Effects of the chemotherapeutic agent doxorubicin on the protein C anticoagulant pathway

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Abstract

Although chemotherapy treatment is associated with an increased risk of thrombosis, the pathogenic mechanisms for the thrombogenic effect of chemotherapeutic drugs are poorly understood. We hypothesize that exposure of vascular endothelial cells to chemotherapeutic agents results in the loss of a thromboresistant phenotype. In this study, we examined the effects of the chemotherapeutic agent doxorubicin on the endothelium-based protein C anticoagulant pathway. The endothelial cell protein C receptor (EPCR) and thrombomodulin are two endothelial cell surface receptors required for the conversion of zymogen protein C to the anticoagulant enzyme activated protein C. Exposure of human umbilical vein endothelial cells (HUVEC) to doxorubicin resulted in a dose- and time-dependent decrease in cell surface EPCR levels. This decrease occurred as a result of receptor shedding as well as from a down-regulation in EPCR mRNA levels. In contrast, doxorubicin treatment of HUVECs resulted in a dose- and time-dependent increase in cell surface thrombomodulin attributed to an up-regulation of thrombomodulin mRNA levels. The net effect of the doxorubicin-induced changes in EPCR and thrombomodulin levels was a decrease in the capacity of HUVECs to convert protein C to activated protein C. Preliminary studies suggest that doxorubicin free radical metabolites mediate the doxorubicin-induced changes in EPCR expression but not those of thrombomodulin expression. In summary, these results suggest that doxorubicin alters the hemostatic balance of endothelial cells by down-regulating the endothelium-based protein C anticoagulant pathway. [Mol Cancer Ther 2006;5(12):3303–11]

Introduction

Thromboembolic disease is the second most frequent cause of death in cancer patients (1, 2). Up to 90% of all cancer patients manifest laboratory evidence of hypercoagulation and an estimated 15% of cancer patients present with venous thromboembolism during the progression of their disease (3, 4). The pathogenesis of this hypercoagulable state is complex and reflects, in part, defects in blood flow due to local tumor growth, direct and indirect activation of the coagulation cascade by tumor procoagulant factors, and the thrombogenic properties of tumor-associated blood vessels (5).

In addition to the tumor itself, chemotherapeutic treatment is an independent risk factor for thrombotic complications in cancer patients. Perhaps the most reliable data on the incidence of thrombosis in patients receiving chemotherapy are derived from studies of breast cancer patients. One study reported a 5% incidence of venous thrombosis in patients with early (stage II) breast cancer receiving postmastectomy combination chemotherapy, whereas no patient developed thrombosis after the chemotherapy was completed (6). The rate of thrombosis in patients with metastatic (stage IV) breast cancer treated with a five-drug chemotherapy regimen was 17.6%, and thrombosis occurred early in the course of therapy (7). In a randomized trial that compared 12 weeks of chemohormonal therapy with 36 weeks of chemotherapy in stage II breast cancer patients, a 6.8% incidence of thrombosis was observed during the months of chemotherapy, whereas no thrombotic events occurred during the months without therapy (8). In women with early breast cancer who were treated with one short intensive course of perioperative chemotherapy, there was a 3-fold greater risk for developing thrombotic complications compared with women who received surgery alone (9).

Doxorubicin (Adriamycin) is a widely prescribedchemotherapeutic drug used to treat a variety of cancers, including breast cancer, prostate cancer, and multiple myeloma (10). In the above-mentioned breast cancer studies, doxorubicin was often one of several drugs in a cocktail regimen of chemotherapy drugs (8, 9). In recent studies of patients with multiple myeloma, thalidomide given in combination with doxorubicin-containing multi-agent chemotherapy was associated with a 7-fold increase in the risk of deep vein thrombosis (11). All episodes of deep vein thrombosis occurred during the first 3 weeks of chemotherapy induction (11). Another study reported a
strong association between deep vein thrombosis and exposure to doxorubicin in patients with multiple myeloma receiving thalidomide therapy (12). Deep vein thrombosis developed in 16% of patients treated with doxorubicin-containing chemotherapy regimen (doxorubicin, thalidomide, dexamethasone, cisplatin, cyclophosphamide, and etoposide) compared with 2.5% in the control arm (thalidomide, dexamethasone, cisplatin, cyclophosphamide, and etoposide).

Although a direct relationship between chemotherapy and increased risk of thrombosis has been shown, the molecular mechanisms by which chemotherapeutic drugs induce a prothrombotic state are poorly understood. Because the vascular endothelium plays an active role in inhibiting blood coagulation under physiologic conditions, one possible mechanism is that exposure of vascular endothelial cells to chemotherapeutic agents results in the loss of a thromboresistant phenotype. A major anticoagulant system that involves endothelium-associated molecules is the protein C pathway (13, 14). Patients with congenital or acquired deficiencies in this pathway have an increased risk of thrombotic events. Indeed, even a 2-fold decrease in protein C concentration results in an increased risk of venous thromboembolism (15).

