MODULATION OF HEMOSTATIC PATHWAYS BY BREAST CANCER CHEMOTHERAPY AGENTS

MODULATION OF HEMOSTATIC PATHWAYS BY BREAST CANCER CHEMOTHERAPY AGENTS

By:
LAURA L. SWYSTUN, B.Sc. (Hons)

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
Doctor of Philosophy

McMaster University

© Copyright by Laura L. Swystun, August, 2011

DOCTOR OF PHILOSOPHY (2011)	McMaster University
(Department of Medical Sciences)	Hamilton, Ontario

TITLE:	Modulation of hemostatic pathways by breast cancer chemotherapy agents
AUTHOR:	Laura L. Swystun B.Sc. (Honours) Biology Co-op (Genetics Specialization) (McMaster University)
SUPERVISOR:	Patricia C. Liaw, Ph.D.
NUMBER OF PAGES:	xx, 223

ABSTRACT

Thrombosis is a common complication of chemotherapy for breast cancer patients. While the overall risk for thrombosis in these patients varies in relation to the stage of disease, chemotherapeutic regimen employed, and presence of other risk factors such as use of hormonal therapies, between 3 – 17% of patients will develop thrombotic complications during treatment. Clinical studies have demonstrated the activation of thrombin generation (as measured by increased thrombin-antithrombin (TAT) complex formation) in breast cancer patients receiving chemotherapy. As well, breast cancer chemotherapy is associated with decreased plasma levels of protein C and activated protein C. However, the specific mechanisms by which chemotherapy agents modulate these hemostatic pathways in breast cancer patients are not well understood. In this thesis, we investigated the mechanism(s) by which chemotherapy agents (doxorubicin, epirubicin, methotrexate, 5-fluorouracil, and cyclophosphamide) can upregulate procoagulant pathways and impair the protein C anticoagulant pathway, using *in vitro* and *in vivo* methods.

We first investigated the ability of breast cancer chemotherapy agents to modify cell surface procoagulant activity (tissue factor (TF) activity and phosphatidylserine exposure) on endothelial cells, smooth muscle cells, and blood monocytes. We found that treatment with the chemotherapy drugs doxorubicin, epirubicin, and the cyclophosphamide metabolite acrolein induced an increase in phosphatidylserine exposure and TF activity on treated endothelial cells. We also found that doxorubicin and epirubicin treatment of monocytes isolated from venous whole blood and treatment of smooth muscle cells with acrolein increased TF activity on both cell types. This corresponded to an increase in thrombin generation on chemotherapy-treated endothelial and smooth muscle cells exposed to recalcified, defibrinated plasma. Treatment of healthy mice with doxorubicin, epirubicin, acrolein, cyclophosphamide, and 5-fluoruracil increased plasma TAT levels, indicating that these agents promote thrombin generation *in vivo*. These studies demonstrate that chemotherapy agents can promote thrombin generation through activation of the TF pathway and by increasing phosphatidylserine exposure.

The second goal of our studies was to investigate the effects of chemotherapy on the release of cell free DNA (CFDNA) from treated blood and vascular cells, and to determine whether or not this CFDNA was procoagulant. We found that chemotherapy can increase CFDNA release from breast cancer chemotherapy patients and healthy mice, which corresponds to an increase in TAT levels. Treatment of venous whole blood and isolated neutrophils with doxorubicin and epirubicin increased CFDNA release. In addition, CFDNA released from doxorubicin and epirubicin-treated whole blood had decreased association with histone protein. We found that exposure of recalcified plasma to CFDNA isolated from epirubicin-treated whole blood increased thrombin generation by activating the contact pathway. These studies suggest that thrombin generation in breast cancer patients and healthy mice treated with chemotherapy agents may be triggered through activation of contact and TF pathways.

The third aim of these studies was to investigate the effects of breast cancer chemotherapy agents on the protein C (PC) anticoagulant pathway. Doxorubicin has been previously demonstrated to decrease endothelial protein C receptor (EPCR) levels on treated endothelial cells, which corresponds to impaired generation of activated protein C (APC). We found that acrolein also decreases EPCR while increasing thrombomodulin expression on treated endothelial cells. A corresponding decrease in APC generation was measured on acrolein-treated endothelial cells exposed to recalcified, defibrinated plasma. Healthy mice treated with acrolein and cyclophosphamide had increased PC antigen levels, but no measurable increase in plasma APC levels. These studies suggest that chemotherapy can modify EPCR and thrombomodulin expression on endothelial cells, thereby impairing the conversion of PC to APC, which may contribute to increased thrombin generation in breast cancer patients receiving chemotherapy.

Our fourth aim was to investigate the role that reactive oxygen species (ROS) might play in mediating the procoagulant effects exerted by chemotherapy drugs. We found that the ROS hydrogen peroxide increased TF activity and phosphatidylserine exposure on endothelial and smooth muscle cells. Preincubation of cells with the antioxidant glutathione attenuated doxorubicin and acrolein-induced increases in TF activity on endothelial and/or smooth muscle cells. As well, glutathione treatment inhibited phosphatidylserine exposure on acrolein-treated endothelial cells. Preincubation with glutathione also inhibited the epirubicin-induced increase in CFDNA release in venous whole blood. Finally, hydrogen peroxide also modified the protein C anticoagulant pathway by decreasing EPCR and increasing thrombomodulin expression on endothelial cells. These studies suggest that ROS generated by chemotherapy drugs may mediate the procoagulant effects observed in our first three aims.

In summary, breast cancer chemotherapy drugs elevate thrombin generation by activating coagulation through the TF and contact pathways, and by promoting phosphatidylserine exposure. In addition, chemotherapy drugs may impair protein C activation, an endogenous anticoagulant mechanism, by decreasing endothelial cell surface expression of EPCR. These effects may be mediated by the generation of ROS by chemotherapy drugs. These studies provide insight into the mechanisms of breast cancer chemotherapy-induced hypercoagulation.

ACKNOWLEDGEMENTS

I am extremely grateful to my supervisor, Dr. Patricia Liaw, for her wisdom, generosity, and support over the past five years. I feel that I have grown significantly as a scientist and as a person with her guidance and encouragement. She has been a fantastic role model, and her mentorship, in particular over the past year as I have prepared to move on from graduate studies, has been invaluable. I am extremely proud of the work that we have accomplished together, and I will certainly carry the passion for science that I found in her lab with me throughout my career.

I truly appreciate the input that my committee members, Dr. Jeffrey Weitz and Dr. Geoff Werstuck, have given me throughout the course of my studies. Their valuable feedback and insight have helped to guide and strengthen my research. I am grateful to Dr. Werstuck for the advice and encouragement he provided during the course of my comprehensive examination, and to Dr. Weitz for his critical review of work-in-progress during Weitz lab meetings.

I would like to thank the past and present members of the Liaw lab, Suzanne Beaduin, Dr. Dhruva Dwivedi, Travis Gould, Zahar Lysov, Safiah Mai, Ellen McDonald, Laura Pepler, Lucy Shin, Dr. Lisa Toltl, and Dr. Cynthia Wu. I greatly appreciate both the camaraderie and support they have provided over the years, and will miss working in such a high-spirited environment. Go Team Laura!

I would also like to thank the other members of TaARI for their expertise and technical support. In particular, I'd like to acknowledge Beverly Leslie, Dr. Jim Fredenburgh, Alan Stafford, and Leslie Berry. I'd also like to thank Dr. Peter Gross and his lab members for helping me to start up my animal experiments, and Dr. Howard Chan for his excellent phlebotomy skills. I'd also like to thank our collaborators Dr. Som Mukherjee and Dr. Mark Levine for patient recruitment and valuable critical feedback on manuscripts, Dr. Charles Esmon and Dr. Naomi Esmon for generously providing antibodies, recombinant proteins, and translating protocols, and Dr. Nigel Mackman for helpful discussions and critical review of manuscripts.

I would like to thank my parents, William and Karen, my sister Victoria, and my brothers Daniel and Thomas for their continued encouragement and support over the course of my studies. I would also like to express my appreciation to my good friends and fellow scientists Andrew Chen, Ran Ni, Nima Vaezzadeh, Trang Vu and Dr. Xi Wang. Thank you for all of your technical (and non-technical) support, all of our scientific (and non-scientific) conversations, and for making graduate school such a productive and enjoyable period.

I would finally like to acknowledge all of the agencies that have financially supported my graduate studies: McMaster University – Medical Sciences Program (Entrance award Master's and PhD), Ontario Graduate Scholarship Program (Master's award), Natural Sciences and Engineering Research Council (Canadian Graduate Scholarship (Master's) and Postgraduate Scholarship (PhD)), Hemostasis Reference Laboratory, CIBC Breast Cancer (Graduate bursary 2007 - 2009), McMaster University – Graduate Student's Association (GSA) (Travel award), International Society on Thrombosis and Haemostasis (Young Investigator's award 2007 and 2011).

TABLE OF CONTENTS

ABSTRACT…………………………………………………………………… iii

ACKNOWLEDGEMENTS………………………………………………….. vii

LIST OF FIGURES…………………………………………………………... xv

LIST OF ABBREVIATIONS………………………………………………… xvii

DECLARATION OF ACADEMIC ACHIEVEMENT………………………….. xx

CHAPTER 1. General Introduction……………………………………………… 1

1.1 Introduction to hemostasis and thrombosis…………………………….. 1

1.1.1 Overview of hemostasis……………………………….…………….. 1

1.1.2 Overview of thrombosis……………………………….…………….. 5

1.2 Tissue factor in hemostasis and thrombosis…………………………….. 7

1.2.1 The tissue factor pathway……………………………….……………. 7

1.2.2 Regulation of tissue factor procoagulant activity………………… 8

1.2.3 Role of tissue factor in hemostasis and thrombosis……………… 9

1.3 Phosphatidylserine in hemostasis and thrombosis……………………… 11

1.3.1 Regulation of phosphatidylserine asymmetry…………………….. 11

1.3.2 Role of phosphatidylserine in hemostasis and thrombosis……….. 12

1.4 The contact pathway in hemostasis and thrombosis…………………… 13

1.4.1 The contact pathway…………………...…………………………….. 13

1.4.2 Role of the contact pathway in hemostasis and thrombosis……… 14

1.4.3 Cell free nucleic acids and coagulation activation……………… 15

1.5 Regulation of thrombin generation and fibrin formation……………… 16

1.5.1 Thrombin generation and fibrin formation……………………….. 16

1.5.2 Thrombin generation and fibrin formation in hemostasis and thrombosis……………………..…………………..……… 18

1.5.3 The thrombogram…………………..………………………….. 19

1.6 The protein C anticoagulant pathway in hemostasis and thrombosis… 23

1.6.1 The protein C pathway……………………..……………………… 23

1.6.2 Dysregulaton of the protein C pathway in hemostasis and thrombosis…………………..………………………..…….. 25

1.7 Regulation of hemostasis by the blood and vasculature……………….. 27

1.7.1 Role of the vascular endothelium in regulating hemostasis……… 27

1.7.2 Role of vascular smooth muscle cells in regulating hemostasis…. 29

1.7.3 Role of monocytes in blood coagulation…………………………. 29

1.8 Thrombosis and malignancy………………..……………...…………... 30

1.8.1 Overview of association between thrombosis and cancer……..…. 30

1.8.2 Dysregulation of coagulation and fibrinolysis in cancer patients… 32

1.9 Chemotherapeutic treatment for breast cancer……………..………. 34

1.9.1 Introduction to treatment of breast cancer……………………… 34

1.9.2 Anthracycline chemotherapeutic agents…………………………. 36

1.9.3 Nitrogen mustard alkylating agents……………………………… 37

1.9.4 Antimetabolite chemotherapeutic agents…………………………. 38

1.10 Association between breast cancer chemotherapy and thrombosis……. 38

1.10.1 *Introduction to chemotherapy and thrombosis……………………* 38

1.10.2 Chemotherapy for breast cancer and thrombosis………………… 40

1.10.3 Modulation of hemostatic pathways by breast cancer chemotherapy agents – clinical studies………………………….. 40

1.10.4 Modulation of hemostatic pathways by chemotherapy agents – in vitro studies……………………….……………………………. 45

1.10.5 Modulation of hemostatic pathways by chemotherapy agents – animal models……………………….…………………………. 46

1.10.6 Association between other cancer therapies and thrombosis……. 47

1.11 Thromboprophylaxis in cancer chemotherapy patients…….……..……. 49

1.12 Relationship between oxidative stress and hypercoagulability……..…… 50

CHAPTER 2: Hypothesis and Aims…………………………………………………….. 52
2.1 Aim and Hypothesis……………………………………………………….. 52
2.2 Objectives……………………………………………………………………. 53

CHAPTER 3: Chemotherapeutic agents doxorubicin and epirubicin induce a procoagulant phenotype on endothelial cells and blood monocytes……………… 54
Foreword……………………………………………………………………………. 54
3.1 Abstract…………………………………………………………………………. 55
3.2 Introduction…………………………………………………………………….. 56
3.3 Materials and Methods…………………………………………………………. 57
3.3.1 Materials…………………………………………………………………… 57
3.3.2 Plasma concentrations of chemotherapeutic drugs used 58
3.3.3 Cell culture………………………………………………………………… 59
3.3.4 Flow cytometric analysis of HUVEC TF expression and phosphatidylserine exposure…………………………………………. 59
3.3.5 Isolation of peripheral human monocytes from whole blood……… 60
3.3.6 Thrombin generation assays…………………………………………. 60
3.3.7 Tissue factor activity assays…………………………………………….. 61
3.3.8 Western blot analysis of EPCR, thrombomodulin and TF in HUVEC whole-cell lysates…………………………………………… 61
3.3.9 Caspase 3-activity assay……………………………………………… 62
3.3.10 Statistical analyses…………………………………………………… 62
3.4 Results……………………………………………………………………………. 62
3.4.1 The effects of chemotherapy drugs on the hemostatic balance of endothelial cells………………………………………………………. 62
3.4.2 Effects of chemotherapeutic drugs on endothelial cell and monocyte TF……………………………………………………………….. 66
3.4.3 Effect of chemotherapeutic drugs on apoptosis…………………… 69

3.4.4 Mechanisms of chemotherapy-induced TF activity and phosphatidylserine exposure……………………….………… 69
3.4.5 Effect of chemotherapeutic drugs on the PC anticoagulant pathway 75
3.5 Discussion……..……..……..……..……..……..……………..……..… **75**

CHAPTER 4: The chemotherapy metabolite acrolein upregulates thrombin generation and impairs the protein C anticoagulant pathway in animal-based and cell-based models……..……..……..……..……..……..………..…. **82**

Foreword……..……..……..……..……..……..……………..……. **82**
4.1 Abstract……..……..……..……..……..……..……………..………… **83**
4.2 Introduction……..……..……..……..……..……..……………..……. **84**
4.3 Materials and Methods……..……..……..……..……..……..………… **87**
4.3.1 Materials……………………….…………………………………… 87
4.3.2 Cell culture……………………….………………………………… 87
4.3.3 Thrombin generation, PC activation and TF activity assay………. 88
4.3.4 Flow cytometric analysis of EPCR, thrombomodulin, TF expression and phosphatidylserine exposure……….……..…….. 88
4.3.5 sEPCR ELISA……………………….……………………………… 89
4.3.6 Mouse model……………………….……………………………… 89
4.3.7 Mouse TAT, PC ELISA, APC capture assay, quantification of circulating microparticles………………….………………. 89
4.3.8 Plasma and media concentrations of acrolein and cyclophosphamide…………………….………………………. 90
4.3.9 Statistical analyses…………………….…………………………… 90
4.4 Results……..……..……..……..……..……..……………..……………. **91**
4.4.1 The effects of cyclophosphamide and acrolein on procoagulant properties of endothelial and smooth muscle cells……….……….. 91
4.4.2 Effects of acrolein on the anticoagulant properties of endothelial cells……………………….……………………………………… 103

4.4.3 *The effects of cyclophosphamide and acrolein on pro- and anticoagulant markers in a mouse model*……….………..………. 106

4.5 Discussion……….……….……….……….……….……….………. **107**

CHAPTER 5: Breast cancer chemotherapy induces the release of cell free DNA, a novel procoagulant stimulus……….……….……….………. **112**

Foreword……….……….……….……….……….……….………. **112**

5.1 Abstract……….……….……….……….……….……….………. **113**

5.2 Introduction……….……….……….……….……….……….………. **114**

5.3 Materials and Methods……….……….……….……….………. **116**

5.3.1 *Materials*……….……….……….……….……….……. 116

5.3.2 *DNA isolation and electrophoresis*……….……….……. 116

5.3.3 *Neutrophil isolation*……….……….……….……….……. 117

5.3.4 *Thrombin generation assay*……….……….……….……. 117

5.3.5 *Animal studies*……….……….……….……….……. 118

5.3.6 *Analysis of murine TAT complexes*……….……….……. 118

5.3.7 *Concentrations of chemotherapy agents*……….……….……. 119

5.3.8 *Collection of plasma from breast cancer patients receiving chemotherapy*……….……….……….……….……. 119

5.3.9 *DNA/histone ELISA*……….……….……….……….……. 120

5.3.10 *Statistical analyses*……….……….……….……….……. 120

5.4 Results……….……….……….……….……….……….………. **120**

5.4.1 *The effects of chemotherapy on the release of CFDNA in breast cancer patients and healthy mice*……….……….……….……. 120

5.4.2 *The effects of chemotherapy on the release of CFDNA from venous whole blood and neutrophils*……….……….……….……. 125

5.4.3 *The effects of CFDNA on thrombin generation*……….……….. 128

5.4.4 *Mechanisms of chemotherapy-induced CFDNA release*……….. 134

5.5 Discussion……….……….……….……….……….……….………. **134**

CHAPTER 6: General Discussion……………………………………………… 141
6.1 Effects of breast cancer chemotherapy agents on the TF pathway……. 143
6.2 Effects breast cancer chemotherapy agents on phosphatidylserine exposure and apoptosis………………………………………………………. 148
6.3 Effects of breast cancer chemotherapy agents on the release of CFDNA 152
6.5 Effects of breast cancer chemotherapy agents on the PC pathway…… 153
6.6 Effects of breast cancer chemotherapy agents on thrombin generation 157
6.6 Contribution of ROS to breast cancer chemotherapy agent-induced procoagulant activity……………………………………………………….. 167
6.7 Future directions……………………………………………………….. 169
  6.7.1 Confirm the role of CFDNA in promoting thrombin generation in response to breast cancer chemotherapy………………………………… 169
  6.7.2 Investigate the effects of breast cancer chemotherapy agents on breast tumour cells in vitro and in vivo………………………………… 173
  6.7.3 Investigate the effects of thalidomide and dexamethasone on hemostatic pathways in vivo…………………………………………... 175
6.8 Conclusions…………………………………………………………………. 190

7.0 REFERENCES……………………………………………………………. 192

LIST OF FIGURES

Figure 1.1 The coagulation cascade and fibronolysis………………………….. 4
Figure 1.2 Modulation of hemostatic pathways by chemotherapy agents……... 21
Figure 1.3 The thrombogram……………………..…………………… 44
Figure 3.1 Effects of chemotherapeutic agents on the ability of HUVECs to trigger thrombin generation in defibrinated plasma.……..……...... 65
Figure 3.2 Effects of doxorubicin and epirubicin on HUVEC and monocyte TF……………………..……………………………………. 68
Figure 3.3 Effects of doxorubicin and epirubicin on endothelial cell apoptosis.. 71
Figure 3.4 Mechanisms of doxorubicin-induced TF activation and phosphatidylserine exposure………………………..…………… 74
Figure 3.5 EPCR and thrombomodulin expression on epirubicin-treated HUVECs……………………..……………………………… 77
Figure 4.1 Effects of acrolein and cyclophosphamide on the ability of vascular cells to trigger thrombin generation in defibrinated plasma……….. 93
Figure 4.2 Effects of acrolein on vascular phosphatidylserine exposure……… 95
Figure 4.3 Effects of acrolein on HUVEC and UASMC tissue factor………… 97
Figure 4.4 Effects of acrolein on the ability of endothlelial cells to trigger APC generation in defibrinated plasma.……..……....................... 100
Figure 4.5 Effects of acrolein on the endothelial protein C pathway………….. 102
Figure 4.6 Effects of acrolein and cyclophosphamide on measures of pro- and anticoagulant pathways in mouse plasma.……..……..................... 105
Figure 5.1 Effects of chemotherapy on CFDNA release in breast cancer patients.……..……......……..……......……..……................... 122
Figure 5.2 Effects of chemotherapy on CFDNA release and TAT formation in healthy mice.……..……......……..……......……..……............ 124
Figure 5.3 Effects of chemotherapy on the release of CFDNA *in vitro*……….. 127
Figure 5.4 Effects of CFDNA on thrombin generation……………………….. 130

Figure 5.5 Effects of CFDNA on the contact pathway………………………… 132
Figure 5.6 Mechanism of CFDNA release…………………………………... 136
Figure 6.1 Effects of chemotherapy agents on MDA-MB-231 TF activity……. 146
Figure 6.2 Effects of chemotherapy agents on microparticle generation in mice. ……………………..…………………………………… 151
Figure 6.3 Quantification of activated protein C levels in plasma of acrolein and cyclophosphamide-treated mice…………………………….. 156
Figure 6.4 Sensitivity of murine TAT levels to method of blood collection.… 163
Figure 6.5 Mechanisms by which breast cancer chemotherapy agents modulate thrombin generation. ……………………………………. 166
Figure 6.6 Generation of NETS by chemotherapy-treated neutrophils……….. 172
Figure 6.7 Effects of thalidomide and dexamethasone on endothelial cell procoagulant activity……………………..……………………… 179
Figure 6.8 Effects of thalidomide and dexamethasone on the endothelial cell protein C pathway……………………..…………………………. 181
Figure 6.9 Effects of thalidomide and dexamethasone-treated cells on thrombin generation in defibrinated plasma……………………….. 183
Figure 6.10 Effects of thalidomide and dexamethasone on monocyte TF activity 185
Figure 6.11 Effects of dexamethasone and thalidomide on UASMC TF activity. 187
Figure 6.12 Effects of dexamethasone and thalidomide on human myeloma cell TF activity……………………..………………………………. 189

LIST OF ABBREVIATIONS

II – prothrombin

IIa – thrombin

5FU – 5-fluorouracil

α2M – α2-macroglobulin

ADP – adenosine diphosphate

ANOVA – analysis of variance

APC – activated protein C

AT – antithrombin III

AUC – area under the curve

BSA – bovine serum albumin

$CaCl_2$ – calcium chloride

CAF – cyclophosphamide, adriamycin (doxorubicin), 5-fluorouracil

CAT – calibrated automated thrombin generation

CEF – cyclophosphamide, epirubicin, 5-fluorouracil

CFDNA – cell free DNA

CMF – cyclophosphamide, methotrexate, 5-fluorouracil

CP – cancer procoagulant

CPA – cyclophosphamide

DIC – disseminated intravascular coagulation

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

DNAse – deoxyribonuclease

DVT – deep vein thrombosis

EDTA – Ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

EGF – endothelial growth factor

EGM – endothelial growth medium

EPCR – endothelial protein C receptor

ESA – erythropoietin stimulating agents

F – factor

F1+2 – prothrombin fragment 1+2

FBS – fetal bovine serum

FITC – Fluorescein isothiocyanate

FPA – fibrinopeptide A

FPB – fibrinopeptide B

Gla – γ carboxyglutamic acid

Glu – glutathione

HBSS – Hank's balanced salt solution

HK – high-molecular weight kininogen

HMWK – high molecular weight kininogen

HRP – horseradish peroxidase

HUVEC – human umbilical vein endothelial cell

IL – interleukin

IVC – inferior vena cava

kDa – kiloalton

LPS – lipopolysaccharide

LMWH – low molecular weight heparin

M199 – growth medium 199

$MgCl_2$ – magnesium chloride

MP – microparticle

mRNA – messenger ribonucleic acid

NET – neutrophil extracellular trap

NO – nitric oxide

OD – optical density

PAI – plasminogen activator inhibitor

PAR – protease-activated receptor

PBS – phosphate buffered saline

PC – protein C
PDI – protein disulfide isomerase
PE – pulmonary embolism
PGI_2 – prostacyclin
PMA – phorbol 12-myristate 13-acetate
PS – protein S
RBC – red blood cell
RNA – ribonucleic acid
RNAse – ribonuclease
ROS – reactive oxygen species
RPMI – Roswell Park Memorial Institute growth medium
SD – standard deviation
SE – standard error
sEPCR – soluble endothelial protein C receptor
SERPIN – serine protease inhibitor
sTM – soluble thrombomodulin
TACE – tumor necrosis factor α converting enzyme
TAFI – thrombin activatable fibronolysis inhibitor
TAPI - tumor necrosis factor α protease inhibitor
TAT – thrombin-antithrombin
TF – tissue factor
TFPI – tissue factor pathway inhibitor
Thal/dex – thalidomide/dexamethasone combination treatment
TNF – tumor necrosis factor
tPA – tissue-type plasminogen activator
UASMC – umbilical arterial smooth muscle cells
uPA – urokinase-type plasminogen activator
VTE – venous thromboembolism
vWF – von Willebrand Factor

DECLARATION OF ACADEMIC ACHIEVEMENT

Laura L. Swystun contributed to conception and design of studies, performed all experiments presented in Chapters 3, 4, 5, and 6, analyzed and interpreted the data, and performed statistical analyses. Laura Swystun wrote all manuscripts chapters.

Dr. Patricia C. Liaw contributed to conception and design of studies, obtained funding to support the studies, and critically reviewed the manuscripts presented in Chapters 3, 4, and 5.

Lucy Y. Y. Shin performed pilot studies which formed the basis for the studies presented in Chapter 3.

Suzanne Beaudin provided technical assistance for Chapter 3.

Dr. Som Mukherjee and Dr. Mark Levine critically reviewed the manuscripts presented in Chapters 4 and 5, and recruited patients for study presented in Chapter 5.

CHAPTER 1. General Introduction

1.1 Introduction to hemostasis and thrombosis

1.1.1 Overview of hemostasis

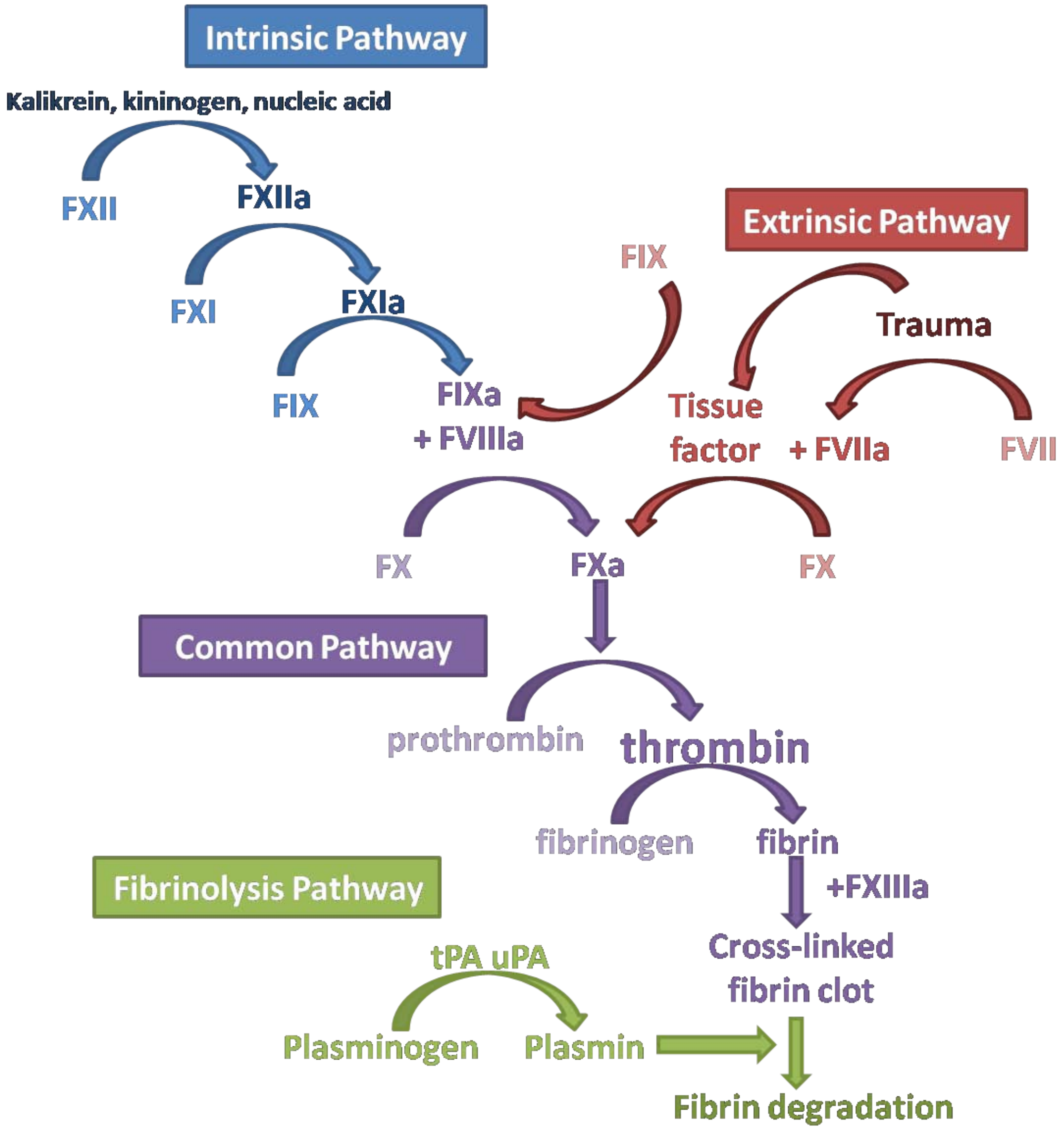
Under physiological conditions, blood is maintained in a fluid state within the circulation, but upon tissue injury, the hemostatic system is rapidly activated to minimize blood loss. This involves the rapid conversion of circulating zymogens to activated coagulation factors, and is highly regulated by cellular components of the blood and the vessel wall (reviewed in (Hoffman et al. 2001)). Upon vascular damage, subendothelial collagen and von Willebrand factor (vWF) are exposed to the blood, which promote platelet adhesion and activation. Activated platelets release the contents of storage granules, including adenosine diphosphate (ADP), and thromboxane A_2, which serve to activate additional platelets, and results in the formation of a platelet plug (reviewed in (Wagner et al. 2003)).

Simultaneous to platelet activation, the coagulation cascade is initiated by two pathways: (1) the extrinsic (tissue factor) pathway, and (2) the intrinsic (contact) pathway. Under normal physiological conditions, tissue factor (TF), a transmembrane glycoprotein, is localized primarily in the medial and adventitial layers of blood vessels. Upon vascular injury, underlying TF is exposed to the blood and initiates coagulation by complexing with low levels of circulating activated factor (F) VII and the zymogen factor X, known as the "extrinsic tenase" complex. TF functions as the regulatory subunit, while factor VIIa catalytically activates factor X to Xa or factor IX to IXa (reviewed in (Owens, III et al. 2010)). Coagulation through the contact system is initiated by the activation of factor XII by high molecular weight kininogen (HMWK) and prekallikrein, or by auto-activation through the interaction with polyphosphates or nucleic acids. Factor XIIa activates factor XI, which in turn activates factor IX (reviewed in (Woodruff et al. 2011)). Factor IXa can activate FX, thereby initiating the common pathway.

Factor Xa, generated either by the intrinsic or extrinsic pathway, converts prothrombin into thrombin in the presence of its cofactor FVa and phosphatidylserine (the "prothrombinase complex"). Small amounts of thrombin generated on the surface of TF-positive cells activates platelets, as well as factors V, VIII, XI, increasing prothrombinase and "intrinsic tenase" (IXa, cofactor VIIIa, and factor X) activity, which in turn results in the amplification of thrombin generation. Thrombin converts soluble fibrinogen into an insoluble fibrin clot, and activates FXIII. Factor XIIIa cross-links fibrin to form polymers, contributing to the strength of the clot and its resistance to lysis (reviewed in (Wolberg et al. 2008)).

Excess thrombin generation is regulated by anticoagulant mechanisms such as tissue factor pathway inhibitor (TFPI), antithrombin, and the protein C (PC) anticoagulant pathway, while fibrin deposition is regulated by fibrinolysis. In the PC pathway, excess thrombin binds to thrombomodulin on the endothelial cell surface. PC binds to the endothelial cell PC receptor (EPCR) and is cleaved by thrombin, generating activated protein C (APC). APC dampens thrombin generation by inactivating coagulation factors Va and VIIIa (reviewed in (Esmon 2003)). Fibrinolysis is initiated by the release of tissue-type plasminogen activator

Figure 1.1 – The clotting cascade. Vascular injury initiates the extrinsic pathway by exposing TF to the blood. TF complexes with low levels of circulating factor VIIa, to convert factor IX to IXa, or X to Xa. The contact or intrinsic pathway is initiated by the activation of prekallikrein to kallikrein and factor XII to XIIa. Factor XIIa activates factor XI, which in turn activates factor IX. Factor Xa converts small amounts of prothrombin (factor II) to thrombin (IIa), the central component of the coagulation cascade. Thrombin converts soluble fibrin into an insoluble fibrin clot, which is cross-linked by factor XIIIa. Dissolution of a fibrin clot is regulated by the activation of liver-produced plasminogen into plasmin by tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA).


Intrinsic Pathway
Kalikrein, kininogen, nucleic acid
FXII
FXIIa
FXI
FXIa
FIX
FIXa
+ FVIIIa
Extrinsic Pathway
FIX
Trauma
Tissue
factor
+ FVIIa
FVII
FX
FXa
FX
Common Pathway
prothrombin
thrombin
fibrinogen
fibrin
+FXIIIa
Cross-linked
fibrin clot
Fibrinolysis Pathway
tPA uPA
Plasminogen
Plasmin
Fibrin degradation

(tPA) and urokinase-type plasminogen activator (uPA), which convert plasminogen to plasmin, which degrades fibrin. Fibrinolysis can be impaired by plasminogen activator inhibitor-1 (PAI-1), which is a direct inhibitor of tPA and uPA. As well, thrombin activatable fibrinolysis inhibitor (TAFI) increases the resistance of fibrin to fibrinolysis by tPA (reviewed in (Cesarman-Maus et al. 2005)).

1.1.2 Overview of thrombosis

The maintenance of the hemostatic balance is important for the prevention of both thrombosis and hemorrhage. Under physiological conditions, components of the coagulation and fibrinolytic pathways are strictly regulated, and hemostatic clots are localized to the vessel wall. Upon pathological stimulation (including vascular injury, inflammation, or malignancy), dysregulation of coagulation can result in the formation of a thrombus which can partially or completely obstruct the flow of blood to tissues and organs. Arterial thrombosis is most frequently the result of atherosclerotic plaque rupture or endothelial denudation with superimposed thrombus formation. Arterial thrombi present in large and medium-sized arteries and can embolize to organs and tissues, such as the heart or limbs, resulting in ischemia or infarction. Common risk factors for arterial thrombosis include exposure to cigarette smoke, diabetes mellitus, and hyperlipidemia (Previtali et al. 2011).

Venous thromboembolism (VTE) is commonly thought to result from impaired circulation (stasis), and changes in blood thrombogenicity (coagulation factor levels), and changes to the vessel wall (endothelial dysfunction). VTE typically has two clinical presentations: deep vein thrombosis (DVT) and pulmonary embolism (PE). DVT most frequently presents in the femoral and popliteal veins or the deep veins of the pelvis. Embolization of a venous clot can produce PE, blockage of the pulmonary arteries, leading to hypoxia. 25% of individuals with DVT suffer from chronic, long-term effects, while 10% of deaths in hospitalized patients can be attributed to PE (Michota 2005). Venous clots are generally larger in size than arterial clots, fibrin-rich, and red in colour due to high levels of erythrocytes (Franchini et al. 2008). This suggests that levels of plasma proteases which promote fibrin deposition strongly influence the risk for VTE (Gailani et al. 2007). Risk factors for VTE tend to increase levels of prothrombotic factors, decrease antithrombotic factors, and affect organs where clotting factors are synthesized or degraded, such as in liver disease and endothelial dysfunction (Rosendaal 2005). Common inherited risk factors for thrombosis include the Factor V Leiden mutation, the prothrombin G20210A mutation, and deficiencies or mutations in anticoagulant molecules such as antithrombin, protein C, and protein S. Acquired risk factors for VTE include stasis, surgery, malignancy, and hormonal therapies such as oral contraceptives or hormone replacement therapy (Previtali et al. 2011).

While arterial thrombosis and VTE affect the large vessels, disseminated intravascular coagulation (DIC) is characterized by the extensive formation of microvascular thrombi and hemorrhage (Levi 2007). Microvascular clot formation and impaired fibrinolysis may occlude blood flow to the organs, leading to organ dysfunction, while consumption of coagulation factors and platelets can result in hemorrhage from the skin, respiratory tract, and gastrointestinal tract. DIC is the result of unregulated systemic activation of inflammatory pathways, and is typically associated with pathological conditions such as severe sepsis and trauma, but may also be a complication of malignancy (Al-Mondhiry 1975).

1.2 Tissue factor in hemostasis and thrombosis

1.2.1 The tissue factor pathway

Tissue factor is an integral membrane glycoprotein, which, when complexed with activated factor VIIa, initiates the extrinsic pathway of the coagulation cascade upon exposure to the blood. FVII circulates in the plasma as either a zymogen coagulation factor, or in lower amounts (approximately 1% of total) as the active form, FVIIa (Morrissey et al. 1993), and binds to the extracellular domain of TF with high affinity. TF-bound FVII is converted to FVIIa by limited proteolysis (Nemerson et al. 1985). TF functions as the regulatory subunit of the TF:FVIIa enzymatic complex. The γ-carboxyglutamic acid (Gla) domain of FVIIa facilitates binding to phospholipids on the cell surface (Bach et al. 1986), while the serine protease domain binds TF and catalyzes the activation of downstream coagulation factors (Dickinson et al. 1996). The predominant substrate of TF:FVIIa is factor X (FX), however, TF:FVIIa can also activate factor IX (FIX), which complexes with cofactor factor VIIIa (FVIIIa) to activate FX, leading to thrombin generation and fibrin deposition (Osterud et al. 1977). In addition to its hemostatic function, the TF:FVIIa complex can activate inflammatory pathways by signalling through protease activated receptor (PAR)-2 and to a lesser extent PAR-1 (Rao et al. 2005).

1.2.2 Regulation of tissue factor procoagulant activity

TF activity is regulated *in vivo* by three distinct mechanisms: (1) plasma or cell surface inhibitors of TF:FVIIa, (2) control of TF expression and exposure to the blood, and (3) regulation of TF procoagulant activity through de-encryption. Tissue factor pathway inhibitor (TFPI) is the primary inhibitor of TF activity *in vivo*, and is constitutively expressed by endothelial cells (ECs), smooth muscle cells (SMCs), platelets, and monocytes. TFPI is comprised of 3 Kunitz type domains which tightly bind and inhibit a target protease. The K1 domain of TFPI binds the active site of FVIIa, (Girard et al. 1989) while the K2 domain binds and inhibits FXa (Petersen et al. 1996). In addition to TFPI, the TF pathway can be inhibited by antithrombin (AT) in the presence of heparin, which prevents the activation of FX or FIX by FVIIa when it is complexed with TF (Rao et al. 1995).

Under normal physiological conditions, TF expression is low or absent on cells that are in direct contact with blood such as the endothelium (Osterud et al. 2006). Circulating TF may be present at very small levels within the blood, located primarily on monocytes or microparticles (MPs), anucleated fragments of cytoplasm derived from apoptotic or activated cells (Owens, III et al. 2011). TF is highly expressed on cells which comprise the tunica media including fibroblasts and SMCs (Osterud et al. 2006). Vascular injury exposes the TF-rich subendothelial layer to the blood, thereby initiating coagulation (Osterud et al. 2006). Additionally, pathological conditions such as sepsis, cancer, and atherosclerosis may elevate levels of blood borne TF (Shantsila et al. 2009; Owens, III et al. 2011).

TF exists in two different functional populations on the cell surface. A small population of TF is procoagulant, with the ability to complex with factor VIIa, and activate factor X and IX. However, the majority of TF is "cryptic" and while it retains the ability to bind factor VIIa, the TF/VIIa complex is unable to activate zymogen coagulation factors (Bach 2006). The initiation of the extrinsic cascade can thus be regulated by inducing the "de-encryption" of non-coagulant TF (Osterud et al. 2006). *In vitro* studies have demonstrated that TF can be de-encrypted upon exposure of the cell to a calcium ionophore (Wakita et al. 1994; Bach 2006), by repeated freeze-thaw cycles (Le et al. 1992), or by apoptosis (Bach 2006; Bombeli et al. 1997). While the specific mechanism of TF encryption/de-encryption is unclear, it has been hypothesized that it is related to increased phosphatidylserine exposure (Bach 2006), association with lipid rafts (Dietzen et al. 2004), interaction with GRP78 (Pozza et al. 2005), and/or the formation of an intramolecular disulfide bond (Kothari et al. 2010; Ruf et al. 2010) which may involve the release of protein disulfide isomerase (PDI) (Popescu et al. 2010).

1.2.3 Role of tissue factor in hemostasis and thrombosis

Humans with deficiencies in TF have not been reported, suggesting that the TF pathway is necessary for the maintenance of vascular integrity. In addition, TF knockout mice exhibit an embryonic lethal phenotype due to abnormal blood vessel development (Toomey et al. 1996; Bugge et al. 1996; Carmeliet et al. 1996). The creation of a "low TF" mouse with a human minigene that expresses TF at less than 1% of physiological levels has demonstrated the critical role for TF in the initiation of coagulation. Such mice exhibit severe bleeding during surgery, lengthened tail bleeding times, and have a shorter lifespan relative to wild type mice (Parry et al. 1998).

In addition to revealing the critical role of TF in mediating hemostasis, mouse models have also confirmed the role of TF in initiating arterial, venous, and microvascular thrombosis (Kretz et al. 2010; Owens, III et al. 2010). However, the relative contribution of different cellular sources of TF remains less well understood. Models in which the integrity of the arterial vessel wall is compromised, such as laser-induced injury, suggest that the major source of procoagulant TF is located on the subendothelium (Wang et al. 2009b). However, circulating TF located on blood monocytes or MPs may drive venous or microvascular coagulation. For example, in endotoxemic mice, MP TF activity correlates with elevated TAT formation (Wang et al. 2009a). In addition, the selective inhibition of TF expression on hematopoetic and non-hematopoietic cells revealed that myeloid cells (likely monocytes) and unspecified non-hematopoietic cells (but not endothelial cells or SMCs) contribute equally to coagulation activation in a mouse model of endotoxemia (Pawlinski et al. 2010b).

The role of endothelial TF in thrombosis is less clear. Stimulation of endothelial cells *in vitro* with pro-inflammatory agents such as thrombin, lipopolysaccharide (LPS), and tumour necrosis factor (TNF) α upregulates TF mRNA synthesis and/or TF antigen levels (Colucci et al. 1983; Stahli et al. 2006; Bevilacqua et al. 1986); however, *in vivo* evidence of endothelial TF expression is uncertain. Endothelial cells of healthy individuals have been reported to be TF negative. However, it has been postulated that pathological conditions may induce TF expression on the endothelium. Several studies of endotoxemia using animal models have reported the detection of endothelial TF, while others have failed to do so (Drake et al. 1993; Semeraro et al. 1993). This may be due to methodological differences, or the potential transfer of TF to the endothelium via leukocyte-derived MPs (Pawlinski et al. 2010a). In addition, the knock-out of endothelial TF in an endotoxemia mouse model did not diminish coagulation activation, suggesting that the contribution of endothelial TF was low (Pawlinski et al. 2010b). However, endothelial TF expression may be regulated by specific pathology. In invasive breast cancer, a condition associated with elevated risk for thrombosis, TF expression was reported on the tumour endothelium, and expression correlated with elevated fibrin deposition (Contrino et al. 1996).

1.3 Phosphatidylserine in hemostasis and thrombosis

1.3.1 Regulation of phosphatidylserine asymmetry

Phosphatidylserine is a negatively charged phospholipid commonly localized to the inner leaflet of the plasma membrane (Gordesky et al. 1973). While it represents a small fraction of total phospholipids present in the eukaryotic cell membrane, it is the most abundant phospholipid with anionic properties (Leventis et al. 2010). The structure of phosphatidylserine is similar to the majority of phospholipids; it is typically comprised of two acyl chains of 16 or more carbon atoms, located at the *sn*-1 and *sn*-2 positions of the glycerol moiety, with serine linked at position *sn*-3 (Folch 1948). As serine is neutral, this confers a negative overall charge to the phosphatidylserine molecule.

Phosphatidylserine asymmetry is maintained through the action of the lipid transporter flippase, which transports phosphatidylserine from the extracellular leaflet to the cytosolic side (Leventis et al. 2010). Disruption of the plasma membrane, apoptosis, and cell stimulation may promote migration of phosphatidylserine to the membrane outer leaflet. Localization of phosphatidylserine to the cell surface is thought to result from an inactivation of flippase and an activation of scramblase, which randomizes phospholipid distribution throughout the plasma membrane (Verhoven et al. 1995). Cell surface phosphatidylserine is an early marker of apoptosis and facilitates phagocytosis (Fadok et al. 1992).

1.3.2 Role of phosphatidylserine in hemostasis and thrombosis

Phosphatidylserine exposure is a potent facilitator of coagulation, and promotes cell surface assembly of positively charged coagulation factors such as prothrombin, FVII, and FX via the Gla domain, or FV and FVIII via the C2 domain. Coagulation enzyme complexes such as tenase and prothrombinase are more efficiently activated on the phosphatidylserine positive surface, resulting in enhanced thrombin generation (Bevers et al. 1982). The exposure of phosphatidylserine on the surface of activated platelets is thought to be the major source of anionic phospholipid during normal hemostasis (Lentz 2003).

Impaired phosphatidylserine exposure is associated with impaired hemostasis. Deficiency in scramblase results in Scott syndrome, a moderate bleeding condition characterized by reduced phosphatidylserine exposure on the surface of activated platelets and impaired coagulation (Dekkers et al. 1998). As well, the inhibition of platelet phosphatidylserine by lactadherin in a mouse model abolished both thrombosis and hemostasis (Shi et al. 2008).

Dysregulation of physiological mechanisms which control phosphatidylserine exposure may promote thrombosis. Antiphospholipid syndrome, an auto-immune disease defined by the presence of antibodies which bind phospholipids is associated with increased risk for venous and arterial thrombosis, as well as pregnancy loss (Lim 2009). Although the precise mechanism of thrombosis is uncertain, it is hypothesized that these antibodies may interfere with endogenous anticoagulant mechanisms, such as interrupting the binding of annexin V to phosphatidylserine (Rand et al. 2008). Pathological conditions which upregulate phosphatidylserine exposure to the blood from various sources may increase the risk for thrombosis. Apoptotic or activated vascular cells may be a source for phosphatidylserine exposure (Bombeli et al. 1997; Flynn et al. 1997). As well, apoptotic cells and activated platelets shed phosphatidylserine-positive MPs, which are elevated in pathological conditions such as sepsis and cancer (Owens, III et al. 2011).

