE) were from Pharmingen (San Diego, CA).

Preparation of human washed platelets. With an approval from the Ethics Committee of the Health Service Center at Seoul National University, human blood was obtained from healthy male donors (18–25 years old) on the day of experiments using acid-citrate-dextrose (ACD) as an anticoagulant in the presence of prostaglandin E1 (PGE1; 1 μM). Human washed platelets were prepared by differential centrifugation as previously described (Bae et al., 2007). Briefly, after isolation of platelet-rich plasma (PRP) by centrifugation of blood at 150 × g for 15 min, and platelets were pelleted by centrifugation at 500 × g for 10 min. Pellet was resuspended with Tyrode buffer (134 mM NaCl, 2.9 mM KCl, 1.0 mM MgCl2, 10.0 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 5.0 mM glucose, 12.0 mM NaHCO3, 0.34 mM Na2HPO4, and 0.3% BSA, pH 7.4) containing 1 μM PGE1 and 10% ACD.

After centrifugation at 500 × g for 10 min, platelets were resuspended in Tyrode buffer to a cell concentration of 3 × 108 cells/ml and final CaCl2 concentration was adjusted to 2 mM prior to use. Isolation of rat washed platelets is principally same, with a minor modification of exclusion of PGE1.

Measurement of activation of prothrombinase complex. DOX-treated platelets were incubated with 5 nM of factor Xa and 10 nM of Va in Tyrode buffer supplemented with 2 mM CaCl2 for 3 min at 37°C. Thrombin formation was initiated by the addition of 2 mM prothrombin. Exactly 3 min after the addition of prothrombin, an aliquot of the suspension was transferred to a tube containing stop buffer (50 nM Tris-HCl, 120 mM NaCl, and 2 mM EDTA, pH 7.9). Thrombin activity was determined using the chromogenic substrate S2238. The rate of thrombin formation was calculated from the time-based change in absorbance at 405 nm, using a calibration curve generated with active site–titrated thrombin.

Determination of PS exposure and MP generation. Platelets were identified by anti-GP Ib-PE. Negative controls for annexin V binding were stained with annexin V-FITC in the presence of 4 mM EDTA. Platelets were incubated with annexin V-FITC and GP Ib-PE for 20 min and analyzed on the FACSCalibur (BD Biosciences, San Jose, CA) equipped with argon laser (λex = 488 nm). Data from 5000 events were collected and analyzed using Cell Quest software. Cells were considered to be positive when fluorescence intensity was >99% of the signal from EDTA-negative control group. In experiments using ethylene glycol tetraacetic acid (EGTA), platelets were preincubated with EGTA (final concentration 5 μM) for 5 min and exposed to DOX for 1 h. MPs were identified based on forward scatter characteristics (FSC) after calibration using 1-μm standard beads.

Observation of MPs using confocal microscopy. To prepare the samples for microscopic observation, platelets were immobilized as described previously (Heemskerk et al., 2001). Platelets (3 × 107 cells/ml) suspended in HEPES buffer (10 mM HEPES, 136 mM NaCl, 5 mM glucose, 2.7 mM KCl, 2 mM MgCl2, and 0.05% BSA, pH 7.45) were attached to Laboratory-Tek coverslips (Nunc, Roskilde, Denmark) coated with fibrinogen (10 mg/ml) for 20 min. Adhered platelets were exposed to DOX for 1 h. After being labeled with platelet-specific GP Ib antibodies, platelets and MPs were analyzed on a confocal microscope (Leica, Wetzlar, Germany) equipped with an argon laser (λex = 488 nm).

Isolation of generated MPs. In order to isolate the MPs, DOX-treated platelets were pelleted by centrifugation at 1000 × g for 10 min, and the supernatant was collected. After further centrifugation at 2000 × g for 10 min, the MP-containing supernatant was isolated, and an aliquot was subjected to the prothrombinase assay and PS exposure as described above.

Phospholipid translocation measurement. Phospholipid translocation was measured according to the methods previously described by Hilarius et al. (2004). Briefly, platelets were incubated with DOX and then loaded with 0.5 μM C6-NBD-PS or C6-NBD-PC. Aliquots from the cell suspension were removed at the indicated time intervals and placed on cold Tris buffer and incubated on the ice for 10 min in the presence or absence of 1% BSA, respectively. The amount of internalized probe was determined by comparing the fluorescence intensity associated with the cells before and after back-extraction. Samples were analyzed on the flow cytometer FACSCalibur.