The protein C pathway provides an “on demand” anticoagulant response whenever thrombin is generated. Briefly, vascular injury, endotoxin, or proinflammatory cytokines initiate the coagulation cascade, ultimately resulting in thrombin generation and blood clot formation. Excess thrombin then complexes with thrombomodulin, a receptor found on the surface of vascular endothelial cells. The thrombin-thrombomodulin complex activates zymogen protein C to generate the anticoagulant enzyme activated protein C. Activated protein C, in combination with its cofactor protein S, acts as an anticoagulant by inactivating factors Va and VIIIa, which are key cofactors in coagulation. Protein C activation is augmented by the endothelial cell protein C receptor (EPCR), an endothelial cell surface receptor that binds protein C and activated protein C with comparable affinity (16). Thus, thrombomodulin and EPCR are two endothelial receptors required for the conversion of protein C to the anticoagulant enzyme activated protein C.

In this study, we examined the effects of doxorubicin on endothelial cell hemostasis. Specifically, we used human umbilical vein endothelial cells (HUVEC) to study the effects of doxorubicin on the endothelial-based protein C anticoagulant pathway. Our studies reveal potential mechanisms by which doxorubicin induces a hypercoagulable state in cancer patients.

Materials and Methods

Materials

HUVECs were purchased from Cambrex Biosciences (Walkersville, MD). M199 growth medium, trypsin in EDTA, and penicillin-streptomycin were purchased from Invitrogen (Burlington, Ontario, Canada). Fetal bovine serum was purchased from Hyclone (Logan, UT). Endothelial cell growth factor was purchased from MP Biomedicals, Inc. (Aurora, OH). Heparin was purchased from Leo Pharma, Inc. (Thornhill, Ontario, Canada). Doxorubicin, bovine serum albumin, FITC, tetramethyl benzamidine, formamide, formaldehyde, diethyl pyrocarbonate, 1,10-phenanthroline, and hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO). S-2366 chromogenic substrate was purchased from DiaPharma (West Chester, OH). Goat anti-human thrombomodulin polyclonal antibody (gt261), murine monoclonal antibodies against human EPCR (JRK 1535, JRK 1495, and JRK 1513) and against human thrombomodulin (CTM 1009), recombinant soluble EPCR, and recombinant soluble thrombomodulin were generously provided by Dr. Charles Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). Iodo-beads were purchased from Pierce (Rockford, IL). Protein C was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). [α-32P]dCTP was purchased from Perkin-Elmer (Boston, MA). SDS-PAGE precast 4% to 15% gradient gels, Triton X-100, and alkaline phosphatase–conjugated goat anti-mouse immunoglobulin G were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Pure mouse immunoglobulin G was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Recombinant human antithrombin was a gift from Genzyme Transgenic (Framingham, MA). Thrombin was purchased from Enzyme Research Laboratories (South Bend, IN).

Cell Culture

HUVECs were used on passages 1 through 4 exclusively. HUVECs were cultured in 2% gelatin–coated six-well plates, 24-well plates, or 1-cm-diameter Petri dishes in HUVEC medium (M199 medium supplemented with 10 μg/mL endothelial cell growth factor containing 20% heat-inactivated fetal bovine serum, 100 units/mL penicillin-streptomycin, and 12.6 units/mL heparin) at 37°C and 5% CO2. On reaching 80% to 90% confluency, cells were treated with doxorubicin concentrations ranging from 0.1 to 10 μg/mL.

Fluorescent Labeling of Antibodies

Monoclonal antibodies against human EPCR (JRK 1535) and human thrombomodulin (CTM 1009) were labeled with FITC as described by Goding (17). Briefly, 10 μL of 100 μg/mL FITC were added to 500 μL of a 50 μmol/L antibody stock. The reaction was carried out in the dark at 4°C and stopped after 1 h with 10 μL of 1 mol/L NH4Cl. Labeled antibodies were separated from free FITC using a PD10 column. PBS [1.9 mmol/L NaHPO4, 8.1 mmol/L Na2HPO4 (pH 7.4), 150 mmol/L NaCl] was then used to elute 0.5-mL aliquots, and A280 readings were obtained to calculate antibody concentration.

Flow Cytometric Analysis of Cell Surface EPCR and Thrombomodulin on HUVECs

HUVEC monolayers were cultured on gelatinized six-well plates, washed twice with PBS, detached with trypsin/EDTA, and resuspended in 1 mL of binding buffer (HBSS containing 25 mmol/L HEPES (pH 7.5), 1% bovine

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serum albumin, 0.02% NaN₃. Previous experiments have shown that treatment of HUVECs with trypsin/EDTA does not affect cell surface levels of EPCR and thrombomodulin (data not shown). Cells (200 μL) were incubated at room temperature in the dark with 50 nmol/L of FITC-JRK 1535 or FITC-CTM 1009 for 15 min. The cells were then washed once with binding buffer and cell-bound fluorescence was determined by flow cytometry with 5,000 events counted per sample. Data acquisition was done on a FACScan flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA).