1.4 The contact pathway in hemostasis and thrombosis

1.4.1 The contact pathway

The intrinsic or contact pathway of blood coagulation is initiated by the activation of coagulation factor XII (FXII), which leads to the proteolytic activation of factors FXI and FIX. Factor XII can be converted into its active form, FXIIa via two distinct methods (Schmaier 2008). The first is auto-activation upon exposure to negatively charged surfaces such as platelet polyphosphate (Smith et al. 2006) and nucleic acid (Kannemeier et al. 2007). The heavy chain of factor XII binds to the negatively charged surface, and induces a conformational change (Cochrane et al. 1973). This promotes auto-activation to factor XIIa by proteolytic cleavage at amino acid residues 353-354 (McMullen et al. 1985). FXIIa is comprised of a heavy chain which mediates binding to anionic surfaces, linked with a disulfide bond to a light chain which contains the serine protease domain (Cool et al. 1985; Citarella et al. 1996). FXII may also be activated on the surface of endothelial cells by kallikrein and high-molecular weight kininogen (HK) (Cochrane et al. 1973). FXIIa then activates FXI, (Kurachi et al. 1980), a disulfide linked homo-dimer which circulates in the plasma complexed with HK (Thompson et al. 1977). In addition to activation by FXIIa, FXIa can be generated by auto-activation and thrombin by cleavage of amino acid residues 369-370 (Naito et al. 1991). FXIa activates FIX which in turn activates FX, the point at which the intrinsic and extrinsic pathways converge (Osterud et al. 1978).

1.4.2 Role of the contact pathway in hemostasis and thrombosis

While coagulation through the contact pathway can be easily activated *in vitro,* its relevance *in vivo* is less certain. Individuals with deficiencies in FXII do not present with elevated bleeding tendencies, while patients with FXI deficiencies have mild to moderate bleeding tendencies and reduced rates of VTE (Woodruff et al. 2011). Mouse models with deficiencies in FXII (Pauer et al. 2004) and FXI (Gailani et al. 1997) are compatible with normal life and reproduction, and do not display hemostatic abnormalities. However, in models of arterial thrombosis, FXII or FXI deficiencies attenuate thrombus formation (Renne et al. 2005; Rosen et al. 2002), suggesting that dysregulation of the contact pathway may contribute to a prothrombotic phenotype.

1.4.3 Cell free nucleic acids and coagulation activation

Recent studies have suggested that plasma or "cell free" nucleic acids can activate coagulation through several different mechanisms. In a purified system, cell free nucleic acids (DNA and RNA) have been shown to decrease clotting time by promoting auto-activation of coagulation factors XI and XII (Kannemeier et al. 2007). In addition, a mouse model of ferric chloride-induced arterial thrombosis demonstrated that RNA is associated with arterial thrombi, and treatment with RNAse or a FXIIa inhibitor can attenuate clot formation. Although this model failed to demonstrate the contribution of cell free DNA (CFDNA) to thrombus formation, the authors speculate this may be due to the complex of DNA with histone proteins which impaired the ability of DNA to promote activation of contact phase coagulation factors (Kannemeier et al. 2007). In addition, administration of exogenous RNA has been demonstrated to induce a rapid upregulation of coagulation in rabbits (Kannemeier et al. 2007), and in the greater wax moth, host-derived DNA has been shown to lead to phenoloxidase (serine protease cascade) activation and clotting of the hemolymph (Altincicek et al. 2008). Increased levels of CFDNA are commonly found in pathological conditions associated with increased thrombotic risk, such as sepsis and cancer (Saukkonen et al. 2008; Jahr et al. 2001).

In addition to release of nucleic acid from cellular injury, the formation of neutrophil extracellular traps (NETs) is another potential source of CFDNA (Clark et al. 2007; Brinkmann et al. 2004). NETs are actively released upon exposure to pathogenic stimuli including LPS, PMA, and IL-8. NETs are comprised of strands of chromatin with histones and antimicrobial agents which capture and kill invading microbial organisms and provide a physical barrier to prevent the spread of infection. In addition, NETs may also promote thrombus formation. NETs induce platelet aggregation and adhesion, which may be attributed to histone H3 and H4 (Fuchs et al. 2010). NETs also promote adhesion of red blood cells, bind von Willebrand Factor, fibronectin, and fibrinogen, and promote thrombin-dependent fibrin formation. In a baboon model of DVT, levels of CFDNA increased 48 hours post-DVT induction (Fuchs et al. 2010), which paralleled D-dimer formation in the same model (Meier et al. 2008). Histological examination revealed extracellular DNA and histones localized within the thrombi, but not within the contralateral control vessels, suggesting a role for CFDNA in promoting thrombus formation.

1.5 Regulation of thrombin generation and fibrin formation

1.5.1 Thrombin generation and fibrin formation

Thrombin, the enzymatic endpoint of the coagulation cascade, is a serine protease synthesized in the liver as the zymogen prothrombin. The conversion of prothrombin to thrombin (thrombin generation) occurs in three distinct phases: initiation, amplification, and propagation (Hoffman et al. 2001). The initiation phase of thrombin generation involves the activation of coagulation through either the tissue factor (extrinsic) or contact (intrinsic) pathways. At the amplification stage, low levels of FXa along with its cofactor Va, prothrombin, and calcium (termed the prothrombinase complex) assemble on a surface containing phosphatidylserine (Rosing et al. 1980). FVa mediates the binding of prothrombin and Xa to the anionic surface (Tracy et al. 1983), while FXa cleaves prothrombin at two sites to produce thrombin (Rodgers et al. 1983).

Simultaneous to the initiation of thrombin generation, platelets localize to the vessel wall by binding exposed collagen and vWF (Ruggeri et al. 1983). Thrombin cleaves PAR1 (at low levels) and PAR4 (at high levels) on the platelet surface, resulting in platelet activation (Kahn et al. 1999). Platelet activation and subsequent granule release exposes partially activated FVa, (Monkovic et al. 1990) and low levels of thrombin activate factor V, XI, and VIII, thereby amplifying the rate of coagulation (Hoffman et al. 2001). The propagation phase of thrombin generation involves large scale assembly of tenase and prothrombinase complexes on the cell surface which facilitates a high level of thrombin generation, catalyzing the conversion of fibrinogen to fibrin (Lorand et al. 1964), and resulting in the arrest of blood flow.

Fibrinogen is a soluble glycoprotein that circulates in the plasma and is converted by thrombin to form an insoluble fibrin clot. Fibrinogen is comprised of symmetrically arranged pairs of polypeptide chains, Aα, Bβ, and γ (Medved et al. 1983). Thrombin binds the central domain of fibrinogen, and cleaves the Aα and Bβ chains to release fibrinopeptide A (FPA) and B (FPB) (Ferry et al. 2011). This results in the end-to-end and lateral association of cleaved fibrinogen to produce fibrin. In addition to converting fibrinogen to fibrin, thrombin also promotes clot strength and resistance to fibrinolysis by activating factor XIII, a transglutaminase which cross-links fibrin (Weisel et al. 1993), increasing its stability and resistance to degradation by plasmin. Thrombin also attenuates plasmin-induced clot lysis by activating TAFI (thrombin activatable fibrinolysis inhibitor) when bound to the endothelial cell surface receptor thrombomodulin (Wang et al. 1998).

Thrombin generation in plasma can be inhibited by several endogenous anticoagulant mechanisms. When thrombin is bound to thrombomodulin, it no longer retains its procoagulant effects, and activates the protein C anticoagulant pathway, which attenuates thrombin generation by inactivating FVa and FVIIIa (reviewed in detail in the following section). As well, direct thrombin inhibitors inactivate thrombin by binding to exosite I, the site at which thrombin interacts with its substrates, and/or by inhibition of the thrombin active site. Antithrombin is a serine protease inhibitor (serpin) which forms a 1:1 complex with the active site of its target protease, preventing the protease from accessing its substrate. Antithrombin physiologically targets multiple coagulation factors including FXa, FIXa, FXIa, FXIIa, FVIIa, and thrombin (Roemisch et al. 2002). The inactivation of thrombin by antithrombin is accelerated in the presence of the anticoagulant drug heparin (Machovich et al. 1975) or heparan sulphate localized to the endothelial cell surface (Ofosu et al. 1984). α_2-macroglobulin is also an important physiological inhibitor of thrombin. It neutralizes thrombin activity by promoting thrombin cleavage of a "bait" region, which induces a conformational change which entraps thrombin inside the α_2-macroglobulin molecule (Barrett et al. 1973). Thrombin bound to α_2-macroglobulin retains its ability to cleave small substrates, although its proteolytic acitivity against macromolecular substrates is abolished.

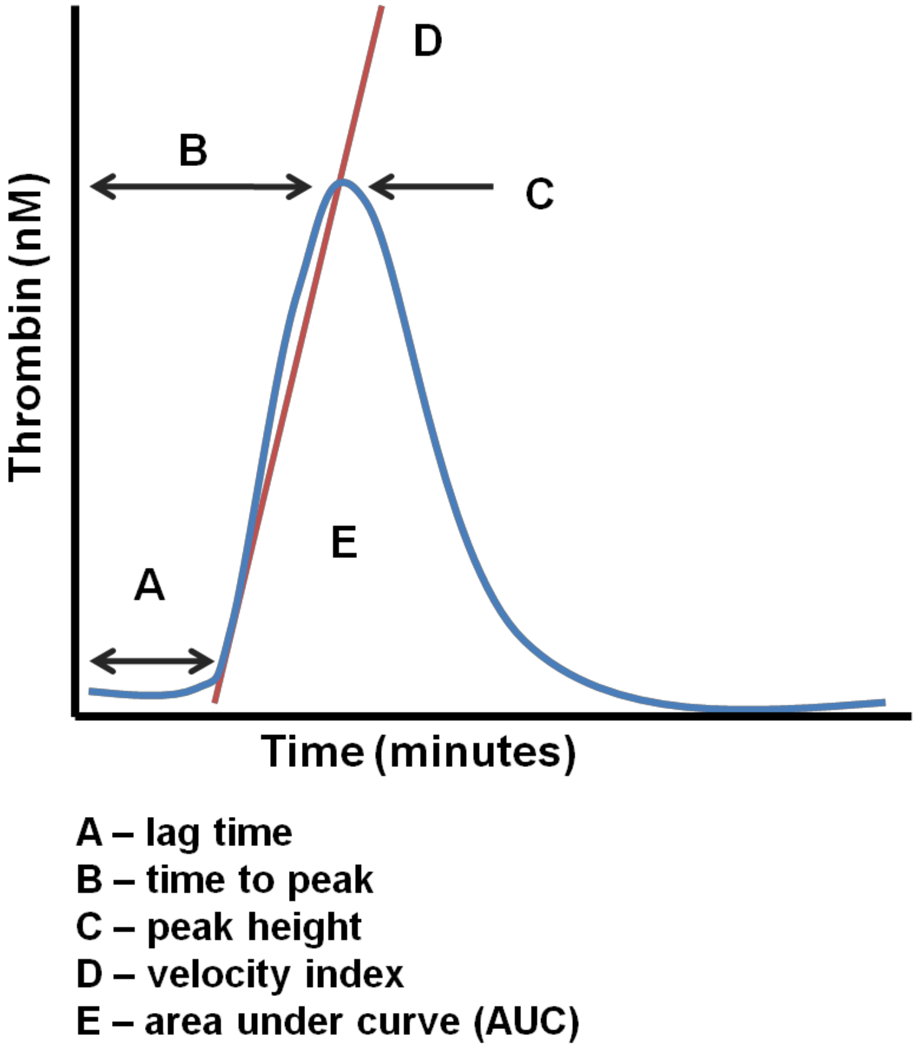
1.5.2 Thrombin generation and fibrin formation in hemostasis and thrombosis

Mutations which affect thrombin generation or the inhibition of thrombin have confirmed the central role of thrombin in hemostasis and thrombosis. Disorders which impair thrombin activation from prothrombin are rare; hypoprothrombinemia involves reduced synthesis of prothrombin, while dysprothrombinemia results in the synthesis of a non-functional prothrombin protein (Meeks et al. 2008). Both conditions are associated with elevated risks of bleeding. In contrast, hyperprothrombinemia significantly increases the risk for thrombosis in affected individuals. The most commonly characterized is the prothrombin 20210a mutation, where adenine is substituted for guanine at position 20210 of the prothrombin gene (Vicente et al. 1999). This mutation elevates plasma levels of prothrombin and increases the risk for developing venous and arterial thrombosis. In addition, inherited deficiencies in antithrombin that decrease antithrombin production, or impair antithrombin function, are associated with an increased risk for thrombotic events (Patnaik et al. 2008). Mouse models which modify thrombin function have revealed its central role in thrombosis and hemostasis. Total prothrombin deficiency results in approximately 75% embryonic lethality; 100% of the mice that survive to term expire within several days due to hemorrhage (Sun et al. 1998; Xue et al. 1998).

1.6.3 The thrombogram

The thrombin generation curve is a research tool established to measure the overall function of the coagulation system (Hemker et al. 2003). The reaction is initiated by the recalcification of citrated plasma, and the conversion of prothrombin to thrombin is measured by cleavage of a chromogenic or fluorogenic substrate, producing a characteristic pattern termed a "thrombogram" (Hemker et al. 2003). Very small amounts of thrombin, generated by TF or the contact pathway, are produced during the initiation/amplification phase and are not detectable by the assay. This is known as the "lag" phase of the profile.

Figure 1.2 – The thrombogram. The thrombin generation curve can be described in terms of lag time (period of undetectable thrombin generation before propagation phase), peak (rate of thrombin formation and inhibition reach an equilibrium), velocity index (rate of thrombin generation during propagation phase), and AUC/ETP (total amount of free thrombin generated during the course of the assay).


D
B
C
Thrombin (nM)
E
A
Time (minutes)
A – lag time
B – time to peak
C – peak height
D – velocity index
E – area under curve (AUC)

A large burst of thrombin generation occurs at the propagation phase, and this can be measured by the velocity index, the rate of thrombin generation. When the rate of thrombin formation and thrombin inhibition reach an equilibrium state, thrombin generation reaches its peak, which can be measured by peak height (in nM thrombin) or time to peak (in units of time). After the peak, thrombin generation decreases and eventually returns to baseline due to inhibition by anticoagulant factors such as antithrombin and in some systems activated protein C. The total amount of free thrombin generated during the course of the assay, the integration of the thrombin generation curve, is known as area under the curve (AUC) or endogenous thrombin potential (ETP) (Hemker et al. 2006).

Many protocols for measuring thrombin generation exist. An early method for thrombin generation analysis was a subsampling technique, whereby thrombin generation is initiated in recalcified, defibrinated plasma, aliquots are removed at regular intervals, and thrombin levels are quantified using a chromogenic assay. In this method, α_2-macroglobulin-bound thrombin, which inhibits exosite I of thrombin but not its active site, is able to cleave the chromogenic substrate leading to an overestimation of thrombin generation (Baglin 2005). The concentration of α_2-macroglobulin-bound thrombin is measured by mixing plasma aliquots with antithrombin and heparin to neutralize free thrombin. Both total thrombin and α_2-macroglobulin-bound thrombin levels must be measured in parallel by cleavage of a chromogenic substrate for thrombin; free thrombin levels are calculated by subtracting α_2-macroglobulin-bound thrombin from total thrombin levels (described in more detail in Chapter 3).

More recently, calibrated automated thrombin generation (CAT) systems have become widely available. In this method, the activation of a fluorogenic substrate for thrombin in clotting plasma is measured relative to a parallel sample with known thrombin activity (Hemker et al. 2003). In platelet poor plasma, the CAT generation assay developed by Hemker et al. (2003) can measure deficiencies in all clotting factors except FXIII, as well as the effects of anticoagulants on thrombin generation. Using these methods, α_2-macroglobulin-bound thrombin can be corrected mathematically; however, not all methods perform this calculation as peak thrombin is not significantly influenced by α_2-macroglobulin (Chandler et al. 2009) and plasma levels of α_2-macroglobulin can vary significantly in different patient groups and by age (neonates and children versus adults) (Ignjatovic et al. 2007). As well, the formation of α_2-macroglobulin-bound thrombin complexes can vary based on the presence of anticoagulants in the plasma (Lau et al. 2007), and α_2-macroglobulin-bound thrombin levels are decreased in plasma samples with normal fibrinogen relative to defibrinated plasma (Hemker et al. 2003).

1.6 The protein C anticoagulant pathway in hemostasis and thrombosis

1.6.1 The protein C pathway

The protein C anticoagulant pathway is a negative feedback mechanism which attenuates thrombin generation in response to excess thrombin formation. The PC zymogen is synthesized in the liver and circulates in the blood as a heterodimer, with a light chain and a heavy chain linked by a disulfide bond (Kisiel 1979). PC is comprised of two EGF-like domains, a serine protease domain, and Gla domain that mediates binding to phospholipids and EPCR on the endothelial cell surface (Plutzky et al. 1986). To initiate PC activation, excess thrombin binds to thrombomodulin, an integral membrane protein expressed on the endothelium (Esmon et al. 1981). This interaction is mediated through the anion binding exosite 1 of thrombin, which binds to EGF 5 and 6 of thrombomodulin (Kurosawa et al. 1988). This alters the substrate specificity of thrombin such that it loses its ability to activate coagulation factors and cleave fibrinogen. Thrombomodulin functions as a cofactor while thrombin proteolytically activates protein C by cleaving a 12 amino acid activation peptide at the N-terminus of the PC heavy chain (Kisiel 1979). The interaction between thrombomodulin and thrombin enhances the generation of APC by up to 1000-fold (Esmon et al. 1982), and the binding of PC to EPCR increases the rate of APC generation by up to 20-fold *in vivo* (Taylor, Jr. et al. 2001).

Upon activation by thrombin, APC dissociates from EPCR and complexes with its cofactor protein S (PS) at the endothelial cell or platelet surface (Walker 1980). Protein S binds to phospholipids on the cell surface membrane via its Gla domain, and serves to localize APC in close proximity to the prothrombinase complex (Hackeng et al. 1993). The interaction between APC and protein S results in a repositioning of the active site of APC (Yegneswaran et al. 1999), and enhances the cleavage of Arg306 and Arg506 of FVa by the serine protease domain of APC, resulting in inactivation of FVa (Egan et al. 1997). APC bound to Protein S also proteolytically inactivates FVIIIa at Arg336 and Arg552 (Regan et al. 1994). Inhibition of FVa and FVIIIa attenuates thrombin generation.

1.6.2 Dysregulation of the protein C pathway in hemostasis and thrombosis

Genetic defects in the protein C anticoagulant pathway increase the risk for developing VTE. The factor V Leiden mutation is the most common and clinically prevalent procoagulant genetic mutation. It involves a point mutation in the coding sequence of factor V which creates an arginine to glutamine substitution at position 506 (Koster et al. 1993). This mutation occurs at one of the three sites cleaved by the anticoagulant APC, and thus renders factor V Leiden more resistant to degradation, resulting in an inhibition of anticoagulation. Genetic deficiencies which result in decreased PC and PS antigen levels, or normal antigen levels but decreased function have been described and are associated with increased risk for VTE (Reitsma 1996; Gandrille et al. 2000). Acquired defects in the protein C pathway are also associated with impaired APC generation and may elevate the risk for thrombosis. PC deficiencies have been documented in individuals with liver disease (Walker 1990) or receiving the anticoagulant warfarin which inhibits the vitamin K-dependent synthesis of protein C (Vigano et al. 1986). Decreased levels of PC, likely due to consumption, have been reported in patients with inflammatory conditions such as sepsis (Liaw et al. 2004) and in patients receiving cancer chemotherapy (Mukherjee et al. 2010). Defects in EPCR are also associated with elevated risk for VTE. For example, a 23 base pair insertion in EPCR which leads to a premature stop codon at Arg96, and prevents the localization of EPCR to the cell surface has been documented in patients with thrombophilia (Biguzzi et al. 2001). Reported mutations in thrombomodulin are fairly rare, and the pathological relevance of identified mutations are uncertain (Faioni et al. 2002).

Although they are expressed on the endothelial cell surface, soluble forms of EPCR (sEPCR) and thrombomodulin (sTM) are also found in plasma. Patients with sepsis (Kurosawa et al. 1998; Liaw et al. 2004), systemic lupus erythromatosis (Kurosawa et al. 1998), and the EPCR A3 haplotype (Saposnik et al. 2004) have been reported to have elevated levels of sEPCR. sEPCR may impair APC generation by several mechanisms: (1) increased EPCR shedding can decrease cell surface EPCR levels (Xu et al. 2000) and (2) at elevated concentrations, sEPCR competes with membrane-bound EPCR for binding to PC/APC (Regan et al. 1997). Both of these factors can contribute to decreased APC generation. In addition, (3) sEPCR may also impair the interaction of APC with the anionic cell surface, impairing efficient inactivation of Va and VIIIa (Liaw et al. 2000). The shedding of EPCR from the endothelium can be induced *in vitro* by hydrogen peroxide (H_2O_2), TNF-α, PMA, thrombin, and IL-1β (Xu et al. 2000). The release of sEPCR induced by PMA is mediated by tumour necrosis factor-α converting enzyme/ADAM17 (TACE), a disintegrin and metalloproteinase (Qu et al. 2007).

Elevated levels of sTM are widely used as a marker of endothelial damage, and are associated with a number of pathological conditions including diabetic angiopathy, trauma, and sepsis (Boffa et al. 1998). Similar to the shedding of EPCR, it is thought that generation of sTM can result in decreased surface levels of thrombomodulin, and therefore impair APC generation. However, the release of sTM into the plasma may also have an anticoagulant function, as it binds to thrombin, promotes APC generation and inhibits fibrin deposition (Gomi et al. 1990; Mohri et al. 1999). Pro-inflammatory factors such as LPS, IL-1, and TNF-α can decrease endothelial thrombomodulin expression by decreasing thrombomodulin mRNA synthesis, increasing thrombomodulin degradation, or increasing thrombomodulin shedding from the cell surface (Nawroth et al. 1986; Moore et al. 1989; Moore et al. 1987).

Mouse models featuring complete disruption of PC, EPCR, or thrombomodulin expression result in embryonic lethality, demonstrating the crucial role of the protein C pathway in maintaining an anticoagulant phenotype (Jalbert et al. 1998; Gu et al. 2002; Healy et al. 1995). Mice with a severe PC deficiency are viable (1 – 18% of normal) but display reduced lifespan due to proinflammatory and prothrombotic phenotypes which vary in severity depending on plasma PC levels (Lay et al. 2005). Selective deletion of endothelial thrombomodulin results in 60% viability after birth, however, mice display decreased survival due to thrombosis and consumptive coagulopathy (Isermann et al. 2001). A mouse model bearing point mutation in thrombomodulin that impairs its ability to participate in protein C activation results in elevated fibrin deposition (Weiler-Guettler et al. 1998) and increased mortality in response to endotoxin treatment (Weiler et al. 2001). Low EPCR expressing mice have diminished survival in response to LPS challenge (Iwaki et al. 2005). Conversely, treatment of mice overexpressing EPCR with LPS results in the generation of more APC, less thrombin, and improves survival relative to wildtype controls (Li et al. 2005).

1.7 Regulation of hemostasis by the blood and vasculature

1.7.1 Role of the vascular endothelium in regulating hemostasis

The vascular endothelium is a specialized epithelial tissue which forms a barrier between blood in the lumen and the tissues of the vessel wall (Becker et al. 2000). Under normal physiological conditions, the vascular endothelium maintains blood in a fluid state by synthesizing anticoagulant factors which inhibit thrombin generation such as EPCR (Fukudome et al. 1995), thrombomodulin (Esmon et al. 1982), heparan sulfate (Mertens et al. 1992), and TFPI (Ameri et al. 1992). The endothelium releases prostacyclin (PGI_2) and nitric oxide (NO), which promote vasodilation and inhibition of platelet activation (Becker et al. 2000). The endothelium also produces ecto-ADPase which degrades ADP, a participant in platelet recruitment during thrombus formation (Marcus et al. 1997). The endothelium supports fibrinolysis by releasing tPA (Levin et al. 1984) and uPA (Gualandris et al. 1995).

Endothelial dysfunction or damage can result in the formation of a localized procoagulant environment. Mechanical injury resulting in endothelial denudation or shedding of endothelial cells into the plasma exposes the procoagulant subendothelium to the blood. The subendothelium is a rich source of TF, and other procoagulant factors such as collagen (Weiss et al. 1989). Stimulation of the endothelium by apoptotic or pro-inflammatory agents can lead to endothelial activation, whereby the endothelium participates in a rapid initiation of coagulation by upregulating expression of procoagulant factors while simultaneously downregulating synthesis of anticoagulant molecules (Levi et al. 2002). Endothelial cells in tissue culture can be induced to increase TF expression and/or activity; however the relevance of this *in vivo* is uncertain (Osterud et al. 2006). The endothelium also synthesizes mediators of platelet activation such as collagen (Howard et al. 1976) and vWF (McCarroll et al. 1985), which are deposited in the subendothelial space. Stimulation of endothelial cells with agents such as thrombin or proinflammatory cytokines can increase synthesis of PGI_2, while other inflammatory agents such as C-reactive protein can impair NO and PGI_2 synthesis thereby promoting platelet aggregation (Venugopal et al. 2002; Venugopal et al. 2003). Endothelial cells undergoing apoptosis display a rapid exposure of phosphatidylserine on the membrane outer leaflet (Bombeli et al. 1997). As well, the endothelium expresses plasminogen activator inhibitor (PAI) (Hekman et al. 1985), the physiological inhibitor of tPA and uPA, thereby impairing fibrinolysis.

1.7.2 Role of vascular smooth muscle cells in regulating hemostasis

Vascular smooth muscle cells (SMCs) are a non-striated muscle found within the media of arteries and veins. SMCs serve to regulate vascular tone, including the volume of blood vessels and local blood pressure (Fisher 2010). Under normal conditions, SMCs are not in contact with the blood; however injury or denudation of the endothelium can expose procoagulant factors expressed by SMCs to the blood and contribute to localized coagulation. SMCs express high levels of active TF (Weiss et al. 1989), and in mouse models, TF from vascular SMCs initiates arterial thrombotic events (Wang et al. 2009b). In addition, apoptotic SMCs have been demonstrated to enhance thrombin generation via the exposure of phosphatidylserine (Pathak et al. 2006; Flynn et al. 1997).

1.7.3 Role of monocytes in blood coagulation

Monocytes are a subset of leukocytes produced in the bone marrow which have roles in phagocytosis, inflammation, and coagulation (Shantsila et al. 2009). Monocytes have been demonstrated to express TF at low baseline levels *in vivo*, however, inflammatory stimuli such LPS markedly elevate monocyte TF expression and activity (Lwaleed et al. 2001). Similar to endothelial and smooth muscle cells, apoptotic monocytes display increased phosphatidylserine exposure (Gockel et al. 2004; Henriksson et al. 2005). Activated monocytes also express Mac-1 and cathepsin G, which may play a role in enhancing the activation of FX independent of TF:FVIIa (Plescia et al. 1996); monocytes can also activate factor V on cell surfaces (Allen et al. 1995). Monocytes can impair fibrinolysis by secreting plasminogen activator inhibitor-2 (PAI-2), which inhibits plasmin generation by directly interacting with uPA (Ritchie et al. 1999). Finally, monocytes also express EPCR (Galligan et al. 2001) and thrombomodulin (McCachren et al. 1991), and may therefore play a role in APC-mediated anticoagulation.

1.8 Thrombosis and malignancy

1.8.1 Overview of association between thrombosis and cancer

Thrombosis is a well-recognized complication of cancer. Between 2.7 – 25.7% of patients presenting with idiopathic thrombosis have an underlying malignancy (Lee et al. 2003), and between 4 – 20% of individuals with cancer will develop clinically diagnosed thrombosis (Lyman 2007). Thrombosis significantly increases mortality in cancer patients. A single episode of VTE is associated with a 2.2-fold increased risk of death (Sorensen et al. 2000), making thromboembolic disease the second leading cause of death in cancer patients (Ambrus JL et al. 1975; Shen et al. 1980; Rickles et al. 1983a). As well as its high cost in terms of morbidity and mortality, thrombosis has a high financial cost. In 2002 in the

United States, the average length of hospitalization after DVT diagnosis was 11 days, with a cost of over $20,000 USD (Elting et al. 2004).

The risk for thrombosis in cancer patients can vary depending on the age of the patient, stage of the disease, the type of cancer, and the presence of metastases. Brain, pancreatic and renal cancers have the highest risk for developing VTE (Sallah et al. 2002). However, out of all cancer patients presenting with VTE, breast, lung, ovarian, prostate, and colon cancers are also common (Sallah et al. 2002; Rickles et al. 1983a; Lee et al. 2003; Levitan et al. 1999); this is likely reflective of the higher incidences of these cancers in the general population (Haddad et al. 2006). In addition, patients with late stage cancers (III/IV) are more likely to develop VTE than those with early stage cancers (I/II) (Sallah et al. 2002). Thrombosis in cancer patients most often presents as VTE; however arterial thrombosis can present in patients undergoing chemotherapy, and DIC is often observed in patients with hematological malignancies or metastases (Prandoni et al. 2005). In addition, cancer patients also develop catheter-related thrombosis, non-bacterial thrombotic (marantic) endocarditis, migratory thrombophelbitis, and hepatic veno-occlusive disease (Haddad et al. 2006).

Malignancy can increase the risk of thrombosis by several different mechanisms. Tumour cells synthesize procoagulant factors, induce inflammation and promote cell death, which contributes to a procoagulant phenotype (reviewed in the following section). As well, cancer patients are more likely be hospitalized or experience prolonged immobilization (stasis), undergo surgery, or employ the use of central venous catheters (Prandoni et al. 2005). Pharmacological interventions such as hormonal therapy, anti-angiogenic therapy, erythropoietin stimulating agents, and chemotherapy have all been documented to increase the risk for thrombosis in cancer patients (reviewed in a following section).

1.8.2 Dysregulation of coagulation and fibrinolysis in cancer patients

More than 50% of all cancer patients and 90% of patients with metastatic disease display evidence of dysregulation of blood coagulation (Sun et al. 1979; De Cicco 2004). The hypercoagulable state in cancer is fuelled by increased levels of procoagulant factors coupled with diminished anticoagulation. Plasma markers for activation of coagulation in cancer patients include elevated levels of prothrombin fragments (F1+2) (which indicates thrombin activation), thrombin-antithrombin (TAT) complex formation (which indicates increased thrombin inactivation), and fibrinopeptide A (FPA) (which indicates the conversion of fibrinogen to fibrin by thrombin) (Rickles et al. 1983b; Sagripanti et al. 1993; Lindahl et al. 1990).

Thrombin generation in cancer patients is primarily thought to be initiated by TF. TF expression has been reported on many types of tumours, including pancreatic, lung, breast, renal, colorectal, and ovarian cancer cells (Rickles et al. 1995). TF levels on tumour cells generally correlate with tumour progression (Kakkar et al. 1995). Patients with tumours that express high levels of TF, such as brain and pancreatic cancers, have a greater risk for thrombosis than patients with low TF expressing tumours such as breast cancers (Kasthuri et al. 2009; White et al. 2007). TF has also been detected on the endothelium of the tumour vasculature (Contrino et al. 1996). As well, cancer patients typically have elevated levels of circulating TF. Monocytes and MPs derived from activated host cells such as monocytes, and endothelial cells may be a major source of procoagulant blood borne TF in cancer patients (Khorana et al. 2008; Lwaleed et al. 2000). In mouse tumour models, circulating levels of TF correlates with increased tumour size, relative TF expression on tumour cells, and thrombin-antithrombin complex formation (Yu et al. 2005; Davila et al. 2008).

In addition to TF, tumour cells also express cancer procoagulant (CP), a cysteine protease which directly activates FX independent of FVIIa (Gordon et al. 1975). CP is expressed on tumour cells derived from lung, breast, kidney, and hematopoietic cancers. Tumour cells may also express mucin, which can directly activate factor X and promote platelet aggregation (Wahrenbrock et al. 2003; Pineo et al. 1974), as well as factor V receptor which accelerates prothrombinase activity (VanDeWater et al. 1985). Tumour cells may also promote platelet activation or aggregation by releasing ADP (Boukerche et al. 1994). In addition, cancer is also associated with elevated plasma levels of fibrinogen, factor VIII, and von Willebrand factor (Sweeney et al. 1990; Vormittag et al. 2009; Edwards et al. 1987).

The procoagulant state in cancer may be exacerbated by impairment of endogenous anticoagulant and fibrinolytic mechanisms. Complications from malignancy such as hepatic metastases and increased consumption may decrease plasma levels of antithrombin (Rubin et al. 1980). In addition, acquired APC deficiencies are commonly associated with increased risk for VTE in cancer patients (Haim et al. 2001). As a result of elevated clotting, fibrinolytic pathways are activated in cancer patients, resulting in increased D-dimer levels (Sagripanti et al. 1993). However, tumour cells synthesize PAI-1, which impairs tPA and uPA mediated fibrinolysis (Meryet-Figuieres et al. 2007); aberrant expression of these factors may lead to dysregulation of fibrinolytic processes.

1.9 Chemotherapeutic treatment for breast cancer

1.9.1 Introduction to treatment of breast cancer

Breast cancer is the most common type of cancer among women, and the fifth leading cause of cancer-related death world-wide (WHO 2003; Ferlay et al. 2010). Like many forms of cancer, the etiology of breast cancer is complex and multifactorial, and there are well characterized environmental and genetic risk factors. Between 5 and 10% of all breast cancers can be directly attributed to germline mutations in several fairly well-characterized genes including BRCA1 and BRCA2 (Lynch et al. 2008). In addition, environmental factors can contribute to breast cancer development including age, sex, obesity, alcohol consumption, and nulliparity (Singletary 2003).

Currently, treatment for breast cancer involves surgery to remove cancerous tissue (lumpectomy), hormonal therapy, chemotherapy, radiation, and immunotherapy (Hortobagyi 1998). While surgery is the most effective method of breast cancer treatment, radiation therapy is frequently given as an adjuvant to lumpectomy and is thought to reduce the recurrence of breast cancer (Morris et al. 1997). In addition, chemotherapy may be administered in a neoadjuvant fashion to reduce the tumour size prior to surgery, adjuvant after surgery to reduce the risk of tumour recurrence, or palliative to control but not cure metastatic disease (Hortobagyi 1998). Chemotherapy in breast cancer patients is most often administered as multi-drug regimens which are more effective than administration of single agents (EBCTCG 1988). Clinical trials have shown that chemotherapeutic regimens containing anthracycline agents (doxorubicin and epirubicin) such as CAF (cyclophosphamide, Adrimycin/doxorubicin, 5-fluorouracil), and CEF (cyclophosphamide, epirubicin, 5-fluorouracil) are more effective than those that do not, precipitating a shift away from methotrexate-based regimens such as CMF (cyclophosphamide, methotrexate, 5-fluorouracil) in recent years (EBCTCG 2005). Such regimens are typically administered every 3 – 4 weeks for 4 – 6 cycles (Hortobagyi 1998). Determination of the appropriate chemotherapeutic regimen is related to patient characteristics such as tumour character, health of the patient, and lymph node status.

The most common forms of breast cancer include ductal and lobular carcinomas, and despite origin of disease, prognosis is typically classified into stages (Singletary et al. 2002). Stage 0 represents a precancerous condition (ductal carcinoma in situ or lobular carcinoma in situ) where the cancerous cells have not migrated into surrounding tissues. Stages I through III are early forms of breast cancer associated with generally good prognosis. Stage IV is advanced or metastatic carcinoma, and is associated with poor prognosis. Breast cancers can also be classified based on presence or absence of estrogen receptor (ER) and human epidermal growth factor receptor (HER2) expression (McArthur et al. 2007). ER-positive tumours are estrogen-dependent, and can benefit from treatment with hormonal therapies such as tamoxifen (EBCTCG 2005). HER2-positive tumours are more aggressive and associated with a worse prognosis than HER2-negative tumours, and respond well to treatment with trastuzumab (Herceptin) (Smith et al. 2007).

1.9.2 Anthracycline chemotherapeutic agents

Both doxorubicin and epirubicin are members of the anthracycline family of chemotherapeutic agents, and are used in the treatment of hematological malignancies as well as solid tumours. Doxorubicin was originally isolated from *Streptomyces peucetius*, and epirubicin is a semi-synthetic structural isomer of doxorubicin, varying only at the orientation of a hydroxyl group on the 4'-C atom (Minotti et al. 2004). Although structurally and mechanistically similar, there are several pharmacokinetic and metabolic differences between doxorubicin and epirubicin. Epirubicin has a shorter half life, increased volume of distribution and enhanced clearance relative to doxorubicin (Robert et al. 1985). The primary metabolites of doxorubicin and epirubicin are doxorubicinol and aglycone, and epirubicinol respectively (Misiti et al. 2003; Robert et al. 1985). Doxorubicin metabolites are thought to be responsible for doxorubicin-associated cardiotoxicities, while patients treated with epirubicin have a lower risk of developing these effects (Cersosimo et al. 1986).

Doxorubicin and epirubicin (and other anthracycline agents) have similar mechanisms of action in terms of their pro-apoptotic and anti-cancer activities. Doxorubicin and epirubicin generate reactive oxygen species (ROS) (Kebieche et al. 2009; Damrot et al. 2006), which induce macromolecular damage and/or cell signalling. Anthracyclines also intercalate between DNA base pairs and impair DNA transcription and translation by inhibiting helicase, polymerase and topoisomerase II (Belloc et al. 1992; Bachur et al. 1992; Binaschi et al. 1997). These effects lead to the induction of apoptosis in tumour cells (Lorenzo et al. 2002).

1.9.3 Nitrogen mustard alkylating agents

Cyclophosphamide is a nitrogen mustard alkylating agent widely administered for its anticancer and immunosuppressive activities (de Jonge et al. 2005). Cyclophosphamide is a prodrug and requires liver cytochrome P450 oxidative metabolism to become 4-hydroxycyclophosphamide (Chang et al. 1993). 4-hydroxycyclophosphamide diffuses into the cell, where it undergoes spontaneous decay into phosphoramide mustard and acrolein (de Jonge et al. 2005). Phosphoramide mustard is an alkylating agent which can form inter- and intra-stranded DNA cross-links, and as well cross-link DNA with protein resulting in the obstruction of DNA replication and apoptotic cell death (O'Connor et al. 1991). It is thought that phosphoramide mustard conveys many of cyclophosphamide’s therapeutic effects, while acrolein is cause of its toxicity.

In addition to cyclophosphamide metabolism, acrolein is also a byproduct of organic combustion and elevated plasma levels have been reported in smokers (Nazaroff et al. 2004). Other conditions such as renal failure, bacterial infection, and chemotherapy are also associated with elevated levels of acrolein (Sakata et al. 2003; Gugliucci et al. 2007). Acrolein is a strong electrophile, and reacts with cellular nucleophiles, including thiols, cysteine, histidine, lysine residues of proteins, and with nucleophilic DNA sites (Kehrer et al. 2000). Acrolein can directly modify activity of transcription factors (Horton et al. 1999), and form inter-stranded DNA and DNA-protein cross-links (Crook et al. 1986) thereby altering gene expression. In addition, acrolein may contribute to oxidative stress by depleting intracellular glutathione stores and generating ROS (Gurtoo et al. 1981; O'Toole et al. 2009). Acrolein is known to induce cell death largely by oncosis (Kehrer et al. 2000), and apoptosis can also be induced by the intermediate generation of NO (Misonou et al. 2006).

1.9.4 Antimetabolite chemotherapeutic agents

Antimetabolite chemotherapeutic agents function by inhibiting biosynthetic processes, or serve as analogs for cellular components, becoming incorporated into macromolecules, and inhibit their function. These agents can have toxic effects on cells, inhibiting growth, division and DNA synthesis, particularly of rapidly growing cells. 5-fluorouracil (5FU) is an analogue of uracil with a fluorine atom substitution at the C-5 position (Longley et al. 2003). It can be misincorporated into RNA, thereby inhibiting protein synthetic pathways (Kufe et al. 1981). 5FU is also an inhibitor of thymidylate synthase, an enzyme that methylates deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) (Van der Wilt et al. 1997), thereby disrupting DNA synthesis and repair. Methotrexate is an analog of folate and inhibits dihydrofolate reductase (DHFR). DHFR converts dihydrofolate (DHF) to tetrahydrofolate (THF), which is required for biosynthesis of thymidine and purines (Genestier et al. 2000).

1.10 Association between breast cancer chemotherapy and thrombosis

1.10.1 Introduction to chemotherapy and thrombosis

While the presence of malignancy increases the risk for thrombosis 4.1-fold above baseline levels, the treatment of cancer patients with chemotherapy can further increase this risk by up to 6.5-fold (Heit 2003). Sixty-five percent of cancer patients who develop thrombosis do so during treatment with chemotherapy (Sallah et al. 2002), and the overall risk for thrombosis while receiving chemotherapy is 11% (Otten et al. 2004). Venous and arterial thrombosis account for over 9% of mortalities in cancer patients receiving outpatient chemotherapy (Khorana et al. 2007), making it the second leading cause of death. Thrombosis typically presents early on (in the first 2 – 3 months) of the chemotherapy treatment (Sallah et al. 2002; Otten et al. 2004). The most common thrombotic complication of chemotherapy treatment is VTE, although arterial thrombosis has also been reported (Saphner et al. 1991).

The risk for thrombosis during chemotherapy treatment varies depending on the type of cancer and the chemotherapy regimen an individual receives (Haddad et al. 2006). For example, non small cell lung cancer patients treated with cisplatin and gemcitabine have a risk of thrombosis of 17.6% (Numico et al. 2005). Ovarian cancer patients receiving cisplatinum, epirubicin, and cyclophosphamide have a risk of 10.6% (von Tempelhoff et al. 1997). Colorectal cancer patients treated with 5FU and leucovorin calcium have a rate of thrombosis of 15% (Otten et al. 2004), and adult patients with acute lymphoblastic leukemia receiving L-asparaginase-based therapy have a rate of thrombosis of 4.2% (Gugliotta et al. 1992). These courses of therapy seek to maximize upon synergistic anti-cancer activities and unrelated toxicities of drug combinations (Ershler 2006), however the frequent use of combination chemotherapy, and presence of additional risk factors makes the prothrombotic risk of individual drugs difficult to assess.

1.10.2 Chemotherapy for breast cancer and thrombosis

Perhaps the best characterized relationship between cancer and chemotherapy is in breast cancer patients; the baseline risk of thrombosis in women with early stage (I – III) breast cancer is less than 1% (Saphner et al. 1991). However, the risk of thrombosis increases to 5% in patients with early (stage II) breast cancer receiving postmastectomy adjuvant chemotherapy (Weiss et al. 1981). A randomized trial comparing 12 weeks of chemohormonal therapy (cyclophosphamide, methotrexate, fluorouracil, vincristine, prednisone, doxorubicin, and tamoxifen) with 36 weeks of chemotherapy in stage II breast cancer patients, noted a 6.8% incidence of thrombosis during the months of chemotherapy treatment with no thrombosis occurring during the months without therapy (Levine et al. 1988). The background rate of thrombosis may be enhanced with cancer progression, and the procoagulant effects of chemotherapy may also increase as a result of this status. The rate of thrombosis in patients with metastatic (stage IV) breast cancer treated with a 5-drug chemotherapy regimen (cyclophosphamide, methotrexate, 5-fluorouracil, vincristine, and prednisone) is considerably higher (17.6%), with thrombosis occurring early in the course of therapy (Goodnough et al. 1984).

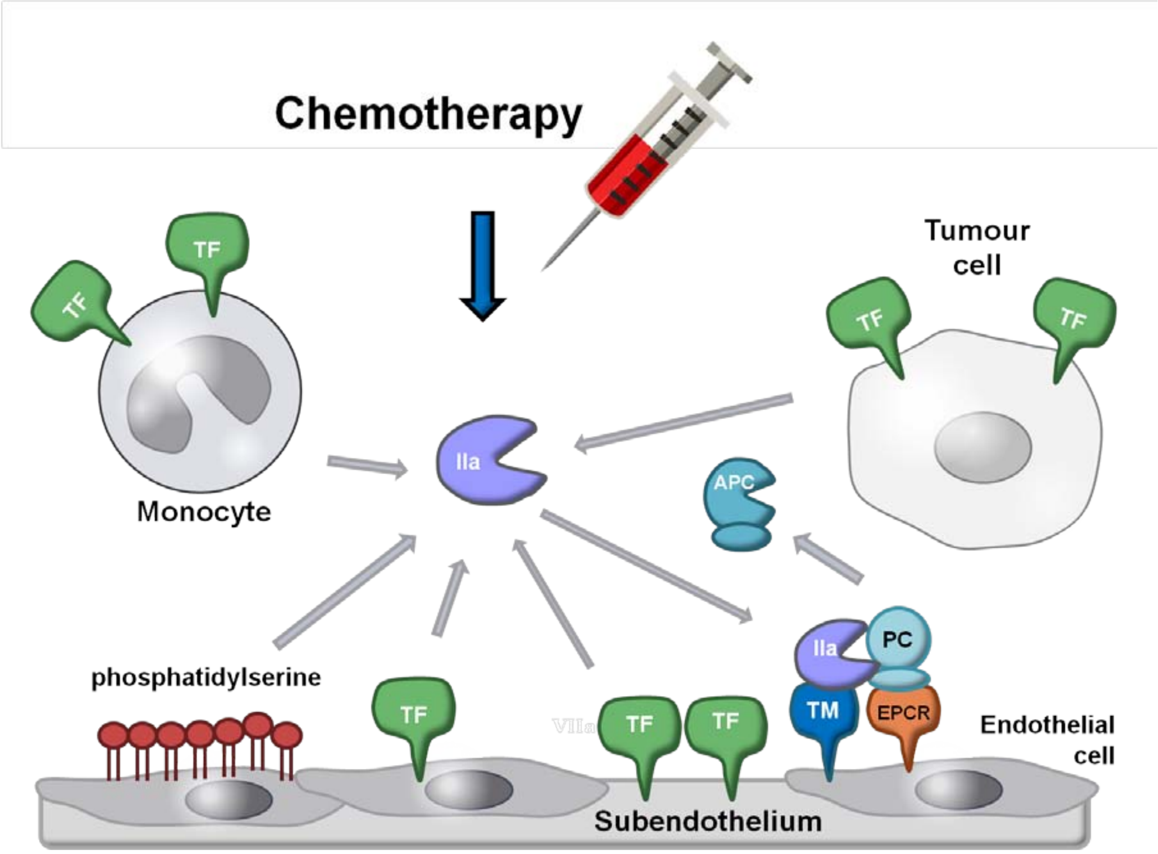
1.10.3 Modulation of hemostatic pathways by breast cancer chemotherapy agents – clinical studies

Several clinical studies have been conducted to identify the hemostatic abnormalities induced by chemotherapeutic agents in breast cancer patients. While these studies vary widely based on sample size, cancer stage, chemotherapeutic regimens, and timeline for blood collection post-chemotherapy treatment, some general trends can be identified. Breast cancer chemotherapy drugs appear to induce a strong procoagulant response shortly (within 24 – 48 hours) post chemotherapy, which can be measured as elevated TAT complex formation (Weitz et al. 2002; Mukherjee et al. 2010; Kirwan et al. 2008). TAT levels appear to normalize 4 – 8 days post chemotherapy (Kirwan et al. 2008; Mukherjee et al. 2010). This may be associated with a small elevation in cancer procoagulant (Kirwan et al. 2008), as well as decreased activated partial thromboplastin time (aPTT) 24-hours post-chemotherapy (Kirwan et al. 2008). Other marks of coagulation activation including plasma TF antigen, microparticle TF activity, F1+2, and fibrinogen levels are not altered by chemotherapy (Rella et al. 1996; Mukherjee et al. 2010; Kirwan et al. 2008).