Measurement of intracellular calcium. Intracellular calcium levels were determined using the methods described previously (Heemskerk et al., 2001). After preparation of immobilized platelets, fluo-3 (5 μM) was loaded in the presence of apyrase (0.2 unit/ml) for 45 min at 37°C. The platelets were washed twice with HEPES buffer and incubated with DOX and then calcium increases were observed using a confocal microscope and quantified in terms of changes in fluorescence intensity.

Measurement of ATP level. ATP levels were determined using the luciferin/luciferase bioluminescence assay with perchloric acid extracts. For extraction, equal volumes of ice-cold 1 M HClO4, and platelet aliquots were mixed and incubated for 30 min on ice. After the extracts were centrifuged at
12,000 × g for 2 min, the supernatants were neutralized by the addition of 1M K$_3$CO$_3$. Precipitated KClO$_3$ was removed by centrifugation at 12,000 × g for 10 min, and the supernatant was collected as perchloric acid extracts. After dilution with TAE buffer (100mM Tris-acetate and 2mM EDTA, pH 7.8), samples were applied to luciferin/luciferase assay in Luminoskan (Labsystems, Franklin, MA) using an ATP assay kit. The ATP concentrations were calculated based on the ATP standard curve.

Detection of reactive oxygen species. Generation of reactive oxygen species (ROS) was measured with DCF using flow cytometry, as previously described (Begonja et al., 2005). After loading with DCF (5µM, 30 min), platelets were washed twice with Tyrode buffer and exposed to DOX. Change of intracellular DCF fluorescence was detected by flow cytometry, in which platelets were identified as being within the platelet window defined by forward and side light scatter characteristics. ROS generation was expressed as mean fluorescence intensity of intracellular DCF.

Determination of mitochondrial membrane potential. Changes in mitochondrial membrane potential (ΔΨm) were determined with the cationic fluorochrome JC-1, which accumulates in mitochondria as red fluorescent aggregates at intact ΔΨm but exists in green fluorescent monomeric form at decreased ΔΨm. After treatment with DOX, platelets were incubated with JC-1 (final 2 µg/ml) for 20 min at room temperature. Fluorescence signal from JC-1 aggregates (FL-2) or JC-1 monomers (FL-1) was measured by flow cytometry. The ratio of mean fluorescence intensity from FL-1 and FL-2 (FL-2/FL-1) was used as the representative parameter for decrease of ΔΨm.

Detection of caspase-3 activity. The activity of caspase was determined using caspase-3 intracellular activity assay kit (Calbiochem). After centrifugation of DOX-treated platelets at 500 × g for 10 min, pellets were mixed with 10µM PhiPhiLux-G1D2 and incubated for 1 h. The samples were diluted with the dilution buffer included in the kit and immediately analyzed in flow cytometer. Data are expressed as percentage of positive cells, of which fluorescence intensity was >99% of the signal from negative control group without PhiPhiLux-G1D2.

Measurement of cytochrome c release and bax translocation. Measurement of cytochrome c release and Bax translocation was measured according to the methods previously described by Lin et al. (2009). Washed platelets (2 × 10^9 cells/ml) were incubated with DOX (0, 30, 60, and 100µM) for 1h at 37°C. After incubation with DOX, cytosolic and mitochondrial fractions of platelets were isolated using a mitochondria isolation kit (Thermo Scientific, Rockford, IL). The cytosolic fraction and mitochondrial fraction were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto Immob-Blot PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked with Tris-buffered saline with 0.5% Tween 20 (TBST) containing 3% BSA for 1 h at room temperature and then probed with primary antibodies (diluted 1:1000 in TBST) specific for cytochrome c, Bax, α-tubulin CoxIV (Cell Signaling, Danvers, MA) for overnight at 4°C. Membranes were washed and the incubated with HRP-linked anti-mouse IgG or anti-rabbit IgG (diluted 1:3000) for 2 h at room temperature. The immunoreactive bands was detected using film exposure with Super Signal West Femto (Thermo Scientific, Rockford, IL), and the density of protein bands was determined by TINA software (Raytest, Germany).

Ex vivo experiments. After iv administration of DOX, blood was collected from the abdominal aorta. Platelets were counted using a Celldyne 3500R blood cell counter (Abbott Diagnostics, Abbott Park, IL). For measurement of PS exposure, an aliquot of the blood sample was diluted with Tyrode buffer containing 2mM CaCl$_2$ and stained with anti-rat GP IIIa-FITC and annexin V-PE for 20 min in dark. PS exposure was measured as described above. To determine mitochondrial membrane potential, PRPs were isolated and incubated with JC-1 (final 2 µg/ml) for 20 min at room temperature. Measurement of mitochondrial membrane potential was done as described above.