Quantification of EPCR and Thrombomodulin Levels on the Surface of HUVECs

Mouse anti-human EPCR monoclonal antibody (JRK 1535), mouse anti-human thrombomodulin monoclonal antibody (CTM 1009), and mouse anti-human immunoglobulin G were radiolabeled with iodine using Iodobeads antibody (CTM 1009), and mouse anti-human immunoglobulin G were radiolabeled with iodine using Iodobeads according to the manufacturer (Pierce). Confluent monolayers of HUVECs in 24-well plates were washed twice in PBS. The cells were incubated at 4°C for 30 min in binding buffer containing increasing concentrations (0–5,000 nmol/L) of a 400:1 ratio of unlabelled to 125I-labeled antibody (JRK 1535 or CTM 1009). After washing thrice with 200 μL of ice-cold binding buffer, the cells were lysed with 200 μL of PBS containing 1% Triton X-100, and the bound radioactivity was measured in a gamma counter. To determine nonspecific binding, 125I-labeled human immunoglobulin G1 was used instead of radiolabeled JRK 1535 or CTM 1009. Specific binding was determined by subtracting bound radioactivity using radiolabeled human immunoglobulin G from that using either radiolabeled JRK 1535 or CTM 1009. The counts bound on cells were plotted against antibody concentration, and data were analyzed by nonlinear regression analysis of the curve by using the equation \( y = a + bx/(c+x) \) (TableCurve, Jandel Scientific, San Rafael, CA). The experiment was repeated using confluent monolayers of HUVECs treated for 24 h with 10 μg/mL doxorubicin.

Western Blot Analysis of EPCR and Thrombomodulin mRNA Content in HUVECs

HUVECs were grown to 80% to 90% confluency on 10-cm-diameter Petri plates and treated with 0, 1, and 10 μg/mL doxorubicin for 6, 12, and 24 h. Total RNA was isolated using an RNasy mini prep kit (Qiagen). Ten micrograms of total RNA were separated on 2% formaldehyde-agarose gels, transferred to nylon ZetaGT Genomic Tested Transfer Membranes (Bio-Rad) using 10× SSC buffer, and fixed to the membrane using UV cross-linking. [α-32P]dCTP–labeled probes were synthesized using the Random Primed DNA Labeling Kit (Roche Diagnostics, Laval, Quebec, Canada), excess nucleotides were removed using the QiaQuick Nucleotide Removal Kit (Qiagen), and the probes were hybridized to the membrane at 43°C overnight in a solution containing 40% formamide, 5 mol/L NaCl, 0.96 mol/L Na2HPO4, and 1% SDS. The membranes were then washed for 15 min at room temperature in 2× SSC with 0.1% SDS, followed by 0.5× SSC with 0.1% SDS, and finally in 0.1× SSC and 0.1% SDS at 43°C. The blots were exposed to imaging film for 2 to 7 days, depending on the radioactive strength of the probe.

Protein C Activation Assay on HUVEC Monolayers

HUVECs were grown to confluency in six-well plates and washed twice with PBS. Protein C activation was initiated by the incubation of HUVECs in 1-mL incubation buffer [HBSS containing 25 mmol/L HEPES (pH 7.5), 0.1% bovine serum albumin, 5 mmol/L CaCl2, 0.6 mmol/L MgCl2] containing 100 nmol/L protein C and 10 nmol/L thrombin. After 45 min at 37°C, the reaction was stopped by the addition of 16 mmol/L EDTA and 5 μmol/L antithrombin for 15 min at room temperature. Fifty microliters of the reaction mixture were transferred into a 96-well plate and activated protein C amidolytic activities were determined toward a 2 mmol/L activated protein C substrate (S-2366) in 20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl. The rates of substrate cleavage were measured with a SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm in kinetic mode. Under the described (19), except that the coating and detecting antibodies were JRK 1535 and JRK 1495, respectively. Levels of soluble thrombomodulin were quantified by ELISA as previously described (20), except that the coating antibody was CTM 1009 and the detecting antibody was goat anti-mouse polyclonal antibody 261.

Northern Blot Analysis of EPCR and Thrombomodulin mRNA Content in HUVECs

PCR was done on the cDNA of human EPCR and human thrombomodulin. A 511-bp PCR product for EPCR was obtained by using the following primers: EPCR sense, 5'-ATGTTGACACATTGCTGCGATA-3'; EPCR antisense, 5'-TACGGTTGAGCTGCTGAGGG-3'. A 538-bp PCR product for thrombomodulin was obtained by using the following primers: thrombomodulin sense, 5'-ATGGCTGGGTCCTGTGCTCCGTG-3'; thrombomodulin antisense, 5'-GAAAGTTGACACTGCGAGGAGCC-3'. After 35 cycles of 40 s at 94°C, 40 s at 50°C, and 80 s at 72°C, PCR products were separated on a 1.5% agarose gel and purified using the QIAEX II gel extraction kit (Qiagen, Valencia, CA).

HUVECs were grown to 80% to 90% confluency in 10-cm-diameter Petri plates and treated with 0, 1, and 10 μg/mL doxorubicin for 6, 12, and 24 h. Total RNA was isolated using an RNasy mini prep kit (Qiagen). Ten micrograms of total RNA were separated on 2% formaldehyde-agarose gels, transferred to nylon ZetaGT Genomic Tested Transfer Membranes (Bio-Rad) using 10× SSC buffer, and fixed to the membrane using UV cross-linking. [α-32P]dCTP–labeled probes were synthesized using the Random Primed DNA Labeling Kit (Roche Diagnostics, Laval, Quebec, Canada), excess nucleotides were removed using the QiaQuick Nucleotide Removal Kit (Qiagen), and the probes were hybridized to the membrane at 43°C overnight in a solution containing 40% formamide, 5 mol/L NaCl, 0.96 mol/L Na2HPO4, and 1% SDS. The membranes were then washed for 15 min at room temperature in 2× SSC with 0.1% SDS, followed by 0.5× SSC with 0.1% SDS, and finally in 0.1× SSC and 0.1% SDS at 43°C. The blots were exposed to imaging film for 2 to 7 days, depending on the radioactive strength of the probe.