Modulation of endogenous anticoagulant pathways by chemotherapeutic agents in breast cancer is well established, and such acquired deficiencies can persist for many weeks post-chemotherapy. Several studies have reported a decrease in protein C antigen (Rella et al. 1996; Mukherjee et al. 2010; Rogers et al. 1988; JABCSG and JCOG 1993), and protein C activity (Rella et al. 1996; Feffer et al. 1989) in response to breast cancer chemotherapy. We have previously demonstrated that despite elevated thrombin generation, APC levels decrease post-chemotherapy in breast cancer patients; this decrease was associated with decreased levels of PC. However, sEPCR and sTM levels were unaltered (Mukherjee et al. 2010). The anticoagulant function of APC may be further impaired by reported post-chemotherapy deficiencies in protein S (Rella et al. 1996; Rogers et al. 1988). Breast cancer chemotherapy may also modulate fibrinolytic pathways. Elevated levels of D-dimers have been reported immediately post chemotherapy administration (1 – 48 hours) (Weitz et al. 2002), but may normalize after several days (Rella et al. 1996; Feffer et al. 1989; Kirwan et al. 2008), similar to TAT values. Chemotherapy does not appear to significantly modulate levels of tPA (JABCSG and JCOG 1993; Rella et al. 1996), but may elevate levels of PAI-1 antigen (Rella et al. 1996), thereby inhibiting fibrinolysis of formed clots.

The procoagulant effects of chemotherapeutic agents commonly used to treat breast cancer may be revealed in clinical studies of patients with other types of cancer. A study of patients with cervical, lung, colon, breast cancer, or Hodgkin's disease receiving one of methotrexate, doxorubicin, or 5-fluorouracil demonstrated that FPA levels were elevated within 45 minutes of chemotherapy administration (Edwards et al. 1990). Children with acute lymphoblastic leukemia treated with high dose methotrexate had elevated levels of fibrin degradation products accompanied by decreased levels of PC, antithrombin, and protein S compared to baseline (Totan et al. 2001). Gastrointestinal or head and neck cancer patients infused with 5-fluorouracil with or without cisplatin increased FPA levels and decreased PC activity levels 24 hours post-chemotherapy (Kuzel et al. 1990). However the procoagulant effects of chemotherapy drugs may also be related to the type of malignancy, or specific chemotherapy regimen administered. For example, lymphoma patients receiving combination chemotherapy (using cyclophosphamide, methotrexate, fluorouracil, vincristine, prednisone, doxorubicin, and tamoxifen) displayed elevated levels of PC, antithrombin, and protein S mid-therapy (Bairey et al. 1997).

Figure 1.3 – Mechanisms by which chemotherapy agents may increase the risk for thrombosis in cancer patients. Chemotherapy treatment may increase TF activity on monocytes, tumour cells, and/or tumour cells. Chemotherapy induced endothelial apoptosis may increase phosphatidylserine exposure, or promote endothelial denudation which exposes the procoagulant subendothelium to the blood. This may result in increased thrombin generation. Chemotherapy may impair the PC anticoagulant pathway by decreasing PC antigen levels in plasma, or by modulating expression on EPCR or thrombomodulin on the endothelial cell surface. This may result in impaired APC generation, leading to increased thrombin generation.


Chemotherapy
TF
TF
Monocyte
IIa
Tumour cell
TF
TF
APC
phosphatidylserine
TF
VIIa
TF
TF
IIa
PC
TM
EPCR
Endothelial cell
Subendothelium

1.10.4 Modulation of hemostatic pathways by chemotherapy agents – in vitro studies

While clinical studies have identified hemostatic pathways that are modified by chemotherapy, relatively few studies have characterized the specific mechanisms by which these drugs can exert their procoagulant effects. In general, chemotherapy has been found to have a cytotoxic and procoagulant effect on endothelial and tumour cells. Cisplatin and gemcitabine both increase TF activity on human umbilical venous endothelial and microvascular cells (HUVEC and HMVEC respectively) (Ma et al. 2005). Etoposide and daunorubicin both exert a procoagulant effect on malignant (NB4) cells by increasing TF activity and/or phosphatidylserine exposure (Zhou et al. 2010; Fu et al. 2010; Zhu et al. 1999). Both cisplatin and daunorubicin induce the release of phosphatidylserine positive microparticles from the endothelium and promote the exposure of phosphatidylserine on red blood cells, resulting in decreased clotting time and increased prothrombinase activity in plasma (Fu et al. 2010; Lechner et al. 2007). Doxorubicin and cisplatin increase TF activity but not antigen levels on human blood monocytes (Walsh et al. 1992). Bladder carcinoma T24/83 cells treated with vincristine, doxorubicin, and L-asparaginase increase thrombin generation in recalcified, defibrinated plasma (Paredes et al. 2003). In addition, chemotherapy may also influence platelet-endothelial cell interactions; treatment of healthy endothelial cells with plasma from stage II breast cancer patients increases platelet adhesion to the endothelium via upregulation of IL-1 and vitronectin levels (Bertomeu et al. 1990).

In contrast, relatively few studies have examined the *in vitro* effects of chemotherapeutic agents on anticoagulant pathways. The cyclophosphamide metabolite acrolein has been associated with a direct decrease in the activity of antithrombin (Gugliucci

2008). In addition to increasing TF activity on HUVEC and HMVEC, cisplatin and gemcitabine both decrease cell surface TFPI activity (Ma et al. 2005). We have previously demonstrated that the anthracycline chemotherapy drug doxorubicin modulates the protein C anticoagulant pathway by modestly upregulating thrombomodulin expression while significantly downregulating EPCR expression (Woodley-Cook et al. 2006). The chemotherapeutic agent vincristine also markedly decreases EPCR expression, and modestly downregulates thrombomodulin expression on endothelial cell surfaces (Mewhort-Buist et al. 2008). This results in impaired protein C generation, and may thereby contribute to enhanced coagulation.

1.10.5 Modulation of hemostatic pathways by chemotherapy agents – animal models

Few animal-based studies have been performed to examine the effects of chemotherapeutic drugs on markers of the hemostatic system. In one study, a single tail vein injection of doxorubicin did not modulate hemostatic properties between 24 to 48 hours post-injection (Poggi et al. 1979). However, decreased platelet levels, increased concentrations of fibrinogen and factor VIII, shorter aPTT, and decreased antithrombin levels and fibrinolytic activity were measured between two and five weeks post-injection. This was associated with a decreased time to occlusion in an arterial thrombosis model. The authors suggest the delayed procoagulant effects of doxorubicin may be related to tissue damage, including nephrotoxicity, induced by the chemotherapeutic agent (Poggi et al. 1979; Bertani et al. 1982).

Animal studies have also suggested that cyclophosphamide has procoagulant effects *in vivo*. TF proficient tumour-bearing mice have a mortality rate of approximately 50% when treated with high dose cyclophosphamide, which was associated with elevated kidney fibrin deposition (Yu et al. 2008). In contrast, low TF expressing tumour-bearing mice were protected from cyclophosphamide-related death. Healthy mice treated with cyclophosphamide had elevated levels of circulating endothelial cells, suggestive of systemic endothelial damage (Zeng et al. 2008). In addition, there was evidence of increased thrombogenesis and necrosis in liver tissues. In contrast, no thrombosis was detected in methotrexate-treated mice. These studies suggest that cyclophosphamide upregulates TF activity and endothelial cell death *in vivo,* however, a specific mechanism has not been identified.

1.10.6 Association between other cancer therapies and thrombosis

In addition to chemotherapy, other antineoplastic agents such as angiogenesis inhibitors and hormonal therapy are associated with elevated risk for thrombosis. Supportive therapies such as erythropoietin stimulating agents (ESAs) may also further augment this risk. When tamoxifen, an estrogen receptor antagonist with partial estrogen agonist effects, is administered as a single agent to otherwise healthy women, a 2-3-fold increase in VTE has been observed (Deitcher et al. 2004). When tamoxifen was administered to breast cancer patients in combination with chemotherapy, the risk for thrombosis is elevated up to 20-fold compared to healthy control groups (Deitcher et al. 2004; Saphner et al. 1991). This risk is further elevated in post-menopausal women, and generally increases with age (Deitcher et al. 2004; Saphner et al. 1991). Tamoxifen is associated with an increased concentration of fibrinogen and decreased levels of anticoagulant factors such as antithrombin and TFPI (Erman et al. 2004; Jones et al. 1992). Novel selective estrogen receptor modulators such as raloxifene have similar effectiveness in reducing breast cancer, but are associated with decreased risk for thrombosis (Vogel et al. 2006).

Angiogenesis inhibitors which prevent the formation of new blood vessels from pre-existing vasculature are effective at preventing tumour growth and metastasis; however, they are associated with significant thrombotic risks. In breast cancer, treatment with the VEGF inhibitor bevacizumab (Avastin) is currently being investigated as a therapeutic agent co-administered with chemotherapy (Rugo 2004). Though the mechanism of action of these agents is not currently understood, it is hypothesized that the inhibition of VEGF may impair endothelial injury repair, thereby exposing the procoagulant subendothelium to the blood leading to platelet adhesion and thrombin generation (Elice et al. 2009). In addition, immune complexes formed by bevacizumab may activate platelets by complexing with VEGF and activating the platelet FcγRIIa receptor (Meyer et al. 2009).

Recombinant human erythropoietin is commonly prescribed in cancer patients to treat chemotherapy-associated anemia. These agents mimic the endogenous hormone erythropoietin, which stimulates red blood cell production. Administration of ESAs to cancer patients with anemia is associated with a 1.7-fold increased risk for VTE compared to controls, as well as an elevated risk for death (Bohlius et al. 2006). It is thought that elevated RBC levels can result in hyperviscosity syndrome, promote vasoconstriction elevate blood pressure, and increase platelet counts and reactivity (Lippi et al. 2010). ESAs have also been shown to upregulate thrombin generation (as measured by TAT and F1+2) in healthy subjects, and decrease levels of antithrombin, PC and PS in hemodialyzed patients (Clyne et al. 1995; Malyszko et al. 1995).

1.11 Thromboprophylaxis in cancer chemotherapy patients

Prophylactic anticoagulant therapy may inhibit activation of coagulation and prevent thrombosis during the course of chemotherapy treatment. Several studies have examined the effects of heparin and warfarin on attenuating the risk of chemotherapy-induced thrombosis, and the effects of these drugs on markers of coagulation. The administration of very low dose warfarin to metastatic breast cancer patients receiving chemotherapy reduced the relative risk of thrombosis by 85% when compared with placebo (Levine et al. 1994), and was associated with overall lower levels of TAT, F1+2, and D-dimers when compared to placebo (Falanga et al. 1998). However, no significant difference in survival benefits between the two treatment groups was identified. The infusion of chemotherapy patients with unfractionated heparin has been reported to reduce the chemotherapy-induced increase in FPA generation (Edwards et al. 1990). In addition, administration of the low molecular weight heparin (LMWH) dalteparin sodium (Fragmin) attenuated chemotherapy-induced increases in D-dimer and TAT levels in both lung and breast cancer patients (Weitz et al. 2002). However, in a larger cohort of advanced breast cancer patients who received the LMWH certoparin, no decrease in thrombotic episodes was found relative to the placebo group, and significant bleeding risks were reported (Haas SK et al. 2005). More recently, the LMWH nadroparin was demonstrated to reduce the rate of thromboembolic events in ambulatory cancer patients with metastatic or locally advanced cancers receiving chemotherapy without increasing the risk for bleeding (Agnelli et al. 2009).

The use of prophylactic anticoagulation in chemotherapy patients must be carefully weighed. The baseline risk for thrombosis, the extent of thrombotic risk reduction, and the increased risk for bleeding must all be considered (Levine 2010). It is difficult to predict the risk for thrombosis of an individual patient, and current anticoagulant regimens are not optimal for long-term treatment on an out-patient basis. The American Society of Clinical Oncology (ASCO) guideline for venous thromboembolism prevention in cancer patients recommends that hospitalized patients with cancer and cancer patients undergoing surgery receive prophylactic anticoagulation (Lyman 2007). However, it does not recommend anticoagulation for ambulatory cancer patients undergoing chemotherapy except for treatment with thalidomide or lenalidomide with either chemotherapy or dexamethasone.

1.12 Relationship between oxidative stress and hypercoagulability

Reactive oxygen species are a family of highly reactive molecules containing oxygen, and include peroxides, superoxides, and hydroxyl radicals. During normal cellular metabolism, ROS are produced in the electron transport chain when excess, unpaired electrons leak from the mitochondria and interact with oxygen to form superoxide (Boveris et al. 1972). Physiologically, ROS can participate in redox cell signalling, and are detoxified by endogenous antioxidants such as superoxide dismutase, catalase, peroxidise, as well as small molecules such as vitamin C and glutathione (Hancock 2009). Oxidative stress represents an imbalance between increased ROS production, and/or decreased antioxidant availability. Elevated ROS can have numerous pathological consequences such as the induction of damage to DNA, oxidation of lipids and proteins, and dysregulation of redox signalling, thereby promoting apoptotic or necrotic cell death (Berlett et al. 1997; Martindale et al. 2002; Dizdaroglu 1991).

Limited evidence suggests that oxidative stress may result in the modification of hemotatic pathways. The formation of ROS by platelets induces platelet activation and aggregation (Iuliano et al. 1997). Oxidative stress may promote endothelial dysfunction (Loscalzo 2002), which may affect pro- and anticoagulant pathways expressed on endothelial cells. For example, H_2O_2 has been shown to increase TF activity on smooth muscle cells without modifying TF antigen levels (Penn et al. 1999). Both H_2O_2 (Xu et al. 2000) and doxorubicin (Woodley-Cook et al. 2006) (which produces ROS) decrease EPCR expression on endothelial cells; the antioxidant glutathione can attenuate the doxorubicin-induced EPCR decrease. The generation of ROS by exposure to chemotherapy drugs may thereby promote the formation of procoagulant phenotype.

CHAPTER 2: Hypotheses and Aims

2.1 Aim and Hypothesis

Treatment with chemotherapy can increase the risk for thrombosis in breast cancer patients. Studies have demonstrated that breast cancer chemotherapy agents can activate thrombin generation (as measured by thrombin-antithrombin complex formation), and impair the protein C anticoagulant pathway (as measured by decreased PC/APC levels). However, the mechanisms by which chemotherapy agents modulate these hemostatic pathways are largely unidentified. Therefore, the overall aim of the research performed for this thesis is to identify the mechanisms by which chemotherapy agents increase the risk for thrombosis in breast cancer patients.

We hypothesize that chemotherapy agents can increase the risk for thrombosis in breast cancer patients by (A) increasing tissue factor activity and phosphatidylserine exposure on cell surfaces, (B) increasing the release of procoagulant cell free DNA, and (C) impairing the protein C anticoagulant pathway. Furthermore, we hypothesize that these effects may be exerted in part through the generation of reactive oxygen species by chemotherapy agents.

To test our hypotheses, we measured the effects of these agents on cell surface tissue factor activity, phosphatidylserine exposure, expression of EPCR and thrombomodulin, and the release of cell free DNA, and how modulations of these pathways can contribute to increasing thrombin generation in vitro and in vivo. We also examined the effects of the reactive oxygen species H_2O_2 on modulating these pathways, as well as the ability of the antioxidant glutathione to attenuate the procoagulant activities of chemotherapy agents.

2.2 Objectives

Our specific objectives were as follows:

1. To measure the effects of breast cancer chemotherapy agents on procoagulant (thrombin generating) pathways such as TF activity and phosphatidylserine exposure on endothelial, blood monocyte, and smooth muscle cell surfaces
2. To examine the effects of breast cancer chemotherapy agents on the release of cell free DNA from chemotherapy-injured cells, and to assess its effects on thrombin generation
3. To investigate the effects of breast cancer chemotherapy agents on the expression of EPCR and thrombomodulin expression as well, as protein C activation, on the endothelial cell surface
4. To investigate the role that reactive oxygen species generation may play in mediating the procoagulant effects exerted by chemotherapy agents

CHAPTER 3: Chemotherapeutic agents doxorubicin and epirubicin induce a procoagulant phenotype on endothelial cells and blood monocytes

Foreword

These studies investigate the mechanisms by which the breast cancer chemotherapy agents doxorubicin and epirubicin modulate procoagulant pathways on endothelial cells and blood monocytes. They demonstrate that doxorubicin and epirubicin increase tissue factor activity on isolated blood monocytes and tissue factor activity and phosphatidylserine exposure on treated endothelial cells. This results in an increase in thrombin generation on chemotherapy-treated endothelial cells exposed to recalcified, defibrinated plasma.

This work was published in the Journal of Thrombosis and Haemostasis (Volume 7, Issue 4, pages 619–626, April 2009). The authors are the following: Laura L. Swystun, Lucy Y.Y. Shin, Suzanne Beaudin, and Patricia C. Liaw. The corresponding author is Dr. Liaw. The experiments in this manuscript were performed by Laura Swystun. The manuscript was written by Laura Swystun and Dr. Liaw. Lucy Shin performed pilot studies. Chapter 3 is a direct representation of the above manuscript. (Copyright 2009. International Society on Thrombosis and Haemostasis)

Link to the online article: http://onlinelibrary.wiley.com/doi/10.1111/j.1538-7836.2009.03300.x/abstract;jsessionid=870340DA74D66D0C9B3DF0D1B2F17BB6.d02t03

3.1 Abstract

Background: Although chemotherapy is associated with an increased risk of thrombosis, the pathogenic mechanisms by which chemotherapeutic agents exert prothrombotic effects are unclear. **Objectives:** In this study we explored the possibility that chemotherapeutic agents doxorubicin, epirubicin, 5-fluorouracil, and methotrexate induce a procoagulant phenotype on vascular endothelial cells and/or on blood monocytes. **Methods:** Thrombin generation was measured in defibrinated plasma exposed to chemotherapy-treated human umbilical vein endothelial cells (HUVECs). Tissue factor activity assays were performed on chemotherapy-treated HUVECs and blood monocytes. The effects of chemotherapy drugs on phosphatidylserine exposure and the protein C pathway were also measured. **Results:** Exposure of defibrinated plasma to either doxorubicin or epirubicin-treated HUVECs resulted in an increase in plasma thrombin generation. The procoagulant activity of doxorubicin- and epirubicin-treated HUVECs reflects an increase in tissue factor activity and phosphatidylserine exposure. Doxorubicin-mediated increase in tissue factor activity is related to increased levels of phosphatidylserine rather than to protein disulfide isomerase activity, and likely involves reactive oxygen species generation. Unlike doxorubicin, epirubicin does not impact the protein C anticoagulant pathway. Interestingly, neither methotrexate nor 5-fluorouracil altered endothelial or monocyte hemostatic properties. **Conclusions:** These studies suggest that the doxorubicin and epirubicin have the greatest “prothrombotic potential” by virtue of their ability to alter endothelial and monocyte hemostatic properties.

3.2 Introduction

Thrombosis is a well-recognized complication of cancer (Prandoni et al. 2005). One out of every seven cancer-related deaths is attributed to pulmonary embolism (Shen et al. 1980), making thromboembolic disease the second leading cause of death in cancer patients (Ambrus JL et al. 1975; Rickles et al. 1983a). The activation of blood coagulation in cancer patients is multifactorial and reflects, in part, clot-promoting properties of tumour cells (Prandoni et al. 2005). In addition to the tumour itself, certain chemotherapy agents or combinations thereof contribute to the prothrombotic state in cancer patients.

The most reliable data on the incidence of thrombosis in patients receiving chemotherapy are derived from studies of breast cancer patients. The baseline risk of thrombosis in women with early stage breast cancer is generally less than one percent (Saphner et al. 1991; Clahsen et al. 1994). A randomized trial which compared 12 weeks of chemohormonal therapy with 36 weeks of chemotherapy in stage II breast cancer patients observed a 6.8% incidence of thrombosis during the months of chemotherapy while no thrombosis occurred during the months without therapy (Levine et al. 1988). In another study, the rate of thrombosis in patients with metastatic (stage IV) breast cancer treated with a 5-drug chemotherapy regimen was 17.6%, and thrombosis occurred early in the course of therapy (Goodnough et al. 1984).

Although a direct relationship between chemotherapy treatment and the occurrence of thrombosis has been established, the molecular mechanisms by which cytotoxic drugs trigger a prothrombotic state are poorly understood. We previously demonstrated that exposure of vascular endothelial cells to doxorubicin, an anthracycline used to treat solid tumours and malignant hematologic disease, impairs the endothelium-based protein C anticoagulant pathway (Woodley-Cook et al. 2006). Specifically, doxorubicin decreases cell surface levels of EPCR, a receptor that is essential for the efficient conversion of zymogen protein C to the anticoagulant enzyme activated protein C (APC). The doxorubicin-induced changes in EPCR expression are mediated by free radical metabolites.

Since chemotherapeutic agents are often administered in combination to maximize the synergistic anti-cancer activities, the prothrombotic risk of individual drugs are difficult to assess. Thus, the objective of this study is to examine the procoagulant activities of individual chemotherapeutic agents commonly used in the treatment of patients with early stage breast cancer (doxorubicin, epirubicin, methotrexate, 5-fluorouracil). We examined the ability of these agents to induce a procoagulant phenotype on vascular endothelial cells and on blood monocytes.

3.3 Materials and Methods

3.3.1 Materials

Human umbilical vein endothelial cells (HUVECs) were purchased from Cascade Biologicals (Portland, OR, USA). M199 and RPMI 1640 growth medium, trypsin-EDTA and penicillin-streptomycin were from Invitrogen (Carlsbad, CA, USA). Endothelial cell growth factor was purchased from VWR (West Chester, PA, USA). Heparin was purchased from Leo Pharma (Thornhill, Canada). Fetal bovine serum, doxorubicin, epirubicin, 5-fluorouracil, methotrexate and BSA were from Sigma-Aldrich (St Louis, MO, USA). Chromogenic substrates were purchased from DiaPharma (West Chester, OH, USA). JRK 1535 and HPC 1555 antibodies were generously provided by C. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA). Anti-human TF monoclonal antibody SATF was from Affinity Biologicals (Ancaster, ON, Canada), while HTF1 was from BD Pharmigen (San Jose, CA, USA). Anti-human thrombomodulin (C-17) and Caco-2 cell lysates were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), anti-PDI (RL77) was from AbCam, and anti-human β-actin was from Biolegend (San Diego, CA, USA). Annexin V and APC- (allophycocyanin) conjugated annexin V were from BD Biosciences (San Jose, CA, USA). AsPC-1 cells were from ATCC (Manassas, VA, USA). Thrombin, factor (F) VIIa, FXa and FX were from Haematologic Technologies (Essex Junction, VT, USA). Batroxobin was purchased from Centerchem (Norwalk, CT, USA). MACS columns and microbeads were from Miltenyi Biotec (Auburn, CA, USA).

3.3.2 Plasma concentrations of chemotherapeutic drugs used

Plasma concentrations of chemotherapeutic drugs in our *in vitro* experiments approximate the maximal initial plasma concentrations (c_{max}) observed in chemotherapy patients. Doxorubicin, has a detected c_{max} between 2 and 6 μg/mL (Robert et al. 1985; Bramwell et al. 2002), while liposome-encapsulated doxorubicin (Doxil) displays higher plasma concentrations (c_{max} of 10 μg/mL) and lower clearance (8 μg/mL after 24 hours) (Fracasso et al. 2005). Pharmacokinetic studies have indicated that the c_{max} of epirubicin is approximately 2 - 3.7 μg/mL (Robert et al. 1985; Danesi et al. 2002), methotrexate is 5.6 μg/mL (Paredes et al. 2003), and 5-fluorouracil is 0.4 μg/mL (Reigner et al. 2003).

3.3.3 Cell culture

HUVEC were cultured in 2% gelatin-coated plates on passages 1-4 exclusively in HUVEC medium (M199 supplemented with 10 μg/mL EGF, 20% heat-inactivated FBS, 100 units/mL penicillin-streptomycin, and 12.6 units/mL heparin) at 37ºC and 5% CO_2. Upon reaching 80-90% confluency, HUVEC were treated with fresh media and chemotherapy drugs at therapeutic concentrations. All drugs were diluted in milliQ water (dH_2O) and sterilized by filtration prior to exposure to HUVEC monolayer.

3.3.4 Flow cytometric analysis of HUVEC TF expression and phosphatidylserine exposure

HUVECs were cultured on gelatinized six-well plates, washed twice with PBS, detached with trypsin/EDTA, washed and resuspended in PBS. Cells were incubated at room temperature in the dark with 2 μg/mL of FITC-conjugated anti-human TF antibody for 30 minutes. Annexin V staining was performed by flow cytometry using APC-conjugated annexin V protein as recommended by the supplier. Cell-bound fluorescence was determined with a FACSCaliber flow cytometer (Becton Dickenson) with 3500 events counted per sample in duplicates. Data acquisition was performed with CellQuest Pro software.

3.3.5 Isolation of peripheral human monocytes from whole blood

Peripheral human monocytes were isolated from the whole blood of healthy volunteers by MACS (magnetic cell sorting) as previously described (Stephenson et al. 2006). Isolated monocytes were resuspended in RPMI 1640 (10% FBS and 100 U/ml penicillin-streptomycin). Cell density of $1x10^6$ cells/ml determined on a hemocytometer was used for monocyte culture experiments.

3.3.6 Thrombin generation assays

Thrombin generation assays were performed as previously described (Paredes et al. 2003). Plasma pooled from normal healthy volunteers collected into citrate was defibrinated prior to use by incubating with 50 U/mL batroxobin at 37ºC for 10 minutes followed by the removal of the fibrin clot, and then incubated 10 minutes on ice. Confluent HUVECs grown in a 24-well plate were treated with anti-cancer drug for 8 - 24h at appropriate concentration. Cells maintained at 37ºC were washed twice with HBS and incubated for 3 minutes with HBS and defibrinated pooled citrated plasma. 0.01 mM $CaCl_2$ was added to initiate thrombin generation. To measure total thrombin generation at 5 minute intervals, reaction mixture was removed from monolayer and added to 0.005 M Na2-EDTA on ice. To measure α_2Macroglobulin (α_2M) bound-thrombin complex generation, reaction mixture was removed at same time points and incubated for 1 minute in the presence of 0.5 U heparin and 0.084 U antithrombin, and then added to 0.005 M Na_2-EDTA on ice. A sample of defibrinated plasma in Na_2-EDTA from each time point was incubated with 0.16 mM S-2238 chromogenic substrate at 37ºC for 10 minutes.

Amidolytic reaction was terminated with the addition of 50% acetic acid, and absorbance was read on a SpectraMax 340 PC microplate reader (Molecular Devices) at 405 nm. Total amount of free thrombin generated was found by subtracting α_2M-thrombin complex generation from total thrombin generated. Free thrombin levels were normalized by cell count and expressed as thrombin concentration (nM per 10,000 cells) in relation to time.

3.3.7 Tissue factor activity assays

Tissue factor (TF) activity was measured as previously described (Nishibe et al. 2001). Briefly, HUVEC grown to confluency in a 24-well dish were treated with chemotherapy drug for 24h. Cells were washed with TBS, and incubated with 5nM Factor VIIa, 150 nM Factor X, and 0.005 mM $CaCl_2$. Cells were incubated at 37ºC for 30 minutes, reaction mixture was removed and added chromogenic substrate S2765 and incubated for 3 minutes at room temperature. The reaction was terminated with the addition of acetic acid. Absorbance was read at 405 nM.

3.3.8 Western blot analysis of EPCR, thrombomodulin and TF in HUVEC whole-cell lysates

HUVECs were grown to confluency on 6-well dishes and treated with 1 – 10 μg/mL doxorubicin or epirubicin for 24 hours. The cells were trypsinized, washed twice with PBS, and lysed in 50 μL of PBS containing 1% Triton X-100 for 45 min. After centrifugation to pellet the insoluble nuclear fraction, total protein was quantified using a bicinchoninic acid protein assay reagent kit (Pierce). 30 μg of each sample was loaded onto 4 - 15% gradient SDS-polyacrylamide gels under reducing conditions, electrophoresed and transferred to nitrocellulose. Western blot analysis was performed using antibodies against human EPCR (JRK 1513), thrombomodulin (C-17), TF (SATF) and β-actin.

3.3.9 Caspase 3-activity assay

HUVECs were treated for 8 – 24 hours with doxorubicin or epirubicin (10 μg/mL). Caspase-3 activity assays were performed using the RediPlate 96 EnzChek Caspase-3 Assay kit according to the protocol supplied by the manufacturer (Molecular Probes).

3.3.10 Statistical analyses

Statistical analysis was performed on experiments with an n=3 or greater. One-way ANOVA and Tukey's pair-wise comparisons or t-tests were performed using GraphPad Software. Values are expressed as relative means ± standard error. Figures denote $p < 0.05$ with * and $p < 0.001$ with **.

3.4 Results

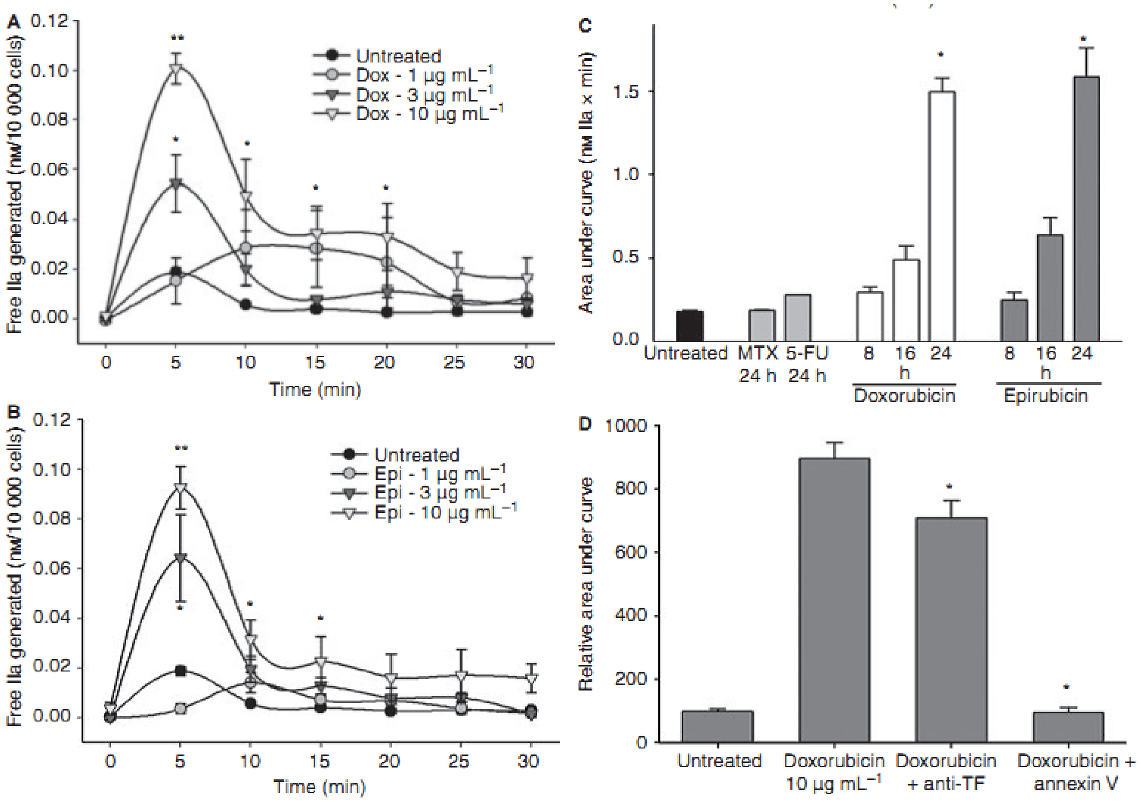
3.4.1 The effects of chemotherapy drugs on the hemostatic balance of endothelial cells

To explore the effects of chemotherapeutic agents on the hemostatic balance of endothelial cells we employed a global coagulation assay that provides a net assessment of pro- and anti-coagulant activities of these cells. Specifically, we measured the ability of drug-treated HUVECs to generate thrombin in recalcified and defibrinated plasma. HUVECs were treated with therapeutic levels (see Methods) of either doxorubicin (1-10 µg/ml), epirubicin (1-10 µg/ml), methotrexate (12.5 µg/ml), or 5-fluorouracil (12.5 µg/ml) for 8 - 24 hours. The drugs were then removed and the cells were incubated with recalcified, defibrinated plasma.

As shown in figures 3.1A and 1B, exposure of defibrinated plasma to untreated HUVECs (labelled as the "untreated" condition) resulted in a small amount of thrombin generation which peaked at approximately 5 minutes and is followed by a decline in thrombin levels back to baseline values. When defibrinated plasma was added to either doxorubicin- or epirubicin-treated HUVECs, we observed a rapid increase in thrombin levels (figure 3.1A and B) compared to untreated cells. The area under the curve (AUC), which indicates the total amount of thrombin generated, confirmed the differences between drug-treated and untreated cells over an 8 to 24 hour time course (figure 3.1C). Interestingly, cells treated with either methotrexate or 5-fluorouracil did not increase the amount of thrombin produced from defibrinated plasma (figure 3.1C) compared to untreated cells. Taken together, these studies indicate that both doxorubicin and epirubicin induce a procoagulant phenotype in endothelial cells.

The ability of doxorubicin and epirubicin to shift the hemostatic balance of HUVECs may reflect a variety of factors including an increase in tissue factor activity, exposure of phosphatidylserine, and/or the downregulation of the protein C (PC) anticoagulant pathway on these cells. To examine the contribution of TF, we performed

Figure 3.1 – Effects of chemotherapeutic agents on the ability of HUVECs to trigger thrombin generation in defibrinated plasma. HUVEC were treated for 24 hours with 1 – 10 µg/mL doxorubicin (A) or epirubicin (B) and thrombin generation was measured as described in methods. (C) Area under the curve (AUC) analysis of thrombin generation for methotrexate and 5-fluororuacil (12.5 µg/mL for 24 hours), and doxorubicin and epirubicin time courses (10 µg/mL for 8 – 24 hours) treated HUVECs. (D) Relative AUC analysis of doxorubicin-treated HUVECs blocked with annexin V or anti-TF (HTF1). $*p \leq 0.05$, $** p \leq 0.001$ ($n = 3$ to 5).


A
Free IIa generated (nM/10 000 cells)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Untreated
Dox - 1 µg mL⁻¹
Dox - 3 µg mL⁻¹
Dox - 10 µg mL⁻¹
0
5
10
15
20
25
30
Time (min)
**
*
C
Area under curve (nM IIa × min)
1.5
1.0
0.5
0.0
Untreated
MTX 24 h
5-FU 24 h
8
16
24
h
Doxorubicin
Epirubicin
B
Free IIa generated (nM/10 000 cells)
Untreated
Epi - 1 µg mL⁻¹
Epi - 3 µg mL⁻¹
Epi - 10 µg mL⁻¹
Time (min)
D
Relative area under curve
1000
800
600
400
200
0
Untreated
Doxorubicin 10 µg mL⁻¹
Doxorubicin + anti-TF
Doxorubicin + annexin V

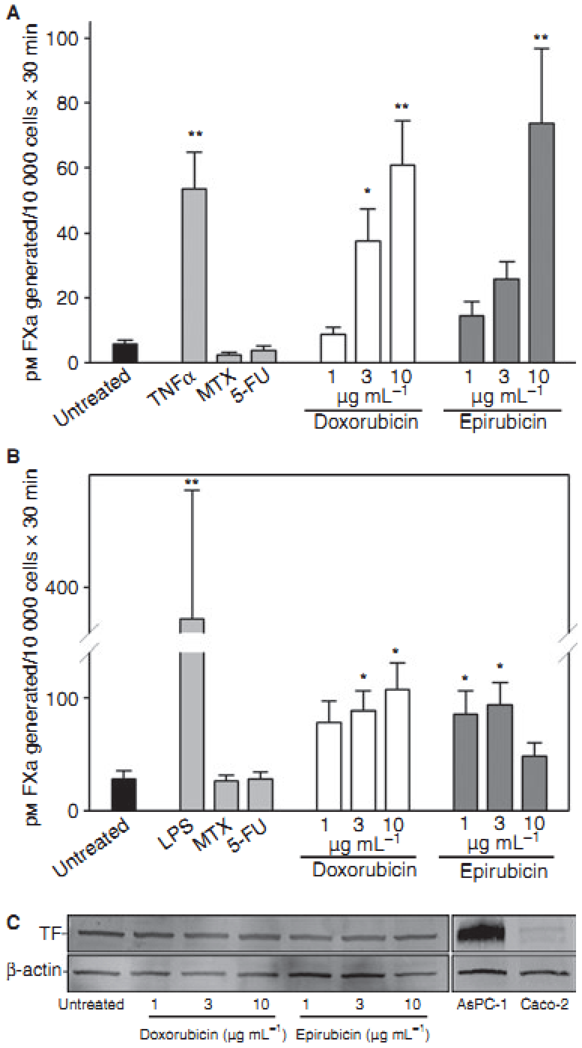
thrombin generation assays on doxorubicin-treated HUVECs which had been blocked with a TF-inhibiting antibody (HTF1). To assess the role of phosphatidylserine, we blocked doxorubicin-treated HUVECs with annexin V and measured thrombin generation. We found that inhibition of TF modestly decreased the amount of thrombin generated in defibrinated plasma exposed to doxorubicin-treated HUVECs, while blocking phosphatidylserine markedly decreased thrombin generation (figure 3.1D). This suggests that both TF and phosphatidylserine are involved in mediating thrombin generation.

3.4.2 Effects of chemotherapeutic drugs on endothelial cell and monocyte TF

Since the increase in thrombin generation in defibrinated plasma exposed to doxorubicin is due in part to TF, we examined the effects of doxorubicin and epirubicin on TF activity on endothelial cells. Endothelial cell surface TF activity was measured by the conversion of factor X to Xa in the presence of factor VIIa and calcium. As shown in figure 3.2A, incubation of HUVECs with either doxorubicin or epirubicin upregulated TF activity. Similarly, treatment of blood monocytes with either doxorubicin or epirubicin upregulates TF activity. In contrast, neither methotrexate nor 5-fluorouracil modulate monocyte or endothelial cell TF activity (figures 3.2A and 3.2B).

Current thinking is that most of the TF expressed on cell surfaces exists in a cryptic form that can be fully activated (ie. de-encrypted) (Bach 2006). Although doxorubicin and epirubicin enhance TF activity on HUVECs, TF antigen levels remained unchanged (figure 3.2C), and TF mRNA levels are actually downregulated by

Figure 3.2 – Effects of doxorubicin and epirubicin on HUVEC and monocyte TF. (A) Confluent HUVECs were treated with either doxorubicin or epirubicin (1 – 10 μg/mL), or with methotrexate or 5FU (12.5 μg/mL) for 24 hours. (B) Monocytes were treated with either doxorubicin or epirubicin (1 – 10 μg/mL), or methotrexate or 5-fluorouracil (10 μg/mL) for 24 hours. As a positive control, monocytes and HUVECs were stimulated with LPS (10 μg/ml) and TNFα (50 ng/mL) respectively for 4 hours. Cell surface TF activity levels on HUVECs and monocytes were determined. (C) TF antigen levels on doxorubicin and epirubicin-treated HUVECs (1 – 10 μg/mL for 24h) was measured by Western blot analysis. * $p \leq 0.05$, ** $p \leq 0.001$ (n = 3 to 4).


A
pM FXa generated/10 000 cells × 30 min
0
20
40
60
80
100
**
*
**
**
Untreated
TNFα
MTX
5-FU
1 3 10
μg mL⁻¹
Doxorubicin
1 3 10
μg mL⁻¹
Epirubicin
B
pM FXa generated/10 000 cells × 30 min
0
100
400
**
*
*
*
*
Untreated
LPS
MTX
5-FU
1 3 10
μg mL⁻¹
Doxorubicin
1 3 10
μg mL⁻¹
Epirubicin
C
TF
β-actin
Untreated
1 3 10
Doxorubicin (μg mL⁻¹)
1 3 10
Epirubicin (μg mL⁻¹)
AsPC-1
Caco-2

doxorubicin and epirubicin (data not shown). This suggests that doxorubicin and epirubicin facilitate the posttranslational de-encryption of TF on the surface of endothelial cells.

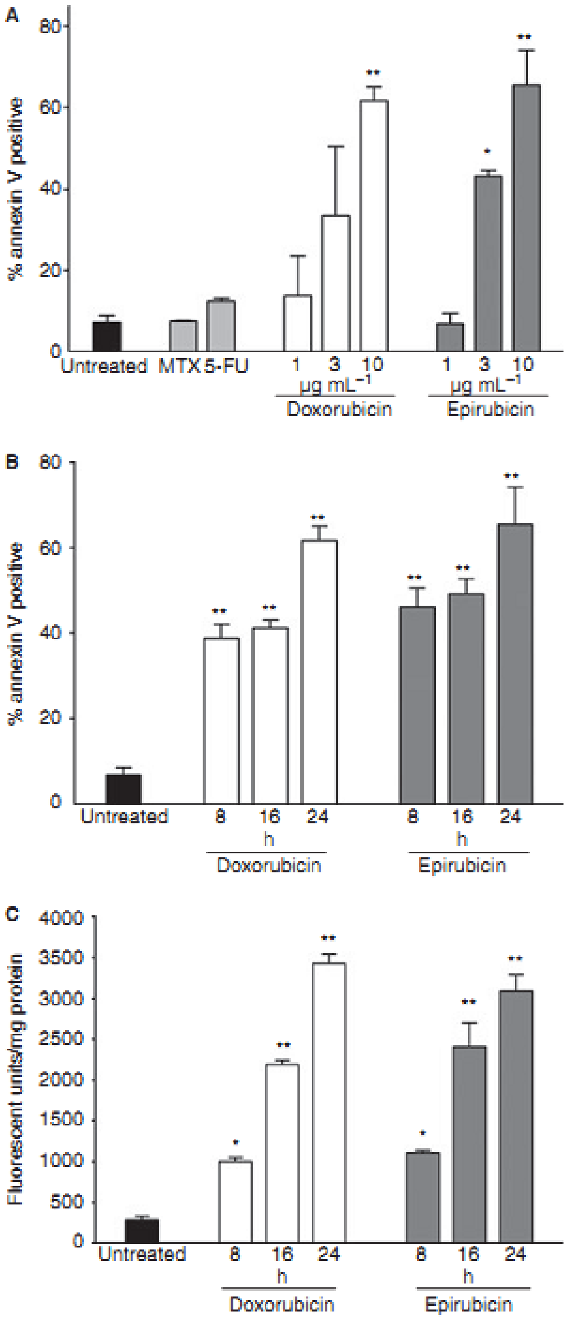
3.4.3 Effect of chemotherapeutic drugs on apoptosis

The procoagulant effects of doxorubicin and epirubicin on endothelial cells may also be due to the ability of these agents to enhance phosphatidylserine exposure. We observed that treatment of HUVECs with either doxorubicin or epirubicin increased phosphatidylserine exposure (as measured by annexin V binding) in a dose- and time-dependent manner (figure 3.3A and B), while neither methotrexate nor 5-fluorouracil enhanced annexin V binding to HUVECs (figure 3.3A). As a marker of cell viability, we measured caspase-3 activation on doxorubicin- and epirubicin-treated HUVECs. As shown in figure 3.3C, both drugs enhanced caspase-3 activity in a time-dependent manner.

3.4.4 Mechanisms of chemotherapy-induced TF activity and phosphatidylserine exposure

De-encryption of TF may occur through a number of postulated mechanisms, including involvement of the ER chaperone protein disulfide isomerase (PDI) (Versteeg et al. 2007), and an increase in phosphatidylserine exposure (Wolberg et al. 1999). As well, anthracycline chemotherapy drugs such as doxorubicin are known to generate reactive oxygen species (ROS) in tissues including endothelial cells (Damrot et al. 2006). The ROS H_2O_2 has been shown to enhance TF activity of smooth muscle cells without an

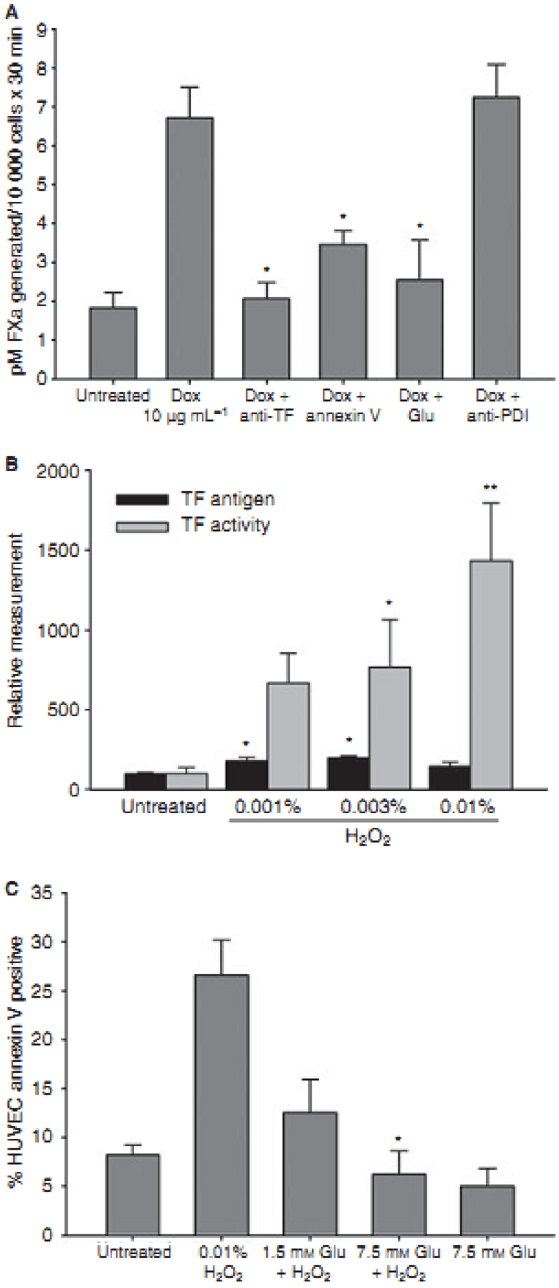
Figure 3.3 – Effects of doxorubicin and epirubicin on endothelial cell apoptosis. (A) HUVECs were treated with doxorubicin or epirubicin (1 – 10 μg/mL) for 24 hours, or (B) 10 μg/mL doxorubicin or epirubicin for 8 – 24 hours. Phosphatidylserine exposure was measured using flow cytometry with APC-conjugated annexin V. (C) Cell viability of doxorubicin and epirubicin-treated HUVECs (10 μg/mL for 8 – 24 hours) was measured by caspase-3 activation. * $p \leq 0.05$, **$p \leq 0.001$ (n = 3 to 5).