Rat venous thrombosis model. All the protocols were approved by the Ethics Committee of the Animal Service Center at Seoul National University.

Male Sprague-Dawley (SD) rats (Dae Han BioLink Co., Chungbuk, Korea) weighing 200–250 g were used in all experiments. Before the experiments, animals were acclimated for 1 week. Food and water were provided ad libitum. For the estimation of thrombus formation, in vivo FeCl$_3$-induced rat venous thrombosis was used based on method of Wang et al. (2006) with a minor modification. After iv administration of DOX, rats were anesthetized with urethane (1.25 g/kg, ip), and abdomen was surgically opened. Loose cotton threads were placed 15 mm apart around the vena cava and all side branches. Ferric chloride-5% in saline) soaked filter paper (3 × 10 mm) was applied to the exposed vena cava for 5 min and removed. Thirty minutes after application of FeCl$_3$, isolated thrombus was blotted to remove excess blood, immediately weighed, and observed under a low power microscope.

Statistical analysis. The means and SEMs were calculated for all treatment groups. The data were subjected to one-way ANOVA followed by Duncan’s multiple range test to determine which means were significantly different from the control. In all cases, a p value of <0.05 was used to determine significance.

RESULTS

To examine whether DOX could affect the platelet activation, freshly isolated human platelets were treated with DOX and then platelet aggregation and procoagulant activity, two major responses of platelet activation were evaluated. Procoagulant activity, which was measured by the unit of thrombin generated, was increased significantly by DOX treatment in a concentration- and time-dependent manner (Figs. 1A and 1B), whereas platelet aggregation was not affected (Fig. 1C, left). A significant increase of procoagulant activity was induced by DOX at the concentrations as low as 60µM by 1-h exposure (Fig. 1B), whereas no platelet cytotoxicity was occurred up to 100µM (Fig. 1C, right).

It is well known that platelet procoagulant activity is accomplished by PS exposure on the outer cellular membrane. To clarify DOX-induced platelet procoagulant activity, we measured the extent of PS exposure using flow cytometry. Treatment with DOX substantially increased the number of PS-positive annexin V-binding platelets (Figs. 1D and 1E), reflecting that DOX induced PS exposure in platelets indeed. When the exposed PS was blocked with purified annexin V, the procoagulant activity of platelets was abolished significantly (Fig. 1F), confirming that PS exposure is essential to DOX-enhanced procoagulant activity. In addition to PS exposure, shedding of PS-bearing MPs could play a role in platelet procoagulant activity and thrombosis (Bidot et al., 2008). Treatment with DOX significantly increased MP generation, as determined in flow cytometry (Figs. 2A and 2B) and confocal microscopy (Fig. 2C). Generated MP also displayed procoagulant activity and PS positivity (Fig. 2D), suggesting that DOX could enhance procoagulant activity by generating PS-bearing MP as well.

PS exposure in platelets can be accomplished by the disturbance of phospholipid transporters like flippase and scramblase (Barber et al., 2009). Flippase maintains the lipid asymmetry in a cell membrane by unidirectional transport of PS to the inner leaflet, whereas scramblase induces a rapid
and nonspecific scrambling of the membrane lipids across the lipid bilayer. In order to measure the effect of DOX on these enzymes, the activities of flippase and scramblase were determined by measuring inward and outward translocation of C6-NBD-PS and C6-NBD-PC, fluorescence-tagged PS and PC, respectively. The activity of scramblase, which can induce phospholipid scrambling and resultant externalization of PS, was significantly enhanced by DOX treatment as measured by increased C6-NBD-PC translocation (Fig. 3A), whereas the activity of flippase, which restores exposed PS into inner membrane, was inhibited as measured by reduced C6-NBD-PS translocation (Fig. 3B).

Because Ca2+ influx into platelets is known to be a key mechanism of agonist-induced PS exposure through the activation of scramblase and inhibition of flippase, the role of Ca2+ in DOX-induced PS exposure was determined by measuring inward and outward translocation of C6-NBD-PS and C6-NBD-PC, fluorescence-tagged PS and PC, respectively. The activity of scramblase, which can induce phospholipid scrambling and resultant externalization of PS, was significantly enhanced by DOX treatment as measured by increased C6-NBD-PC translocation (Fig. 3A), whereas the activity of flippase, which restores exposed PS into inner membrane, was inhibited as measured by reduced C6-NBD-PS translocation (Fig. 3B).