Protein C Activation Assay on HUVEC Monolayers

HUVECs were grown to confluency in six-well plates and washed twice with PBS. Protein C activation was initiated by the incubation of HUVECs in 1-mL incubation buffer [HBSS containing 25 mmol/L HEPES (pH 7.5), 0.1% bovine serum albumin, 5 mmol/L CaCl2, 0.6 mmol/L MgCl2] containing 100 nmol/L protein C and 10 nmol/L thrombin. After 45 min at 37°C, the reaction was stopped by the addition of 16 mmol/L EDTA and 5 μmol/L antithrombin for 15 min at room temperature. Fifty microliters of the reaction mixture were transferred into a 96-well plate and activated protein C amidolytic activities were determined toward a 2 mmol/L activated protein C substrate (S-2366) in 20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl. The rates of substrate cleavage were measured with a SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm in kinetic mode. Under the
conditions used in this assay, <20% of the protein C was activated, as determined by reference to a standard curve of activated protein C amidolytic activity. All determinations were done in triplicate.

**Statistical Analyses**

One-way ANOVA and Tukey’s pairwise comparisons were done using Minitab software (version 12; Minitab, State College, PA).

**Results**

**Effects of Doxorubicin on EPCR Protein and mRNA Levels in HUVECs**

The conversion of circulating zymogen protein C to the anticoagulant enzyme activated protein C occurs on the surface of vascular endothelial cells and is dependent on two endothelial cell surface receptors: EPCR and thrombomodulin. In this study, we explored the hypothesis that exposure of vascular endothelial cells to doxorubicin impairs the endothelium-based protein C anticoagulant pathway by down-regulating EPCR and/or thrombomodulin levels. Clinically, doxorubicin is given to breast cancer patients in doses ranging from 20 to 90 mg/m^2^ (8, 9, 21–23).

Doxorubicin undergoes triexponential decay with successive half-lives in plasma of ~5 min, 2 h, and 40 h (24, 25). Maximum initial plasma concentrations (C_max) detected range from ~2 to 6 μg/mL (23, 26, 27). At 24 h, the plasma concentration of doxorubicin is ~0.05 μg/mL (23, 27).

In this study, HUVECs were treated with increasing concentrations of doxorubicin (0, 0.1, 1.0, 3.0, and 10 μg/mL) for 6, 12, and 24 h. Apoptosis of HUVECs (as determined by terminal deoxynucleotidyl transferase-mediated nick end-labeling analysis) after a 24-hour incubation with 0, 0.1, 1.0, 3.0, and 10 μg/mL doxorubicin was 0.3%, 0.9%, 1.7%, 4.4%, and 16%, respectively. Doxorubicin-induced changes in cell surface EPCR levels on HUVECs were determined by flow cytometry using a fluorescently labeled monoclonal antibody directed against human EPCR (FITC-JRK 1535).

As shown in Fig. 1A, flow cytometry studies revealed that treatment of HUVECs with doxorubicin resulted in a dose- and time-dependent decrease in cell surface EPCR levels. One-way ANOVA identified the presence of statistically significant differences within the data (P = 0.013). Tukey’s pairwise comparisons identified that these significant differences had occurred between the following groups: EPCR cell surface levels on untreated HUVECs are significantly different from those on HUVECs treated with 3 and 10 μg/mL doxorubicin. To confirm that doxorubicin down-regulates cell surface EPCR, Scatchard binding analysis was done using 125I-labeled anti-EPCR monoclonal antibodies and monolayers of HUVECs. Scatchard analysis indicated that untreated HUVECs expressed ~4.95 × 10^4 ± 0.29 × 10^5 molecules of EPCR per cell, whereas HUVECs treated with 10 μg/mL doxorubicin for 24 h expressed ~9.08 × 10^4 ± 1.34 × 10^5 molecules of EPCR per HUVEC.

Next, Western blot analysis was done to determine if treatment of HUVECs with doxorubicin results in a decrease in total cellular EPCR protein. As shown in Fig. 1B, there is a dose-dependent decrease in total EPCR protein levels in HUVECs upon treatment with doxorubicin for 24 h. To determine if the doxorubicin-dependent down-regulation of EPCR protein is attributed to alterations at a transcriptional level, Northern blot analysis of EPCR mRNA was done. HUVECs were treated with 0, 0.1, or 10 μg/mL doxorubicin for 0, 6, 12, or 24 h. Interestingly, HUVEC EPCR mRNA levels follow a bimodal pattern of expression in response to doxorubicin (Fig. 1C). Within the first few hours after doxorubicin treatment, EPCR transcript levels transiently increase, whereas after 24 h, EPCR transcript levels decline.