A
% annexin V positive
0
20
40
60
80
Untreated
MTX
5-FU
1
3
10
μg mL⁻¹
Doxorubicin
1
3
10
μg mL⁻¹
Epirubicin
*
**
**
B
% annexin V positive
0
20
40
60
80
Untreated
8
16
24
h
Doxorubicin
8
16
24
h
Epirubicin
**
C
Fluorescent units/mg protein
0
500
1000
1500
2000
2500
3000
3500
4000
Untreated
8
16
24
h
Doxorubicin
8
16
24
h
Epirubicin
*
**

increase in TF mRNA or cell surface antigen levels (Penn et al. 1999). We thus investigated the role of PDI, phosphatidylserine exposure, and ROS generation on doxorubicin-induced TF activation. HUVECs were pre-incubated with 10 µg/mL of a PDI-neutralizing antibody (RL77) dialyzed to remove sodium azide, or with 7.5 mM glutathione. All HUVECs were then treated with doxorubicin (10 µg/mL) for 4 hours. Phosphatidylserine and TF on doxorubicin-treated HUVECs was blocked with annexin V (50 µg/mL) and anti-TF antibody HTF1 (12.5 µg/mL), respectively, and TF activity assays were performed. Doxorubicin-induced TF activation was attenuated by HTF1, annexin V, and glutathione, however, neutralization of PDI did not affect TF activation (figure 3.4A).

Since doxorubicin-induced TF activation can be attenuated by the antioxidant glutathione, we further investigated the role of ROS in mediating TF activation. As shown in figure 3.4B, incubation of HUVECs with increasing concentrations of H_2O_2 markedly increased TF activity, whereas TF antigen levels (as measured with flow cytometry) were only modestly increased. Furthermore, we demonstrate that H_2O_2 enhances phosphatidylserine exposure on HUVECs, which was attenuated by the pre-incubation of HUVECs with the antioxidant glutathione (figure 3.4C). Taken together, these studies suggest that the ability of doxorubicin and epirubicin to upregulate TF activity is mediated by the generation of ROS, which facilitates an upregulation of phosphatidylserine exposure on the endothelial cell surface.

Figure 3.4 – Mechanisms of doxorubicin-induced TF activation and phosphatidylserine exposure. (A) HUVEC were pretreated with PDI-neutralizing antibody, or glutathione, and then incubated with 10 μg/mL doxorubicin for 4 hours. TF and phosphatidylserine were blocked post doxorubicin-treatment with annexin V or an antibody which blocks TF activity (HTF1). TF activity was measured as described in methods. (B) HUVEC were treated with H_20_2 for 4h. TF expression was measured with flow cytometry and TF activity was measured with a factor X activation assay. (C) HUVECs were pretreated for 3 h with 0, 1.5, or 7.5 mM glutathione (Glu), followed by a 4-hour treatment with 0.01% H_20_2. Phosphatidylserine exposure was measured by the binding of annexin V. $*p \leq 0.05$, $**p \leq 0.001$ (n = 3 to 4).


A
pM FXa generated/10 000 cells x 30 min
0
1
2
3
4
5
6
7
8
9
Untreated
Dox 10 µg mL⁻¹
Dox + anti-TF
Dox + annexin V
Dox + Glu
Dox + anti-PDI
*
*
*
B
Relative measurement
0
500
1000
1500
2000
TF antigen
TF activity
Untreated
0.001%
0.003%
0.01%
H₂O₂
*
*
*
**
C
% HUVEC annexin V positive
0
5
10
15
20
25
30
35
Untreated
0.01% H₂O₂
1.5 mM Glu + H₂O₂
7.5 mM Glu + H₂O₂
7.5 mM Glu
*

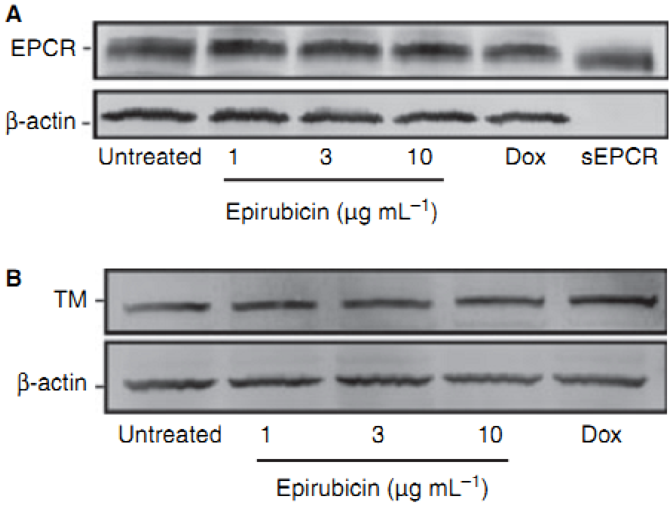
3.4.5 Effect of chemotherapeutic drugs on the protein C anticoagulant pathway

We also investigated the effects of doxorubicin and epirubicin on protein C activation. We have previously shown that exposure of HUVECs to doxorubin results in a significant dose- and time-dependent downregulation of EPCR, and a modest upregulation of thrombomodulin. The net effect is an impaired capacity to convert zymogen protein C to the anticoagulant enzyme APC (Woodley-Cook et al. 2006). Although epirubicin differs from doxorubicin only in the orientation of the 4'-hydroxyl group, exposure of HUVECs to epirubicin does not downregulate EPCR expression to the extent of doxorubicin (figure 3.5A), while both doxorubicin and epirubicin modestly upregulate thrombomodulin expression (figure 3.5B).

3.5 Discussion

One of the most serious problems related to the use of certain anti-cancer agents is the increased incidence of adverse clotting events, some of which prove to be fatal. Although anticoagulation with low-molecular weight heparin (LMWH) or warfarin is commonly used in hospitalized medical and surgical patients to prevent thrombosis, these agents are not optimal for long term treatment in out-patient cancer patients receiving anti-cancer drugs. There is a need to identify the mechanism by which certain anti-cancer agents induce a prothrombotic effect. This information may be helpful in "customizing" anti-thrombotic prophylaxis tailored to a specific anti-cancer regimen that a patient receives using newer anticoagulants which target specific steps in the coagulation or anticoagulation pathways (Bates et al. 2006).

Figure 3.5 – EPCR and thrombomodulin expression on epirubicin-treated HUVECs. HUVECs were treated with 1 – 10 μg/mL epirubicin and 10 μg/mL doxorubicin for 24 hours. (A) EPCR and (B) thrombomodulin levels were measured using Western blot analysis. (n = 3).


A
EPCR
β-actin
Untreated
1
3
10
Dox
sEPCR
Epirubicin (μg mL⁻¹)
B
TM
β-actin
Untreated
1
3
10
Dox
Epirubicin (μg mL⁻¹)

Since chemotherapeutic agents are commonly administered as multi-drug regimens, the objective of this paper was to identify the procoagulant potentials of chemotherapeutic agents commonly used in the treatment of patients with early stage breast cancer. We found that doxorubicin and epirubicin-treated HUVECs enhance the generation of thrombin in defibrinated plasma. Most of the doxorubicin-induced thrombin generation can be attributed to increases in phosphatidylserine exposure, however TF activation also appears to play a role. In contrast, methotrexate and 5-fluorouracil did not upregulate endothelial cell or monocyte procoagulant activity.

As doxorubicin and epirubicin enhance TF activity without modulating TF antigen levels, they likely enhance the de-encryption of a latent pool of cell surface TF. Doxorubicin-induced TF activation appears to be dependent on exposure of phosphatidylserine, rather than on PDI. This is consistent with studies which have demonstrated no evidence for localization of PDI at the HUVEC cell surface, or association between HUVEC TF and PDI (Pendurthi et al. 2007). We further demonstrated that ROS may be responsible for this increase in TF activity levels on endothelial cells. Consistent with our observations, H_2O_2 has been shown to enhance TF activity of smooth muscle cells without an increase in TF mRNA or cell surface protein (Penn et al. 1999).

Our studies are consistent with other investigations of the procoagulant effects of anti-cancer agents. Plasma thrombin generation was increased when plasma was exposed to doxorubicin-treated human bladder carcinoma cells (Paredes et al. 2003). Another study found that treatment of microvascular endothelial cells with a combination of anti-angiogenic and chemotherapy drugs enhanced TF activity and modestly upregulated TF antigen levels (Ma et al. 2005). As well, the chemotherapeutic drug cisplatin increased the formation of endothelial microparticles with procoagulant activity related to phosphatidylserine exposure rather than TF activity (Lechner et al. 2007). In our study, we demonstrated that doxorubicin exerts its procoagulant effects on HUVECS mainly by increasing phosphatidylserine exposure. Increased levels of phosphatidylserine promotes blood coagulation by enhancing the activity of the TF:VIIa complex as well as the prothrombinase complex.

In parallel with the current *in vitro* studies, we have performed a pilot prospective study to evaluate markers of hemostatic activation in 20 early stage breast cancer patients receiving adjuvant chemotherapy. These patients received chemotherapy regimens of either CEF, CAF, or CMF (C=cyclophosphamide, E=epirubicin, F=5-fluorouracil, M=methotrexate, A=doxorubicin). Patients were followed for two courses of chemotherapy treatment, and blood samples were collected on day 1 (baseline), 2, and 8 of each cycle. Plasma thrombin levels were elevated and protein C activation was impaired at cycle 1 day 2, cycle 2 day 2, and cycle 2 day 8 compared to pre-chemotherapy levels (Mukherjee et al. 2006. American Society of Clinical Oncology Meeting abstract). It should be noted that increases in thrombin generation in these patients does not necessarily imply that there is an increase in endothelial cell or monocyte procoagulant activity (e.g. increases in phosphatidylserine exposure and TF activity). Anthracycline-based chemotherapeutic agents such as doxorubicin and epirubicin promote apoptosis by a variety of mechanisms (Muller et al. 1998). In addition to exposing phosphatidylserine on endothelial cells, doxorubicin and epirubicin-induced endothelial denudation may also trigger coagulation by exposing blood to the subendothelium, which expresses procoagulant factors such as collagen. Interestingly, two patients in the study developed thrombosis during the course of their treatment, and both women had received epirubicin-containing chemotherapy (CEF). Current studies with a larger sample size are underway to determine if an increased incidence of thrombosis is related to the type of chemotherapy regimen that the patient receives.

Our studies do not exclude the possibility that methotrexate and 5-fluorouracil can modulate hemostasis through other mechanisms. For example, inhibition of folate metabolism by methotrexate is known to induce moderate hyperhomocysteinemia, an independent risk factor for venous thromboembolism (Cattaneo 2006). Also, circulating levels of protein C and antithrombin are decreased in leukemia patients treated with high-dose methotrexate (Fisgin et al. 2004), suggesting that liver production of these anticoagulants is decreased due to the cytotoxic effects of methotrexate on dividing liver cells.

In summary, our studies provide novel insight into the prothrombotic effects of commonly used anti cancer agents. Our *in vitro* studies suggest that doxorubicin and epirubicin have the greatest “prothrombotic potential” by virtue of their ability to induce a procoagulant phenotype on endothelial cells and on blood monocytes. Thus, these agents can contribute to the dissemination of local as well as systemic procoagulant potentials. In contrast, neither methotrexate nor 5-fluorouracil altered the hemostatic balance of these cells. The results of this study may warrant future clinical studies of “customized” anticoagulant therapy tailored to the specific chemotherapy regimen that the patient receives.

CHAPTER 4: The chemotherapy metabolite acrolein upregulates thrombin generation and impairs the protein C anticoagulant pathway in animal-based and cell-based models

Foreword

These studies investigate the mechanisms by which the cyclophosphamide and its metabolite acrolein modulate pro- and/or anticoagulant pathways on endothelial and smooth muscle cells. They demonstrate that acrolein increases tissue factor activity and/or phosphatidylserine exposure on treated cells, which results in an increase in thrombin generation when cells were exposed to recalcified, defibrinated plasma. Acrolein also impairs APC generation in recalcified, defibrinated plasma which is associated with decreased levels of EPCR. These studies demonstrate that treatment of healthy mice with cyclophosphamide and acrolein increases TAT formation.

This work was published in the Journal of Thrombosis and Haemostasis (Volume 9, Issue 4, pages 767–775, April 2011). The authors are the following: Laura L. Swystun, Som Mukherjee, Mark L. Levine, and Patricia C. Liaw. The corresponding author is Dr. Liaw. The experiments in this manuscript were performed by Laura Swystun. The manuscript was written by Laura Swystun. Chapter 4 is a direct representation of the above manuscript. (© 2011. International Society on Thrombosis and Haemostasis)

Link to the online article: http://onlinelibrary.wiley.com/doi/10.1111/j.1538-7836.2011.04232.x/abstract

4.1 Abstract:

Background: Thrombosis is a common complication in cancer patients receiving chemotherapy regimens which include cyclophosphamide. However, the mechanisms by which these agents increase this risk are largely uncharacterized. **Objectives:** We examined the effects of cyclophosphamide and its metabolite acrolein on procoagulant and anticoagulant pathways using both cell- and animal-based models. **Methods:** Thrombin and activated protein C (APC) generation were measured in defibrinated plasma exposed to acrolein-treated endothelial and smooth muscle cells. Tissue factor (TF) activity was measured on acrolein-treated cells. Cell surface levels of phosphatidylserine, TF, endothelial cell protein C receptor (EPCR), and thrombomodulin were measured. Healthy Balb/C mice received injections of saline (control), acrolein or cyclophosphamide; blood was collected and plasma thrombin-antithrombin (TAT), protein C (PC), and APC levels were analyzed. **Results:** Exposure of acrolein-treated endothelial and smooth muscle cells to defibrinated plasma increased thrombin generation in the plasma. This was associated with enhanced phosphatidylserine exposure and/or increased TF activity on acrolein-treated cells. Despite elevated levels of thrombin generation, plasma APC levels were not elevated. *In vivo*, treatment of mice with cyclophosphamide and acrolein resulted in elevations of plasma TAT levels while APC levels remained low. **Conclusions:** This is the first study which examines thrombin generation and the APC pathway in chemotherapy-treated mice. Cyclophosphamide and acrolein appear to upregulate procoagulant pathways, while impairing endogenous

anticoagulant pathways. This may explain, in part, the increased risk of thrombosis observed in cancer patients receiving cyclophosphamide-containing chemotherapy.

4.2 Introduction:

Venous and arterial thrombosis account for over 9% of mortalities in cancer patients receiving outpatient chemotherapy, making it the second leading cause of death (Khorana et al. 2007). A single episode of VTE is associated with a 2.2-fold increased risk of death in cancer patients (Sorensen et al. 2000). The chemotherapy drug cyclophosphamide is commonly used to treat hematological cancers such as lymphomas, as well as solid tumours such as breast and ovarian cancers. It is difficult to assess the direct toxicity of cyclophosphamide treatment as it is commonly administered as part of a multi-drug regimen. However, chemotherapy regimens containing cyclophosphamide have been associated with an elevated risk of developing thrombosis. For example, a study of stage I – IV ovarian cancer patients receiving cisplatinum, epirubicin, and cyclophosphamide reported a thrombosis rate of 10.6% during treatment (von Tempelhoff et al. 1997). Treatment of multiple myeloma with thalidomide as a single agent has a thrombosis rate of about 1%; however the addition of chemotherapy (combinations of cyclophosphamide, dexamethasone, vincristine, doxorubicin, etoposide, cisplatin) increased this risk to 28% (Zangari et al. 2001). Ten percent of breast cancer patients who received epirubicin and cyclophosphamide developed thrombotic complications after the second and third cycles of treatment (von Tempelhoff et al. 1996). Although thrombosis typically presents as deep vein thrombosis or pulmonary embolism in most patients, arterial thrombosis has also been reported in breast cancer patients receiving cyclophosphamide (Wall et al. 1989).

Animal studies have suggested that cyclophosphamide has procoagulant effects. TF proficient tumour-bearing mice have a mortality rate of approximately 50% when treated with high dose cyclophosphamide, which was associated with elevated kidney fibrin deposition (Yu et al. 2008). In contrast, low TF expressing tumour-bearing mice were protected from cyclophosphamide-related death. Healthy mice treated with cyclophosphamide were found to have elevated levels of circulating endothelial cells, suggestive of systemic endothelial damage. In addition, there was also evidence of increased thrombogenesis and necrosis in liver tissues (Zeng et al. 2008). These studies suggest that cyclophosphamide upregulates TF activity and endothelial cell death *in vivo.* However, a specific mechanism by which cyclophosphamide induces a procoagulant phenotype has not been previously identified.

Cyclophosphamide is a prodrug, and requires metabolism by cytochrome P450 enzymes in the liver to become biologically active (de Jonge et al. 2005). The two primary by-products of cyclophosphamide metabolism are phosphoramide mustard, and acrolein (2-propenal); it is hypothesized that acrolein conveys the majority of the toxic side effects of cyclophosphamide (de Jonge et al. 2005). Acrolein is a strong electrophile, and is known to mediate cellular processes such as the induction of apoptosis, regulation of gene expression, and DNA synthesis (Kehrer et al. 2000). Acrolein may also contribute to oxidative stress by decreasing levels of the antioxidant glutathione (Rudra et al. 1999) and increasing the generation of reactive oxygen species (ROS) (Misonou et al.

2005), which we hypothesize may promote a prothrombotic phenotype (Swystun et al. 2009). While common side effects of acrolein exposure include hemorrhagic cystitis, nephrotoxicity, and neurotoxicity, (Giraud et al. 2010) pathological conditions in which acrolein levels are elevated, such as chemotherapy, cigarette smoking, and renal failure, are also associated with an increased risk of thrombotic disease (Gugliucci 2008).

We and others have previously demonstrated that chemotherapeutic agents can perturb the hemostatic balance by upregulating procoagulant mechanisms on endothelial, tumour, and blood cells (Swystun et al. 2009; Paredes et al. 2003; Ma et al. 2005) and/or impairing the protein C anticoagulant pathway (Swystun et al. 2009; Woodley-Cook et al. 2006). Chemotherapy may also induce microparticle formation from host or tumour cells (Fu et al. 2010; Lechner et al. 2007), which have been demonstrated to contribute to the pathogenesis of thrombosis in cancer models (Thomas et al. 2009; Davila et al. 2008). As there is an association between cyclophosphamide administration and the development of arterial and venous thrombosis, and cyclophosphamide has been shown to induce endothelial cell shedding *in vivo* (Zeng et al. 2008) which may expose smooth muscle cells to the blood, we examined the effects of acrolein on pro- and anticoagulant properties of endothelial and smooth muscle cells. To demonstrate that our *in vitro* results were relevant *in vivo*, we also examined the effects of these drugs on thrombin activation and the protein C pathway in a mouse model.

4.3 Materials and Methods:

4.3.1 Materials

Human umbilical venous endothelial cells (HUVECs), umbilical arterial smooth muscle cells (UASMCs), endothelial growth media (EGM-2), and smooth muscle growth media (SMGM-2) bullet kits were from Lonza (Basel, Switzerland). Cyclophosphamide and acrolein were from Sigma-Aldrich (St. Louis, MO, USA). Chromogenic substrates S2238, S2365, and S2166 were from DiaPharma (West Chester, OH, USA). Antibodies to EPCR thrombomodulin, PC and APC, as well as recombinant murine PC (mPC) and murine APC (mAPC) were generously provided by Dr. Charles Esmon (Oklahoma Medical Research Foundation, Oklahoma City). Anti-human TF monoclonal antibody maTF-FITC was from Affinity Biologicals (Ancaster, ON, Canada), while HTF1, annexin V and FITC- conjugated annexin V were from BD Biosciences (San Jose, CA, USA). Thrombin, Factor VIIa, Factor Xa, and Factor X were from Haematologic Technologies (Essex Junction, VT, USA). Batroxobin was purchased from Centerchem (Norwalk, CT, USA). TNF-α protease inhibitor (TAPI-1) was from Calbiochem (Darmstadt, Germany).

4.3.2 Cell culture

HUVECs were cultured in 2% gelatin-coated plates on passages 1-4 exclusively in EGM-2 at 37ºC and 5% CO_2. UASMC were cultured on passages 1-4 in SMGM-2 media.

4.3.3 Thrombin generation, PC activation and TF activity assay

HUVECs were treated with acrolein for 5 hours, and UASMCs were treated for 7 hours, blocked with inhibitory antibodies to EPCR or TF or with annexin V for 20 minutes, and washed with HBS. Thrombin generation was initiated in defibrinated plasma and quantified as described previously (Swystun et al. 2009). To measure APC generation, plasma was defibrinated, recalcified, and exposed to acrolein-treated HUVECs as in the thrombin generation assay. Aliquots were removed at 5 minute intervals and incubated in benzamidine with 2 U/mL heparin, 20 mM HEPES pH 7.5, and 10 mM $CaCl_2$. Levels of APC were quantified using a human enzyme capture assay previously described (Liaw et al. 2003). TF activity was measured as previously described (Swystun et al. 2009).

4.3.4 Flow cytometric analysis of EPCR, thrombomodulin, TF expression and phosphatidylserine exposure

Cells were cultured and prepared as previously described (Swystun et al. 2009), and incubated with 2 μg/mL of FITC-conjugated anti-human TF, EPCR, or thrombomodulin antibody for 30 minutes. Annexin V staining was performed by flow cytometry using FITC-conjugated annexin V protein as recommended by the supplier. Cell-bound fluorescence was determined with a FACSCaliber flow cytometer (Becton Dickenson, San Jose, CA, USA) with 3500 events counted per sample in duplicates. Data acquisition was performed with CellQuest Pro software (San Jose, CA, USA).

4.3.5 sEPCR ELISA

HUVECs were incubated with acrolein (0.4 – 4.9 µg/mL) or hydrogen peroxide (0.01%) for 5 hours. Levels of sEPCR in HUVEC media was measured using ELISA as previously described (Woodley-Cook et al. 2006).

4.3.6 Mouse model

All animal studies were approved by the McMaster University Research Ethics Board (AUP 08-06-30). Eight – 10 week old (18 – 21 gram) female Balb/C mice (Charles River, Wilmington, MA, USA) received IP injections of saline (100 µL), cyclophosphamide (50, 100, 140 mg/kg), on days 1, 3, and 5, or acrolein (1.1, 2.2, 4.4 mg/kg) on days 1 and 3. Blood was collected on day 6 for saline or cyclophosphamide treatment, or on day 4 for acrolein treatment via carotid artery cannulation into citrate benzamidine as previously performed (Gross et al. 2005).

4.3.7 Mouse TAT, PC ELISA, APC capture assay, quantification of circulating microparticles

Murine TAT levels were assessed using the Enzygnost TAT ELISA kit (Siemens Healthcare Diagnostics, Deerfield, IL, USA) according to the manufacturer's protocols. Mouse PC levels were quantified using ELISA as previously described using recombinant mPC as a standard (Li et al. 2005). Mouse APC levels were analyzed with an enzyme capture assay; recombinant mAPC was used for a standard curve (Li et al. 2005).

Circulating microparticles were quantified using the Zymuphen MP activity assay (Aniara, Mason, Ohio, USA).

4.3.8 Plasma and media concentrations of acrolein and cyclophosphamide

Plasma concentrations of cyclophosphamide are difficult to measure due to variable rates of absorption and metabolism by P450 cytochromes. The elimination half life of cyclophosphamide ranges between 5 – 9 hours, and maximal plasma concentrations are estimated to be between 10 - 100 µM (2.5 – 25 µg/mL) (de Jonge et al. 2005). Animal studies using cyclophosphamide typically employ high dose concentration, between 60 – 140 mg/kg (Yu et al. 2008; Zeng et al. 2008). Serum concentrations of acrolein-modified proteins in healthy individuals may be as high as 50 µM (2.8 µg/mL) (Satoh et al. 1999); pathological concentrations may reach 50 – 100 µM (2.8 – 5.62 µg/mL) (Eiserich et al. 1995). Cell culture studies of acrolein employ a wide range of concentrations, from 1 µM to 100 µM (0.056 – 5.62 µg/mL) (Lovell et al. 2001; Grafstrom et al. 1988) while animal studies using acrolein typically employ concentrations around 5 mg/kg (Conklin et al. 2010).

4.3.9 Statistical analyses

Statistical analysis was performed on experiments with an $n = 3$ or greater. One-way ANOVA and Tukey’s pair-wise comparisons or t-tests were performed using GraphPad Software (San Diego, CA, USA). Values are expressed as relative means ± standard error. Figures denote $p < 0.05$ with * and $p < 0.001$ with **.

4.4 Results

4.4.1 The effects of cyclophosphamide and acrolein on procoagulant properties of endothelial and smooth muscle cells

To better understand the mechanisms by which cyclophosphamide and acrolein may induce a prothrombotic effect in patients receiving chemotherapy, we examined the effects of these drugs on vascular tissues using a coagulation assay which measures net procoagulant activity (TF activity and phosphatidylserine exposure). HUVECs or UASMCs treated with acrolein, exposed to recalcified, defibrinated plasma, and free thrombin generation was measured at 5 minute intervals.

When HUVECS or UASMCs were treated with acrolein (0.4 – 4.9 µg/mL), we observed a significant increase in thrombin generation relative to the untreated control (figure 4.1A and 1C). The enhanced thrombin generation in the presence of acrolein-treated HUVECs appeared to be related to an increase in phosphatidylserine exposure on the endothelial cell surface, as thrombin generation was inhibited by annexin-V (figure 4.1A). Enhanced thrombin generation in the presence of acrolein-treated UASMCs was related to TF activation and the presence of phosphatidylserine, as the addition of either TF-blocking antibody HTF-1 or annexin V significantly impaired thrombin generation (figure 4.1C). Comparisons of area under the curve (AUC) confirmed the differences in thrombin generation in the presence of acrolein-treated vascular cells (figures 4.1B and 1D). The cyclophosphamide parent compound (CPA) was used as a dead agent control in these experiments.

Figure 4.1 – Effects of acrolein and cyclophosphamide on the ability of vascular cells to trigger thrombin generation in defibrinated plasma. HUVECs (figure 4.1A) or UASMCs (figure 4.1C) were treated with acrolein (0.4 – 4.9 µg/mL) for 5 and 7 hours respectively. Cells were blocked with an inhibitory antibody to TF (HTF-1), or annexin V for 20 minutes. Thrombin generation was measured in recalcified, defibrinated plasma. Area under the curve (AUC) analysis was performed (figures 4.1B and 4.1D). *denotes $p<0.05$; **denotes $p<0.001$

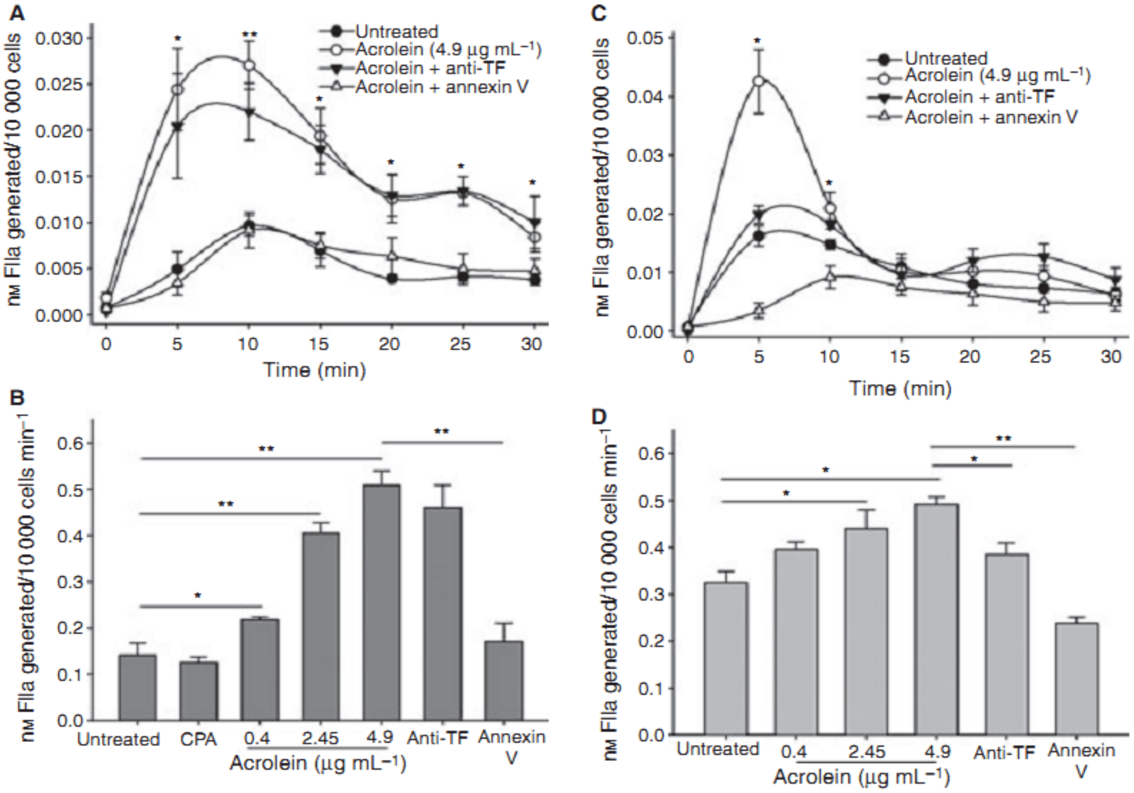

A
nM FIIa generated/10 000 cells
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0
5
10
15
20
25
30
Time (min)
Untreated
Acrolein (4.9 μg mL⁻¹)
Acrolein + anti-TF
Acrolein + annexin V
*
**
B
nM FIIa generated/10 000 cells min⁻¹
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Untreated
CPA
0.4
2.45
4.9
Anti-TF
Annexin V
Acrolein (μg mL⁻¹)
C
nM FIIa generated/10 000 cells
0.05
0.04
0.03
0.02
0.01
0.00
0
5
10
15
20
25
30
Time (min)
Untreated
Acrolein (4.9 μg mL⁻¹)
Acrolein + anti-TF
Acrolein + annexin V
D
nM FIIa generated/10 000 cells min⁻¹
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Untreated
0.4
2.45
4.9
Anti-TF
Annexin V
Acrolein (μg mL⁻¹)

Figure 4.2 – Effects of acrolein on vascular phosphatidylserine exposure. HUVECs were treated with acrolein (0.4 – 4.9 µg/mL) or H_20_2 (0.01%) for 5 hours (figure 4.2A). UASMCs were treated with acrolein 0.4 – 4.9 µg/mL for 7 hours or H_20_2 (0.003%) for 7 hours (figure 4.2B). Phosphatidylserine exposure was measured using flow cytometry with FITC-conjugated annexin V. *denotes $p<0.05$; **denotes $p<0.001$

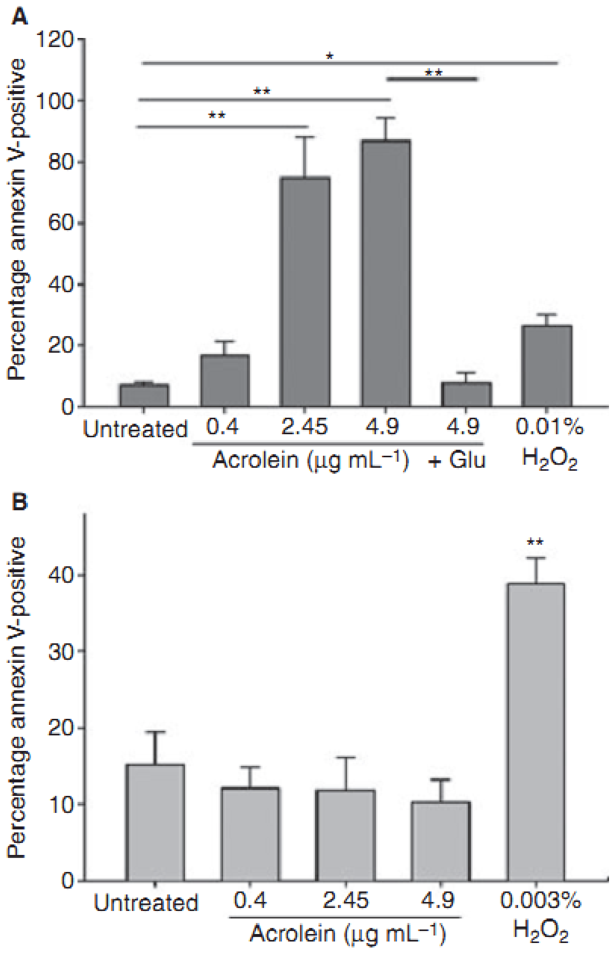
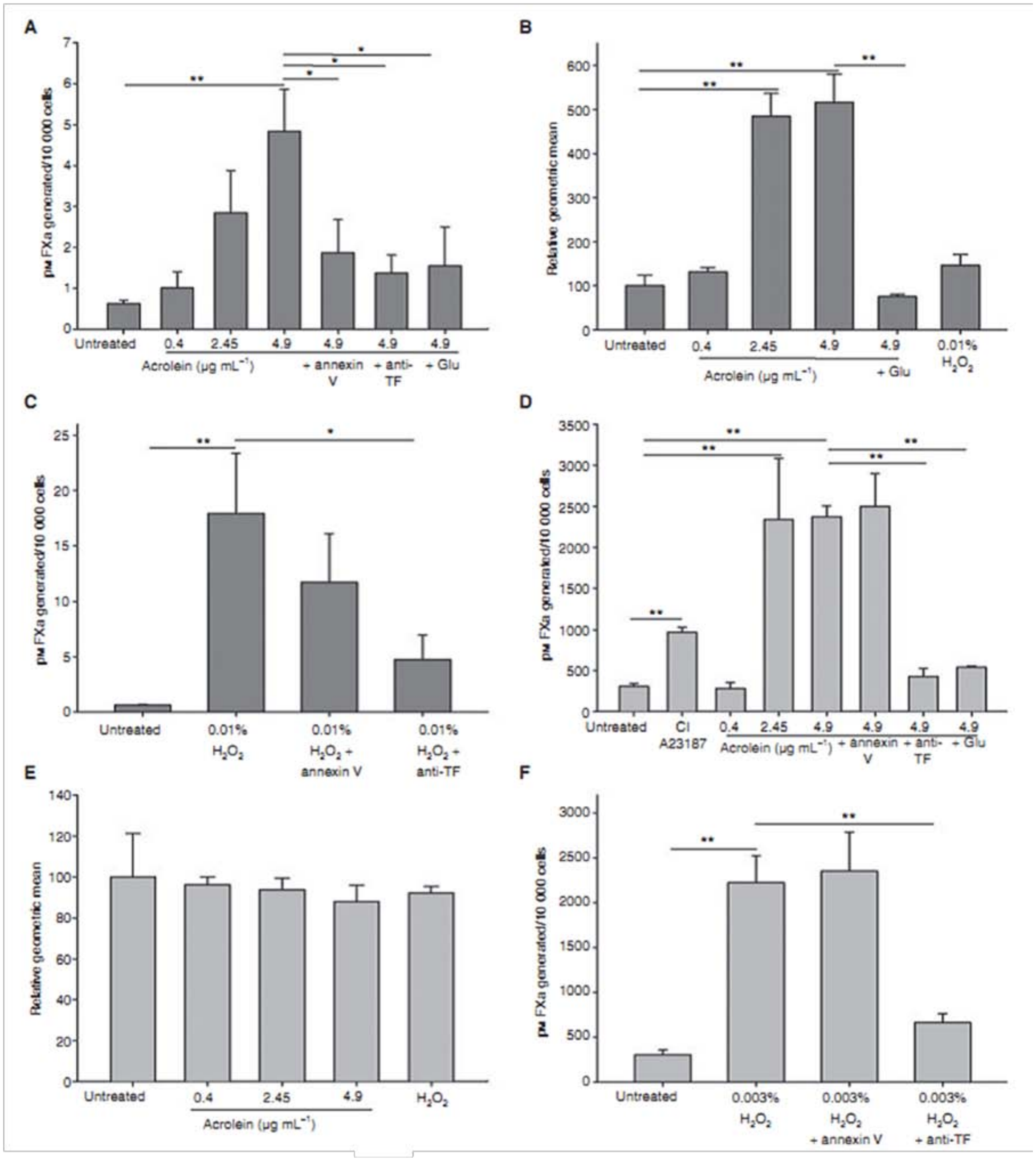

A
Percentage annexin V-positive
0
20
40
60
80
100
120
*
**
**
**
Untreated
0.4
2.45
4.9
4.9
0.01%
Acrolein (μg mL⁻¹)
+ Glu
H₂O₂
B
Percentage annexin V-positive
0
10
20
30
40
**
Untreated
0.4
2.45
4.9
0.003%
Acrolein (μg mL⁻¹)
H₂O₂

Figure 4.3 – Effects of acrolein on HUVEC and UASMC tissue factor. HUVECs (figure 4.3A) and UASMCs (figure 4.3D) were exposed to acrolein (0.4 – 4.9 μg/mL) for 5 and 7 hours respectively, or with H_2O_2 for 5 hours. Cells were preincubated with 1.5 mM glutathione (Glu) for 1 hour and treated with acrolein (4.9 μg/mL) or calcium ionophore (CIA23187) for 10 minutes. TF was blocked with HTF-1 and phosphatidylserine was blocked with annexin V for 20 minutes. TF activity was assessed as described in Methods. TF antigen levels on acrolein-or H_2O_2-treated HUVECs (figure 4.3B) and UASMCs (figure 4.3E) were assessed using flow cytometry. HUVECs (figure 4.3C) and UASMCs (figure 4.3F) were treated with H_2O_2 for 5 hours. *denotes $p<0.05$; **denotes $p<0.001$


A
pM FXa generated/10 000 cells
0
1
2
3
4
5
6
7
**
*
*
*
Untreated
0.4
2.45
4.9
4.9
4.9
4.9
Acrolein (μg mL⁻¹)
+ annexin V
+ anti-TF
+ Glu
B
Relative geometric mean
0
100
200
300
400
500
600
**
**
**
Untreated
0.4
2.45
4.9
4.9
0.01% H₂O₂
Acrolein (μg mL⁻¹)
+ Glu
C
pM FXa generated/10 000 cells
0
5
10
15
20
25
**
*
Untreated
0.01% H₂O₂
0.01% H₂O₂ + annexin V
0.01% H₂O₂ + anti-TF
D
pM FXa generated/10 000 cells
0
500
1000
1500
2000
2500
3000
3500
**
**
**
**
**
Untreated
CI A23187
0.4
2.45
4.9
4.9
4.9
4.9
Acrolein (μg mL⁻¹)
+ annexin V
+ anti-TF
+ Glu
E
Relative geometric mean
0
20
40
60
80
100
120
140
Untreated
0.4
2.45
4.9
H₂O₂
Acrolein (μg mL⁻¹)
F
pM FXa generated/10 000 cells
0
500
1000
1500
2000
2500
3000
**
**
Untreated
0.003% H₂O₂
0.003% H₂O₂ + annexin V
0.003% H₂O₂ + anti-TF

To confirm that acrolein enhances phosphatidylserine exposure on the endothelial cell surface, we measured annexin-V binding to acrolein-treated HUVECs and UASMCs with flow cytometry. Interestingly, acrolein enhanced phosphatidylserine exposure on HUVECs (figure 4.2A), but not on UASMCs (figure 4.2B). The ROS hydrogen peroxide (H_2O_2) increased phosphatidylserine exposure on both HUVECs and UASMCs. Interestingly, acrolein did not activate caspase 3 on either cell type (data not shown).

We then measured TF activity by the generation of factor Xa on acrolein-treated vascular cells. Acrolein increased TF activity on both HUVECs (figure 4.3A) and UASMCs (figure 4.3D) in a dose-dependent manner, however, the total amount of Xa generated by acrolein-treated UASMCs was approximately 400-fold higher than that generated by acrolein-treated HUVECs. H_2O_2 also increased FXa generation on HUVECs and UASMCs (figure 4.3C and 3F). The anti-TF antibody HTF-1, and the antioxidant glutathione attenuated FXa generation on both cell types, while annexin V impaired FXa generation on HUVECs but not UASMCs. We next examined the effects of acrolein on TF expression on both endothelial and smooth muscle cells by flow cytometry. Acrolein induced a dose-dependent increase in TF expression on HUVECs (figure 4.3B), but did not modulate TF expression on UASMCs (figure 4.3E). These results were confirmed with a TF antigen ELISA (IMUBIND, American Diagnostica).

Figure 4.4 – Effects of acrolein on the ability of endothelial cells to trigger APC generation in defibrinated plasma. HUVECs were treated with acrolein (0.4 – 4.9 μg/mL) for 5 hours respectively. Cells were blocked with an inhibitory antibody to EPCR (JRK1535), or thrombin was incubated with 1 μM hirudin. APC generation was measured in recalcified, defibrinated plasma (figure 4.4A). AUC analysis was performed (figure 4B). *denotes $p<0.05$; **denotes $p<0.001$

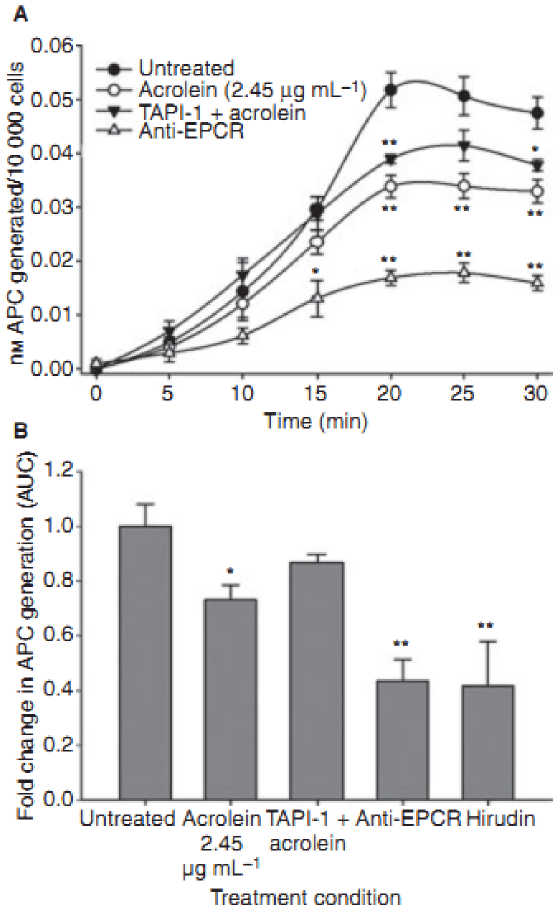
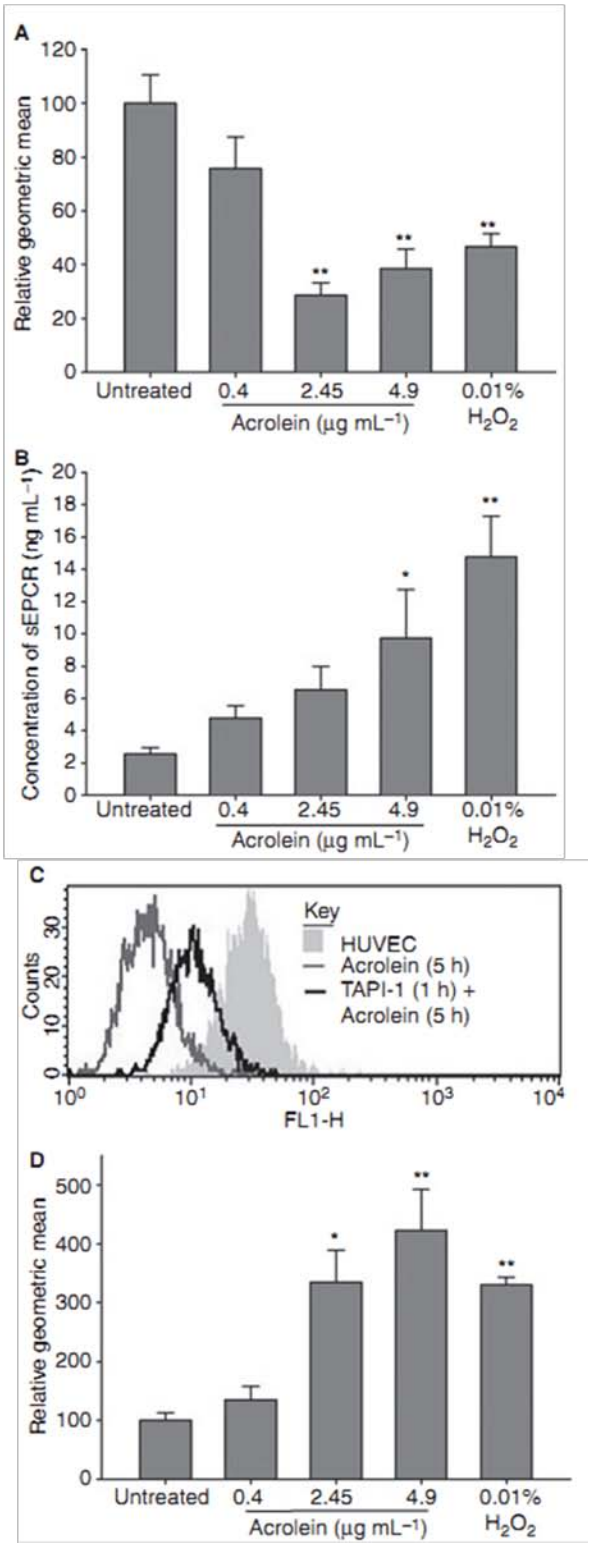

A
0.06
0.05
0.04
0.03
0.02
0.01
0.00
nM APC generated/10 000 cells
Untreated
Acrolein (2.45 µg mL⁻¹)
TAPI-1 + acrolein
Anti-EPCR
*
**
0
5
10
15
20
25
30
Time (min)
B
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fold change in APC generation (AUC)
Untreated
Acrolein 2.45 µg mL⁻¹
TAPI-1 + acrolein
Anti-EPCR
Hirudin
Treatment condition

Figure 4.5 – Effects of acrolein on the endothelial protein C pathway. HUVECs were treated with acrolein (0.4 – 4.9 µg/mL) for 5 hours and EPCR (figure 4.5A) and thrombomodulin (figure 4.5D) expression was assessed with flow cytometry. Soluble EPCR shed from the endothelial cell surface was measured using ELISA (figure 4.5D). HUVECs were pretreated with TAPI-1, and then treated with acrolein (2.81 µg/mL) for 5 hours; EPCR levels were assessed with flow cytometry (figure 4.5C). *denotes $p<0.05$; **denotes $p<0.001$

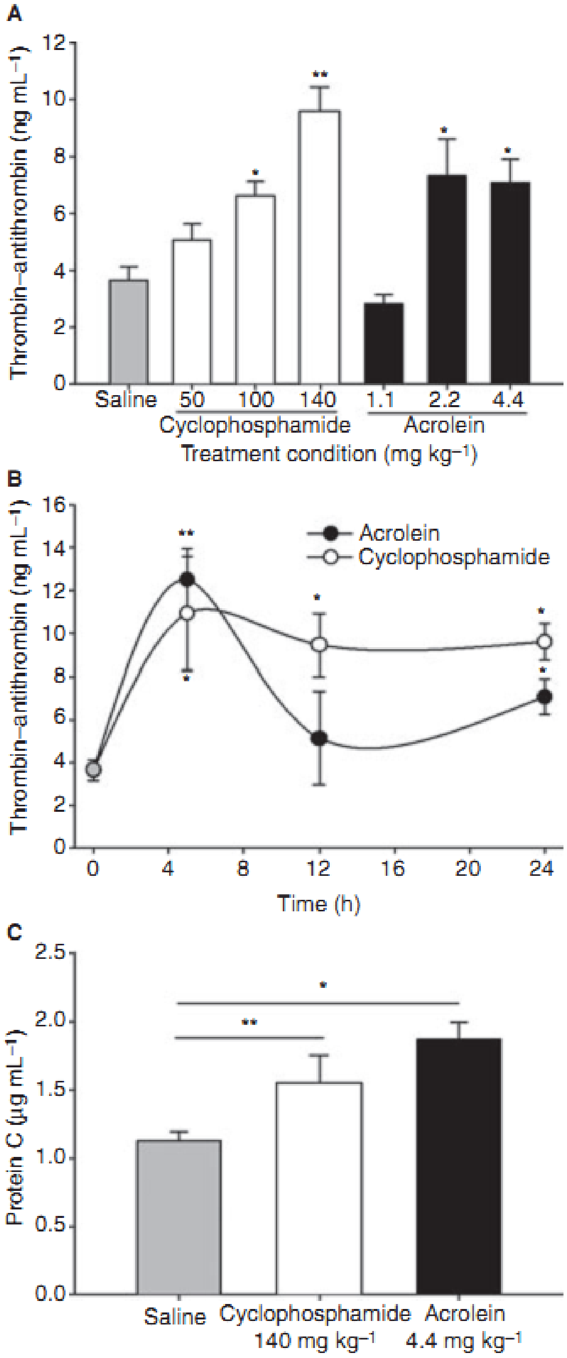

A
Relative geometric mean
120
100
80
60
40
20
0
Untreated
0.4
2.45
4.9
0.01%
Acrolein (µg mL−1)
H2O2
**
**
**
B
Concentration of sEPCR (ng mL−1)
20
18
16
14
12
10
8
6
4
2
0
Untreated
0.4
2.45
4.9
0.01%
Acrolein (µg mL−1)
H2O2
*
**
C
Counts
0
10
20
30
Key
HUVEC
Acrolein (5 h)
TAPI-1 (1 h) +
Acrolein (5 h)
10^0
10^1
10^2
10^3
10^4
FL1-H
D
Relative geometric mean
500
400
300
200
100
0
Untreated
0.4
2.45
4.9
0.01%
Acrolein (µg mL−1)
H2O2
*
**
**

4.4.2 Effects of acrolein on the anticoagulant properties of endothelial cells

To measure the effects of acrolein on the protein C anticoagulant pathway on endothelial cells, we performed an APC enzyme capture assay on acrolein-treated treated HUVECs exposed to defibrinated, recalcified plasma. Plasma was removed at 5 minute intervals, and levels of APC generated were measured as previously described by Liaw et a. (Liaw et al. 2003). We demonstrated that EPCR and free thrombin are both necessary for APC generation in this assay as shown by the reduction in APC levels when the assay was performed in the presence of an anti-EPCR antibody (JRK 1535) or hirudin (1 µM). Treatment of HUVECs with acrolein decreased the total amount of APC generated in this assay (figure 4.4A). *In vivo,* APC is generated "on demand" in response to thrombin formation. As our thrombin generation assay demonstrated there was more free thrombin available to activate protein C (figure 4.1A and 1B), this suggests that APC generation was impaired on acrolein-treated endothelial cells.