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Interestingly, both procoagulant activity and PS exposure by DOX were reversed by the pretreatment of caspase inhibitor, Q-VD-OPh (QVD) as well as antioxidants, Vitamin C, and Trolox (Fig. 4E), suggesting that ROS and apoptotic pathway may be deeply involved in the DOX-mediated procoagulant activity and PS exposure in platelets.

In order to investigate the association of DOX-enhanced platelet procoagulant activity with thrombosis in vivo, we examined the effects of DOX on platelets ex vivo and venous thrombosis in rats after iv administration of DOX. Although DOX administration (2.5–7.5 mg/kg, iv) did not affect platelet counts (Fig. 5A), \( \Delta \psi_m \) decreased and PS exposure increased significantly in a good accordance with the in vitro findings (Figs. 5B and 5C). Most notably, thrombus formation was significantly enhanced by DOX treatment (Figs. 5D and 5E), implying that DOX could increase thrombosis indeed through increased platelet procoagulant activity.

**DISCUSSION**

In the present study, we demonstrated that although platelet aggregation was not affected, DOX significantly increased PS exposure and PS-bearing MP generation and subsequently induced platelet procoagulant activity, which could ultimately contribute to the increased thrombus formation (Fig. 6). DOX-generated ROS and \( \text{Ca}^{2+} \) increase were determined to mediate DOX-induced PS exposure through the alteration of scramblase and flippase activity. Apoptotic like events like \( \Delta \psi_m \) decrease, cytochrome c release, and Bax translocation, which culminated in the activation of caspase-3 also contributed to DOX-induced PS exposure. These in vitro results were further confirmed in in vivo rat models where DOX administration iv increased PS exposure on platelets and venous thrombosis significantly. Considering the paucity of mechanistic data for the thrombotic risk associated with chemotherapy, we believe that this study might provide an important insight into the understanding of the chemotherapy-associated thrombosis.

DOX-induced thrombosis has been suggested to be mediated by platelet activation (Bernat and Herbert, 1994), but the exact mechanism has not been fully illustrated, to our best knowledge. It is conspicuous that DOX selectively induced platelet procoagulant activity, whereas platelet aggregation, another important response of platelet activation, was not affected (Fig. 1C). Both platelet aggregation and procoagulant activity are important for the participation of platelets in thrombosis and hemostasis (Siljander et al., 2001). Aggregation provides the basic building blocks of a thrombus and...
Procoagulant activity promotes thrombin and fibrin generation that further enlarge it. Platelet aggregation and procoagulant activity also share intracellular Ca\(^{2+}\) increase as a common signaling pathway. However, procoagulant activity requires a prolonged rise in intracellular Ca\(^{2+}\) level (Heemskerk et al., 2002), and distinct mechanisms such as scramblase activation, flippase inhibition, and caspase activation can increase procoagulant activity through the mediation of PS exposure (Forsberg et al., 2004; Lentz, 2003). It is well established that increased PS exposure in platelets and/or generation of PS-bearing MPs can contribute to the increased thrombus formation (Bidot et al., 2008; Mallat et al., 2000; Vidal et al., 2001) through the acceleration of coagulation cascades and promoting thrombin formation, which can induce fibrin clot formation and stimulate platelet aggregation in itself (Kumar et al., 1994; Ruggeri, 2002). In many CVDs including myocardial infarction, hypertension, atherosclerosis, and pulmonary thrombosis, enhanced platelet procoagulant activity and thrombotic events are frequently observed (Lentz, 2003; Zwaal et al., 2005). In addition, the people with high risks of CVDs, such as diabetes mellitus, chronic uremia, and hyperlipidemia patients, exhibit elevated levels of platelet procoagulant activity, supporting its role in thrombosis. As with these diseases, we believe that the procoagulant activation of platelets might contribute substantially to the increased thrombotic risks in the patients treated with DOX.

In this study, ROS generation was determined to be a key mediator for the DOX-induced PS exposure and procoagulant activity. Many studies demonstrated that DOX can generate a large amount of ROS (Kotamraju et al., 2002), which can induce ATP depletion, intracellular Ca\(^{2+}\) increase, and apoptosis (Santos et al., 2002; Zhou et al., 2001a,b). ROS can modify SH-groups in adenine nucleotide transporter (Berthiaume and Wallace, 2007), leading to the opening of permeability transition pore and the inhibition of mitochondrial respiration. ROS-induced functional perturbation of mitochondrial respiration and mitochondrial membrane potential ultimately results in the impairment of ATP production and more importantly, cytosolic release of proapoptotic proteins like cytochrome c (cyt c), which culminates in the activation of caspase-3 (Orrenius et al., 2011). Important role of ROS could be further evidenced by the complete inhibition of DOX-induced PS exposure with antioxidants, such as vitamin C and trolox (Fig. 4E). ROS generation and the disruption of
mitochondrial membrane potential are a major target mechanism of action for the anticancer effect of DOX and many other chemotherapeutic agents (Fulda et al., 2010). Indeed, increased complication of thrombotic diseases is well documented in the patients treated with many ROS-generating chemotherapeutic agents, including bleomycin, cisplatin, and etoposide (Cantwell et al., 1988; Strumberg et al., 2002; Weijl et al., 2000). In this regard, it would be necessary to investigate whether other ROS-generating chemotherapy might result in enhanced PS exposure and ultimately procoagulant activity in platelets.