![Figure 1](Image Link)

**Figure 1.** EPCR cell surface, total protein, and mRNA levels in doxorubicin-treated HUVECs. **A,** cell surface levels of EPCR in doxorubicin (dox)–treated HUVECs were determined by flow cytometry using FITC-conjugated anti-EPCR (JRK 1535) monoclonal antibody. **B,** Western blot analysis of whole-cell EPCR levels in doxorubicin-treated HUVECs. Lane 1, molecular weight markers; lanes 2 to 6, whole-cell lysates from HUVECs treated for 24 h with 0, 0.1, 1.0, 3.0, and 10.0 μg/mL doxorubicin, respectively. **C,** Northern blot analysis of EPCR mRNA levels in doxorubicin-treated HUVECs. Lane 1, untreated HUVECs; lanes 2 and 3, HUVECs treated for 6 h with 0.1 and 10 μg/mL doxorubicin, respectively; lanes 4 and 5, HUVECs treated for 12 h with 0.1 and 10 μg/mL doxorubicin, respectively; lanes 6 and 7, HUVECs treated for 24 h with 0.1 and 10 μg/mL, respectively. The levels of 28S and 18S rRNA visualized on the formaldehyde-agarose gel are shown as loading controls.
Effects of Doxorubicin on Thrombomodulin Protein and mRNA Levels in HUVECs

We next investigated the effects of doxorubicin on thrombomodulin protein and mRNA levels in HUVECs. In contrast to EPCR, flow cytometry studies revealed that cell surface levels of thrombomodulin in doxorubicin-treated HUVECs increased in a dose- and time-dependent manner (Fig. 2A). One-way ANOVA identified the presence of statistically significant differences within the data ($P < 0.001$). Tukey’s pairwise comparisons identified that these significant differences had occurred between the following groups: thrombomodulin cell surface levels on untreated HUVECs are significantly different from those on HUVECs treated with 1, 3, and 10 µg/mL doxorubicin. Scatchard analysis using $^{125}$I-labeled antihuman thrombomodulin monoclonal antibody indicated that untreated HUVECs expressed $4.45 \times 10^3 \pm 0.16 \times 10^3$ molecules of thrombomodulin molecules per cell. After treatment of HUVECs with 10 µg/mL doxorubicin for 24 h, the cells expressed $9.56 \times 10^3 \pm 1.18 \times 10^3$ molecules of thrombomodulin per cell. Western blot analysis of thrombomodulin protein levels in whole-cell lysates of doxorubicin-treated HUVECs indicates that thrombomodulin levels increase upon exposure of the cells to increasing concentrations of doxorubicin (Fig. 2B). The increase in thrombomodulin protein levels is due to an increase in thrombomodulin mRNA levels based on Northern blot analysis of HUVECs treated with 0, 0.1, or 10 µg/mL doxorubicin for 0, 6, 12, or 24 h (Fig. 2C).

The Effect of Doxorubicin on Receptor Shedding from the Surface of HUVECs

To determine if the doxorubicin-induced down-regulation of cell surface EPCR on HUVECs was also attributed to receptor shedding, we measured soluble EPCR release from HUVECs following doxorubicin treatment. As shown in Fig. 3 (top), treatment of HUVECs with doxorubicin results in a dose- and time-dependent increase in soluble EPCR levels in the conditioned medium of the HUVECs. In contrast, soluble thrombomodulin levels in the conditioned medium of HUVECs do not increase upon exposure of the cells to doxorubicin (Fig. 3, bottom).

Effect of Doxorubicin on Protein C Activation on the Surface of HUVECs

Because both EPCR and thrombomodulin are involved in the conversion of protein C to activated protein C, we investigated the net effect of EPCR down-regulation and thrombomodulin up-regulation on protein C activation rates on doxorubicin-treated HUVECs. Confluent HUVEC monolayers were treated with doxorubicin concentrations ranging from 0 to 10 µg/mL for 6, 12, or 24 h. Protein C activation on the surface of HUVECs was initiated by the addition of protein C and thrombin to the HUVEC monolayer. The concentration of activated protein C generated was measured after HUVECs were exposed to protein C and thrombin for 45 min. As shown in Fig. 4, there is a dose- and time-dependent reduction in protein C activation rates on HUVECs upon treatment with doxorubicin. These results indicate that the net effect of doxorubicin-mediated EPCR down-regulation and thrombomodulin up-regulation is a decrease in the capacity of endothelial cells to support the conversion of protein C to the anticoagulant enzyme activated protein C.

Preliminary Analysis of Potential Mechanisms by which Doxorubicin Modulates EPCR and Thrombomodulin Expression

One of the proposed mechanisms for the cytotoxic effect of doxorubicin is the formation of reactive oxygen species. Doxorubicin undergoes a one-electron reduction to a semiquinone free radical, which, in the presence of O$_2$, is
reoxidized in a process that generates reactive oxygen species such as $\text{H}_2\text{O}_2$ (28, 29). In this study, we investigated whether the antioxidant glutathione attenuates doxorubicin-induced changes in EPCR expression in HUVECs. As shown in Fig. 5, pretreatment of HUVECs with glutathione (5 or 7.5 mmol/L) for 3 h attenuated the doxorubicin-induced decrease in cell surface EPCR, suggesting that doxorubicin-induced down-regulation of cell surface EPCR is due to the generation of reactive oxygen species. In contrast, exposure of HUVECs to glutathione did not affect doxorubicin-induced up-regulation of cell surface thrombomodulin (data not shown).