We next examined the effects of acrolein treatment on HUVEC EPCR and thrombomodulin antigen levels with flow cytometry. Acrolein decreases EPCR expression on HUVECs (figure 4.5A) while increasing thrombomodulin expression (figure 4.5B) in a dose-dependent manner. Real-time PCR analysis indicated that mRNA levels for both EPCR and thrombomodulin were increased upon acrolein treatment (data not shown). However, a soluble EPCR (sEPCR) ELISA demonstrated that EPCR shedding was upregulated upon acrolein treatment (figure 4.5C). It has been previously demonstrated that TNF-α converting enzyme (TACE) can mediate the shedding of EPCR upon stimulation with PMA (Qu et al. 2007). We found that the inhibition of TACE with

Figure 4.6 – Effects of acrolein and cyclophosphamide on measures of pro- and anticoagulant pathways in mouse plasma. Healthy female Balb/C mice received IP injections of saline, cyclophosphamide, or acrolein as described in Methods. TAT complex (figures 4.6A and 4.6B) and protein C levels (figure 4.6C) were measured using ELISA. *denotes $p<0.05$; **denotes $p<0.001$


A
Thrombin–antithrombin (ng mL⁻¹)
0
2
4
6
8
10
12
*
**
*
*
Saline
50
100
140
1.1
2.2
4.4
Cyclophosphamide
Acrolein
Treatment condition (mg kg⁻¹)
B
Thrombin–antithrombin (ng mL⁻¹)
0
2
4
6
8
10
12
14
16
Acrolein
Cyclophosphamide
**
*
*
*
*
0
4
8
12
16
20
24
Time (h)
C
Protein C (µg mL⁻¹)
0.0
0.5
1.0
1.5
2.0
2.5
*
**
Saline
Cyclophosphamide
140 mg kg⁻¹
Acrolein
4.4 mg kg⁻¹

TNF-α protease inhibitor-1 (TAPI-1) partially inhibited EPCR shedding by acrolein. In addition, preincubation acrolein-treated HUVECs with TAPI-1 partially restored APC generation in our functional assay.

4.4.3 The effects of cyclophosphamide and acrolein on pro- and anticoagulant markers in a mouse model

To assess whether our *in vitro* results were relevant *in vivo*, we examined the effects of acrolein and cyclophosphamide on pro- and anticoagulant pathways in a mouse model. We injected healthy Balb/C mice with saline or cyclophosphamide on 3 occasions (days 1, 3, and 5), or acrolein on 2 occasions (days 1, and 3). Blood was collected via carotid artery cannulation into trisodium citrate ± benzamidine. We measured the effects of these drugs on the thrombin generation pathways by measuring TAT levels. Both cyclophosphamide and acrolein upregulated TAT levels in mice relative to the saline control (figure 4.6A, 4.6B). However, no corresponding increase in circulating microparticles was observed (data not shown). We also investigated the effects of these drugs on the protein C anticoagulant pathway. Levels of protein C increased slightly upon treatment with cyclophosphamide and acrolein (relative to the saline control) (figure 4.6C). Despite elevations in TAT levels, APC levels remained below the assay limit of detection (0.78 ng/mL) (data not shown) in the mice treated with either cyclophosphamide or acrolein.

4.5 Discussion:

The American Society of Clinical Oncology (ASCO) guideline for venous thromboembolism prevention in cancer patients recommends that hospitalized patients with cancer and cancer patients undergoing surgery receive prophylactic anticoagulation (Lyman 2007). However, it does not recommend prophylactic anticoagulation for ambulatory cancer patients undergoing chemotherapy except for treatment with thalidomide or lenalidomide with either chemotherapy or dexamethasone. The guidelines also call for further research to identify biomarkers that could help clinicians select patients for VTE prophylaxis. Understanding the mechanism(s) through which commonly used chemotherapy drugs modify the thrombotic risk may lead to better screening for at-risk patients and prophylactic anticoagulation. As well, the identification of which chemotherapeutic agents, or chemotherapy combinations, serve to increase this risk is of great interest.

In these studies, our aim was to determine if the common chemotherapeutic agent cyclophosphamide could induce a prothrombotic state. We first examined the effects of the cyclophosphamide metabolite acrolein on pro- and anticoagulant pathways on vascular cells. When HUVECs and UASMCs were treated with the acrolein, we observed an increase in thrombin generation in recalcified, defibrinated plasma (figure 4.1). We hypothesize that the increased thrombin generation on acrolein-treated HUVECs is related to increased phosphatidylserine exposure, as treatment with annexin V impairs thrombin generation, whereas inhibiting TF does not. Although acrolein does increase TF activity and antigen levels on HUVECs, the small amount of FXa produced did not appear to be significant enough to elevate thrombin generation. Interestingly, blocking both phosphatidylserine exposure and TF on acrolein-treated UASMCs impairs thrombin generation. However, although treatment of UASMCs with acrolein increases TF activity (but not TF antigen levels), it does not increase phosphatidylserine exposure. This suggests that phosphatidylserine present on the surface of untreated UASMCs cells is necessary to facilitate thrombin generation in both treated and untreated conditions. As evidence for this, when annexin V is used to block phosphatidylserine exposure on acrolein-treated UASMCs, thrombin generation is actually decreased to levels below the levels generated on untreated cells.

As treatment of UASMCs with acrolein increased TF activity but not antigen levels, we examined the possibility that acrolein induced TF de-encryption on vascular cells. When HUVECs or UASMCs treated with either acrolein or H_2O_2 were blocked with annexin V, we found that some TF activation was inhibited on HUVECs (figures 4.3A and 4.3C) but not UASMCs. This suggested a potential phosphatidyserine-mediated mechanism of TF de-encryption on HUVECs but not UASMCs. Interestingly, calcium ionophore A23187 (50 µM) increased TF activity on UASMCs after a 10 minute incubation, confirming the presence of cryptic TF on the surface of these cells.

To assess whether our *in vitro* findings were relevant *in vivo*, we examined thrombin-generation (by measuring TAT complex formation) in Balb/c mice treated with saline, acrolein, or cyclophosphamide. We found that TAT levels were significantly increased post-chemotherapy (figure 4.6A). As TAT complexes have a half life of approximately 3 minutes *in vivo* (Shifman et al. 1982), this suggests that chemotherapy-treated animals have active and ongoing thrombin generation. This is consistent with animal studies that have demonstrated that cyclophosphamide administration can increase thrombogenesis and fibrin deposition (Yu et al. 2008). As well, studies of breast cancer patients who receive combination chemotherapy that includes cyclophosphamide display markers of thrombin generation up to 7 days post-chemotherapy (Weitz et al. 2002; Mukherjee et al. 2010; Kirwan et al. 2008).

We next investigated the effects of cyclophosphamide on the protein C anticoagulant pathway. When APC levels were measured in recalcified, defibrinated plasma exposed to HUVECs, we observed a decrease in APC generation on acrolein-treated HUVECs relative to untreated cells, suggesting impairment in the PC pathway (figure 4.4). This effect was likely due to decreased cell surface levels of EPCR due increased shedding mediated by TACE (figure 4.5). We also investigated the protein C pathway in our chemotherapy-treated mice. Interestingly, PC levels in the animals increased upon treatment with our anti-cancer agents (figure 4.6B). Studies of breast cancer patients receiving cyclophosphamide chemotherapy have reported decreased levels of PC (Rella et al. 1996; Mukherjee et al. 2010). However, treatment of lymphoma with cyclophosphamide, doxorubicin, vincristine, and prednisone is associated with increased levels of protein C and protein S (Bairey et al. 1997). This may be the result of interactions with different tumour types, or different combinations of chemotherapy. Although we observed an increase in TAT levels in chemotherapy-treated mice, APC levels remained low. This may indicate impairment in the PC anticoagulant pathway due to decreased endothelial cell surface levels of EPCR and/or thrombomodulin. These animal studies are consistent with our human studies in which we observed that despite elevated TAT levels, breast cancer patients receiving cyclophosphamide do not have a corresponding increase in APC generation (Mukherjee et al. 2010).

As with many other anti-cancer drugs, acrolein has a complex mechanism of action. However, its procoagulant effects on endothelial cells and smooth muscle cells may be related to its ability to induce oxidative stress by decreasing levels of antioxidant glutathione and increasing the generation of ROS. On UASMCs, acrolein and H_2O_2 activate latent TF on smooth muscle cells without modulating antigen levels (figures 4.3D-F), consistent with other published data (Penn et al. 1999). On endothelial cells, both acrolein and H_2O_2 upregulate phosphatidylserine exposure and TF activity, and induce EPCR shedding (figures 4.2, 4.3, and 4.4). Pretreatment of cells with glutathione attenuates the acrolein-mediated increases in TF antigen levels and phosphatidyserine exposure on HUVECs, and TF activity on HUVECs and UASMCs. The generation of ROS appears to be a mechanism of action common to other chemotherapeutic agents, as the anthracycline drug doxorubicin also induces procoagulant activity via the generation of ROS.

In this and previous studies, we suggest that that endothelial cell damage induced by anti-cancer agents such as acrolein, doxorubicin, and epirubicin induce a prothrombotic phenotype. The chemotherapeutic agents doxorubicin, epirubicin, and acrolein enhance endothelial cell procoagulant activity, as a result of increased phosphatidylserine exposure and/or tissue factor activity (Swystun et al. 2009). Endothelial cell injury may also promote exposure of the procoagulant subendothelium to the blood (Zeng et al. 2008), and acrolein may enhance TF activity of subendothelial components such as smooth muscle cells (Penn et al. 1999), thereby promoting thrombin generation. Chemotherapy drugs may also upregulate procoagulant activity of blood monocytes (Swystun et al. 2009), microparticles (Fu et al. 2010; Lechner et al. 2007), or tumour cells (Paredes et al. 2003), which can promote localized or systemic coagulation. Impairment of endogenous anticoagulant mechanisms such as the protein C pathway by acrolein or doxorubicin could exacerbate the prothrombotic effects of these anti-cancer agents (Woodley-Cook et al. 2006; Mukherjee et al. 2010).

In summary, our studies provide novel insight into the prothrombotic effects of cyclophosphamide, a commonly administered chemotherapeutic agent. To our knowledge, this is the first study which demonstrates that anti-cancer agents modulate pro- and anticoagulant pathways in a mouse model. These results parallel translational studies which demonstrate similar hemostatic perturbations in breast cancer chemotherapy patients (Weitz et al. 2002; Mukherjee et al. 2010; Kirwan et al. 2008), and suggest that either acute or chronic exposure to cyclophosphamide and/or acrolein may contribute to an increased risk for venous or arterial thrombosis. The results of our studies suggest that anticoagulants (e.g. FXa inhibitors) or antioxidants (e.g. MESNA) may be potential therapies to avert the prothrombotic effects of certain combinations of chemotherapy agents.

CHAPTER 5: Breast cancer chemotherapy induces the release of cell free DNA, a novel procoagulant stimulus

Foreword

These studies demonstrate that chemotherapy agents induce the release of CFDNA in breast cancer patients and healthy mice, which correspond to a increase in plasma TAT levels. They further demonstrate that doxorubicin and epirubicin induce the release of CFDNA when cultured with venous whole blood or neutrophils *in vitro*. They also demonstrate that CFDNA released from chemotherapy-treated venous whole blood can increase thrombin generation in recalcified plasma through activation of the contact pathway.

This manuscript has been accepted for publication in the Journal of Thrombosis and Haemostasis (Reference number: JTH-2011-00745). The authors are the following: Laura L. Swystun, Som Mukherjee, and Patricia C. Liaw. The corresponding author is Dr. Liaw. The experiments in this manuscript were performed by Laura Swystun. The manuscript was written by Laura Swystun. Chapter 5 is a direct representation of the above manuscript. (Copyright 2011. International Society on Thrombosis and Haemostasis)

5.1 Abstract:

Background*:* Thrombosis is a common complication for breast cancer patients receiving chemotherapy. However, the mechanisms by which breast cancer chemotherapeutic agents increase this risk are largely uncharacterized. Nucleic acids released by injured cells may enhance coagulation via the activation of the contact pathway. **Objectives*:*** In this study, we examined the effects of breast cancer chemotherapy agents on the release of cell free DNA (CFDNA) and its relationship to thrombin generation using *in vitro* and *in vivo* methods. **Methods*:*** CFDNA release and TAT levels were measured in plasma of breast cancer patients and healthy mice receiving chemotherapy. Venous whole blood and cultured cells were exposed to chemotherapy and CFDNA release and levels of DNA-histone complexes were measured. The procoagulant activity of isolated CFDNA was measured with calibrated, automated thrombin generation. **Results*:*** Breast cancer patients receiving chemotherapy had elevated levels of CFDNA 24-hours post-chemotherapy, a timepoint at which elevated thrombin-antithrombin levels have been previously reported. Treatment of healthy mice with doxorubicin, epirubicin, and 5-fluorouracil increased CFDNA release, with a corresponding elevation in TAT complex formation. Venous whole blood and neutrophils incubated with chemotherapeutic agents had elevated CFDNA in plasma or cell supernatants. In addition, incubation of venous whole blood with chemotherapy decreased histone-DNA complex levels. CFDNA released from epirubicin-treated whole blood significantly elevated thrombin generation in a dose-dependent manner, and involved activation of the contact pathway.

Conclusions*:* Release of CFDNA from chemotherapy-injured cells may represent a novel mechanism by which thrombosis is triggered in cancer patients.

5.2 Introduction:

Chemotherapy treatment for cancer is a well-recognized acquired risk factor for thrombosis. In addition to prothrombotic risk factors associated with the presence of a tumour, chemotherapy treatment can further increase the risk by 6.5-fold above baseline (Heit 2003). In patients with breast cancer, the baseline risk for thrombosis is less than 1% (Saphner et al. 1991) but increases significantly following chemotherapy treatment, to 5 – 17% depending on the stage of cancer and type of chemotherapy or hormonal therapy administered (Levine et al. 1988; Goodnough et al. 1984). Several clinical studies have been conducted to identify the hemostatic abnormalities induced by chemotherapeutic agents in breast cancer patients. We and others have demonstrated that breast cancer chemotherapy induces a strong procoagulant response shortly (24 – 48 hours) after chemotherapy administration, which can be measured as an increase in thrombin-antithrombin (TAT) complex formation (Weitz et al. 2002; Mukherjee et al. 2010; Kirwan et al. 2008). This may be associated with a small elevation in cancer procoagulant levels (Kirwan et al. 2008), and decreased aPTT times (Kirwan et al. 2008), but is not associated with elevated plasma tissue factor (TF) levels (Kirwan et al. 2008) or microparticle TF activity (Mukherjee et al. 2010), suggesting a mechanism distinct from the TF pathway may be involved in activating coagulation in these patients.

There is a well-established relationship between the presence of malignancy and elevated levels of plasma or cell free nucleic acids (Schwarzenbach et al. 2011). Cell free DNA (CFDNA) in cancer patients may be either tumour derived, or released from injured host blood or vascular tissues by both apoptotic and necrotic cell death (Jahr et al. 2001). Recent studies have suggested that cell-free nucleic acids may be procoagulant. In a purified system, DNA and RNA have been shown to accelerate plasma clotting time by promoting (auto)-activation of contact phase coagulation factors XI and XII (Kannemeier et al. 2007). In addition, a mouse model of arterial thrombosis demonstrated that RNA is associated with arterial thrombi, and treatment with RNAse, or a factor XIIa inhibitor, can attenuate clot formation in this model. Although this model failed to demonstrate the contribution of CFDNA to thrombus formation, it is hypothesized that this may be due to the complex of DNA with histone proteins which impaired access to contact coagulation factors (Kannemeier et al. 2007). In addition, the release of neutrophil extracellular traps (NETs) represent another potential source of CFDNA (Clark et al. 2007; Brinkmann et al. 2004). NETs promote platelet aggregation, adhesion of red blood cells, thrombin-dependent fibrin formation and bind von Willebrand Factor, fibronectin, and fibrinogen (Fuchs et al. 2010). As well, histone protein released into the plasma can impair the protein C pathway (Ammollo et al. 2011), activate platelets (Fuchs et al. 2010), and promote thrombin generation (Semeraro et al. 2011), thereby contributing to a procoagulant phenotype.

We hypothesized that chemotherapy-injured cells may release DNA into the plasma, which may promote thrombin generation either by activating the contact pathway, or by forming extracellular traps. In this study, we examined the effects of chemotherapeutic drugs doxorubicin, epirubicin, and 5-fluorouracil on the release of CFDNA and its relationship to thrombin generation using *in vitro* and *in vivo* methods.

5.3 Materials and Methods:

5.3.1 Materials:

Doxorubicin, cyclophosphamide, methotrexate, 5-fluorouracil, camptothecin, HistoPaque 1077, phorbol myristate acetate (PMA), and glutathione were from Sigma-Aldrich (St. Louis, MO, USA). Epirubicin was from Pfizer (New York, NY, USA). TF blocking antibody HTF1 was from BD Biosciences (San Jose, CA, USA). Factor XII, factor XI, and corn trypsin inhibitor (CTI) were from Haematologic Technologies (Essex Junction, VT, USA). SynthASil was from Instrument Laboratory Company, Lexington, MA, USA. Z-VAD-FMK was from BD Pharmingen (Franklin Lakes, NJ USA). IM-54 was from EMD Chemicals (Darmstadt, Germany, 2011). Bovine DNAse was from Promega (Madison, WI, USA).

5.3.2 DNA isolation and electrophoresis:

Cells or whole blood were treated with chemotherapy agents for 4 - 24 hours. Samples were spun twice at 1250 x g for 5 minutes to remove cells, and stored at -80°C. For DNA purification, samples were thawed at room temperature, and spun at 10000 x g for 10 minutes to remove cells. DNA from media or plasma was purified with Qiamp DNA blood mini kit (Qiagen, Mississauga, ON) according to manufacturer's protocol.

CFDNA was isolated from 200 µL plasma or supernatant and eluted into 200 µL Buffer AE. Isolated DNA was quantified at A_{260} with a BioPhotometer Plus spectrophotometer (Eppendorf, Mississauga, ON). To visualize CFDNA, DNA was concentrated using a speed-vac and separated on a 2% agarose gel.

5.3.3 Neutrophil isolation:

Venous whole blood was collected into heparin (10 U/mL) from healthy individuals who were drug-free for 72 hours prior to collection. Neutrophils were isolated according to previously established protocols (Oh et al. 2008). Briefly, blood was layered over Lympholyte poly cell separation media (Cedarlane, Burlington, Ontario, Canada) and centrifuged at 500 x g for 40 minutes. The neutrophil layer was collected and residual red blood cells were lysed using an RBC lysis buffer (Roche Applied Science, Basel, Switzerland). Neutrophils were washed and resuspended in HBSS with calcium chloride and magnesium chloride (Gibco, Invitrogen, Carlsbad, CA, USA) and 2% human serum albumin (Canadian Blood Services, Ottawa, ON, Canada).

5.3.4 Thrombin generation assay:

Thrombin generation assays were conducted in pooled, citrated, platelet poor plasma using a calibrated thrombin generation assay (Technothrombin TGA; Technoclone, Vienna, Austria) as previously described with several modifications (Bunce et al. 2011). CFDNA was isolated from the plasma of venous whole blood incubated with epirubicin for 24 hours as described above. Pooled plasma, buffer AE (elution buffer from QIAmp kit) and 5 µL CFDNA were mixed in a black Costar microtitre plate (Lowell, MA, USA). Samples were incubated at 37°C for 5 minutes, followed by the addition of calcium + fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). Fluorescence was measured at 1 minute intervals over a 60 minute period at excitation and emission values of 360 and 460 nM respectively on a Spectramax M5e plate reader (Molecular Devices, Sunnyvale, California, USA). Thrombin generation profiles were analyzed using Technothrombin TGA software (Technoclone).

5.3.5 Animal studies:

All animal studies were approved by the McMaster University Research Ethics Board (AUP 08-06-30). Eight – 10 week old (18 – 21 gram) female Balb/C mice (Charles River, Wilmington, MA, USA) received intraperitoneal injections of saline (100 µL), or chemotherapy agents. Blood was collected 6 – 24 hours post injection by carotid artery cannulation.

5.3.6 Analysis of murine TAT complexes:

Mouse blood collected via carotid artery cannulation was spun twice at 1250 x g for 5 minutes to separate plasma, aliquoted, and stored at -80°C. Murine TAT complexes were analyzed using the Enzygnost TAT ELISA (Siemens Healthcare Diagnostics, Deerfield, IL, USA) according to the manufacturer’s protocol. Supplied human TAT at known concentrations was used as a standard.

5.3.7 Concentrations of chemotherapy agents:

*C*oncentrations of chemotherapeutic drugs in our *in vitro* experiments approximate the maximal initial plasma concentrations (c_{max}) observed in chemotherapy patients, and have been previously discussed (Woodley-Cook et al. 2006; Mukherjee et al. 2010). Concentrations of agents administered to healthy mice are similar to doses administered in previous studies (Chenaille et al. 1990; Cividalli et al. 1998; Kang et al. 2000).

5.3.8 Collection of plasma from breast cancer patients receiving chemotherapy:

Blood was collected from patients with stage I to III breast cancer scheduled to receive adjuvant or neo-adjuvant chemotherapy with any of the following regimens: CMF [oral cyclophosphamide 100 mg/m^2, intravenous methotrexate 40 mg/m^2 and 5-fluorouracil 600 mg/m^2], CEF (oral cyclophosphamide 75 mg/m^2, i.v. epirubicin 60 mg/m^2 and 5-fluorouracil 500 mg/m^2), or CAF (oral cyclophosphamide 100 mg/m^2, i.v. doxorubicin 30 mg/m^2 and 5-fluorouracil 500 mg/m^2). Baseline patient characteristics, inclusion, and exclusion criteria, and detailed treatment regimens have been previously described (Mukherjee et al. 2010). Blood was collected prior to initiation of chemotherapy (baseline), 24 hours post-chemotherapy (day 1), and 8 days post-chemotherapy (day 8) for the first two cycles of chemotherapy by venipuncture into 0.105 M trisodium citrate vacutainer tubes. Plasma was isolated via centrifugation (2000 g for 15 minutes) within 1 hour of blood collection, and stored at –80°C until immediately prior to analysis. This study was approved by the Research Ethics Board of McMaster University and the Hamilton Health Sciences Corporation.

5.3.9 DNA/histone ELISA:

DNA-histone complex levels were measured using the cell death detection ELISA Plus from Roche Applied Science (Basel, Switzerland) according to the manufacturer's instructions.

5.3.10 Statistical analyses:

Statistical analysis was performed on experiments with an $n = 3$ or greater. One-way ANOVA and Tukey's pair-wise comparisons or t-tests were performed using GraphPad Software (San Diego, CA, USA). Values are expressed as relative means ± standard error. Figures denote $p < 0.05$ with * and $p < 0.001$ with **.

5.4 Results

5.4.1 The effects of chemotherapy on the release of CFDNA in breast cancer patients and healthy mice

To measure the effects of chemotherapy drugs on CFDNA release, we quantified plasma DNA levels in early stage breast cancer patients receiving chemotherapy (CMF, CEF, or CAF). Although baseline CFDNA levels were variable between patients, CFDNA levels increased significantly after 24 hours relative to the baseline samples ($p = 0.0486$) (figure 5.1). Overall, 72.7% of patients had elevated CFDNA levels 24 hours

Figure 5.1 – Effects of chemotherapy on CFDNA release in breast cancer patients. Blood samples were collected from early stage breast cancer patients at baseline, 24 hours, and 8 days post chemotherapy over the first two cycles. CFDNA levels were measured and expressed relative to baseline values. Brackets indicated percent of patients with CFDNA levels elevated above baseline. *$P < 0.05$.

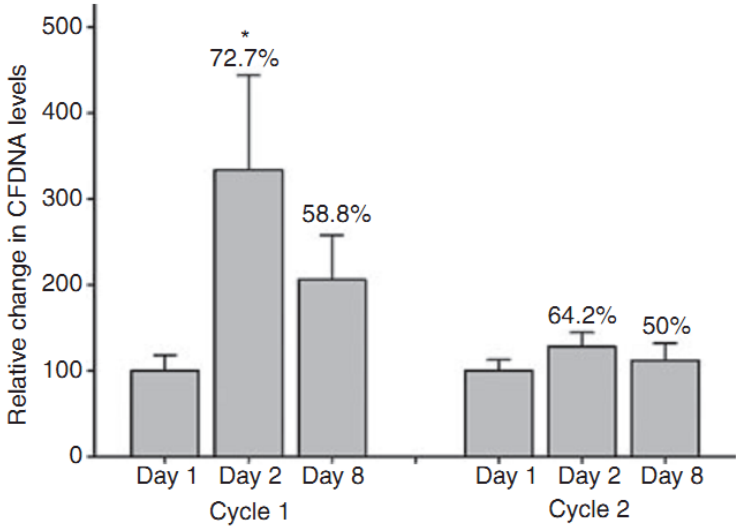
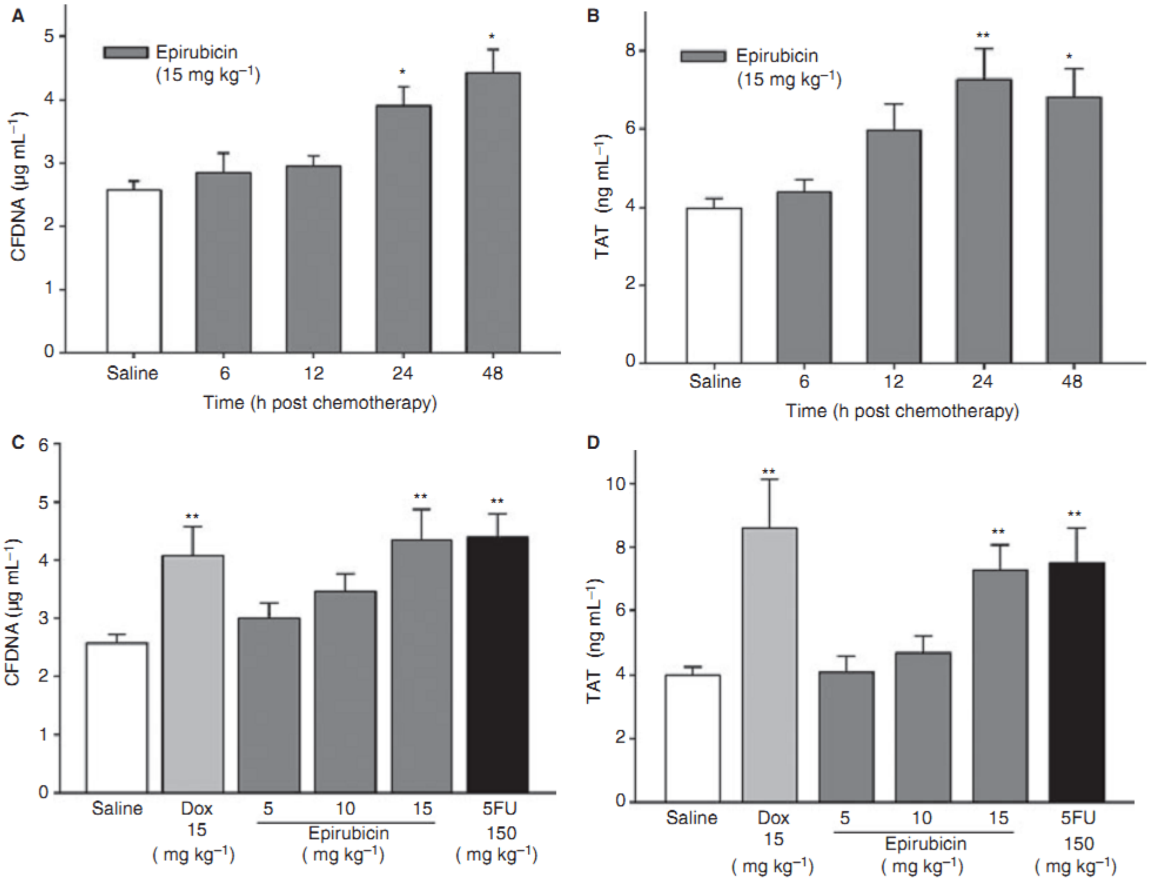

Relative change in CFDNA levels
500
400
300
200
100
0
*
72.7%
58.8%
64.2%
50%
Day 1
Day 2
Day 8
Cycle 1
Day 1
Day 2
Day 8
Cycle 2

Figure 5.2 – Effects of chemotherapy on CFDNA release and TAT formation in health mice. Healthy Balb/C mice were treated with chemotherapy, and blood was collected via cannulation of the carotid artery. CFDNA and TAT levels were measured as described in Methods. TAT and CFDNA levels were measured 6 – 48 hours post chemotherapy in mice treated with epirubicin (15 mg/kg) (figure 5.2A and 5.2B). TAT and CFDNA levels were measured 24 hours post treatment with doxorubicin (15 mg/kg), epirubicin (5 – 15 mg/kg), and 5-fluororuacil (150 mg/kg) (figure 5.2C and 5.2D). *$P < 0.05$; **$P < 0.001$.


A
CFDNA (µg mL^{-1})
0
1
2
3
4
5
Epirubicin
(15 mg kg^{-1})
*
*
Saline
6
12
24
48
Time (h post chemotherapy)
B
TAT (ng mL^{-1})
0
2
4
6
8
Epirubicin
(15 mg kg^{-1})
**
*
Saline
6
12
24
48
Time (h post chemotherapy)
C
CFDNA (µg mL^{-1})
0
1
2
3
4
5
6
**
**
**
Saline
Dox
15
(mg kg^{-1})
5
10
15
Epirubicin
(mg kg^{-1})
5FU
150
(mg kg^{-1})
D
TAT (ng mL^{-1})
0
2
4
6
8
10
**
**
**
Saline
Dox
15
(mg kg^{-1})
5
10
15
Epirubicin
(mg kg^{-1})
5FU
150
(mg kg^{-1})

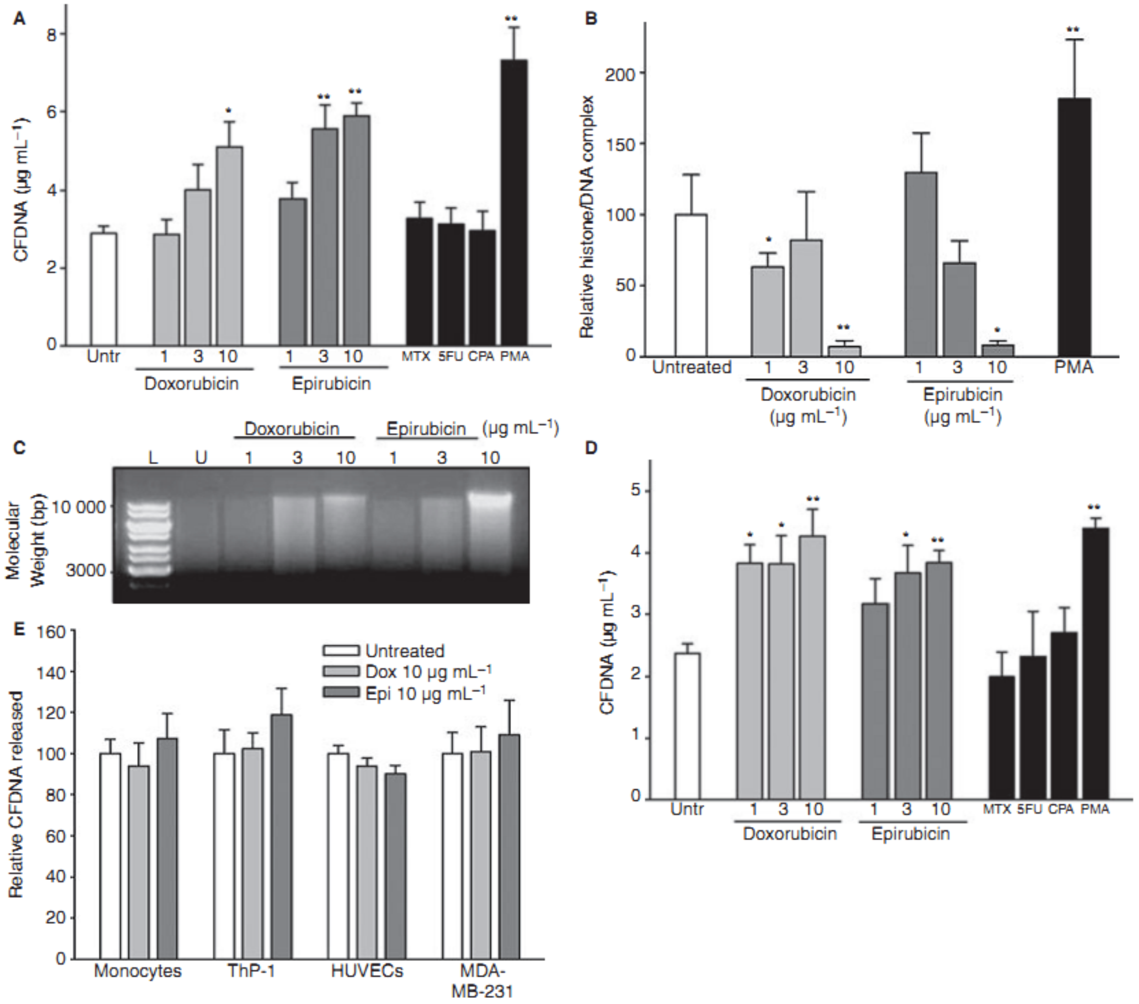
post-chemotherapy, and 58.8% of patients had elevated CFDNA levels 8 days post-chemotherapy of cycle 1. This corresponded with the increase in TAT levels observed in these patients at the 24 hour post-chemotherapy time-point (previously published data (Mukherjee et al. 2010)).

To further demonstrate the relationship between chemotherapy-mediated release of CFDNA and thrombin generation *in vivo*, we treated healthy Balb/C mice with chemotherapeutic agents. Blood was collected post-chemotherapy by carotid artery cannulation, and CFDNA levels and TAT complex levels were measured. Doxorubicin, epirubicin, and 5-fluorouracil increased both CFDNA levels and TAT complex formation in healthy mice (figure 5.2). Chemotherapy treatment did not significantly increase plasma microparticle levels as quantified by the Zymuphen MP Activity assay (Aniara, Mason, Ohio, USA) (data not shown).

5.4.2 The effects of chemotherapy on the release of CFDNA from venous whole blood and neutrophils

To better understand the mechanisms by which chemotherapy induced the release of CFDNA, we collected venous whole blood from healthy volunteers and incubated it with chemotherapy drugs for 24 hours. Plasma was isolated from venous whole blood, and CFDNA was isolated from plasma as described in Methods. We observed a dose-dependent increase in CFDNA release from doxorubicin and epirubicin-treated whole blood. However, methotrexate, 5-fluorouracil, and cyclophosphamide did not increase CFDNA release (figure 5.3A). Gel electrophoresis of isolated CFDNA released from

Figure 5.3 – Effects of chemotherapy on the release of CFDNA *in vitro*. Venous whole blood (figure 5.3A), isolated blood monocytes, ThP-1 cells, HUVECs, and MDA-MB-231 breast tumour cells (figure 5.3E) were treated with chemotherapy for 24 hours. Isolated neutrophils were treated with chemotherapy for 4 hours (figure 5.3D). Cell free plasma or supernatants were isolated, and CFDNA levels were quantified. Histone-DNA complex levels were measured in plasma isolated from venous whole blood treated with doxorubicin and epirubicin or PMA for 24 hours using ELISA (figure 5.3B). CFDNA released from chemotherapy-treated whole blood was concentrated and visualized on a 2% agarose gel (L= ladder, U = untreated) (figure 5.3C). *$P < 0.05$; **$P < 0.001$.


A
CFDNA (μg mL^{-1})
0
2
4
6
8
Untr
1 3 10
Doxorubicin
1 3 10
Epirubicin
MTX 5FU CPA PMA
*
**
**
**
B
Relative histone/DNA complex
0
50
100
150
200
Untreated
1 3 10
Doxorubicin (μg mL^{-1})
1 3 10
Epirubicin (μg mL^{-1})
PMA
*
**
*
**
C
Doxorubicin
Epirubicin
(μg mL^{-1})
L U 1 3 10 1 3 10
Molecular Weight (bp)
10 000
3000
D
CFDNA (μg mL^{-1})
0
1
2
3
4
5
Untr
1 3 10
Doxorubicin
1 3 10
Epirubicin
MTX 5FU CPA PMA
*
*
**
*
**
**
E
Relative CFDNA released
0
20
40
60
80
100
120
140
160
Untreated
Dox 10 μg mL^{-1}
Epi 10 μg mL^{-1}
Monocytes
ThP-1
HUVECs
MDA-MB-231

chemotherapy-treated whole blood revealed that the DNA varied in size between approximately 3,000 and 10,000 base pairs (figure 5.3C). Neutrophils purified from venous whole blood and incubated with doxorubicin or epirubicin for 4 hours induced the release of CFDNA in a dose-dependent manner (figure 5.3D). Treatment of human umbilical venous endothelial cells (HUVECs), breast cancer tumour cells MDA-MB-231, ThP-1 cells, and monocytes isolated from venous whole blood with chemotherapy drugs did not induce the release of CFDNA after a 24 hour treatment (figure 5.3E).

We also investigated the structure of the CFDNA released by chemotherapy drugs. Chromatin levels in plasma isolated from whole blood treated with doxorubicin and epirubicin were measured using an ELISA which measures levels of DNA-histone complex. Interestingly, we found that exposure of venous whole blood to increasing concentrations of doxorubicin and epirubicin decreased histone-bound DNA (figure 5.3B), despite the fact that total DNA levels in these samples significantly increased (figure 5.3A). This was not associated with a significant increase in plasma histone H3 plasma levels (data not shown). The potent NET stimulator PMA (10 nM) increased CFDNA release from whole blood (figure 5.3A) and neutrophils (figure 5.3D), and increased histone/DNA complex levels (figure 5.3B).

5.4.3 The effects of CFDNA on thrombin generation

The release of DNA-histone complexes by doxorubicin and epirubicin may allow CFDNA to interact directly with contact pathway coagulation factors, thereby promoting thrombin generation. We measured the effects of chemotherapy-released CFDNA on

Figure 5.4 – Effects of CFDNA on thrombin generation. CFDNA isolated from plasma of chemotherapy-treated venous whole blood was exposed to recalcified plasma and thrombin generation was measured as described in Methods (figure 5.4A and 5.4B). Lag time (figure 5.4C), velocity index (figure 5.4D), and time to peak (figure 5.4E) analysis was performed with Technothrombin TGA software. Epirubicin concentration (1, 3, 10 µg/mL) indicates the amount of epirubicin incubated with venous whole blood (24 hours). CFDNA values in brackets indicate initial concentration of CFDNA isolated from plasma of epirubicin-treated whole blood added to reaction. * indicates significance relative to plasma only, # indicates significance relative to untreated CFDNA levels, and + indicates significance relative to epirubicin (10 µg/mL) condition. $P < 0.05$.

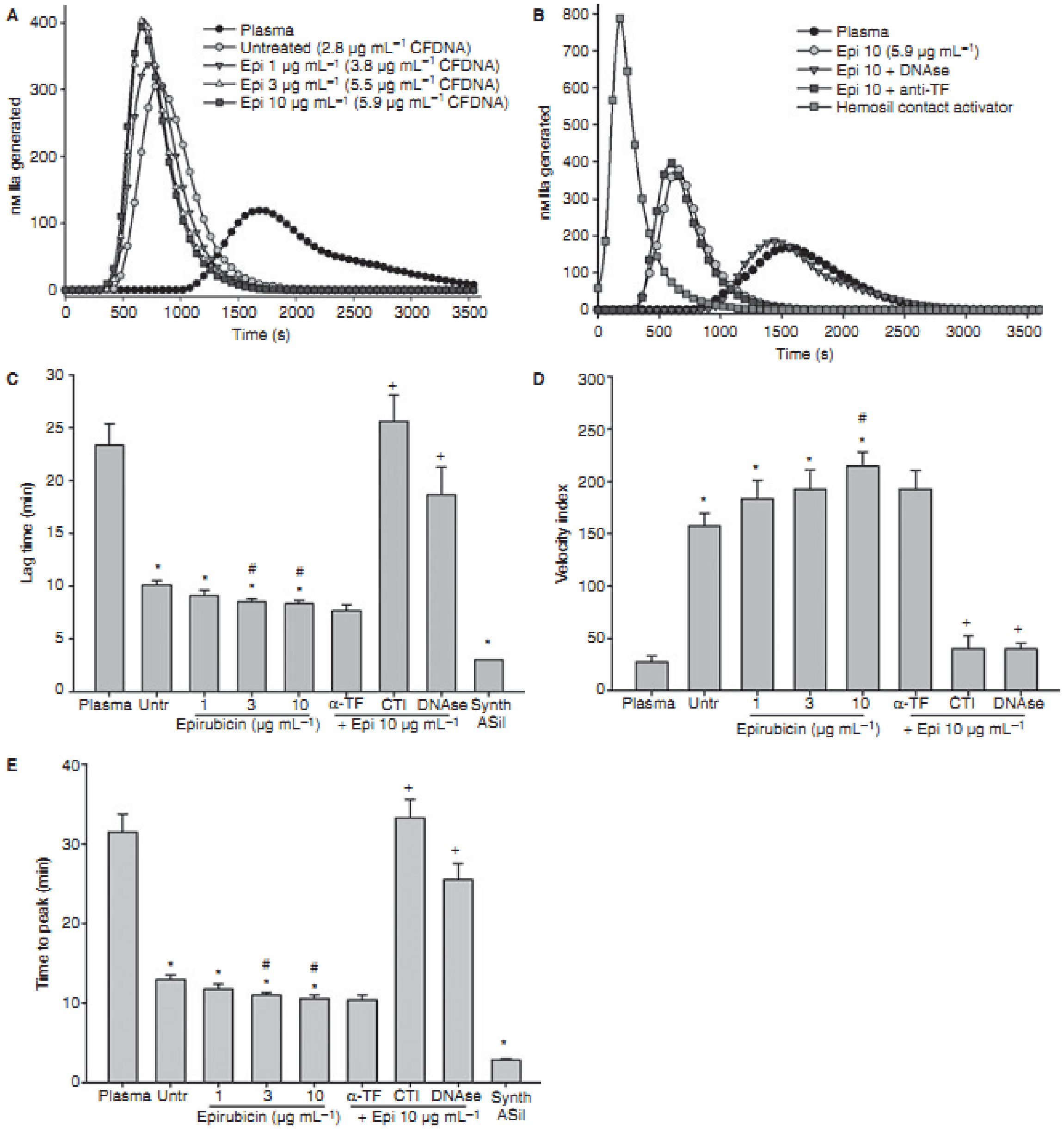
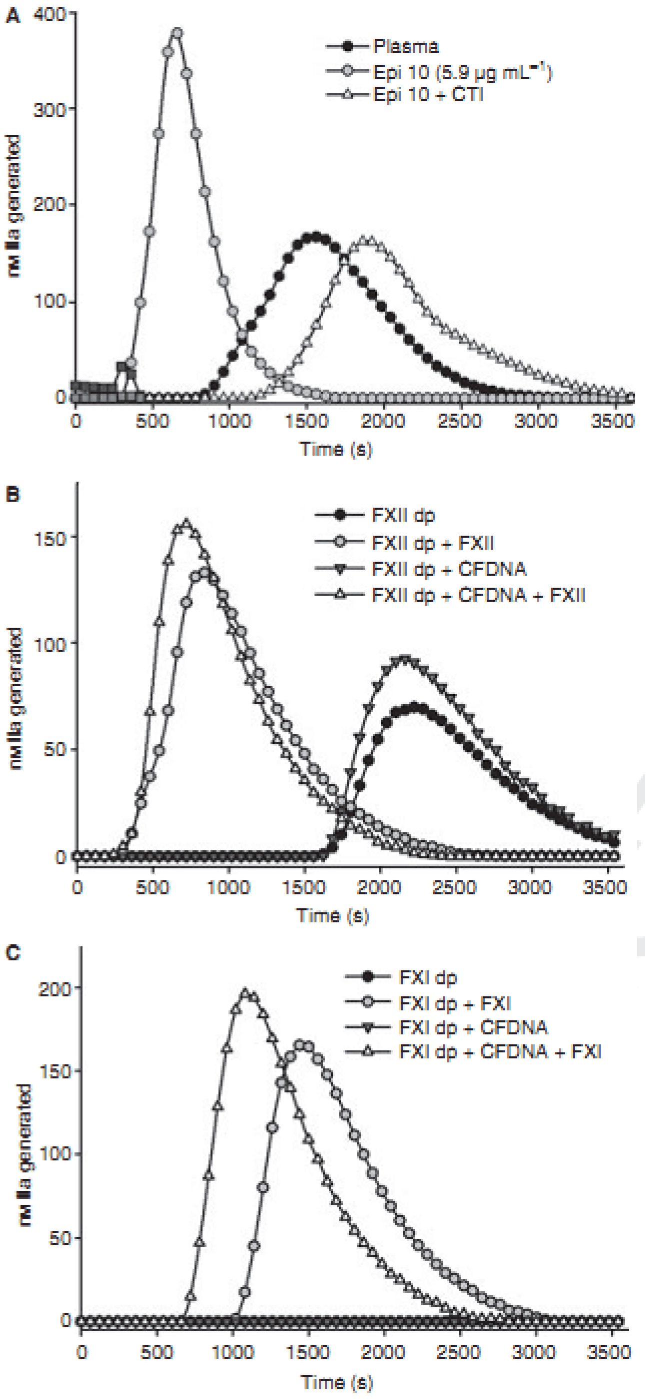

A
nM IIa generated
Time (s)
Plasma
Untreated (2.8 μg mL⁻¹ CFDNA)
Epi 1 μg mL⁻¹ (3.8 μg mL⁻¹ CFDNA)
Epi 3 μg mL⁻¹ (5.5 μg mL⁻¹ CFDNA)
Epi 10 μg mL⁻¹ (5.9 μg mL⁻¹ CFDNA)
B
nM IIa generated
Time (s)
Plasma
Epi 10 (5.9 μg mL⁻¹)
Epi 10 + DNAse
Epi 10 + anti-TF
Hemosil contact activator
C
Lag time (min)
Plasma
Untr
1
3
10
Epirubicin (μg mL⁻¹)
α-TF
CTI
DNAse
+ Epi 10 μg mL⁻¹
Synth ASil
D
Velocity index
Plasma
Untr
1
3
10
Epirubicin (μg mL⁻¹)
α-TF
CTI
DNAse
+ Epi 10 μg mL⁻¹
E
Time to peak (min)
Plasma
Untr
1
3
10
Epirubicin (μg mL⁻¹)
α-TF
CTI
DNAse
+ Epi 10 μg mL⁻¹
Synth ASil

Figure 5.5 – Effects of CFDNA on the contact pathway. Thrombin generation in normal plasma exposed to CFDNA was measured in the presence of corn trypsin inhibitor (figure 5.5A). The effects of CFDNA on thrombin generation in FXII (figure 5.5B) and FXI (figure 5.5C) deficient plasma were also measured.