For the treatment of leukemia, DOX is generally dosed at 15–90 mg/m² body surface area, and the plasma concentration of DOX can be elevated as high as 4–10 μM (Gewirtz, 1999; Greene et al., 1983; Robert et al., 1985), which has not much margin from the effective procoagulant DOX concentrations (60 μM) in our study. Moreover, DOX is administered through iv infusion, putting circulating blood cells including platelets in a direct contact with extremely high concentrations of DOX. This parenteral dosing regimen and high plasma DOX concentrations may place platelets at a greater risk of procoagulant activation. Considering the repetitive use of DOX for the cancer treatment, cumulative DOX concentration might become even higher (Pai and Nahata, 2000) increasing the chance of the platelet procoagulant activation and thrombosis and embolism ultimately. Incidentally, the risk of cardiotoxicity of DOX is being regularly checked by...
monitoring cardiac function during the treatment (Takemura and Fujiwara, 2007). As seen in this study, we suggest that platelet procoagulant activity should be also checked as an important diagnostic marker for monitoring of the risk of DOX-associated thrombosis.

DOX increased not only Ca$^{2+}$-dependent but also the apoptosis-dependent PS exposure through the dissipation of mitochondrial membrane potential ($\Delta \Psi_m$), cyt$c$ release, mitochondrial translocation of Bax, and caspase-3 activation (Figs. 4B–4D). Ca$^{2+}$-dependent PS exposure is from the unidirectional transport of PS to outer membrane through scramblase activation and flippase inhibition. In contrast, apoptotic pathways involve caspase-3-dependent cytoskeleton degradation, membrane blebbing, MP release, and subsequent disruption of lipid asymmetry (Hugel et al., 2005). Recently, Schoenwaelder et al. (2009) reported that physiological agonists induce PS exposure and procoagulant activity through Ca$^{2+}$-dependent pathway, whereas those by proapoptotic agents like BH3 mimetic, ABT-737, are dominated by Bak/Bax-mediated caspase-3 activation. In a good contrast to this theory, in our study, DOX-induced PS exposure procoagulant activities could be attenuated by both EGTA, a Ca$^{2+}$ chelator (Fig. 3E) and QVD, a caspase-3 inhibitor (Fig. 4E), reflecting that apoptosis-dependent and Ca$^{2+}$-dependent PS exposure cannot be clearly separated but to a certain degree, mutually interactive although further studies will be required to clarify it.

During our study, Swystun et al. (2009) reported that DOX can induce procoagulant phenotypes in endothelial cells and blood monocytes. They showed that DOX induces procoagulant...
phenotypes in endothelial cells and blood monocytes, which was achieved by PS exposure and tissue factor increase through the mediation of ROS formation. However, they did not delineate the involvement of ATP depletion, flippase, or scramblase as the mechanisms for the accomplishment of PS exposure. In addition, in vivo evidence of DOX-mediated PS exposure and procoagulant activity in endothelial cells and monocytes was not provided. This information is critical for the verification of the roles of endothelial cells and monocytes in DOX-associated thrombosis because multiple tissues or cells are involved in the development of thrombosis. Most importantly, the effect of DOX on the procoagulant activity of platelets has not been addressed despite the essential and major role of platelets in thrombosis and coagulation. In this regard, we believe that our study provided a clearer picture for DOX-associated thrombosis by describing the mechanism underlying DOX-mediated procoagulant activity and demonstrating DOX-induced PS exposure in vivo.

In conclusion, we demonstrated that DOX can induce platelet procoagulant activity through PS exposure and MP generation by the mediation of ROS, Ca²⁺ increase, ATP depletion, and caspase-3 activation. Increased thrombus formation in vivo after DOX administration strongly suggests that DOX-induced procoagulant activity may contribute to DOX-associated thrombosis indeed, providing important information for the understanding of the thrombotic risks associated with chemotherapy.

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