**Discussion**

One of the most serious problems related to the use of chemotherapeutic agents is the increased incidence of adverse blood clotting events. These events can sometimes be fatal. In addition, when cancer patients develop blood clots, they require anticoagulant therapy, which increases the risk of major clinical bleeding. Thus, it is important to understand the mechanisms by which chemotherapeutic agents induce a prothrombotic state in cancer patients.

Although a direct relationship between chemotherapy treatment and the occurrence of thrombosis has been established, little is known about the mechanisms by which cytotoxic drugs trigger a prothrombotic state. Plasma samples from breast cancer patients receiving combination chemotherapy (cyclophosphamide, epirubicin, 5-fluorouracil) have been shown to enhance endothelial cell reactivity to platelets (30). Increased thrombin generation, as measured by plasma fibrinopeptide A levels, has been documented in patients with advanced or metastatic cancer receiving various chemotherapy regimens (31). Administration of chemotherapy to patients with breast and lung cancer has been shown to increase plasma thrombin-antithrombin complexes and D-dimer levels (32). Recently, Ma et al. (33) reported that a marked increase in coagulation index (defined as the ratio of tissue factor over tissue factor pathway inhibitor expression) was observed after exposure of cultured endothelial cells to the chemotherapeutic agents gemcitabine and cisplatin in combination with SU5416, a small-molecule antiangiogenic drug.

Doxorubicin is a broad-range chemotherapeutic agent commonly used in the treatment of solid tumors and malignant hematologic disease. The main route of entry of doxorubicin into cells is passive diffusion across the plasma membrane (34, 35). In this study, we investigated the effects of doxorubicin on the endothelium-based protein C anticoagulant pathway. EPCR and thrombomodulin are two endothelial cell surface receptors required for the conversion of protein C to the anticoagulant enzyme activated protein C. We showed that there is a dose- and time-dependent down-regulation of cell surface EPCR in doxorubicin-treated HUVECs (Fig. 1A). Western blot analysis of total EPCR protein levels in HUVEC lysates reveals that there is a dose- and time-dependent decrease in EPCR protein upon treatment with doxorubicin (Fig. 1B).
that the A3 haplotype of the protein C binding. Recently, Saposnik et al. (39) reported by virtue of its ability to compete with cellular EPCR for inglevels of soluble EPCR may impair protein C activation on the endothelial surface. Second, an increase in circulat-endothelium would reduce the rate of protein C activation to 10-fold (38), a reduction of cell surface EPCR on the activation by the thrombin-thrombomodulin complex up because cell surface EPCR enhances the rate of protein C protein C(19,36,37). Thus, doxorubicin potentially impairs zymogen protein C to the anticoagulant enzyme activated for protein C binding, thereby inhibiting the conversion of soluble EPCR and thrombomodulin (41). The finding that doxorubicin decreases cell surface EPCR while up-regulating surface thrombomodulin on HUVECs is intriguing. Because both EPCR and thrombomodulin are components of the “machinery” required to convert the protein C zymogen to the anticoagulant enzyme activated protein C (19,36,37). Thus, doxorubicin potentially impairs protein C activation on endothelial cells in two ways. First, because cell surface EPCR enhances the rate of protein C activation by the thrombin-thrombomodulin complex up to 10-fold (38), a reduction of cell surface EPCR on the endothelium would reduce the rate of protein C activation on the endothelial surface. Second, an increase in circulating levels of soluble EPCR may impair protein C activation by virtue of its ability to compete with cellular EPCR for protein C binding. Recently, Saposnik et al. (39) reported that the A3 haplotype of the EPCR gene results in higher soluble EPCR levels and is a risk factor for venous thrombosis.

Our studies also indicate that the decrease in endothelial cell surface EPCR that occurs in response to doxorubicin treatment is attributed, in part, to a decrease in EPCR gene expression (Fig. 1C). HUVEC EPCR mRNA levels follow a bimodal pattern of expression in response to doxorubicin. EPCR transcript levels transiently increase within the first few hours after doxorubicin treatment, whereas after 24 h, EPCR transcript levels decline. Gu et al. (40) have reported that the constitutive expression of murine EPCR is dependent on AP4 and SP1 binding sites in the 5′ promoter region of the EPCR gene, and that a thrombin response element upstream of the AP4 and SP1 binding sites is required for thrombin-dependent up-regulation of EPCR mRNA. To date, negative regulatory elements have not yet been identified in the EPCR gene.