A
400
300
200
100
0
nM IIa generated
Plasma
Epi 10 (5.9 μg mL⁻¹)
Epi 10 + CTI
0
500
1000
1500
2000
2500
3000
3500
Time (s)
B
150
100
50
0
nM IIa generated
FXII dp
FXII dp + FXII
FXII dp + CFDNA
FXII dp + CFDNA + FXII
0
500
1000
1500
2000
2500
3000
3500
Time (s)
C
200
150
100
50
0
nM IIa generated
FXI dp
FXI dp + FXI
FXI dp + CFDNA
FXI dp + CFDNA + FXI
0
500
1000
1500
2000
2500
3000
3500
Time (s)

coagulation using a calibrated automated thrombin generation assay. We found that CFDNA isolated from epirubicin-treated venous whole blood increased thrombin generation in a dose-dependent manner (figure 5.4A). CFDNA decreased lag time (figure 5.4C) and time to peak (figure 5.4E), and increased peak height (data not shown), area under curve (AUC) (data not shown), and velocity index (figure 5.4D) relative to the plasma control (indicated by *). When comparing CFDNA released from epirubicin-treated whole blood to CFDNA released from untreated whole blood over a 24 hour period, we found a similar trend; however, only changes in lag time, time to peak, and velocity index were statistically significant relative to the untreated condition (indicated by #). We found that in addition to CFDNA derived from epirubicin-treated whole blood, the contact activator SynthASil (figure 5.4B), DNA isolated from HUVECs (7.2 µg/mL), salmon sperm DNA (250 µg/mL), and RNA (500 µg/mL) all increased thrombin generation (data not show). Inhibition of the extrinsic pathway with the TF blocking antibody HTF1 (5 µg/mL) did not affect thrombin generation (figure 5.4B). Degradation of CFDNA with DNAse (confirmed with gel electrophoresis, data not shown) attenuated thrombin generation (figure 5.4B).

CFDNA has been reported to increase thrombin generation via activation of the contact pathway. The addition of CTI (20 µg/mL) significantly impaired thrombin generation in the presence of CFDNA (figure 5.5A). In addition, thrombin generation assays performed in FXII and FXI deficient plasma revealed that thrombin generation in the presence of CFDNA was impaired, and restored by supplementation with factor XII (50 µg/mL) (figure 5.5B) or FXI (5 µg/mL) (figure 5.5C). The addition of CFDNA elevated thrombin generation relative to control conditions in both FXII and FXI deficient plasmas.

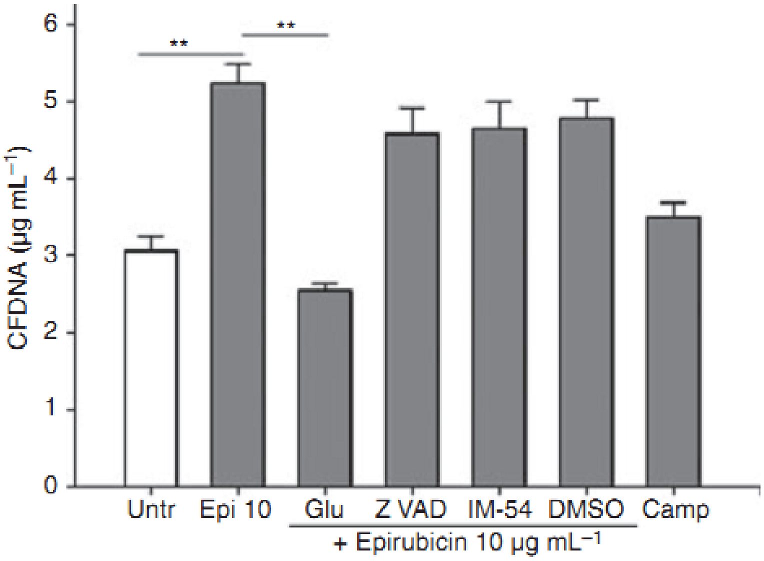
5.4.4 Mechanisms of chemotherapy-induced CFDNA release

To better understand the mechanism by which chemotherapy induces the release of CFDNA *in vitro*, we pretreated venous whole blood with 100 µM Z-VAD (a pan-caspase inhibitor), 3.25 µM IM-54 (a necrosis inhibitor), and 3.75 mM glutathione (an antioxidant) for 1 hour, and then treated whole blood with epirubicin (10 µg/mL) for 24 hours. We also treated blood with 6 µM camptothecin (an apoptosis inducer) for 24 hours. A DMSO condition was included to control for the solvent of Z-VAD and PMA. We found that glutathione but not Z-VAD or IM-54 significantly attenuated CFDNA release in epirubicin-treated whole blood (figure 5.6). We also found that PMA but not camptothecin significantly increased CFDNA release from stimulated whole blood.

5.5 Discussion:

The association between cancer chemotherapy and coagulation initiation through the tissue factor pathway has been well investigated (Kasthuri et al. 2009). Breast cancer cells express high levels of TF, and breast tumours are associated with elevated levels of endothelial TF, which correlates with fibrin deposition (Contrino et al. 1996). Chemotherapeutic agents can increase TF activity on endothelial cells, monocytes,

Figure 5.6 – Mechanism of CFDNA release. Venous whole blood was preincubated with inhibitors (Z-VAD, IM-54, glutathione) for 1 hour, and then with epirubicin for 24 hours. Plasma was isolated from venous whole blood, and CFDNA was isolated from plasma and quantified. $^{**}P < 0.001$.


CFDNA (μg mL⁻¹)
0
1
2
3
4
5
6
**
**
Untr
Epi 10
Glu
Z VAD
IM-54
DMSO
Camp
+ Epirubicin 10 μg mL⁻¹

smooth muscle cells (Swystun et al. 2009; Swystun et al. 2011) and breast tumour cells (Swystun LL and Liaw PC). Although chemotherapy induces a strong procoagulant response in breast cancer patients, no clear mechanism for thrombin generation *in vivo* has been identified. The role of the intrinsic pathway in malignancy-associated thrombosis is not well characterized. While humans and mice with deficiencies in FXII do not present with bleeding tendencies, mice deficient in either FXII or FXI are protected from venous and arterial thrombosis (Woodruff et al. 2011). This suggests a potential role for physiological activators of the contact pathway, including CFDNA, in mediating thrombotic disease.

Previous studies of CFDNA in cancer patients receiving chemotherapy have examined the relationship between plasma DNA levels and therapeutic response or tumour recurrence post-therapy. One study of breast cancer patients found that chemotherapy did not affect levels of plasma DNA, however, samples were not collected serially throughout the course of chemotherapy (Deligezer et al. 2008). In this study, we demonstrated that CFDNA levels are increased 24 hours after chemotherapy treatment in breast cancer patients (figure 5.1). This is consistent with studies which found that docetaxel can increase CFDNA release in ovarian tumour-bearing mice (Kamat et al. 2006). We and others have demonstrated that TAT levels in breast cancer chemotherapy patients are also elevated 24 hours post chemotherapy (Weitz et al. 2002; Mukherjee et al. 2010; Kirwan et al. 2008). We also found that treatment of healthy Balb/C mice with chemotherapy induced a dose- and time-dependent increase in CFDNA levels, with a corresponding increase in TAT complex formation (figure 5.2).

Our *in vitro* studies demonstrated that both doxorubicin and epirubicin stimulated the release of CFDNA in venous whole blood and neutrophils, but not from endothelial cells, blood monocytes, or breast tumour cells (figure 5.3). This suggests that a portion of CFDNA released by chemotherapy agents in breast cancer patients may be neutrophil in origin. Previous studies have demonstrated that 3 – 93% of plasma DNA in cancer patients is tumour derived (Jahr et al. 2001), and we cannot rule tumour cells out as a source of CFDNA measured in our breast cancer chemotherapy patients, even though chemotherapy agents did not release DNA from breast tumour cells *in vitro* (figure 5.3E). In addition to stimulating DNA release into the plasma from injured cells, chemotherapy may also impair deoxyribonuclease (DNAse) synthesis or activity, thereby promoting accumulation of CFDNA in the plasma (Economidou-Karaoglou et al. 1989).

Doxorubicin and epirubicin are both anthracycline chemotherapeutic agents which intercalate between DNA base pairs, thereby leading to chromatin unfolding. These structural alterations interfere with DNA replication and transcription and lead to apoptosis (Rabbani et al. 2004). We found that CFDNA isolated from venous whole blood treated with doxorubicin and epirubicin had decreased association with histones; overall there was increased CFDNA release upon exposure to these drugs (figure 5.3A and 5.3B). This suggests that CFDNA released by cells exposed to doxorubicin and epirubicin *in vivo* may have a greater negative charge density and thus able to promote coagulation activation through the contact pathway. We found that thrombin generation was increased when pooled, recalcified plasma was exposed to increasing concentrations of CFDNA isolated from the plasma of epirubicin-treated whole blood (figure 5.4).

Thrombin generation was inhibited with CTI, and assays performed in FXII and FXI-deficient plasma confirmed activation through the contact pathway (figure 5.5). We acknowledge that the DNA purification protocol that was used to isolate CFDNA samples prior to thrombin generation artificially removed any residual histone and other nuclear proteins from DNA, potentially altering its procoagulant activity in this system.

We also investigated the mechanism(s) by which chemotherapy-exposed cells may release CFDNA. Electrophoresis of CFDNA isolated from plasma of venous whole blood, breast cancer chemotherapy samples, and murine samples did not reveal mono-, di-, or trinucleosomal DNA fragments which are characteristic of apoptotic cell death (figure 5.3C) (Jahr et al. 2001). In addition, treatment of HUVECs and MDA-MB-231 cells with doxorubicin and epirubicin at concentrations and incubation periods previously demonstrated to induce apoptosis (Swystun et al. 2009; Koutsilieris et al. 1999), did not significantly increase CFDNA release (figure 5.3E). As well, inhibition of apoptosis and necrosis did not attenuate CFDNA release, while treatment with apoptosis inducer camptothecin did not significantly increase CFDNA release in venous whole blood (figure 5.6). Interestingly, pretreatment of venous whole blood with antioxidant glutathione was able to prevent epirubicin-induced release of CFDNA in venous whole blood. NETosis, a mechanism of neutrophil cell death whereby pathological stimuli leads to vacuolization and chromatin decondensation without phosphatidylserine exposure, followed by NET release, may involve reactive oxygen species generation (Fuchs et al. 2007). Taken together, this suggests that release of CFDNA by doxorubicin and epirubicin may not be through apoptotic or necrotic mechanisms, but may involve the release of NETs.

Our studies demonstrate a relationship between CFDNA release and thrombin generation *in vitro* and *in vivo*. Although we have not established CFDNA as a causative agent in facilitating thrombin generation *in vivo*, CFDNA release may be a marker for of chemotherapy-induced damage to healthy blood and/or vascular cells, which may be associated with elevated risk for thrombosis in these patients. In addition to CFDNA, the release of RNA by chemotherapy-injured cells may also play a role in activating coagulation in breast cancer chemotherapy patients. Furthermore, the release of histones from DNA mediated by anthracycline chemotherapy agents may also contribute to the prothrombotic phenotype observed in these patients (Xu et al. 2009; Ammollo et al. 2011; Semeraro et al. 2011).

Thromboprophylaxis in cancer patients remains a significant challenge given that only a subgroup of cancer patients develop thrombosis during the course of their disease and also because the use of anticoagulants is associated with bleeding. The release of CFDNA from injured host and/or tumour cells may represents a novel mechanism by which thrombosis is triggered in cancer patients receiving chemotherapy. Understanding the mechanism(s) through which chemotherapy drugs modify the thrombotic risk may lead to better screening for at-risk patients and safer prophylactic strategies.

CHAPTER 6: General Discussion

Thrombosis is a well recognized complication of chemotherapy in breast cancer patients. Clinical studies have clearly identified alterations in hemostatic pathways in response to treatment with chemotherapeutic agents. However, few studies recommend prophylactic anticoagulation of ambulatory cancer patients receiving chemotherapy due to the risk of hemorrhage. The identification of patients with the greatest risk for developing chemotherapy-associated thrombosis is critical for the prevention and management of the disease. However, the mechanisms by which chemotherapeutic agents exert procoagulant side effects are not well characterized.

Chemotherapy agents exert their anti-neoplastic effects by inducing apoptosis or inhibiting mitosis in rapidly dividing neoplastic cells. Normal function of host cells which undergo rapid cell division may also be affected by chemotherapy treatment, resulting in the development of negative side effects. For example, damage to the hair follicle may result in alopecia, damage to the myocardium can induce cardiotoxicity, damage to the intestinal lining can produce mucositis, and damage to the bone marrow can result in myelosuppression or immunosuppression . Similarly, chemotherapy treatment may be cytotoxic to cellular components of the blood, such as monocytes and neutrophils, and vascular tissues, such as endothelial and smooth muscle cells, which regulate hemostasis. Phosphatidylserine exposure is a hallmark of apoptotic cell death, and is associated with the facilitation of TF de-encryption. Pathological stimulation of cells can also alter TF, EPCR, and thrombomodulin cell surface expression. As well, several mechanisms of cell death, including apoptosis, necrosis, and NETosis have been associated with release of CFDNA (Jahr et al. 2001; Fuchs et al. 2007).

We conducted the studies described in this thesis in order to better understand the mechanisms by which chemotherapy agents increase the risk for thrombosis in breast cancer patients. We hypothesized that breast cancer chemotherapy agents upregulate procoagulant pathways and dampen anticoagulant mechanisms on vascular and blood cells. This thesis had four main objectives: (1) to measure the effects of breast cancer chemotherapy agents on procoagulant (thrombin generating) pathways on endothelial, blood monocyte, and smooth muscle cell surfaces, (2) to examine the effects of breast cancer chemotherapy agents on the release of cell free DNA from chemotherapy-injured cells, and to assess the effects of chemotherapy-released CFDNA on thrombin generation, (3) to investigate the effects of breast cancer chemotherapy agents on the protein C anticoagulant pathway, (4) to investigate the role that reactive oxygen species may play in mediating the procoagulant effects exerted by chemotherapy agents.

We tested our hypothesis by treating blood and vascular tissues with breast cancer chemotherapy agents (doxorubicin, epirubicin, methotrexate, 5-fluorouracil, and the cyclophosphamide metabolite acrolein). We measured alterations in levels of pro- and anticoagulant factors on cell surfaces (TF, phosphatidylserine, EPCR, TM), and in cell supernatants (CFDNA, sEPCR). We determined the relevance of these alterations to the function of the hemostatic pathways by performing tissue factor activity assays, thrombin generation assays, and protein C activation assays. Furthermore, we demonstrated the relevance of our *in vitro* experiments by examining the effects of chemotherapy treatment on pro- and anticoagulant pathways in a healthy mouse model.

6.1 Effects of breast cancer chemotherapy agents on the TF pathway

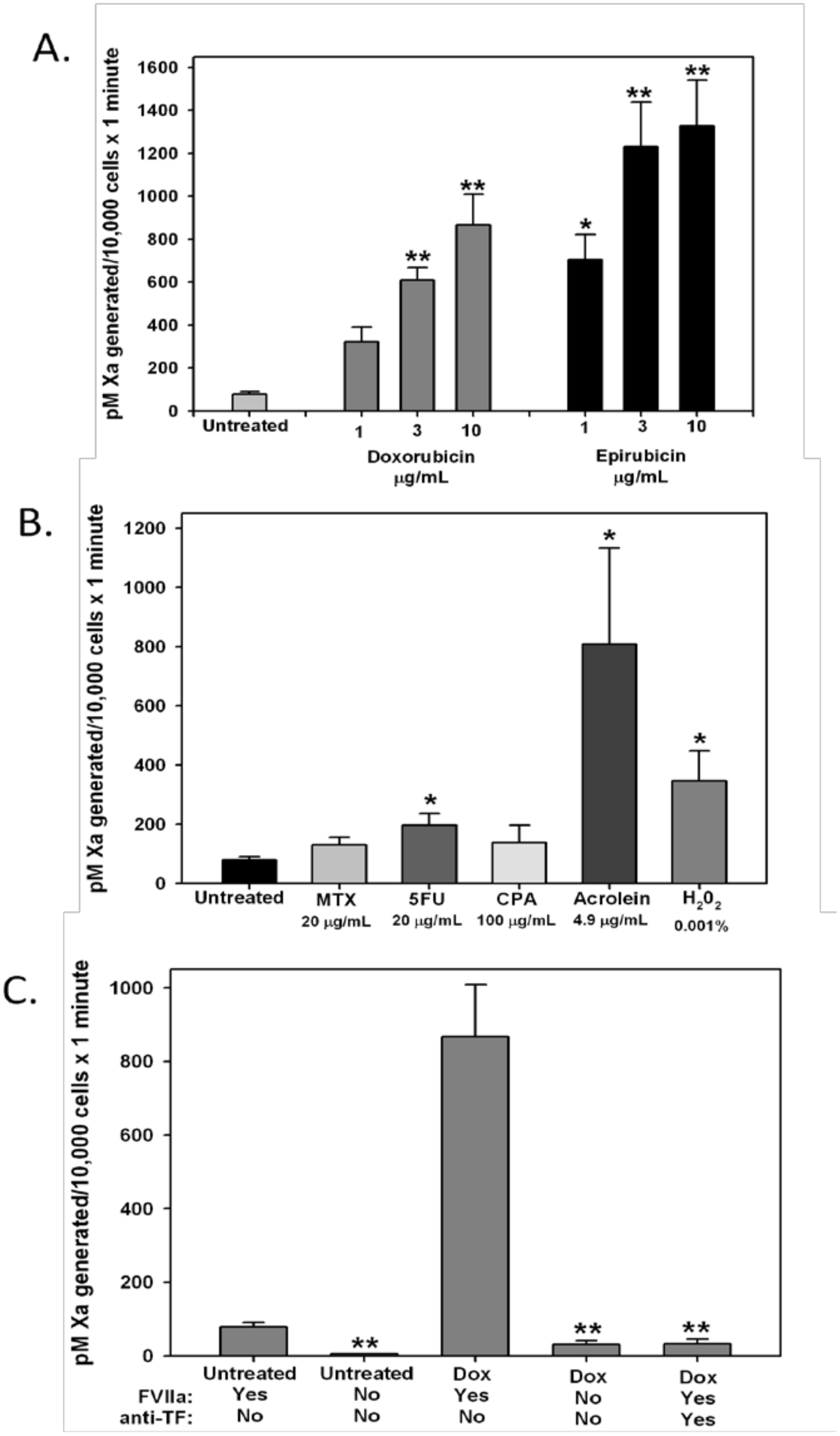
TF, which initiates coagulation upon exposure to the blood, has a well documented relationship with the activation of coagulation in cancer. Tumour cells, including breast cancer cells, express high levels of TF (Rickles et al. 1995). TF-positive microparticles have been observed in several types of cancer including pancreatic (Khorana et al. 2008), colorectal (Hron et al. 2007), and metastatic breast cancer (Tesselaar et al. 2007). As well, monocytes expressing TF have been documented in cancer patients (Lwaleed et al. 2000). Breast tumours are associated with elevated levels of endothelial TF, which correlates with fibrin deposition (Contrino et al. 1996). In Chapters 3 and 4, we investigated the effects of the chemotherapeutic agents doxorubicin, epirubicin, methotrexate, 5-fluororuracil, and the cyclophosphamide metabolite acrolein on TF activity and TF expression on endothelial cells (HUVECs), monocytes, and smooth muscle cells (UASMCs).

We found that doxorubicin, epirubicin and acrolein upregulate TF activity on the endothelial cell surface (figures 3.2A and 4.3A). This was confirmed by blocking TF activity with the inhibitory antibody HTF1. Interestingly, doxorubicin and epirubicin increase TF activity on isolated blood monocytes (figure 3.2B), but acrolein does not (data not shown). In contrast, acrolein increases TF activity on UASMCs (figure 4.3D), although doxorubicin and epirubicin do not (data not shown). In addition, we found that doxorubicin, epirubicin, acrolein, and 5-fluorouracil can increase TF activity on the MDA-MB-231 breast tumour cell line (figure 6.1A).

Tissue factor activity can be modified by increasing antigen levels (expression), or by increasing the de-encryption of TF from an existing cryptic population. We investigated the mechanisms by which chemotherapy agents modulate endothelial and SMC TF activity. We found that doxorubicin and epirubicin increased endothelial TF activity without modifying TF antigen levels in whole cell lysates, as measured by Western blot analysis (figure 4.2C). Interestingly, we found that acrolein increased both TF activity and antigen levels as measured by flow cytometry (figure 3.3B); changes in TF antigen levels induced by acrolein were confirmed by ELISA (data not shown). Interestingly, treatment of UASMCs with acrolein did not modify TF antigen levels (figure 4.3E).

Regulation of TF encryption/de-encryption may be modified by increased phosphatidylserine exposure (Bach 2006), and/or the formation of an intramolecular disulfide bond which may involve the release of protein disulfide isomerase (PDI) (Popescu et al. 2010). To test the involvement of PDI on mediating TF de-encryption on doxorubicin-treated endothelial cells, we blocked endothelial cells with a PDI-inhibitory antibody (RL77), then treated with doxorubicin, and measured factor Xa generation. We found that inhibition of PDI did not modulate doxorubicin-induced TF activity (figure 3.4A). To measure the contribution of phosphatidylserine exposure in mediating TF de-encryption on endothelial cells, we blocked the cells with annexin V, which binds to exposed phosphatidylserine, and then measured TF activity. On doxorubicin and

Figure 6.1 – Effects of chemotherapy agents on MDA-MB-231 TF activity. MDA-MB-231 Breast tumour cells were grown to confluency and treated with chemotherapy drugs for 24 hours. Cells were exposed to FVIIa and FX for 60 seconds, and FXa generation was quantified as described in Chapter 3. (A) TF activity on doxorubicin and epirubicin-treated MDA-MB-231 cells. (B) TF activity on chemotherapy-treated MDA-MB-231 cells. (C) Inhibition assays on untreated and doxorubicin-treated MDA-MB-231 cells. N = 3 – 8 independent experiments. P> 0.05 *, P>0.001 **


A.
pM Xa generated/10,000 cells x 1 minute
1600
1400
1200
1000
800
600
400
200
0
Untreated
1
3
10
Doxorubicin
μg/mL
1
3
10
Epirubicin
μg/mL
*
**
B.
pM Xa generated/10,000 cells x 1 minute
1200
1000
800
600
400
200
0
Untreated
MTX
20 μg/mL
5FU
20 μg/mL
CPA
100 μg/mL
Acrolein
4.9 μg/mL
H202
0.001%
C.
pM Xa generated/10,000 cells x 1 minute
1000
800
600
400
200
0
FVIIa:
anti-TF:
Untreated
Yes
No
Untreated
No
No
Dox
Yes
No
Dox
No
No
Dox
Yes
Yes

acrolein-treated HUVECs, we found that annexin V was able to partially attenuate the chemotherapy-mediated increase in TF activity (figure 3.4A and 4.3A). This suggests that the increase in TF activity on acrolein-treated endothelial cells may be due to increased antigen levels and de-encryption. Inhibition of phosphatidylserine with annexin V on acrolein-treated UASMCs did not attenuate factor Xa generation (figure 4.3D), suggesting a phosphatidylserine-independent mechanism of TF de-encryption. The release of TFPI from acrolein-treated HUVECs and UASMCs was also measured (data not shown); however, no significant effects were observed.

These studies are consistent with others which have demonstrated that chemotherapy agents can increase TF activity on monocytes (Wheeler et al. 1990; Walsh et al. 1992), and endothelial cells (Ma et al. 2005). These studies are also consistent with studies which suggest that TF de-encryption is related to phosphatidylserine exposure, and do not support the hypothesis that PDI mediates activation of cryptic TF on chemotherapy-treated endothelial cells. In breast cancer patients receiving chemotherapy, no increases in plasma soluble TF (Kirwan et al. 2008), or TF-positive microparticles (Mukherjee et al. 2010) were observed at timepoints with elevated TAT levels. However, other cellular sources of TF were not quantified in these studies.

Our studies are the first to demonstrate that doxorubicin, epirubicin, and acrolein can increase endothelial cell TF activity. To our knowledge these studies were also the first to examine the effects of chemotherapeutic agents on SMC procoagulant activity. Overall, they suggest that breast cancer chemotherapy agents may increase TF procoagulant activity on blood and vascular tissues in cancer patients. Although these agents generally do not promote *de novo* TF antigen synthesis, they may serve to de-encrypt existing TF pools (located on endothelial cells, monocytes, and/or tumour cells) in cancer patients. These studies may in part provide an explanation for the increased TAT levels observed in breast cancer patients receiving chemotherapy.

6.2 Effects of breast cancer chemotherapy agents on phosphatidylserine exposure and apoptosis

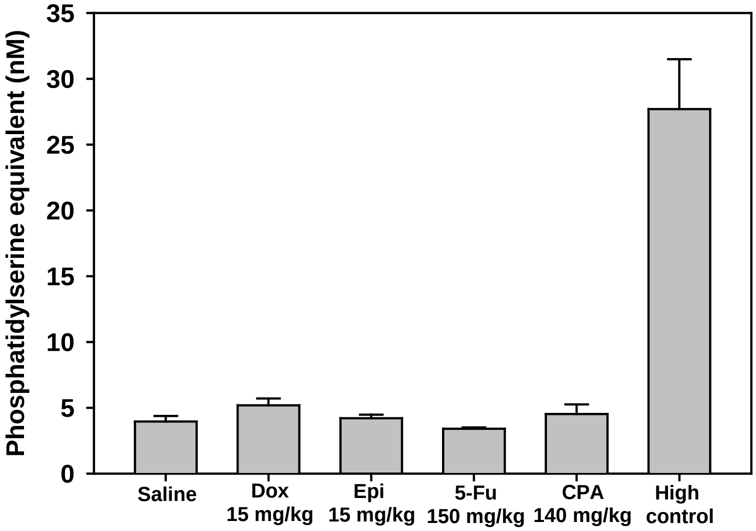
Uncontrolled endothelial cell death may upregulate procoagulant pathways by a number of distinct processes. Phosphatidylserine exposure, an early hallmark of apoptotic cell death, can enhance coagulation by allowing for efficient activation of tenase and prothrombinase complexes (Bombeli et al. 1997), and phosphatidylserine exposure may facilitate the de-encryption of TF as previously discussed (Wolberg et al. 1999). Apoptotic endothelial cells may be shed into the plasma, thereby exposing the blood to the procoagulant subendothelium (Zeng et al. 2008). Finally, apoptotic or activated cells may release procoagulant microparticles into the plasma (Jimenez et al. 2003).

We measured phosphatidylserine exposure on doxorubicin, epirubicin, and acrolein-treated endothelial cells, as well as acrolein-treated smooth muscle cells. We found that a 24 hour treatment of HUVECs with doxorubicin and epirubicin significantly increases phosphatidylserine exposure, which corresponded to an increase in caspase 3 activity, confirming induction of apoptosis (figure 3.3). Interestingly, acrolein increased phosphatidylserine exposure on HUVECs (figure 4.2A), but did not increase caspase 3 activity after a 5 hour incubation (data not shown). Treatment of UASMCs with acrolein did not increase phosphatidylserine exposure (figure 4.2B) or caspase 3 activation (data not shown).

Our studies are consistent with other investigations on the procoagulant effects of chemotherapeutic agents *in vitro*. For example, daunorubicin and cisplatin increase endothelial cell phosphatidylserine exposure and generation of phosphatidylserine-positive MPs (Fu et al. 2010; Lechner et al. 2007) and increase phosphatidylserine exposure on erythrocytes (Lu et al. 2008; Zhou et al. 2010). However, few studies directly measure phosphatidyserine exposure on vascular cells *in vivo*. Quantification of microparticle levels in plasma may be indicative of endothelial activation or apoptosis, and as well represent another source of phosphatidylserine in the blood. We measured plasma microparticles in chemotherapy-treated mice with a prothrombinase activity assay. No increase in microparticle levels was observed in chemotherapy-treated healthy mice (figure 6.2).

To our knowledge, these studies were the first to demonstrate that the breast cancer chemotherapy agents increase phosphatidylserine exposure on endothelial cells. These studies also suggest that that breast cancer chemotherapy agents do not significantly increase microparticle generation *in vivo*. The increase in endothelial phosphatidylserine exposure by chemotherapy agents may represent an additional mechanism to by which these drugs increase thrombin generation in breast cancer patients receiving chemotherapy.

Figure 6.2 – Effects of chemotherapy agents on microparticle generation in mice. Levels of phosphatidylserine-positive microparticles in plasma from chemotherapy-treated mice were quantified with the Zymuphen microparticle activity assay (Aniara, Mason, Ohio, USA) according to the manufacturer's instructions. High control is a plasma sample containing high levels of MPs provided by the manufacturer. N = 4 – 6.


Phosphatidylserine equivalent (nM)
35
30
25
20
15
10
5
0
Saline
Dox 15 mg/kg
Epi 15 mg/kg
5-Fu 150 mg/kg
CPA 140 mg/kg
High control

6.3 Effects of breast cancer chemotherapy agents on the release of CFDNA

Recent studies which suggest that cell free nucleic acids play a role in the pathophysiology of prothrombotic diseases such as cancer and sepsis prompted us to investigate the effects of chemotherapy treatment on the release of CFDNA. We found that CFDNA levels are increased in breast cancer patients (figure 5.1) and in healthy mice in response to treatment with breast cancer chemotherapy agents (figure 5.2). Treatment of venous whole blood and neutrophils with doxorubicin and epirubicin can increase CFDNA release (figure 5.3A and 5.3D). However, treatment of monocytes, THP-1 cells, MDA-MB-231 breast tumour cells, and endothelial cells did not significantly increase CFDNA release (figure 5.3E). The DNA released from doxorubicin and epirubicin-treated whole blood has a decreased association with histone protein relative to untreated controls (figure 5.3B).

To our knowledge, we are the first to demonstrate that CFDNA levels increase 24 hours post-chemotherapy in breast cancer patients. While other studies have investigated the effects of chemotherapy on CFDNA release, measurements were only taken at baseline and post-chemotherapy timepoints (Deligezer et al. 2008; Capizzi et al. 2008), rather than serially throughout the course of chemotherapy. Our studies are consistent with an *in vivo* study which demonstrated that treatment of ovarian tumour-bearing mice with docetaxel increases CFDNA release (Kamat et al. 2006). Our studies are also the first to demonstrate that doxorubicin and epirubicin can increase CFDNA release from venous whole blood and isolated neutrophils, and decrease the association of CFDNA with histone protein. These studies provide insight into mechanisms by which CFDNA levels may be increased in breast cancer patients. In addition, they suggest a novel mechanism of chemotherapy-induced neutrophil injury.

6.4 Effects of breast cancer chemotherapy agents on the PC pathway

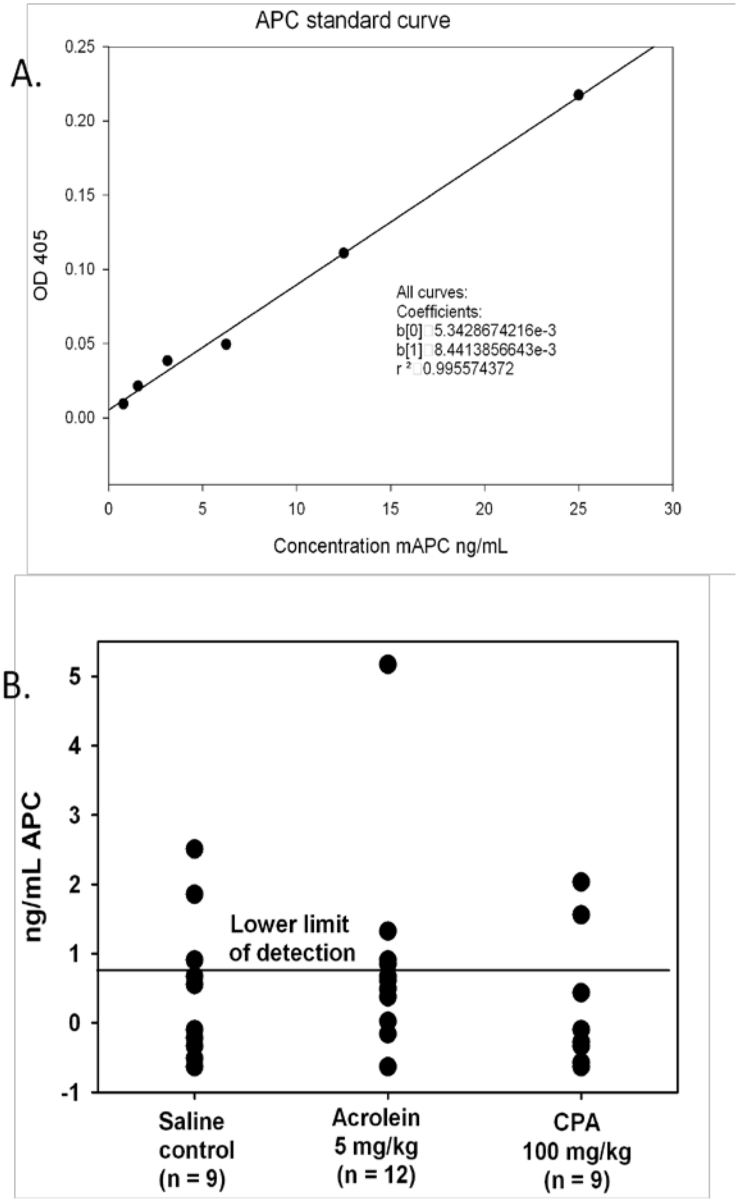
The protein C pathway is a critical anticoagulant mechanism that is activated in response to elevated thrombin generation. Acquired deficiencies in the protein C anticoagulant pathway, such as those associated with breast cancer chemotherapy, may result in an increased risk for thrombosis. We investigated the effects of breast cancer chemotherapy on the protein C pathway *in vitro* and *in vivo*. We also measured modulation of endothelial EPCR and thrombomodulin by chemotherapy agents.

We found that acrolein increased thrombomodulin and decreased EPCR expression on the endothelium (figure 4.5). This was associated with an increase in sEPCR shedding from the endothelial surface. In contrast, we found that epirubicin does not decrease EPCR levels to the same extent as doxorubicin (figure 3.5A). To confirm that decreased EPCR and increased thrombomodulin levels on acrolein-treated HUVECs resulted in impaired APC formation, we measured APC generation on acrolein treated endothelial cells exposed to defibrinated plasma with an enzyme capture assay. We observed a decrease in APC generation on acrolein-treated HUVECs relative to untreated cells, suggesting impairment in the PC pathway (figure 4.4). Our *in vitro* data is consistent with our previous studies which demonstrated that doxorubicin can decrease endothelial cell EPCR and thrombomodulin expression on HUVECs, thereby impairing APC generation on the cell surface (Woodley-Cook et al. 2006). It has also been reported that vincristine can decrease EPCR expression on endothelial cells (Mewhort-Buist et al. 2008).

Chemotherapy has been previously reported to impair the protein C anticoagulant pathway by decreasing plasma PC levels in breast cancer patients, a side effect which persists throughout the course of chemotherapy. To demonstrate that breast cancer chemotherapy agents can modify the PC pathway *in vivo*, we examined the effects of acrolein and cyclophosphamide on PC antigen levels and APC generation in a mouse model. We found that treatment of healthy mice with acrolein and cyclophosphamide increased PC levels in our mouse model (figure 4.6C). There are several potential explanations for this discrepancy. First, the time points at which we measured PC levels in mice (4 – 6 days) may be earlier than the amount of time required to observe significant decreases in PC in breast cancer patients (1 – 2 weeks) (Mukherjee et al. 2010). Regulation of PC synthesis may be affected by procoagulant or proinflammatory properties of different tumour types, or different chemotherapy combinations. In support of this, treatment of lymphoma patients with cyclophosphamide, doxorubicin, vincristine, and prednisone is associated with increased levels of protein C and protein S (Bairey et al. 1997).

Although we observed an increase in TAT levels in cyclophosphamide and acrolein-treated mice, APC levels remained below the limit of detection of the assay (figure 6.3). This suggests that despite elevated thrombin generation (and elevated PC antigen levels) there was no large corresponding increase in APC generation. A more

Figure 6.3 – Quantification of activated protein C levels in plasma of acrolein and cyclophosphamide-treated mice. Mice received IP injections of cyclophosphamide (140 mg/kg), or saline on days 1, 3, and 5, and blood was collected on day 5 via carotid artery cannulation. Mice received IP injections of acrolein on days 1 and 3, and blood was collected on day 4. Activated Protein C were measured using ELISA using mAPC as a standard as described in Chapter 4. (A) Standard curve. (B) Dot plot. N = 9 – 12.


A.
APC standard curve
0.25
0.20
0.15
0.10
0.05
0.00
OD 405
All curves:
Coefficients:
b[0] 5.3428674216e-3
b[1] 8.4413856643e-3
r ² 0.995574372
0
5
10
15
20
25
30
Concentration mAPC ng/mL
B.
5
4
3
2
1
0
-1
ng/mL APC
Lower limit
of detection
Saline
control
(n = 9)
Acrolein
5 mg/kg
(n = 12)
CPA
100 mg/kg
(n = 9)

sensitive method for quantifying murine APC levels is required in order to clarify this issue. However, our results are consistent with previous studies which have demonstrated that chemotherapy decreases levels of APC despite increasing thrombin generation (Mukherjee et al. 2010).

To our knowledge, our studies were the first to quantify APC generation over time on endothelial cells using an enzyme capture assay. These studies were also the first to measure PC and APC levels in mice in response to treatment with chemotherapy. The PC/APC quantification methods employed in this study may help to better characterize the relationship between thrombin generation and production of APC in future investigations. Overall, our results indicate that chemotherapy agents may impair the PC anticoagulant pathway by modulating endothelial cell surface levels of EPCR, resulting in impaired PC activation in response to elevated thrombin generation. Our studies offer novel insight into the mechanisms by which chemotherapy may impair the PC pathway, thereby contributing to a procoagulant phenotype in breast cancer patients.

6.4 Effects of breast cancer chemotherapy agents on thrombin generation

Thrombin is the end product of the coagulation cascade which converts soluble fibrinogen to an insoluble fibrin clot. Measurement of the conversion of prothrombin to thrombin using a thrombin generation assay can reveal how the modulation of various hemostatic pathways can contribute to an overall increase in procoagulant activity. In our studies we demonstrated that breast cancer chemotherapy agents can increase TF activity, phosphatidylserine exposure, CFDNA release, and impair protein C activation, all of which may promote the generation of a procoagulant phenotype. We assessed the effects of these modified pathways on thrombin generation.

Endothelial or smooth muscle cells were grown to confluency, treated with chemotherapy agents, washed, and exposed to recalcified, defibrinated plasma. Levels of thrombin generated at 5 minute intervals were quantified by cleavage of chromogenic substrate using a sub-sampling method. Treatment of endothelial cells with doxorubicin, epirubicin, and acrolein increased thrombin generation when exposed to recalcified, defibrinated plasma (figures 3.1, 4.1A and 4.1B). Treatment of UASMCs with acrolein also increased thrombin generation in recalcified, defibrinated plasma (figures 4.1C and 4.1D). As well, the addition of CFDNA to recalcified plasma increased thrombin generation (figure 4.4). These results were confirmed by comparisons of AUC, lag time, velocity index and/or peak thrombin.

Blocking phosphatidylserine exposure on doxorubicin and acrolein-treated endothelial cells with annexin V significantly attenuated TF activity, which was consistent with the chemotherapy-induced upregulation of phosphatidylserine exposure (figure 3.1D and 4.1A). In contrast, the inhibition of TF with a blocking antibody impaired thrombin generation on doxorubicin-treated endothelial cells (figure 3.1D), but did not modulate thrombin generation on acrolein-treated endothelial cells (figure 4.1A). While both agents significantly increased TF activity on endothelial cells, we believe this difference was due to the fact that at high levels of doxorubicin treatment, the average increase in TF activity (approximately 60 pM Xa generated/10,000 cells) was much greater than the average increase in TF activity (approximately 5 pM Xa generated/10,000 cells) on HUVECs treated with the highest concentration of acrolein (figures 3.2A and 4.3A).

Exposure of acrolein-treated UASMCs to recalcified, defibrinated plasma also increased thrombin generation, which was impaired by blocking TF activity or phosphatidylserine exposure (figure 4.1C and D). Interestingly, while acrolein treatment of UASMCs increased TF activity (figure 4.3D), it did not increase phosphatidylserine exposure (figure 4.2B). As background levels of phosphatidylserine exposure on UASMCs were fairly high (approximately 15%, as measured by fraction of cells annexin V positive cells), this suggests that background phosphatidylserine is necessary to facilitate thrombin generation in both treated and untreated conditions. As evidence for this, thrombin generation was decreased below the levels generated on untreated cells when annexin V was used to block phosphatidylserine exposure on acrolein-treated UASMCs (figure 4.1C).

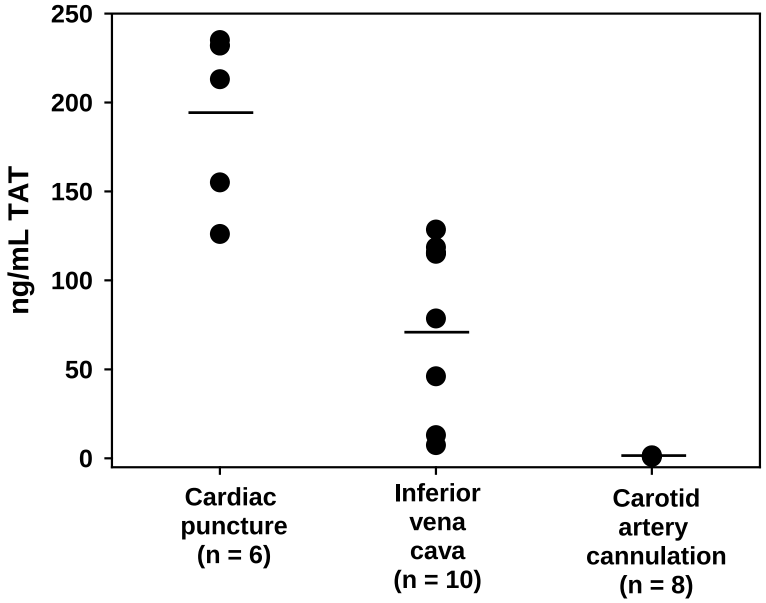
We also examined the role that release of CFDNA may play in activating the contact pathway. We found that treatment of venous whole blood with chemotherapy agents increased release of CFDNA (figure 5.3A), but decreased association of CFDNA with histone proteins (figure 5.3B), suggesting that CFDNA released by cells exposed to doxorubicin and epirubicin *in vivo* may have a greater negative charge density and thus able to promote coagulation activation through the contact pathway. We measured the effects of purified CFDNA on thrombin generation using a calibrated, automated thrombin generation assay. We found that thrombin generation was increased when pooled, recalcified plasma was exposed to increasing concentrations of CFDNA isolated from the plasma of epirubicin-treated whole blood (figure 5.4A). This was inhibited with CTI (figure 5.5A), an inhibitor of FXIIa, but not with a TF blocking antibody (figure 5.4B) suggesting that coagulation activation was through the contact pathway. The effect was confirmed by performing thrombin generation assays in FXII and FXI deficient plasmas (figure 5.5B and 5.5C).

In chapter 3 we hypothesize that increased thrombin generation on chemotherapy-treated endothelial cells exposed to recalcified, defibrinated plasma may be related to impaired APC generation in the system. While *in vivo* APC dampens thrombin generation, and impaired formation of APC can result in increased thrombin generation, our *in vitro* methods were not sensitive enough to capture this effect. Previous studies indicate that blocking EPCR or thrombomodulin with inhibitory antibodies on endothelial cell surfaces does not increase thrombin generation in our model, although APC generation in defibrinated plasma is impaired (Pepler 2009). In addition, thrombin generation assays performed in PC deficient plasma did not increase thrombin generation relative to PC deficient plasma supplemented with 100% PC. This is likely due to the fact that the number of endothelial cells present in our thrombin generation assay compared to the plasma volume used is low relative to the ratio of surface area of the endothelium to the blood *in vivo*.

To confirm the relevance of our *in vitro* thrombin generation methods *in vivo*, we treated healthy Balb/C mice with IP injections of chemotherapy agents. Blood was drawn by carotid artery cannulation rather than cardiac puncture or through IVC to minimize artificial thrombin activation during collection (figure 6.4). Mice treated with 3 injections of cyclophosphamide (day 1, 3, and 5 with blood collection on day 6), or 2 injections of acrolein (day 1, and 3 with blood collection on day 4) had elevated levels of TAT relative to saline controls (figure 4.6A and 4.6B). Mice treated with 1 injection of doxorubicin, epirubicin, or 5-fluororuracil had elevated levels of TAT relative to saline controls (figure 5.2D). Mice treated with 1 injection of methotrexate or cyclophosphamide did not have elevated levels of TAT (data not shown). The chemotherapy-induced release of CFDNA in breast cancer patients (figure 5.1) and mice (figure 5.2) parallels the observed increase in TAT complex formation in chemotherapy-treated breast cancer patients (Mukherjee et al. 2010).

To our knowledge, we are the first to propose that chemotherapy induces the release of CFDNA, a novel mechanism by which coagulation may be activated in breast cancer chemotherapy patients. In addition, the demonstration that chemotherapy increases thrombin generation in defibrinated plasma exposed to endothelial and smooth muscle cells is also a novel finding. We are also the first to directly measure thrombin generation in chemotherapy-treated mice. Our data is consistent with other studies which found that cyclophosphamide administration can increase fibrin deposition and thrombogenesis in mouse models (Yu et al. 2008; Zeng et al. 2008).

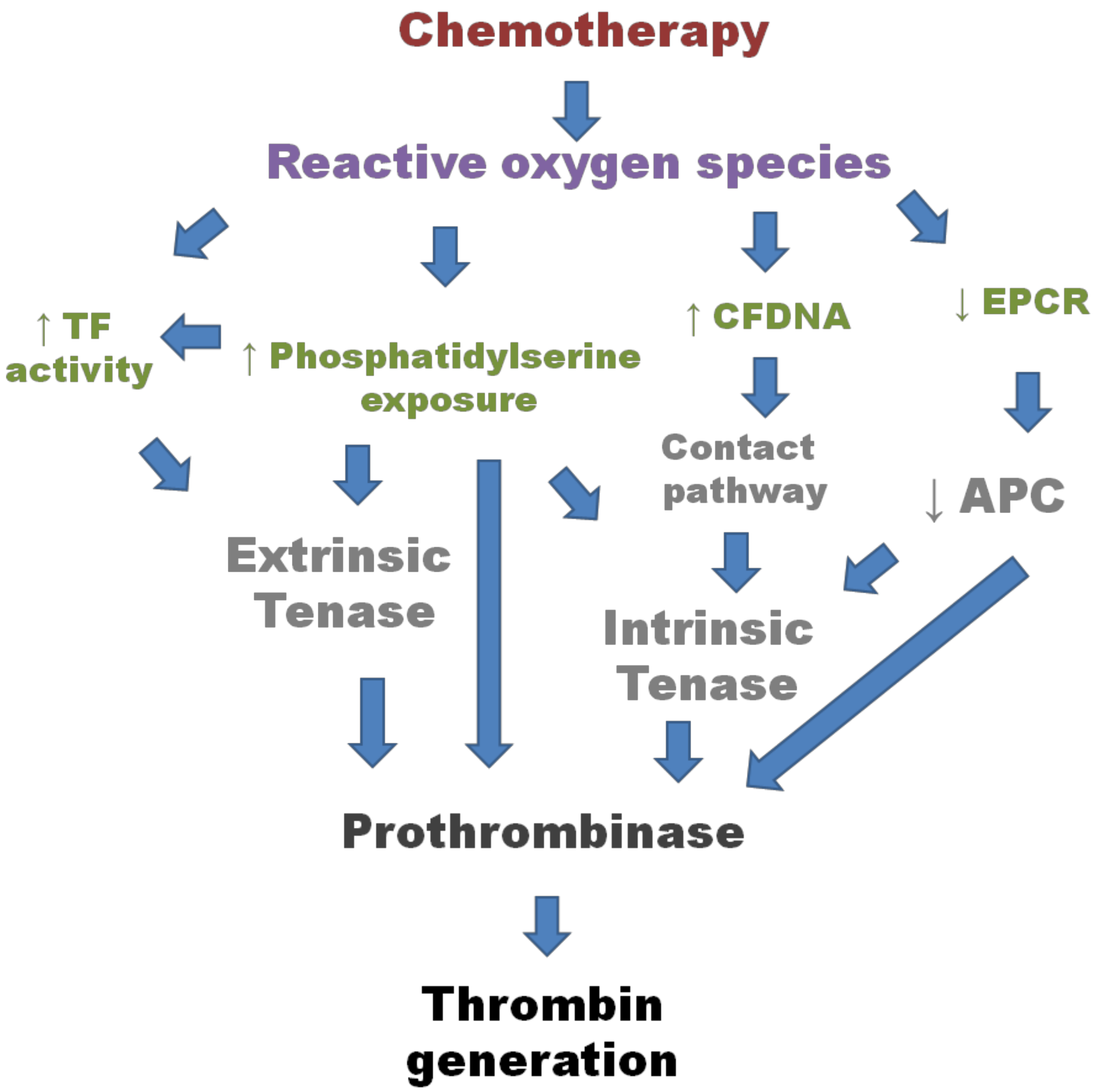
Figure 6.4 – Sensitivity of murine TAT levels to method of blood collection. Blood from healthy Balb/c mice was collected via carotid artery puncture, inferior vena cava, and carotid artery cannulation. TATs were measured using ELISA. N = 6


250
200
150
100
50
0
ng/mL TAT
Cardiac puncture (n = 6)
Inferior vena cava (n = 10)
Carotid artery cannulation (n = 8)

These studies suggest that breast cancer chemotherapy-induced thrombin generation can be initiated by modulation of hemostatic properties of host cells, independent of the presence of tumour procoagulant activity. In addition, our findings indicate that several breast cancer chemotherapy agents, doxorubicin, epirubicin, 5-fluorouracil, and cyclophosphamide, have procoagulant effects *in vitro* and/or *in vivo*, suggesting that the activation of thrombin generation in chemotherapy patients may be the result of additive or synergistic procoagulant effects of multi-drug regimens.

Overall, these studies suggest a mechanism of thrombin generation in breast cancer chemotherapy patients that is initiated through the TF pathway by de-encryption, and the contact pathway by the release of procoagulant CFDNA. Exposure of phosphatidylserine on the endothelial cell surface, or on the surface of other blood or vascular cells, may enhance thrombin generation by increasing tenase and prothrombinase activity. Finally, *in vivo* impairment of APC formation may also contribute to elevated thrombin generation. Taken together, these studies provide an explanation for the chemotherapy-induced increase in TAT generation in breast cancer patients and provide novel insights into the mechanisms by which chemotherapy can induce a procoagulant phenotype *in vivo*.

Figure 6.5 – Mechanisms by which breast cancer chemotherapy agents modulate thrombin generation. Reactive oxygen species generated by chemotherapy agents increase TF activity, phosphatidylserine exposure, CFDNA release and decrease EPCR expression. This leads to elevated tenase and prothrombinase activity, resulting in increased thrombin generation.


Chemotherapy
Reactive oxygen species
↑ TF activity
↑ Phosphatidylserine exposure
↑ CFDNA
↓ EPCR
Contact pathway
↓ APC
Extrinsic Tenase
Intrinsic Tenase
Prothrombinase
Thrombin generation

6.6 Contribution of ROS to breast cancer chemotherapy agent-induced procoagulant activity

Chemotherapy agents such as doxorubicin, epirubicin, and acrolein convey many of their cytotoxic effects through the generation of ROS. We investigated the involvement of ROS in mediating the procoagulant effects exerted by breast cancer chemotherapy drugs. We found that similar to chemotherapy agents, the ROS H_2O_2 increases TF activity as well as phosphatidylserine exposure on endothelial cells (figure 3.4B and 3.4C). As the H_2O_2-induced increase in TF antigen levels were modest (figure 3.4B), and blocking H_2O_2–treated HUVEC with annexin V partially attenuated tissue factor activity (figure 4.3C), we suggest that the H_2O_2-induced increase in TF activity is at least partially mediated by de-encryption associated with phosphatidylserine exposure (figure 3.4C). In contrast, while H_2O_2 increased TF activity on UASMCs without modifying antigen levels (consistent with previous studies (Penn et al. 1999)), blocking of phosphatidylserine with annexin V did not attenuate TF activity (figure 4.3F). This was consistent with the mechanism of TF de-encryption observed on acrolein-treated UASMCs (4.3D). To confirm the role of ROS in mediating TF de-encryption and phosphatidylserine exposure, we demonstrated that the antioxidant glutathione can attenuate the doxorubicin and acrolein-induced increase in endothelial cell TF activity (figure 3.4A, 4.3A), the acrolein-induced increase in UASMC TF activity (figure 4.3D), and H_2O_2 and acrolein-induced increases in endothelial phosphatidylserine exposure (figure 3.4C and 4.2A).

We also found that ROS generation may be involved in CFDNA release from epirubicin-treated whole blood. While CFDNA release could not be impaired by inhibiting apoptosis or necrosis, we found that glutathione attenuated epirubicin-induced CFDNA release (figure 5.6). Although our experiments did not demonstrate that H_2O_2 could release CFDNA from venous whole blood (data not shown), other studies have found that H_2O_2 induces the release of NETs from treated neutrophils, and that ROS generating pathways may be involved in NET formation (Fuchs et al. 2007).

In addition, we demonstrated that ROS generation may modify the protein C anticoagulant pathway. H_2O_2 treatment increased thrombomodulin expression and decreased EPCR expression on the endothelial cell surface (figure 4.5), consistent with previous studies (Xu et al. 2000). We have also previously demonstrated that the antioxidant glutathione can attenuate the doxorubicin-induced decrease in EPCR expression (Woodley-Cook et al. 2006).

Overall, these studies suggest that chemotherapy drugs induce the generation of ROS leading to a state of oxidative stress. ROS likely exert many of the procoagulant effects of these agents, either by participating in cell signalling or by directly damaging proteins or nucleic acids. To our knowledge, we are the first to demonstrate that H_2O_2 can increase TF activity on endothelial cells, and that glutathione can attenuate many procoagulant effects of doxorubicin, epirubicin, and acrolein. These studies provide novel insights into the mechanisms by which chemotherapeutic agents exert their procoagulant effects. They also suggest potential mechanisms by which oxidative stress may result in the generation of a procoagulant phenotype.

6.7 Future directions

6.7.1 Confirm the role of CFDNA in promoting thrombin generation in response to breast cancer chemotherapy

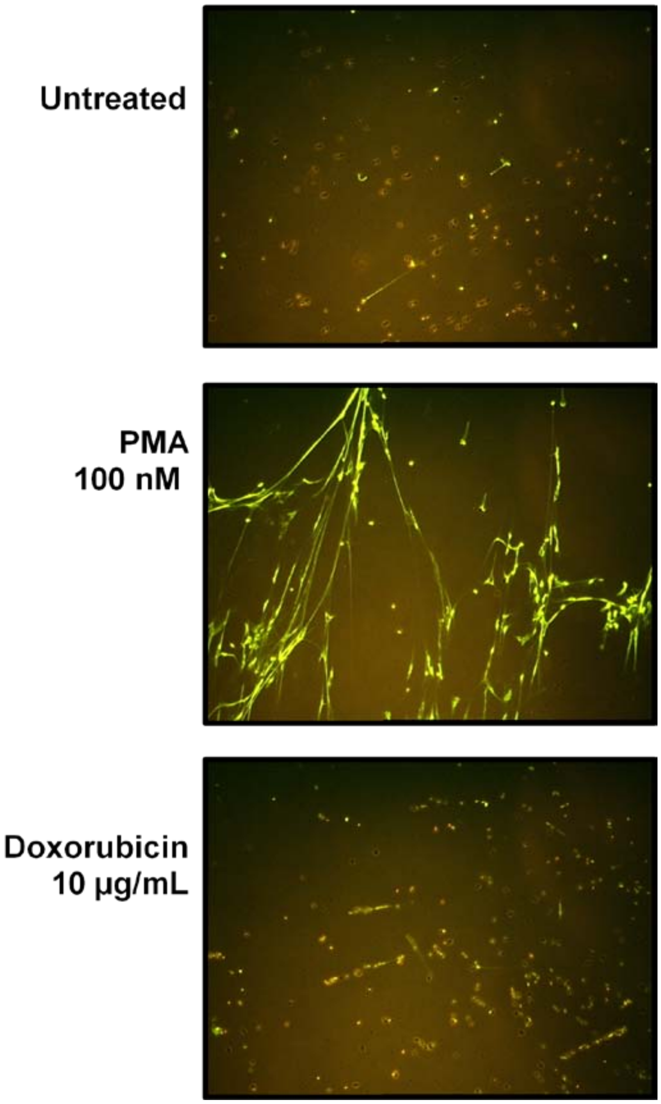
Our studies in Chapter 5 suggest that chemotherapy induces the release of CFDNA in breast cancer patients and in healthy mice, which parallels thrombin generation. We propose two mechanisms by which CFDNA release may be procoagulant *in vivo*: (1) by initiation of coagulation through the contact pathway, and (2) by the generation of NETs, which exhibit a variety of procoagulant influences. Further studies would strengthen the evidence we presented. Our *in vitro* studies suggest that this CFDNA may be, at least in part, neutrophil in origin, and is released by a ROS-dependent mechanism rather than through apoptotic or necrotic cell death. This data is consistent with previous studies of NET formation (Fuchs et al. 2007). However, it should be determined if doxorubicin and epirubicin treatment of neutrophils promotes NET formation.

NET formation by chemotherapy-treated neutrophils was measured with fluorescent microscopy. Briefly, neutrophils isolated from venous whole blood as described in Chapter 5, were incubated in the presence of doxorubicin (10 µg/mL) or PMA (100 nM) on glass cover slips for 4 hours. NETs were stained with sytox green, a nucleic acid stain that does not cross the intact cell membrane, and visualized with fluorescent microscopy (10X magnification). Results generated thus far clearly demonstrate an increase in NET formation by PMA (positive control) (figure 6.6);

however, it is less certain if chemotherapy treatment increases NET formation. These experiments are ongoing.

Although our studies in Chapter 5 established association between CFDNA release and thrombin generation in response to treatment with breast cancer chemotherapy drugs, we did not establish a causative effect. We have shown that the addition of purified CFDNA to recalcified plasma increased thrombin generation, and others have found that administration of exogenous RNA to rabbits increases thrombin activity (Kannemeier et al. 2007). To extend these experiments, it would be interesting to demonstrate that CFDNA isolated from chemotherapy treated whole blood is procoagulant *in vivo*. We propose to administer isolated CFDNA to healthy mice via injection into the jugular vein. Blood would be collected 5 – 30 minutes post treatment though cannulation of the carotid artery. TAT and CFDNA levels would be quantified as described in Chapter 5. Further animal studies could demonstrate a causative relationship between CFDNA release and thrombin generation. Administration of recombinant human DNAse or a FXII inhibitor such as H-D-pro-phe-arg-chloromethylketone (Bachem, Basel, Switzerland) to chemotherapy treated mice may attenuate thrombin generation. Alternatively, studies may also be conducted in FXII or FXI deficient mice and thrombin generation compared to contact pathway proficient controls. These studies would demonstrate a causative effect between CFDNA release and thrombin generation, rather than a corollary relationship.

Figure 6.6 – Generation of NETS by chemotherapy-treated neutrophils. Neutrophils were isolated from venous whole blood as described in Methods, and treated with doxorubicin or PMA for 4 hours. NETS were visualized by staining with Sytox Green using fluorescent microscopy.


Untreated
PMA
100 nM
Doxorubicin
10 µg/mL

6.7.2 Investigate the effects of breast cancer chemotherapy agents on breast tumour cells in vitro and in vivo

Our studies have focused on the ability of breast cancer chemotherapy agents to modify hemostatic pathways on healthy host blood and vascular cells. These studies parallel many of the published clinical studies which investigate the effects of adjuvant chemotherapy on alteration of hemostatic pathways in early stage (I – III) breast cancer patients. These patients have typically undergone lumpectomy and/or radiation, with chemotherapy used to prevent relapse due to occult disease. As a result, these patients have a relatively low tumour burden. However, chemotherapy may also be administered to patients with larger tumours: as neoadjuvant to decrease tumour volume prior to surgery, or in patients with stage IV (metastatic) breast cancer. In these patients in particular, the modulation of hemostatic pathways on tumour cells by chemotherapy may further increase the risk for thrombosis. Supportive of this, clinical data suggests that the risk for thrombosis increases in patients with advanced stage breast cancer receiving chemotherapy relative to those with early stage breast cancer (Goodnough et al. 1984).

We have conducted some preliminary studies on the effects of breast cancer chemotherapy agents on the hemostatic factors expressed by MDA-MB-231 cells, a breast tumour cell line. We found that MDA-MB-231 cells have a high baseline TF activity status relative to endothelial cells, SMCs, and monocytes (figure 6.1). In addition, doxorubicin, epirubicin, acrolein, 5FU, and H_2O_2 significantly increased TF activity on these breast tumour cells. Further *in vitro* studies are warranted to investigate the effects of breast cancer chemotherapy agents on breast tumour cell TF antigen levels, phosphatidylserine exposure, and thrombin generation in recalcified plasma.

In addition, the creation of a mouse breast tumour xenograft model to measure the effects of breast cancer chemotherapy *in vivo* would be novel and relevant to these studies. Briefly, 2 million MDA-MB-231 cells would be diluted in PBS and injected subcutaneously into 6 week old female Cb-17 SCID mice. After 4 – 6 weeks of tumour growth, mice would be treated with saline, or chemotherapy would be administered as single agents or in combination. Blood would be collected by cannulation of the carotid artery, and levels of pro- and anticoagulant factors could be measured as described in Chapters 4 and 5. Interesting hemostatic markers to investigate include TAT, PC, APC, CFDNA, and MP TF activity (as MP TF activity increases in metastatic breast cancer patients). Fibrin deposition in organs could be quantified, and markers of platelet activation such as P-selectin, or fibrinolysis, such as D-dimers or PAI-1, could also be measured.

Additionally, it would be novel and relevant to compare the effects of chemotherapy versus chemohormonal therapy (standard breast cancer chemotherapy plus tamoxifen) on the hemostatic pathway. As MDA-MB-231 cells are ER-negative, an ER-positive breast tumour cell line such as MCF-7 could be used to generate a second group of xenograft animals. This could be particularly interesting, as MDA-MB-231 cells have high levels of TF activity, while MCF-7 levels have lower TF activity levels (Hu et al. 1993).

Measuring phosphatidylserine exposure and thrombin generation in recalcified plasma would more thoroughly investigate hemostatic pathways modulated by breast cancer chemotherapy agents *in vitro*. In addition, these studies would demonstrate the contribution of tumour cells to the activation of coagulation using an *in vivo* model. These studies could also investigate the effects of combination chemotherapy on markers of coagulation relative to administration of single agents. These studies might also elucidate the mechanism(s) by which treatment with tamoxifen plus chemotherapy may further increase the risk for thrombosis in this patient population.

6.7.3 Investigate the effects of thalidomide and dexamethasone on hemostatic pathways in vivo

In addition to investigating the mechanisms by which chemotherapy increases the risk for thrombosis in breast cancer patients, we have also investigated the mechanisms by which the anti-cancer agents thalidomide and dexamethasone increase the risk for thrombosis in multiple myeloma (MM) patients. MM patients treated with thalidomide as a single agent have a risk of thrombosis of less than 3% (Rajkumar et al. 2006). However, when thalidomide treatment is combined with the glucocorticoid dexamethasone (thal/dex), the rate of thrombosis increases to between 8 – 16% (Cavo et al. 2004; Anagnostopoulos et al. 2003). In addition, when thalidomide or thal/dex treatment is combined with chemotherapy, the risk is increased to over 16% (Zangari et al. 2002).

Thalidomide exerts its anti-cancer effects by several mechanisms: (1) inhibition of angiogenesis by decreasing VEGF synthesis, (2) inhibition of myeloma cell growth, (3) decrease in synthesis of pro-inflammatory cytokines and (4) stimulation of T-cell and natural killer-cells (Garcia-Sanz 2006). Some of thalidomide's anti-cancer activities are conveyed by the parent compound, while others are mediated by the effects of thalidomide metabolites (such as PGA (phthaloyl glutamic acid), CPS3, and CPS11), generated by cytochrome P450-dependent metabolism. Dexamethasone is a glucocorticoid steroid hormone with anti-inflammatory and immunosuppressant properties and potentiates thalidomide's anti-cancer effects.

Few clinical studies have investigated the mechanism(s) by which thal/dex treatment can increase the risk for thrombosis in MM patients. One investigation of 5 MM patients receiving thalidomide found elevated levels of platelet surface P-selectin and platelet-leukocyte aggregates, markers of platelet activation (Dunkley et al. 2007). Thal/Dex treatment is also associated with increased plasma levels of fibrinogen (Petropoulou et al. 2008). Recent *in vitro* studies have demonstrated that endothelial cells treated with TNFα, and/or IL-6, and then co-stimulated with thalidomide, can increase endothelial cell TF activity and the generation of TF-positive MPs (Valsami et al. 2011). Another study found that co-stimulation of the MonoMac6 cell line with thalidomide plus TNFα also increased TF activity (Arkel et al. 2003).

We have investigated the effects of thalidomide, thalidomide metabolites CPS3, CPS11, and PGA (kindly provided by Dr. William Figg, National Cancer Institute, Bethesda, MD, USA), and/or dexamethasone on hemostatic pathways on endothelial cells and blood monocytes. We found that at high *in vitro* concentrations, these drugs did not significantly modulate endothelial cell TF, phosphatidylserine exposure (figure 6.6), or expression of EPCR, or thrombomodulin (figure 6.8). Consistent with these studies, we found that thrombin generation was not increased on thalidomide or dexamethasone-treated endothelial cells exposed to recalcified, defibrinated plasma (figure 6.9). Similar to recently-published data (Hoshi et al. 2011), we found that high dose dexamethasone increased TF activity on THP-1 cells (figure 6.10). However, we were uncertain as to the relevance of this data *in vivo*, as only a very modest increase in TF activity was observed on blood monocytes stimulated with thalidomide and/or dexamethasone. In addition, we found that dexamethasone treatment increased TF activity and antigen levels on UASMCs (figure 6.11). This may be relevant *in vivo*, as arterial thrombosis has been documented in MM patients receiving immunomodulatory/chemotherapy including dexamethasone (Libourel et al. 2010). We also found that dexamethasone increases TF activity on the MM cell line NCI-H292 after a 72 hour incubation period, but not on RPMI-8226 or U266 cells (figure 6.12).

As thalidomide requires *in vivo* metabolic activation in order to exert many of its anti-angiogenic and immunomodulatory effects, we propose that further studies on the effects of thalidomide and dexamethasone be conducted using a mouse xenograft tumour model. Tumour-bearing mice would be created with NCI-H292 cells which have elevated TF activity when treated with dexamethasone, and RPMI-8226 cells which do not. Mice would receive IP injections of dexamethasone, and treatments of thalidomide resuspended in carboxy-methylcellulose by oral gavage. Blood would be collected by cannulation of the carotid artery, and levels of pro- and anticoagulant factors would be measured. In particular, it would be interesting to measure TAT levels in these mice in

Figure 6.7 – Effects of thalidomide and dexamethasone on endothelial cell procoagulant activity. HUVECs were grown to confluency, and treated with thalidomide (50 μg/mL), thalidomide metabolites (12.5 μg/mL), or dexamethasone (1 mM) for 24 hours. TF activity and phosphatidylserine exposure were measured as described in Chapter 3. (A) TF antigen levels. (B) Phosphatidylserine exposure.

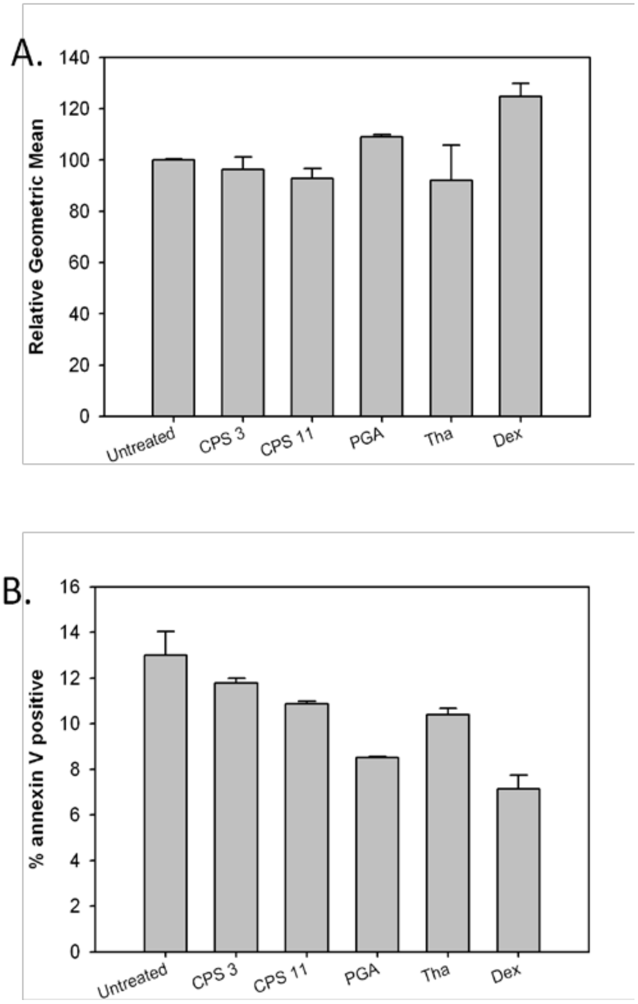

A.
Relative Geometric Mean
0
20
40
60
80
100
120
140
Untreated
CPS 3
CPS 11
PGA
Tha
Dex
B.
% annexin V positive
0
2
4
6
8
10
12
14
16
Untreated
CPS 3
CPS 11
PGA
Tha
Dex

Figure 6.8 – Effects of thalidomide and dexamethasone on the endothelial cell protein C pathway. HUVECs were grown to confluency, and treated with thalidomide (50 μg/mL), thalidomide metabolites (12.5 μg/mL), or dexamethasone (1 mM) for 24 hours. EPCR and thrombomodulin levels were measured as described in Chapter 3. (A) EPCR expression. (B) Thrombomodulin expression.

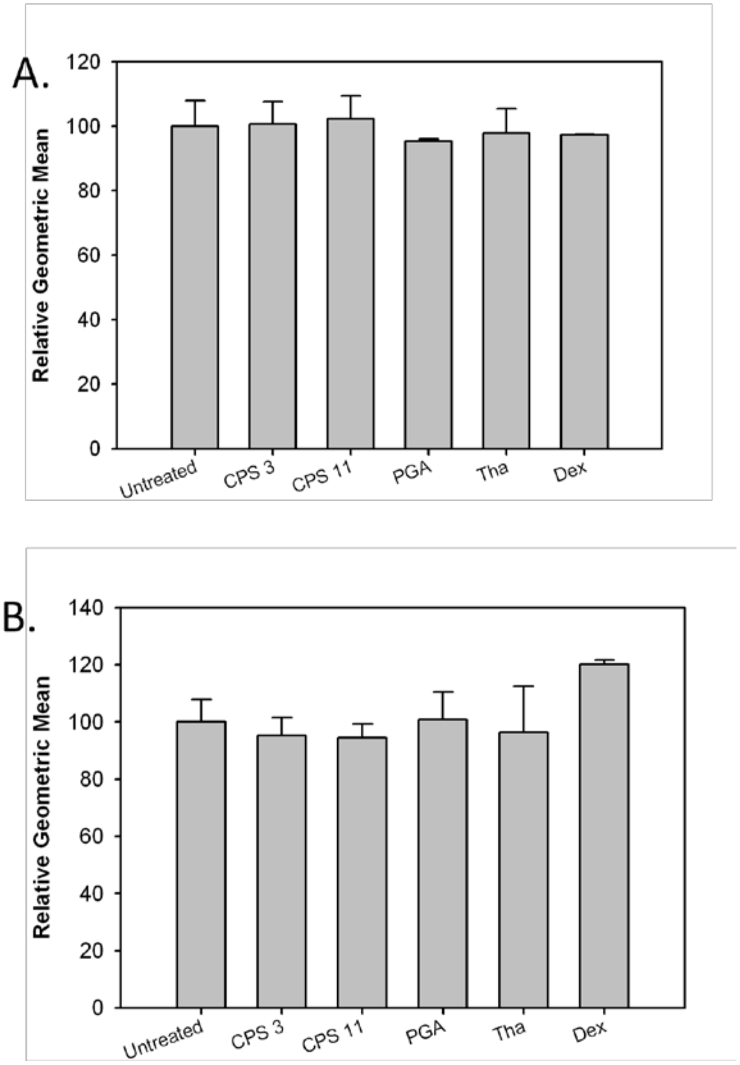
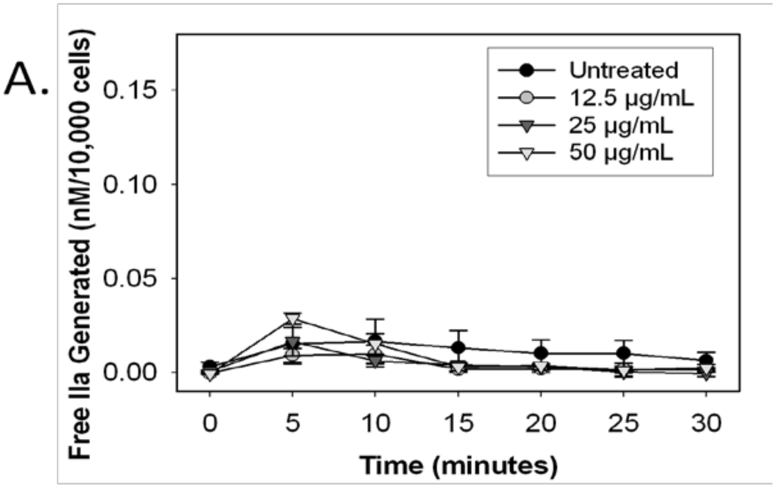
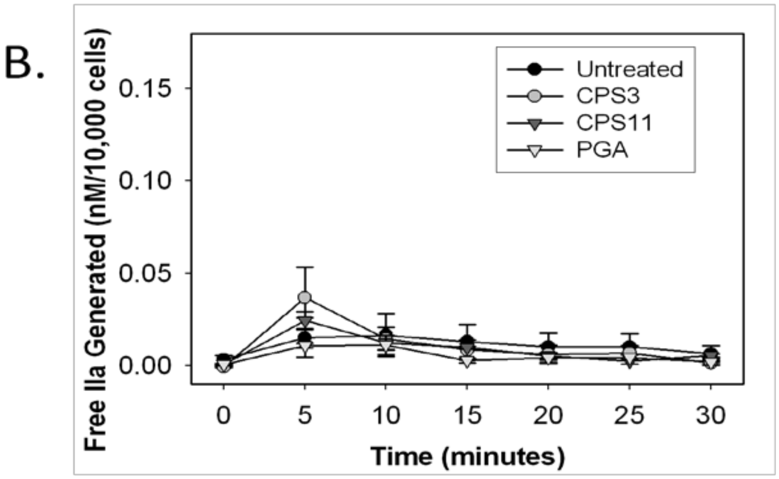

A.
Relative Geometric Mean
0
20
40
60
80
100
120
Untreated
CPS 3
CPS 11
PGA
Tha
Dex
B.
Relative Geometric Mean
0
20
40
60
80
100
120
140
Untreated
CPS 3
CPS 11
PGA
Tha
Dex

Figure 6.9 – Effects of thalidomide and dexamethasone-treated cells on thrombin generation in defibrinated plasma. HUVECs were grown to confluency, and treated with thalidomide (50 µg/mL), thalidomide metabolites (12.5 µg/mL), or dexamethasone (1 mM) for 24 hours. Thrombin generation assays were performed in defibrinated plasma as described in Chapter 3. (A) Thrombin generation in the presence of thalidomide treated HUVECs. (B) Thrombin generation in the presence of thalidomide metabolite treated HUVECs. (C) Thrombin generation in the presence of dexamethasone or dex/thal treated HUVECs.


A.
Free IIa Generated (nM/10,000 cells)
0.15
0.10
0.05
0.00
0
5
10
15
20
25
30
Time (minutes)
Untreated
12.5 µg/mL
25 µg/mL
50 µg/mL


B.
Free IIa Generated (nM/10,000 cells)
0.15
0.10
0.05
0.00
0
5
10
15
20
25
30
Time (minutes)
Untreated
CPS3
CPS11
PGA

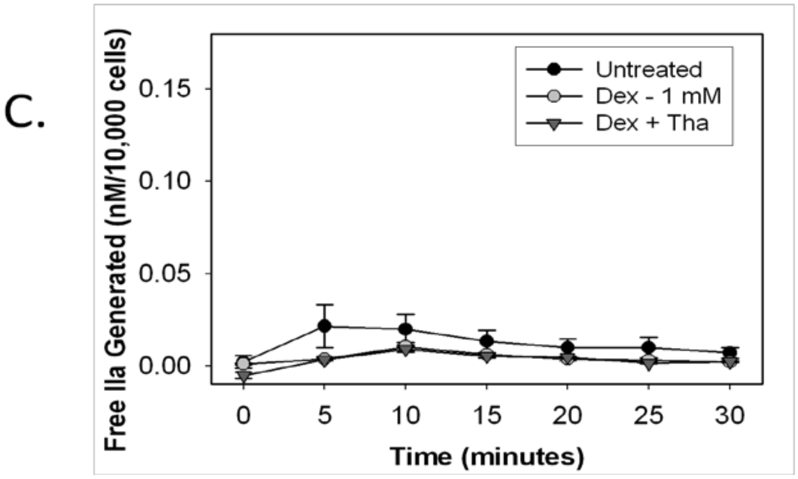

C.
Free IIa Generated (nM/10,000 cells)
0.15
0.10
0.05
0.00
0
5
10
15
20
25
30
Time (minutes)
Untreated
Dex - 1 mM
Dex + Tha

Figure 6.10 – Effects of thalidomide and dexamethasone on monocyte TF activity. Monocytes isolated from venous whole blood or THP-1 cells were treated with thalidomide (50 µg/mL), and/or dexamethasone (1 mM) for 24 hours. TF activity assays were performed in defibrinated plasma as described in Chapter 3. (A) Monocytes. (B) THP-1 cells.

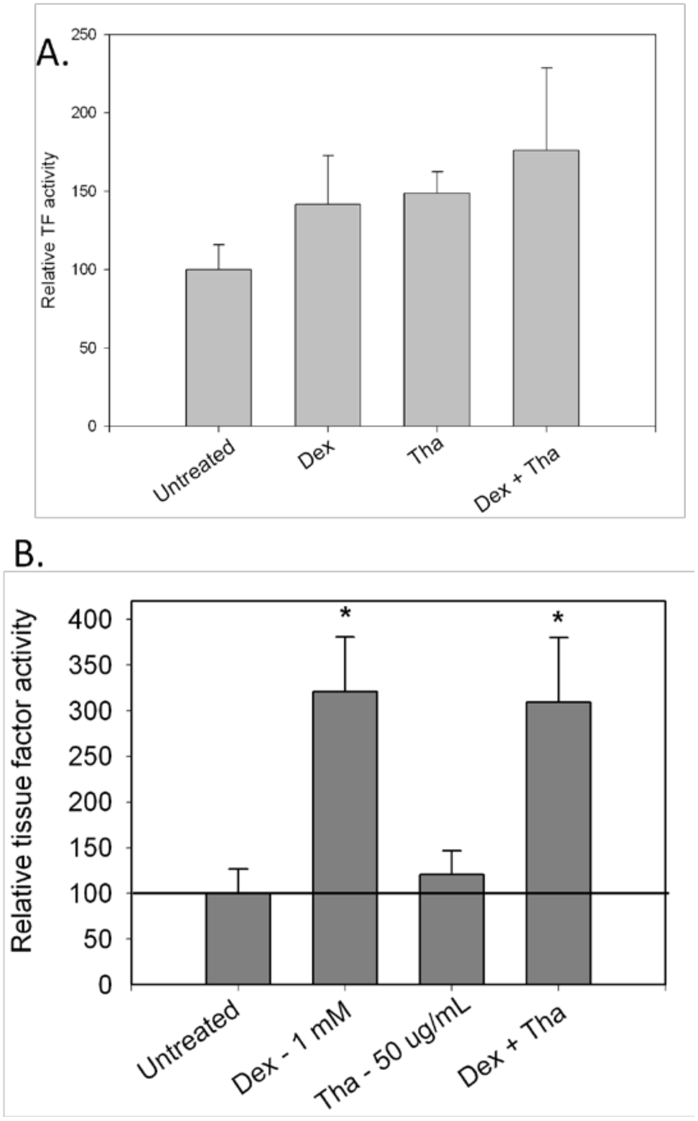

A.
Relative TF activity
0
50
100
150
200
250
Untreated
Dex
Tha
Dex + Tha
B.
Relative tissue factor activity
0
50
100
150
200
250
300
350
400
*
*
Untreated
Dex - 1 mM
Tha - 50 ug/mL
Dex + Tha

Figure 6.11 – Effects of dexamethasone and thalidomide on UASMC TF activity. UASMCs were grown to confluency and treated with dexamethasone and/or thalidomide for 24 hours. TF activity was measured as described in Chapter 3. TF antigen levels were measured by Western blot analysis. (A) TF activity. (B) TF antigen levels (1 mM Dexamethasone).

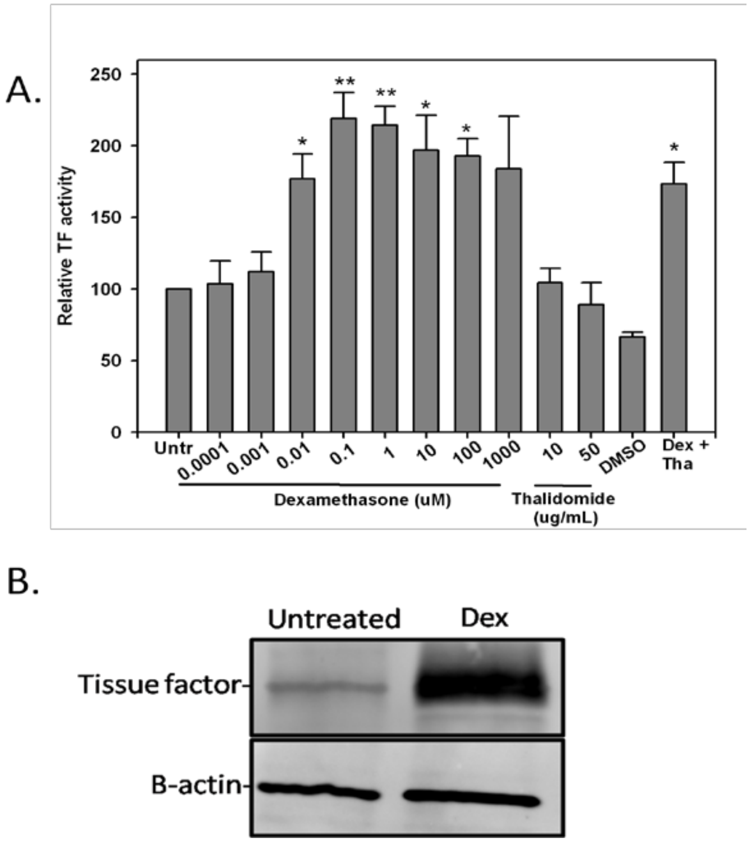
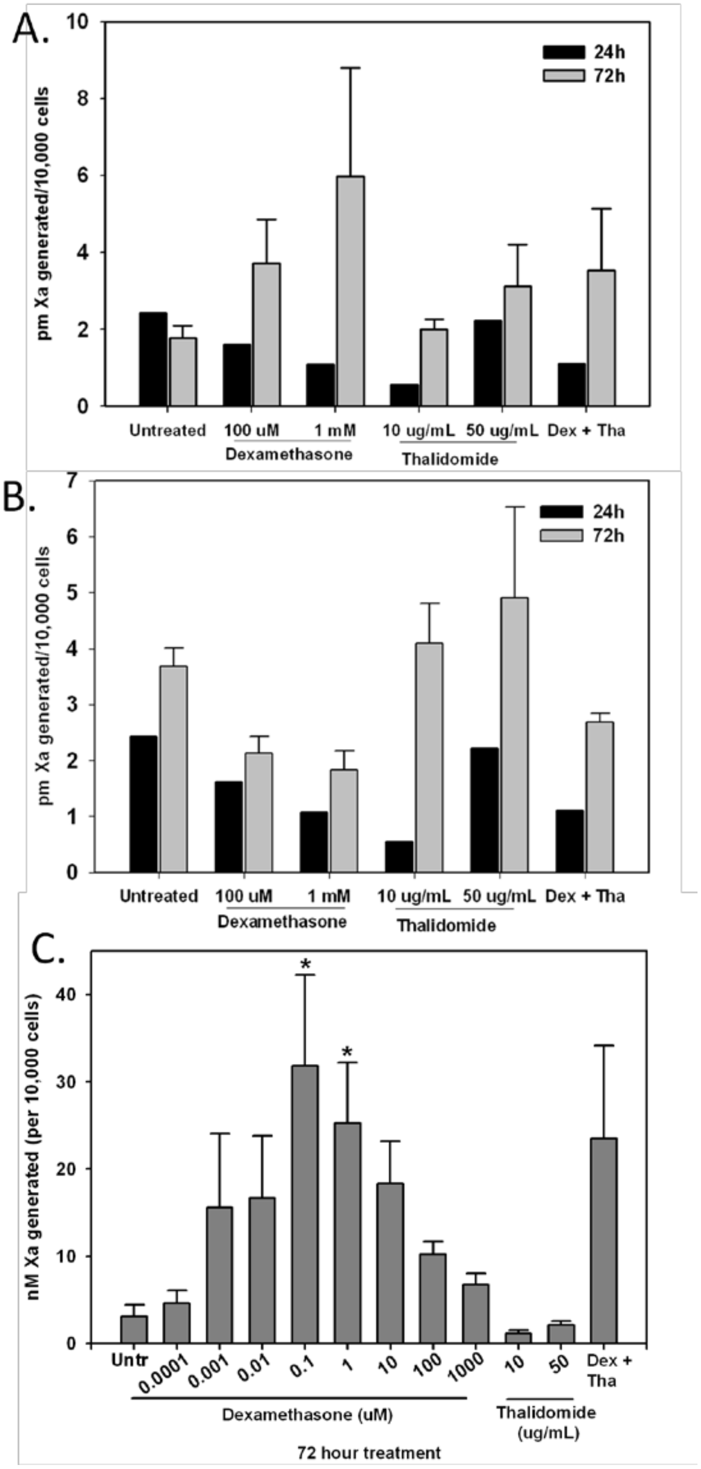

A.
Relative TF activity
250
200
150
100
50
0
**
**
*
*
*
*
Untr
0.0001
0.001
0.01
0.1
1
10
100
1000
10
50
DMSO
Dex + Tha
Dexamethasone (uM)
Thalidomide (ug/mL)
B.
Untreated
Dex
Tissue factor
B-actin

Figure 6.12 – Effects of dexamethasone and thalidomide on human myeloma cell TF activity. Myeloma cells were cultured at $1x10^6$ cells/mL, and treated with 0.1 – 1000 µM dexamethasone or 5 – 50 µg/mL thalidomide for 24 – 72 hours. TF activity was measured as described in Chapter 3. (A) RPMI-8226 – TF activity 24 and 72 hours. (B) U266 – TF activity 24 and 72 hours. (D) NCI -H929 – TF activity 72 hours.


A.
pm Xa generated/10,000 cells
0
2
4
6
8
10
24h
72h
Untreated
100 uM
1 mM
Dexamethasone
10 ug/mL
50 ug/mL
Thalidomide
Dex + Tha
B.
pm Xa generated/10,000 cells
0
1
2
3
4
5
6
7
24h
72h
Untreated
100 uM
1 mM
Dexamethasone
10 ug/mL
50 ug/mL
Thalidomide
Dex + Tha
C.
nM Xa generated (per 10,000 cells)
0
10
20
30
40
*
*
Untr
0.0001
0.001
0.01
0.1
1
10
100
1000
Dexamethasone (uM)
10
50
Thalidomide (ug/mL)
Dex + Tha
72 hour treatment

response to therapy, as activation of coagulation by thal/dex therapy in MM patients has not yet been thoroughly demonstrated. Other markers of coagulation activation, platelet activation or impaired anticoagulation or fibrinolysis could be measured as described in 6.7.1. These studies will provide novel insight into the mechanisms by which anti-cancer agents such as thalidomide and dexamethasone exert an increased risk for thrombosis in MM patients.

6.8 Conclusions

Chemotherapy increases the risk for thrombosis in breast cancer patients. However, the mechanisms by which these agents exert their procoagulant effects are largely uncharacterized. The studies presented in this thesis identify hemostatic pathways modulated by breast cancer chemotherapy agents, which may result in the generation of a prothrombotic phenotype in breast cancer patients. Since chemotherapy increases TAT levels in breast cancer patients, we first investigated the mechanisms by which chemotherapy agents can initiate and propagate coagulation. We found that chemotherapy can increase TF activity and phosphatidylserine exposure on endothelial and/or smooth muscle cells, resulting in elevated thrombin generation in recalcified, defibrinated plasma. We also found that chemotherapy can induce the release of CFDNA, which activates thrombin generation through the contact pathway when exposed to recalcified plasma. This data suggests that activation of coagulation in breast cancer patients receiving chemotherapy may be through TF and contact pathways. Breast cancer chemotherapy patients also present with decreased levels of the anticoagulant APC. We found that chemotherapy agents can impair the protein C anticoagulant pathway by decreasing endothelial EPCR cell surface expression. We also found that many of the procoagulant effects exhibited by breast cancer chemotherapy agents may be mediated by the generation of ROS. In summary, our studies demonstrate novel mechanisms by which chemotherapy agents may increase the risk for thrombosis in breast cancer patients. Our studies contribute to the growing knowledge base on the effects of chemotherapy on blood coagulation. Characterization of these mechanisms may assist in the identification of breast cancer patients with the greatest risk for chemotherapy-associated thrombosis, and suggest targets for customized prophylactic anticoagulant therapy.

7.0 REFERENCES

Agnelli,G., Gussoni G., Bianchini C., Verso M., Mandala M., Cavanna L., Barni S., Labianca R., Buzzi F., Scambia G., Passalacqua R., Ricci S., Gasparini G., Lorusso V., Bonizzoni E., and Tonato M. (2009). Nadroparin for the prevention of thromboembolic events in ambulatory patients with metastatic or locally advanced solid cancer receiving chemotherapy: a randomised, placebo-controlled, double-blind study. Lancet Oncol. *10,* 943-949.