This study is also the first to show that doxorubicin up-regulates cell surface thrombomodulin levels in the endothelium in a dose- and time-dependent manner (Fig. 2A). Western blot analysis of total thrombomodulin content in HUVEC whole-cell lysates (Fig. 2B) reveals that there is a dose-dependent up-regulation of total thrombomodulin protein following treatment with doxorubicin. Scatchard binding analyses revealed that HUVECs treated with 10 μg/mL doxorubicin for 24 h expressed 9.56 × 10^3 ± 1.18 × 10^3 molecules of thrombomodulin, ~200% more thrombomodulin molecules compared with untreated HUVECs, which expressed 4.45 × 10^3 ± 0.16 × 10^3 molecules. Our Scatchard analyses indicate that HUVECs express ~11 times more EPCR than thrombomodulin, consistent with previous reports that HUVECs express ~7 times more EPCR than thrombomodulin (41). Endothelial cell surface thrombomodulin has been shown to be up-regulated by factors including cyclic AMP (42–44), retinoic acid (45), and heat shock elements (46). A cyclic AMP response element has been mapped to the 3′ untranslated region (47) of the human thrombomodulin gene. A retinoic acid response element (48) and heat shock elements (46) have been mapped to the 5′ region of the human thrombomodulin gene. In this study, Northern blot analysis suggests that the doxorubicin-dependent up-regulation of thrombomodulin protein occurs at the transcriptional level (Fig. 2C).

The finding that doxorubicin decreases cell surface EPCR while up-regulating surface thrombomodulin on HUVECs is intriguing. Because both EPCR and thrombomodulin are components of the “machinery” required to convert the protein C zymogen to the anticoagulant enzyme activated protein C, we investigated the net effect of EPCR down-regulation and thrombomodulin up-regulation on protein C activation on doxorubicin-treated HUVEC. Our studies indicate that treatment of HUVECs results in a dose- and time-dependent decrease in the rate of protein C activation on HUVECs (Fig. 4). Previous studies have shown that HUVECs express ~7 times more EPCR than thrombomodulin and that EPCR concentration plays a major role in determining protein C activation (41), an observation consistent with our findings. Based on the current study, doxorubicin-mediated down-regulation of EPCR has a direct effect on protein C activation rates (Fig. 4) and thus has the potential to shift the hemostatic balance in favor of a prothrombotic state.

Scatchard analysis revealed that HUVECs express 4.95 × 10^3 ± 0.29 × 10^4 molecules of EPCR per cell. After treatment with 10 μg/mL doxorubicin for 24 h, HUVECs expressed 9.08 × 10^3 ± 1.34 × 10^3 molecules of EPCR per cell, ~80% fewer EPCR molecules compared with untreated HUVECs.

To investigate the mechanisms responsible for the down-regulation of cell surface EPCR observed in doxorubicin-treated HUVECs, we measured soluble EPCR levels in the conditioned medium of cells before and after doxorubicin treatment to determine if doxorubicin induces shedding of cellular EPCR. Our results suggest that the doxorubicin-induced decrease in cell surface EPCR levels is due, at least in part, to shedding of cell surface EPCR from the endothelium (Fig. 3). In contrast to membrane-bound EPCR, soluble EPCR inhibits protein C activation over endothelial cells in culture (19). This presumably reflects competition between soluble EPCR and cell surface EPCR for protein C binding, thereby inhibiting the conversion of zymogen protein C to the anticoagulant enzyme activated protein C (19,36,37). Thus, doxorubicin potentially impairs protein C activation on endothelial cells in two ways. First, because cell surface EPCR enhances the rate of protein C activation by the thrombin-thrombomodulin complex up to 10-fold (38), a reduction of cell surface EPCR on the endothelium would reduce the rate of protein C activation on the endothelial surface. Second, an increase in circulating levels of soluble EPCR may impair protein C activation by virtue of its ability to compete with cellular EPCR for protein C binding. Recently, Saposnik et al. (39) reported that the A3 haplotype of the EPCR gene results in higher soluble EPCR levels and is a risk factor for venous thrombosis.

Our studies also indicate that the decrease in endothelial cell surface EPCR that occurs in response to doxorubicin treatment is attributed, in part, to a decrease in EPCR gene expression (Fig. 1C). HUVEC EPCR mRNA levels follow a bimodal pattern of expression in response to doxorubicin. EPCR transcript levels transiently increase within the first few hours after doxorubicin treatment, whereas after 24 h, EPCR transcript levels decline. Gu et al. (40) have reported that the constitutive expression of murine EPCR is dependent on AP4 and SP1 binding sites in the 5′ promoter region of the EPCR gene, and that a thrombin response element upstream of the AP4 and SP1 binding sites is required for thrombin-dependent up-regulation of EPCR mRNA. To date, negative regulatory elements have not yet been identified in the EPCR gene.

This study is also the first to show that doxorubicin up-regulates cell surface thrombomodulin levels in the endothelium in a dose- and time-dependent manner (Fig. 2A). Western blot analysis of total thrombomodulin content in HUVEC whole-cell lysates (Fig. 2B) reveals that there is a dose-dependent up-regulation of total thrombomodulin protein following treatment with doxorubicin. Scatchard binding analyses revealed that HUVECs treated with 10 μg/mL doxorubicin for 24 h expressed 9.56 × 10^3 ± 1.18 × 10^3 molecules of thrombomodulin, ~200% more thrombomodulin molecules compared with untreated HUVECs, which expressed 4.45 × 10^3 ± 0.16 × 10^3 molecules. Our Scatchard analyses indicate that HUVECs express ~11 times more EPCR than thrombomodulin, consistent with previous reports that HUVECs express ~7 times more EPCR than thrombomodulin (41).

Endothelial cell surface thrombomodulin has been shown to be up-regulated by factors including cyclic AMP (42–44), retinoic acid (45), and heat shock elements (46). A cyclic AMP response element has been mapped to the 3′ untranslated region (47) of the human thrombomodulin gene. A retinoic acid response element (48) and heat shock elements (46) have been mapped to the 5′ region of the human thrombomodulin gene. In this study, Northern blot analysis suggests that the doxorubicin-dependent up-regulation of thrombomodulin protein occurs at the transcriptional level (Fig. 2C).

The finding that doxorubicin decreases cell surface EPCR while up-regulating surface thrombomodulin on HUVECs is intriguing. Because both EPCR and thrombomodulin are components of the “machinery” required to convert the protein C zymogen to the anticoagulant enzyme activated protein C, we investigated the net effect of EPCR down-regulation and thrombomodulin up-regulation on protein C activation on doxorubicin-treated HUVEC. Our studies indicate that treatment of HUVECs results in a dose- and time-dependent decrease in the rate of protein C activation on HUVECs (Fig. 4). Previous studies have shown that HUVECs express ~7 times more EPCR than thrombomodulin and that EPCR concentration plays a major role in determining protein C activation (41), an observation consistent with our findings. Based on the current study, doxorubicin-mediated down-regulation of EPCR has a direct effect on protein C activation rates (Fig. 4) and thus has the potential to shift the hemostatic balance in favor of a prothrombotic state.

Figure 5. Effect of glutathione on doxorubicin-induced changes in cell surface EPCR levels in HUVECs. HUVECs were treated for 3 h with 0, 5, or 7.5 mmol/L glutathione (Glu) followed by 2-h treatment with 10 μg/mL doxorubicin. Cell surface levels of EPCR were determined by flow cytometry using FITC-conjugated anti-EPCR (JRK 1535) monoclonal antibody. *, P < 0.05 (n = 3).
As mentioned previously, doxorubicin undergoes triexponential decay with successive half-lives in plasma of ~5 min, 2 h, and 40 h (24, 25). Maximum initial plasma concentrations ($C_{\text{Max}}$) detected range from ~2 to 6 µg/mL (23, 26, 27), whereas at 24 h, the plasma concentration of doxorubicin is ~0.05 µg/mL (23, 27). We acknowledge that one limitation of our in vitro study is that the concentrations of doxorubicin used (0.1–10 µg/mL) are more similar to the maximal initial plasma concentrations than to the plasma concentrations at $t = 24$ h. However, the concentrations that we used may still have clinical relevance because liposome-encapsulated formulations of doxorubicin (e.g., Myocet, Doxil) have been shown to be as effective as conventional doxorubicin and have much less toxicity (e.g., cardiotoxicity) in phase II and phase III studies (49). The plasma levels of doxorubicin after infusion of liposomal forms of doxorubicin are substantially higher than those after the administration of the same dose of conventional doxorubicin (23, 50). For example, the initial plasma concentration of doxorubicin and the plasma doxorubicin concentration at $t = 24$ h in patients receiving Doxil are 10 and 8 µg/mL, respectively (50). In a phase II study, deep vein thrombosis was one of the side effects experienced by patients treated with liposomal doxorubicin (Doxil) in combination with vincristine, dexamethasone, and thalidomide (51, 52).

The mechanisms of action of doxorubicin are extremely complex. The cytotoxic effects of this drug include the formation of reactive oxygen species (e.g., H$_2$O$_2$; refs. 28, 29), the formation of alcohol metabolites (e.g., doxorubicinol and aglycones; ref. 53), intercalation of the drug into DNA with subsequent inhibition of DNA and RNA synthesis, DNA cross-linking, and induction of DNA strand breaks with subsequent inhibition of topoisomerase II (10). Doxorubicin has also been shown to induce apoptosis through a number of pathways, such as the accumulation of p53 and the activation of caspase-3 and caspase-9 (54), although apoptosis is likely triggered through upstream events such as induction of DNA damage. Because the antioxidant glutathione attenuated doxorubicin-induced down-regulation of EPCR on HUVECs (Fig. 5), reactive oxygen species may be a signaling intermediate that down-regulates EPCR expression on HUVECs (Fig. 5). This finding is consistent with the studies of Xu et al. (55), which showed that H$_2$O$_2$ induced EPCR shedding in endothelial cells. In contrast, glutathione did not affect doxorubicin-induced decreases in thrombomodulin, suggesting that mechanisms other than the formation of reactive oxygen species may be responsible for alterations in thrombomodulin expression.

In summary, our results provide novel insight into potential mechanisms by which the chemotherapeutic agent doxorubicin induces a hypercoagulable state in cancer patients. Our studies are the first to show that doxorubicin down-regulates cell surface EPCR levels, induces EPCR shedding, and up-regulates cell surface thrombomodulin levels on endothelial cells. The net effect is a decrease in the capacity of endothelial cells to support the conversion of protein C to the anticoagulant enzyme activated protein C. The results of this study are important for two reasons. First, this study has identified markers (e.g., soluble EPCR) that may be useful in assessing the potential of anticancer drugs to impair natural anticoagulant mechanisms, thereby sparing life-threatening blood clotting events. Second, the results of this study may warrant future clinical studies to evaluate “customized” anticoagulant therapy tailored to the specific chemotherapy regimen that the patient receives.

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