Al-Mondhiry,H. (1975). Disseminated intravascular coagulation: experience in a major cancer center. Thromb. Diath. Haemorrh. *34,* 181-193.

Allen,D.H., Tracy P.B. (1995). Human coagulation factor V is activated to the functional cofactor by elastase and cathepsin G expressed at the monocyte surface. J Biol. Chem. *270,* 1408-1415.

Altincicek,B., Stotzel S., Wygrecka M., Preissner K.T., and Vilcinskas A. (2008). Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. J Immunol. *181,* 2705-2712.

Ambrus JL, Ambrus CM, Mink IB, and Pickren JW. (1975). Causes of death in cancer patients. J Med *6,* 61-64.

Ameri,A., Kuppuswamy M.N., Basu S., and Bajaj S.P. (1992). Expression of tissue factor pathway inhibitor by cultured endothelial cells in response to inflammatory mediators. Blood *79,* 3219-3226.

Ammollo,C.T., Semeraro F., Xu J., Esmon N.L., and Esmon C.T. (2011). Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation. J Thromb. Haemost. *9,* 1795-1803.

Anagnostopoulos,A., Weber D., Rankin K., Delasalle K., and Alexanian R. (2003). Thalidomide and dexamethasone for resistant multiple myeloma. Br. J Haematol. *121,* 768-771.

Arkel,Y.S., Ku D.H., and Thurston A.L. (2003). The effect of thalidomide on tissue factor activity in Mono Mac 6 cells and the relationship to tumor necrosis factor (TNF)-alpha-stimulated cells. J Thromb. Haemost. *1,* 2691-2692.

Bach,R., Gentry R., and Nemerson Y. (1986). Factor VII binding to tissue factor in reconstituted phospholipid vesicles: induction of cooperativity by phosphatidylserine. Biochemistry *25,* 4007-4020.

Bach,R.R. (2006). Tissue factor encryption. Arterioscler. Thromb. Vasc. Biol. *26,* 456-461.

Bachur,N.R., Yu F., Johnson R., Hickey R., Wu Y., and Malkas L. (1992). Helicase inhibition by anthracycline anticancer agents. Mol. Pharmacol. *41,* 993-998.

Baglin,T. (2005). The measurement and application of thrombin generation. Br. J. Haematol. *130,* 653-661.

Bairey,O., Gabbay U., Blickstein D., Stark P., Prokocimer M., Epstein O., Shaklai M., and Lahav J. (1997). Levels of proteins C and S do not decline subsequent to first line chemotherapy in lymphoma patients. Hematol. Oncol. *15,* 121-127.

Barrett,A.J., Starkey P.M. (1973). The interaction of alpha 2-macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. Biochem. J *133,* 709-724.

Bates,S.M., Weitz J.I. (2006). The status of new anticoagulants. Br. J. Haematol. *134,* 3-19.

Becker,B.F., Heindl B., Kupatt C., and Zahler S. (2000). Endothelial function and hemostasis. Z. Kardiol. *89,* 160-167.

Belloc,F., Lacombe F., Dumain P., Lopez F., Bernard P., Boisseau M.R., and Reifers J. (1992). Intercalation of anthracyclines into living cell DNA analyzed by flow cytometry. Cytometry *13,* 880-885.

Berlett,B.S., Stadtman E.R. (1997). Protein oxidation in aging, disease, and oxidative stress. J. Biol. Chem. *272,* 20313-20316.

Bertani,T., Poggi A., Pozzoni R., Delaini F., Sacchi G., Thoua Y., Mecca G., Remuzzi G., and Donati M.B. (1982). Adriamycin-induced nephrotic syndrome in rats: sequence of pathologic events. Lab Invest *46,* 16-23.

Bertomeu,M.C., Gallo S., Lauri D., Levine M.N., Orr F.W., and Buchanan M.R. (1990). Chemotherapy enhances endothelial cell reactivity to platelets. Clin. Exp. Metastasis *8,* 511-518.

Bevers,E.M., Comfurius P., van Rijn J.L., Hemker H.C., and Zwaal R.F. (1982). Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. Eur. J. Biochem. *122,* 429-436.

Bevilacqua,M.P., Pober J.S., Majeau G.R., Fiers W., Cotran R.S., and Gimbrone M.A., Jr. (1986). Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. Proc. Natl. Acad. Sci. U. S. A *83,* 4533-4537.

Biguzzi,E., Merati G., Liaw P.C., Bucciarelli P., Oganesyan N., Qu D., Gu J.M., Fetiveau R., Esmon C.T., Mannucci P.M., and Faioni E.M. (2001). A 23bp insertion in the endothelial protein C receptor (EPCR) gene impairs EPCR function. Thromb. Haemost. *86,* 945-948.

Binaschi,M., Capranico G., Dal B.L., and Zunino F. (1997). Relationship between lethal effects and topoisomerase II-mediated double-stranded DNA breaks produced by anthracyclines with different sequence specificity. Mol. Pharmacol. *51,* 1053-1059.

Boffa,M.C., Karmochkine M. (1998). Thrombomodulin: an overview and potential implications in vascular disorders. Lupus *7 Suppl 2,* S120-S125.

Bohlius,J., Wilson J., Seidenfeld J., Piper M., Schwarzer G., Sandercock J., Trelle S., Weingart O., Bayliss S., Djulbegovic B., Bennett C.L., Langensiepen S., Hyde C., and Engert A. (2006). Recombinant human erythropoietins and cancer patients: updated meta-analysis of 57 studies including 9353 patients. J Natl. Cancer Inst. *98,* 708-714.

Bombeli,T., Karsan A., Tait J.F., and Harlan J.M. (1997). Apoptotic vascular endothelial cells become procoagulant. Blood *89,* 2429-2442.

Boukerche,H., Berthier-Vergnes O., Penin F., Tabone E., Lizard G., Bailly M., and McGregor J.L. (1994). Human melanoma cell lines differ in their capacity to release ADP and aggregate platelets. Br. J. Haematol. *87,* 763-772.

Boveris,A., Oshino N., and Chance B. (1972). The cellular production of hydrogen peroxide. Biochem. J. *128,* 617-630.

Bramwell,V.H., Morris D., Ernst D.S., Hings I., Blackstein M., Venner P.M., Ette E.I., Harding M.W., Waxman A., and Demetri G.D. (2002). Safety and efficacy of the multidrug-resistance inhibitor biricodar (VX-710) with concurrent doxorubicin in patients with anthracycline-resistant advanced soft tissue sarcoma. Clin. Cancer Res. *8,* 383-393.

Brinkmann,V., Reichard U., Goosmann C., Fauler B., Uhlemann Y., Weiss D.S., Weinrauch Y., and Zychlinsky A. (2004). Neutrophil extracellular traps kill bacteria. Science *303,* 1532-1535.

Bugge,T.H., Xiao Q., Kombrinck K.W., Flick M.J., Holmback K., Danton M.J., Colbert M.C., Witte D.P., Fujikawa K., Davie E.W., and Degen J.L. (1996). Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc. Natl. Acad. Sci. U. S. A *93,* 6258-6263.

Bunce,M.W., Toso R., and Camire R.M. (2011). Zymogen-like factor Xa variants restore thrombin generation and effectively bypass the intrinsic pathway in vitro. Blood *117,* 290-298.

Capizzi,E., Gabusi E., Grigioni A.D., De I.P., Rosati M., Zamagni C., and Fiorentino M. (2008). Quantification of free plasma DNA before and after chemotherapy in patients with advanced epithelial ovarian cancer. Diagn. Mol. Pathol. *17,* 34-38.

Carmeliet,P., Mackman N., Moons L., Luther T., Gressens P., Van V., I, Demunck H., Kasper M., Breier G., Evrard P., Muller M., Risau W., Edgington T., and Collen D. (1996). Role of tissue factor in embryonic blood vessel development. Nature *383,* 73-75.

Cattaneo,M. (2006). Hyperhomocysteinemia and venous thromboembolism. Semin. Thromb. Hemost. *32,* 716-723.

Cavo,M., Zamagni E., Tosi P., Cellini C., Cangini D., Tacchetti P., Testoni N., Tonelli M., de V.A., Palareti G., Tura S., and Baccarani M. (2004). First-line therapy with thalidomide and dexamethasone in preparation for autologous stem cell transplantation for multiple myeloma. Haematologica *89,* 826-831.

Cersosimo,R.J., Hong W.K. (1986). Epirubicin: a review of the pharmacology, clinical activity, and adverse effects of an adriamycin analogue. J. Clin. Oncol. *4,* 425-439.

Cesarman-Maus,G., Hajjar K.A. (2005). Molecular mechanisms of fibrinolysis. Br. J. Haematol. *129,* 307-321.

Chandler,W.L., Roshal M. (2009). Optimization of plasma fluorogenic thrombin-generation assays. Am. J. Clin. Pathol. *132,* 169-179.

Chang,T.K., Weber G.F., Crespi C.L., and Waxman D.J. (1993). Differential activation of cyclophosphamide and ifosphamide by cytochromes P-450 2B and 3A in human liver microsomes. Cancer Res. *53,* 5629-5637.

Chenaille,P.J., Steward S.A., Ashmun R.A., and Jackson C.W. (1990). Prolonged thrombocytosis in mice after 5-fluorouracil results from failure to down-regulate megakaryocyte concentration. An experimental model that dissociates regulation of megakaryocyte size and DNA content from megakaryocyte concentration. Blood *76,* 508-515.

Citarella,F., Ravon D.M., Pascucci B., Felici A., Fantoni A., and Hack C.E. (1996). Structure/function analysis of human factor XII using recombinant deletion mutants. Evidence for an additional region involved in the binding to negatively charged surfaces. Eur. J. Biochem. *238,* 240-249.

Cividalli,A., Cruciani G., Livdi E., Cordelli E., Eletti B., and Tirindelli D.D. (1998). Greater antitumor efficacy of paclitaxel administered before epirubicin in a mouse mammary carcinoma. J. Cancer Res. Clin. Oncol. *124,* 236-244.

Clahsen,P.C., van de Velde C.J., Julien J.P., Floiras J.L., and Mignolet F.Y. (1994). Thromboembolic complications after perioperative chemotherapy in women with early breast cancer: a European Organization for Research and Treatment of Cancer Breast Cancer Cooperative Group study. J. Clin. Oncol. *12*, 1266-1271.

Clark,S.R., Ma A.C., Tavener S.A., McDonald B., Goodarzi Z., Kelly M.M., Patel K.D., Chakrabarti S., McAvoy E., Sinclair G.D., Keys E.M., len-Vercoe E., Devinney R., Doig C.J., Green F.H., and Kubes P. (2007). Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat. Med. *13,* 463-469.

Clyne,N., Berglund B., and Egberg N. (1995). Treatment with recombinant human erythropoietin induces a moderate rise in hematocrit and thrombin antithrombin in healthy subjects. Thromb. Res. *79,* 125-129.

Cochrane,C.G., Revak S.D., and Wuepper K.D. (1973). Activation of Hageman factor in solid and fluid phases. A critical role of kallikrein. J. Exp. Med. *138,* 1564-1583.

Colucci,M., Balconi G., Lorenzet R., Pietra A., Locati D., Donati M.B., and Semeraro N. (1983). Cultured human endothelial cells generate tissue factor in response to endotoxin. J. Clin. Invest *71,* 1893-1896.

Conklin,D.J., Barski O.A., Lesgards J.F., Juvan P., Rezen T., Rozman D., Prough R.A., Vladykovskaya E., Liu S., Srivastava S., and Bhatnagar A. (2010). Acrolein consumption induces systemic dyslipidemia and lipoprotein modification. Toxicol. Appl. Pharmacol. *243*, 1-12.

Contrino,J., Hair G., Kreutzer D.L., and Rickles F.R. (1996). In situ detection of tissue factor in vascular endothelial cells: correlation with the malignant phenotype of human breast disease. Nat. Med. *2,* 209-215.

Cool,D.E., Edgell C.J., Louie G.V., Zoller M.J., Brayer G.D., and MacGillivray R.T. (1985). Characterization of human blood coagulation factor XII cDNA. Prediction of the primary structure of factor XII and the tertiary structure of beta-factor XIIa. J. Biol. Chem. *260,* 13666-13676.

Crook,T.R., Souhami R.L., and McLean A.E. (1986). Cytotoxicity, DNA cross-linking, and single strand breaks induced by activated cyclophosphamide and acrolein in human leukemia cells. Cancer Res. *46,* 5029-5034.

Damrot,J., Nubel T., Epe B., Roos W.P., Kaina B., and Fritz G. (2006). Lovastatin protects human endothelial cells from the genotoxic and cytotoxic effects of the anticancer drugs doxorubicin and etoposide. Br. J. Pharmacol. *149,* 988-997.

Danesi,R., Innocenti F., Fogli S., Gennari A., Baldini E., Di P.A., Salvadori B., Bocci G., Conte P.F., and Del T.M. (2002). Pharmacokinetics and pharmacodynamics of combination chemotherapy with paclitaxel and epirubicin in breast cancer patients. Br. J. Clin. Pharmacol. *53,* 508-518.

Davila,M., Amirkhosravi A., Coll E., Desai H., Robles L., Colon J., Baker C.H., and Francis J.L. (2008). Tissue factor-bearing microparticles derived from tumor cells: impact on coagulation activation. J. Thromb. Haemost. *6,* 1517-1524.

De Cicco,M. (2004). The prothrombotic state in cancer: pathogenic mechanisms. Crit Rev. Oncol. Hematol. *50,* 187-196.

de Jonge,M.E., Huitema A.D., Rodenhuis S., and Beijnen J.H. (2005). Clinical pharmacokinetics of cyclophosphamide. Clin. Pharmacokinet. *44,* 1135-1164.

Deitcher,S.R., Gomes M.P. (2004). The risk of venous thromboembolic disease associated with adjuvant hormone therapy for breast carcinoma: a systematic review. Cancer *101,* 439-449.

Dekkers,D.W., Comfurius P., Vuist W.M., Billheimer J.T., Dicker I., Weiss H.J., Zwaal R.F., and Bevers E.M. (1998). Impaired Ca2+-induced tyrosine phosphorylation and defective lipid scrambling in erythrocytes from a patient with Scott syndrome: a study using an inhibitor for scramblase that mimics the defect in Scott syndrome. Blood *91,* 2133-2138.

Deligezer,U., Eralp Y., Akisik E.Z., Akisik E.E., Saip P., Topuz E., and Dalay N. (2008). Effect of adjuvant chemotherapy on integrity of free serum DNA in patients with breast cancer. Ann. N. Y. Acad. Sci. *1137,* 175-179.

Dickinson,C.D., Kelly C.R., and Ruf W. (1996). Identification of surface residues mediating tissue factor binding and catalytic function of the serine protease factor VIIa. Proc. Natl. Acad. Sci. U. S. A *93,* 14379-14384.

Dietzen,D.J., Page K.L., and Tetzloff T.A. (2004). Lipid rafts are necessary for tonic inhibition of cellular tissue factor procoagulant activity. Blood *103,* 3038-3044.

Dizdaroglu,M. (1991). Chemical determination of free radical-induced damage to DNA. Free Radic. Biol. Med. *10,* 225-242.

Drake,T.A., Cheng J., Chang A., and Taylor F.B., Jr. (1993). Expression of tissue factor, thrombomodulin, and E-selectin in baboons with lethal Escherichia coli sepsis. Am. J. Pathol. *142,* 1458-1470.

Dunkley,S., Gaudry L. (2007). Thalidomide causes platelet activation, which can be abrogated by aspirin. J Thromb. Haemost. *5,* 1323-1325.

EBCTCG. (1988). Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. Early Breast Cancer Trialists' Collaborative Group. N. Engl. J Med *319*, 1681-1692.

EBCTCG. (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet *365,* 1687-1717.

Economidou-Karaoglou,A., Brasseur F., Lans M., Taper H.S., and Roberfroid M.B. (1989). Variations in serum alkaline DNase activity in rats during growth and treatment of tumors sensitive or resistant to therapy. Int. J. Cancer *43*, 956-959.

Edwards,R.L., Klaus M., Matthews E., McCullen C., Bona R.D., and Rickles F.R. (1990). Heparin abolishes the chemotherapy-induced increase in plasma fibrinopeptide A levels. Am. J. Med. *89,* 25-28.

Edwards,R.L., Rickles F.R., Moritz T.E., Henderson W.G., Zacharski L.R., Forman W.B., Cornell C.J., Forcier R.J., O'Donnell J.F., Headley E., and . (1987). Abnormalities of blood coagulation tests in patients with cancer. Am. J Clin. Pathol. *88,* 596-602.

Egan,J.O., Kalafatis M., and Mann K.G. (1997). The effect of Arg306-->Ala and Arg506-->Gln substitutions in the inactivation of recombinant human factor Va by activated protein C and protein S. Protein Sci. *6,* 2016-2027.

Eiserich,J.P., van d., V, Handelman G.J., Halliwell B., and Cross C.E. (1995). Dietary antioxidants and cigarette smoke-induced biomolecular damage: a complex interaction. Am. J. Clin. Nutr. *62,* 1490S-1500S.

Elice,F., Rodeghiero F., Falanga A., and Rickles F.R. (2009). Thrombosis associated with angiogenesis inhibitors. Best. Pract. Res. Clin. Haematol. *22,* 115-128.

Elting,L.S., Escalante C.P., Cooksley C., Avritscher E.B., Kurtin D., Hamblin L., Khosla S.G., and Rivera E. (2004). Outcomes and cost of deep venous thrombosis among patients with cancer. Arch. Intern. Med. *164,* 1653-1661.

Erman,M., Abali H., Oran B., Haznedaroglu I.C., Canpinar H., Kirazli S., and Celik I. (2004). Tamoxifen-induced tissue factor pathway inhibitor reduction: a clue for an acquired thrombophilic state? Ann. Oncol. *15,* 1622-1626.

Ershler,W.B. (2006). Capecitabine monotherapy: safe and effective treatment for metastatic breast cancer. Oncologist. *11,* 325-335.

Esmon,C.T. (2003). The protein C pathway. Chest *124,* 26S-32S.

Esmon,C.T., Owen W.G. (1981). Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. Proc. Natl. Acad. Sci. U. S. A *78,* 2249-2252.

Esmon,N.L., Owen W.G., and Esmon C.T. (1982). Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. J. Biol. Chem. *257,* 859-864.

Fadok,V.A., Voelker D.R., Campbell P.A., Cohen J.J., Bratton D.L., and Henson P.M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. *148,* 2207-2216.

Faioni,E.M., Franchi F., Castaman G., Biguzzi E., and Rodeghiero F. (2002). Mutations in the thrombomodulin gene are rare in patients with severe thrombophilia. Br. J. Haematol. *118,* 595-599.

Falanga,A., Levine M.N., Consonni R., Gritti G., Delaini F., Oldani E., Julian J.A., and Barbui T. (1998). The effect of very-low-dose warfarin on markers of hypercoagulation in metastatic breast cancer: results from a randomized trial. Thromb. Haemost. *79,* 23-27.

Feffer,S.E., Carmosino L.S., and Fox R.L. (1989). Acquired protein C deficiency in patients with breast cancer receiving cyclophosphamide, methotrexate, and 5-fluorouracil. Cancer *63,* 1303-1307.

Ferlay J, Shin H, Bray F, Forman D, Mathers C, Parkin D. GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide. International Agency for Research on Cancer 2010Available from: URL: http://globocan.iarc.fr

Ferry,J., Morrisson P. (2011). Preparation and properties of serum and plasma proteins; the conversion of human fibrinogen to fibrin under various conditions. J. Am. Chem. Soc. *69,* 388-400.

Fisgin,T., Yarali N., Kara A., Bozkurt C., Birgen D., Erten U., and Duru F. (2004). Hemostatic side effects of high-dose methotrexate in childhood acute lymphoblastic leukemia. Pediatr. Hematol. Oncol. *21,* 77-83.

Fisher,S.A. (2010). Vascular smooth muscle phenotypic diversity and function. Physiol Genomics *42A,* 169-187.

Flynn,P.D., Byrne C.D., Baglin T.P., Weissberg P.L., and Bennett M.R. (1997). Thrombin generation by apoptotic vascular smooth muscle cells. Blood *89,* 4378-4384.

Folch,J. (1948). The chemical structure of phospatidyl serine. J. Biol. Chem. *174,* 439-450.

Fracasso,P.M., Blum K.A., Ma M.K., Tan B.R., Wright L.P., Goodner S.A., Fears C.L., Hou W., Arquette M.A., Picus J., Denes A., Mortimer J.E., Ratner L., Ivy S.P., and McLeod H.L. (2005). Phase I study of pegylated liposomal doxorubicin and the multidrug-resistance modulator, valspodar. Br. J. Cancer *93,* 46-53.

Franchini,M., Mannucci P.M. (2008). Venous and arterial thrombosis: different sides of the same coin? Eur. J. Intern. Med. *19,* 476-481.

Fu,Y., Zhou J., Li H., Cao F., Su Y., Fan S., Li Y., Wang S., Li L., Gilbert G.E., and Shi J. (2010). Daunorubicin induces procoagulant activity of cultured endothelial cells through phosphatidylserine exposure and microparticles release. Thromb. Haemost. *104,* 1235-1241.

Fuchs,T.A., Abed U., Goosmann C., Hurwitz R., Schulze I., Wahn V., Weinrauch Y., Brinkmann V., and Zychlinsky A. (2007). Novel cell death program leads to neutrophil extracellular traps. J. Cell Biol. *176,* 231-241.

Fuchs,T.A., Brill A., Duerschmied D., Schatzberg D., Monestier M., Myers D.D., Jr., Wrobleski S.K., Wakefield T.W., Hartwig J.H., and Wagner D.D. (2010). Extracellular DNA traps promote thrombosis. Proc. Natl. Acad. Sci. U. S. A *107,* 15880-15885.

Fukudome,K., Esmon C.T. (1995). Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR). The structural and functional conservation in human, bovine, and murine EPCR. J. Biol. Chem. *270,* 5571-5577.

Gailani,D., Lasky N.M., and Broze G.J., Jr. (1997). A murine model of factor XI deficiency. Blood Coagul. Fibrinolysis *8,* 134-144.

Gailani,D., Renne T. (2007). Intrinsic pathway of coagulation and arterial thrombosis. Arterioscler. Thromb. Vasc. Biol. *27,* 2507-2513.

Galligan,L., Livingstone W., Volkov Y., Hokamp K., Murphy C., Lawler M., Fukudome K., and Smith O. (2001). Characterization of protein C receptor expression in monocytes. Br. J Haematol. *115,* 408-414.

Gandrille,S., Borgel D., Sala N., Espinosa-Parrilla Y., Simmonds R., Rezende S., Lind B., Mannhalter C., Pabinger I., Reitsma P.H., Formstone C., Cooper D.N., Saito H., Suzuki K., Bernardi F., and Aiach M. (2000). Protein S deficiency: a database of mutations--summary of the first update. Thromb. Haemost. *84,* 918.

Garcia-Sanz,R. (2006). Thalidomide in multiple myeloma. Expert. Opin. Pharmacother. *7,* 195-213.

Genestier,L., Paillot R., Quemeneur L., Izeradjene K., and Revillard J.P. (2000). Mechanisms of action of methotrexate. Immunopharmacology *47,* 247-257.

Girard,T.J., Warren L.A., Novotny W.F., Likert K.M., Brown S.G., Miletich J.P., and Broze G.J., Jr. (1989). Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. Nature *338,* 518-520.

Giraud,B., Hebert G., Deroussent A., Veal G.J., Vassal G., and Paci A. (2010). Oxazaphosphorines: new therapeutic strategies for an old class of drugs. Expert. Opin. Drug Metab Toxicol. *6,* 919-938.

Gockel,H.R., Lugering A., Heidemann J., Schmidt M., Domschke W., Kucharzik T., and Lugering N. (2004). Thalidomide induces apoptosis in human monocytes by using a cytochrome c-dependent pathway. J Immunol. *172,* 5103-5109.

Gomi,K., Zushi M., Honda G., Kawahara S., Matsuzaki O., Kanabayashi T., Yamamoto S., Maruyama I., and Suzuki K. (1990). Antithrombotic effect of recombinant human thrombomodulin on thrombin-induced thromboembolism in mice. Blood *75,* 1396-1399.

Goodnough,L.T., Saito H., Manni A., Jones P.K., and Pearson O.H. (1984). Increased incidence of thromboembolism in stage IV breast cancer patients treated with a five-drug chemotherapy regimen. A study of 159 patients. Cancer *54,* 1264-1268.

Gordesky,S.E., Marinetti G.V. (1973). The asymetric arrangement of phospholipids in the human erythrocyte membrane. Biochem. Biophys. Res. Commun. *50,* 1027-1031.

Gordon,S.G., Franks J.J., and Lewis B. (1975). Cancer procoagulant A: a factor X activating procoagulant from malignant tissue. Thromb. Res. *6,* 127-137.

Grafstrom,R.C., Dypbukt J.M., Willey J.C., Sundqvist K., Edman C., Atzori L., and Harris C.C. (1988). Pathobiological effects of acrolein in cultured human bronchial epithelial cells. Cancer Res. *48,* 1717-1721.

Gross,P.L., Furie B.C., Merrill-Skoloff G., Chou J., and Furie B. (2005). Leukocyte-versus microparticle-mediated tissue factor transfer during arteriolar thrombus development. J. Leukoc. Biol. *78,* 1318-1326.

Gu,J.M., Crawley J.T., Ferrell G., Zhang F., Li W., Esmon N.L., and Esmon C.T. (2002). Disruption of the endothelial cell protein C receptor gene in mice causes placental thrombosis and early embryonic lethality. J. Biol. Chem. *277,* 43335-43343.

Gualandris,A., Presta M. (1995). Transcriptional and posttranscriptional regulation of urokinase-type plasminogen activator expression in endothelial cells by basic fibroblast growth factor. J. Cell Physiol *162,* 400-409.

Gugliotta,L., Mazzucconi M.G., Leone G., Mattioli-Belmonte M., Defazio D., Annino L., Tura S., and Mandelli F. (1992). Incidence of thrombotic complications in adult patients with acute lymphoblastic leukaemia receiving L-asparaginase during induction therapy: a retrospective study. The GIMEMA Group. Eur. J. Haematol. *49,* 63-66.

Gugliucci,A. (2008). Antithrombin activity is inhibited by acrolein and homocysteine thiolactone: Protection by cysteine. Life Sci. *82,* 413-418.

Gugliucci,A., Tsuji M., Kinugasa E., Ogata H., Ikeda H., Shikama Y., and Kimura S. (2007). High free serum acrolein levels in bacterial infection and other disease states associated with oxidative stress: a potential biomarker? Clin. Chem. Lab Med. *45,* 1559-1560.

Gurtoo,H.L., Hipkens J.H., and Sharma S.D. (1981). Role of glutathione in the metabolism-dependent toxicity and chemotherapy of cyclophosphamide. Cancer Res. *41,* 3584-3591.

Haas SK, Kakkar AK, Kemkes-Matthes B, Freund M, Gatzemeier U, Heilmann L, von Tempelhoff GF, Brom J, and Weidinger G. (2005). Prevention of Venous Thromboembolism with Low-Molecular-Weight Heparin in Patients with Metastatic Breast or Lung Cancer - Results of the TOPIC Studies #OR059. Journal of Thrombosis and Haemostasis *3, supplement 1,*

Hackeng,T.M., Hessing M., van '., V, Meijer-Huizinga F., Meijers J.C., De Groot P.G., van Mourik J.A., and Bouma B.N. (1993). Protein S binding to human endothelial cells is required for expression of cofactor activity for activated protein C. J. Biol. Chem. *268,* 3993-4000.

Haddad,T.C., Greeno E.W. (2006). Chemotherapy-induced thrombosis. Thromb. Res. *118,* 555-568.

Haim,N., Lanir N., Hoffman R., Haim A., Tsalik M., and Brenner B. (2001). Acquired activated protein C resistance is common in cancer patients and is associated with venous thromboembolism. Am. J. Med. *110,* 91-96.

Hancock,J.T. (2009). The role of redox mechanisms in cell signalling. Mol. Biotechnol. *43,* 162-166.

Healy,A.M., Rayburn H.B., Rosenberg R.D., and Weiler H. (1995). Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc. Natl. Acad. Sci. U. S. A *92,* 850-854.

Heit,J.A. (2003). Risk factors for venous thromboembolism. Clin. Chest Med. *24,* 1-12.

Hekman,C.M., Loskutoff D.J. (1985). Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. J. Biol. Chem. *260,* 11581-11587.

Hemker,H.C., Al D.R., de S.E., and Beguin S. (2006). Thrombin generation, a function test of the haemostatic-thrombotic system. Thromb. Haemost. *96,* 553-561.

Hemker,H.C., Giesen P., Al D.R., Regnault V., de S.E., Wagenvoord R., Lecompte T., and Beguin S. (2003). Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol. Haemost. Thromb. *33,* 4-15.

Henriksson,C.E., Klingenberg O., Ovstebo R., Joo G.B., Westvik A.B., and Kierulf P. (2005). Discrepancy between tissue factor activity and tissue factor expression in endotoxin-induced monocytes is associated with apoptosis and necrosis. Thromb. Haemost. *94,* 1236-1244.

Hoffman,M., Monroe D.M., III. (2001). A cell-based model of hemostasis. Thromb. Haemost. *85,* 958-965.

Hortobagyi,G.N. (1998). Treatment of breast cancer. N. Engl. J. Med. *339,* 974-984.

Horton,N.D., Biswal S.S., Corrigan L.L., Bratta J., and Kehrer J.P. (1999). Acrolein causes inhibitor kappaB-independent decreases in nuclear factor kappaB activation in human lung adenocarcinoma (A549) cells. J Biol. Chem. *274,* 9200-9206.

Hoshi,A., Matsumoto A., Chung J., Isozumi Y., and Koyama T. (2011). Activation of coagulation by a thalidomide-based regimen. Blood Coagul. Fibrinolysis *22,* 532-540.

Howard,B.V., Macarak E.J., Gunson D., and Kefalides N.A. (1976). Characterization of the collagen synthesized by endothelial cells in culture. Proc. Natl. Acad. Sci. U. S. A *73,* 2361-2364.

Hron,G., Kollars M., Weber H., Sagaster V., Quehenberger P., Eichinger S., Kyrle P.A., and Weltermann A. (2007). Tissue factor-positive microparticles: cellular origin and association with coagulation activation in patients with colorectal cancer. Thromb. Haemost. *97,* 119-123.

Hu,T., Bach R.R., Horton R., Konigsberg W.H., and Todd M.B. (1993). Synthesis of tissue factor messenger RNA and procoagulant activity in breast cancer cells in response to serum stimulation. Thromb. Res. *72,* 155-168.

Ignjatovic,V., Greenway A., Summerhayes R., and Monagle P. (2007). Thrombin generation: the functional role of alpha-2-macroglobulin and influence of developmental haemostasis. Br. J. Haematol. *138,* 366-368.

Isermann,B., Hendrickson S.B., Zogg M., Wing M., Cummiskey M., Kisanuki Y.Y., Yanagisawa M., and Weiler H. (2001). Endothelium-specific loss of murine thrombomodulin disrupts the protein C anticoagulant pathway and causes juvenile-onset thrombosis. J. Clin. Invest *108,* 537-546.

Iuliano,L., Colavita A.R., Leo R., Pratico D., and Violi F. (1997). Oxygen free radicals and platelet activation. Free Radic. Biol. Med. *22,* 999-1006.

Iwaki,T., Cruz D.T., Martin J.A., and Castellino F.J. (2005). A cardioprotective role for the endothelial protein C receptor in lipopolysaccharide-induced endotoxemia in the mouse. Blood *105,* 2364-2371.

JABCSG and JCOG. (1993). Effects of chemoendocrine therapy on the coagulation-fibrinolytic systems in patients with advanced breast cancer. Japan Advanced Breast Cancer Study Group and Japan Clinical Oncology Group. Jpn. J. Cancer Res. *84,* 455-461.

Jahr,S., Hentze H., Englisch S., Hardt D., Fackelmayer F.O., Hesch R.D., and Knippers R. (2001). DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. *61,* 1659-1665.

Jalbert,L.R., Rosen E.D., Moons L., Chan J.C., Carmeliet P., Collen D., and Castellino F.J. (1998). Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J. Clin. Invest *102,* 1481-1488.

Jimenez,J.J., Jy W., Mauro L.M., Soderland C., Horstman L.L., and Ahn Y.S. (2003). Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. Thromb. Res. *109,* 175-180.

Jones,A.L., Powles T.J., Treleaven J.G., Burman J.F., Nicolson M.C., Chung H.I., and Ashley S.E. (1992). Haemostatic changes and thromboembolic risk during tamoxifen therapy in normal women. Br. J. Cancer *66,* 744-747.

Kahn,M.L., Nakanishi-Matsui M., Shapiro M.J., Ishihara H., and Coughlin S.R. (1999). Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J. Clin. Invest *103,* 879-887.

Kakkar,A.K., Lemoine N.R., Scully M.F., Tebbutt S., and Williamson R.C. (1995). Tissue factor expression correlates with histological grade in human pancreatic cancer. Br. J. Surg. *82,* 1101-1104.

Kamat,A.A., Bischoff F.Z., Dang D., Baldwin M.F., Han L.Y., Lin Y.G., Merritt W.M., Landen C.N., Jr., Lu C., Gershenson D.M., Simpson J.L., and Sood A.K. (2006). Circulating cell-free DNA: a novel biomarker for response to therapy in ovarian carcinoma. Cancer Biol. Ther. *5,* 1369-1374.

Kang,Y.J., Zhou Z.X., Wang G.W., Buridi A., and Klein J.B. (2000). Suppression by metallothionein of doxorubicin-induced cardiomyocyte apoptosis through inhibition of p38 mitogen-activated protein kinases. J. Biol. Chem. *275,* 13690-13698.

Kannemeier,C., Shibamiya A., Nakazawa F., Trusheim H., Ruppert C., Markart P., Song Y., Tzima E., Kennerknecht E., Niepmann M., von Bruehl M.L., Sedding D., Massberg S., Gunther A., Engelmann B., and Preissner K.T. (2007). Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation. Proc. Natl. Acad. Sci. U. S. A *104,* 6388-6393.

Kasthuri,R.S., Taubman M.B., and Mackman N. (2009). Role of tissue factor in cancer. J. Clin. Oncol. *27,* 4834-4838.

Kebieche,M., Lakroun Z., Lahouel M., Bouayed J., Meraihi Z., and Soulimani R. (2009). Evaluation of epirubicin-induced acute oxidative stress toxicity in rat liver cells and mitochondria, and the prevention of toxicity through quercetin administration. Exp. Toxicol. Pathol. *61,* 161-167.

Kehrer,J.P., Biswal S.S. (2000). The molecular effects of acrolein. Toxicol. Sci. *57,* 6-15.

Khorana,A.A., Francis C.W., Culakova E., Kuderer N.M., and Lyman G.H. (2007). Thromboembolism is a leading cause of death in cancer patients receiving outpatient chemotherapy. J. Thromb. Haemost. *5,* 632-634.

Khorana,A.A., Francis C.W., Menzies K.E., Wang J.G., Hyrien O., Hathcock J., Mackman N., and Taubman M.B. (2008). Plasma tissue factor may be predictive of venous thromboembolism in pancreatic cancer. J. Thromb. Haemost. *6,* 1983-1985.

Kirwan,C.C., McDowell G., McCollum C.N., Kumar S., and Byrne G.J. (2008). Early changes in the haemostatic and procoagulant systems after chemotherapy for breast cancer. Br. J. Cancer *99,* 1000-1006.

Kisiel,W. (1979). Human plasma protein C: isolation, characterization, and mechanism of activation by alpha-thrombin. J. Clin. Invest *64,* 761-769.

Koster,T., Rosendaal F.R., de R.H., Briet E., Vandenbroucke J.P., and Bertina R.M. (1993). Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. Lancet *342,* 1503-1506.

Kothari,H., Nayak R.C., Rao L.V., and Pendurthi U.R. (2010). Cystine 186-cystine 209 disulfide bond is not essential for the procoagulant activity of tissue factor or for its de-encryption. Blood *115,* 4273-4283.

Koutsilieris,M., Reyes-Moreno C., Choki I., Sourla A., Doillon C., and Pavlidis N. (1999). Chemotherapy cytotoxicity of human MCF-7 and MDA-MB 231 breast cancer cells is altered by osteoblast-derived growth factors. Mol. Med. *5,* 86-97.

Kretz,C.A., Vaezzadeh N., and Gross P.L. (2010). Tissue factor and thrombosis models. Arterioscler. Thromb. Vasc. Biol. *30,* 900-908.

Kufe,D.W., Major P.P. (1981). 5-Fluorouracil incorporation into human breast carcinoma RNA correlates with cytotoxicity. J. Biol. Chem. *256,* 9802-9805.

Kurachi,K., Fujikawa K., and Davie E.W. (1980). Mechanism of activation of bovine factor XI by factor XII and factor XIIa. Biochemistry *19,* 1330-1338.

Kurosawa,S., Stearns D.J., Jackson K.W., and Esmon C.T. (1988). A 10-kDa cyanogen bromide fragment from the epidermal growth factor homology domain of rabbit thrombomodulin contains the primary thrombin binding site. J. Biol. Chem. *263,* 5993-5996.

Kurosawa,S., Stearns-Kurosawa D.J., Carson C.W., D'Angelo A., Della V.P., and Esmon C.T. (1998). Plasma levels of endothelial cell protein C receptor are elevated in patients with sepsis and systemic lupus erythematosus: lack of correlation with thrombomodulin suggests involvement of different pathological processes. Blood *91,* 725-727.

Kuzel,T., Esparaz B., Green D., and Kies M. (1990). Thrombogenicity of intravenous 5-fluorouracil alone or in combination with cisplatin. Cancer *65,* 885-889.

Lau,A., Berry L.R., Mitchell L.G., and Chan A.K. (2007). Effect of substrate and fibrin polymerization inhibitor on determination of plasma thrombin generation in vitro. Thromb. Res. *119,* 667-677.

Lay,A.J., Liang Z., Rosen E.D., and Castellino F.J. (2005). Mice with a severe deficiency in protein C display prothrombotic and proinflammatory phenotypes and compromised maternal reproductive capabilities. J. Clin. Invest *115,* 1552-1561.

Le,D.T., Rapaport S.I., and Rao L.V. (1992). Relations between factor VIIa binding and expression of factor VIIa/tissue factor catalytic activity on cell surfaces. J. Biol. Chem. *267,* 15447-15454.

Lechner,D., Kollars M., Gleiss A., Kyrle P.A., and Weltermann A. (2007). Chemotherapy-induced thrombin generation via procoagulant endothelial microparticles is independent of tissue factor activity. J Thromb. Haemost. *5,* 2445-2452.

Lee,A.Y., Levine M.N. (2003). Venous thromboembolism and cancer: risks and outcomes. Circulation *107,* I17-I21.

Lentz,B.R. (2003). Exposure of platelet membrane phosphatidylserine regulates blood coagulation. Prog. Lipid Res. *42,* 423-438.

Leventis,P.A., Grinstein S. (2010). The distribution and function of phosphatidylserine in cellular membranes. Annu. Rev. Biophys. *39,* 407-427.

Levi,M. (2007). Disseminated intravascular coagulation. Crit Care Med. *35,* 2191-2195.

Levi,M., ten C.H., and van der P.T. (2002). Endothelium: interface between coagulation and inflammation. Crit Care Med *30,* S220-S224.

Levin,E.G., Marzec U., Anderson J., and Harker L.A. (1984). Thrombin stimulates tissue plasminogen activator release from cultured human endothelial cells. J. Clin. Invest *74,* 1988-1995.

Levine,M., Hirsh J., Gent M., Arnold A., Warr D., Falanga A., Samosh M., Bramwell V., Pritchard K.I., Stewart D., and . (1994). Double-blind randomised trial of a very-low-dose warfarin for prevention of thromboembolism in stage IV breast cancer. Lancet *343,* 886-889.

Levine,M.N. (2010). Thromboprophylaxis during chemotherapy: NO. Thromb. Res. *125 Suppl 2,* S16.

Levine,M.N., Gent M., Hirsh J., Arnold A., Goodyear M.D., Hryniuk W., and De P.S. (1988). The thrombogenic effect of anticancer drug therapy in women with stage II breast cancer. N. Engl. J Med *318,* 404-407.

Levitan,N., Dowlati A., Remick S.C., Tahsildar H.I., Sivinski L.D., Beyth R., and Rimm A.A. (1999). Rates of initial and recurrent thromboembolic disease among patients with malignancy versus those without malignancy. Risk analysis using Medicare claims data. Medicine (Baltimore) *78,* 285-291.

Li,W., Zheng X., Gu J., Hunter J., Ferrell G.L., Lupu F., Esmon N.L., and Esmon C.T. (2005). Overexpressing endothelial cell protein C receptor alters the hemostatic balance and protects mice from endotoxin. J. Thromb. Haemost. *3,* 1351-1359.

Liaw,P.C., Esmon C.T., Kahnamoui K., Schmidt S., Kahnamoui S., Ferrell G., Beaudin S., Julian J.A., Weitz J.I., Crowther M., Loeb M., and Cook D. (2004). Patients with severe sepsis vary markedly in their ability to generate activated protein C. Blood *104,* 3958-3964.

Liaw,P.C., Ferrell G., and Esmon C.T. (2003). A monoclonal antibody against activated protein C allows rapid detection of activated protein C in plasma and reveals a calcium ion dependent epitope involved in factor Va inactivation. J. Thromb. Haemost. *1,* 662-670.

Liaw,P.C., Neuenschwander P.F., Smirnov M.D., and Esmon C.T. (2000). Mechanisms by which soluble endothelial cell protein C receptor modulates protein C and activated protein C function. J. Biol. Chem. *275,* 5447-5452.

Libourel,E.J., Sonneveld P., van der H.B., de Maat M.P., and Leebeek F.W. (2010). High incidence of arterial thrombosis in young patients treated for multiple myeloma: results of a prospective cohort study. Blood *116,* 22-26.

Lim,W. (2009). Antiphospholipid antibody syndrome. Hematology. Am. Soc. Hematol. Educ. Program. 233-239.

Lindahl,A.K., Sandset P.M., and Abildgaard U. (1990). Indices of hypercoagulation in cancer as compared with those in acute inflammation and acute infarction. Haemostasis *20,* 253-262.

Lippi,G., Franchini M., and Favaloro E.J. (2010). Thrombotic complications of erythropoiesis-stimulating agents. Semin. Thromb. Hemost. *36,* 537-549.

Longley,D.B., Harkin D.P., and Johnston P.G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. Nat. Rev. Cancer *3,* 330-338.

Lorand,L., Konishi K. (1964). Activation of the fibrin stabilizing factor of plasma by thrombin. Arch. Biochem. Biophys. *105,* 58-67.

Lorenzo,E., Ruiz-Ruiz C., Quesada A.J., Hernandez G., Rodriguez A., Lopez-Rivas A., and Redondo J.M. (2002). Doxorubicin induces apoptosis and CD95 gene expression in human primary endothelial cells through a p53-dependent mechanism. J. Biol. Chem. *277,* 10883-10892.

Loscalzo,J. (2002). Oxidative stress in endothelial cell dysfunction and thrombosis. Pathophysiol. Haemost. Thromb. *32,* 359-360.

Lovell,M.A., Xie C., and Markesbery W.R. (2001). Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures. Neurobiol. Aging *22,* 187-194.

Lu,C.F., Yu H.J., Hou J.X., and Zhou J. (2008). Increased procoagulant activity of red blood cells in the presence of cisplatin. Chin Med. J. (Engl.) *121,* 1775-1780.

Lwaleed,B.A., Bass P.S., and Cooper A.J. (2001). The biology and tumour-related properties of monocyte tissue factor. J Pathol. *193,* 3-12.

Lwaleed,B.A., Francis J.L., and Chisholm M. (2000). Monocyte tissue factor levels in cancer patients. Saudi. Med. J. *21,* 722-729.

Lyman,G.H. (2007). ASCO Clinical Practice Guidelines and Beyond. J. Oncol. Pract. *3,* 330-331.

Lynch,H.T., Silva E., Snyder C., and Lynch J.F. (2008). Hereditary breast cancer: part I. Diagnosing hereditary breast cancer syndromes. Breast J. *14,* 3-13.

Ma,L., Francia G., Viloria-Petit A., Hicklin D.J., du M.J., Rak J., and Kerbel R.S. (2005). In vitro procoagulant activity induced in endothelial cells by chemotherapy and antiangiogenic drug combinations: modulation by lower-dose chemotherapy. Cancer Res. *65,* 5365-5373.

Machovich,R., Blasko G., and Palos L.A. (1975). Action of heparin on thrombin-antithrombin reaction. Biochim. Biophys. Acta *379,* 193-200.

Malyszko,J., Malyszko J.S., Borawski J., Rydzewski A., Kalinowski M., Azzadin A., Mysliwiec M., and Buczko W. (1995). A study of platelet functions, some hemostatic and fibrinolytic parameters in relation to serotonin in hemodialyzed patients under erythropoietin therapy. Thromb. Res. *77,* 133-143.

Marcus,A.J., Broekman M.J., Drosopoulos J.H., Islam N., Alyonycheva T.N., Safier L.B., Hajjar K.A., Posnett D.N., Schoenborn M.A., Schooley K.A., Gayle R.B., and Maliszewski C.R. (1997). The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. J. Clin. Invest *99,* 1351-1360.

Martindale,J.L., Holbrook N.J. (2002). Cellular response to oxidative stress: signaling for suicide and survival. J. Cell Physiol *192,* 1-15.

McArthur,H.L., Hudis C.A. (2007). Breast cancer chemotherapy. Cancer J. *13,* 141-147.

McCachren,S.S., Diggs J., Weinberg J.B., and Dittman W.A. (1991). Thrombomodulin expression by human blood monocytes and by human synovial tissue lining macrophages. Blood *78,* 3128-3132.

McCarroll,D.R., Levin E.G., and Montgomery R.R. (1985). Endothelial cell synthesis of von Willebrand antigen II, von Willebrand factor, and von Willebrand factor/von Willebrand antigen II complex. J. Clin. Invest *75,* 1089-1095.

McMullen,B.A., Fujikawa K. (1985). Amino acid sequence of the heavy chain of human alpha-factor XIIa (activated Hageman factor). J. Biol. Chem. *260,* 5328-5341.

Medved,L.V., Gorkun O.V., and Privalov P.L. (1983). Structural organization of C-terminal parts of fibrinogen A alpha-chains. FEBS Lett. *160,* 291-295.

Meeks,S.L., Abshire T.C. (2008). Abnormalities of prothrombin: a review of the pathophysiology, diagnosis, and treatment. Haemophilia. *14,* 1159-1163.

Meier,T.R., Myers D.D., Jr., Wrobleski S.K., Zajkowski P.J., Hawley A.E., Bedard P.W., Ballard N.E., Londy F.J., Kaila N., Vlasuk G.P., Schaub R.G., and Wakefield T.W. (2008). Prophylactic P-selectin inhibition with PSI-421 promotes resolution of venous thrombosis without anticoagulation. Thromb. Haemost. *99,* 343-351.

Mertens,G., Cassiman J.J., van den B.H., Vermylen J., and David G. (1992). Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. Core protein characterization and antithrombin III binding properties. J. Biol. Chem. *267,* 20435-20443.

Meryet-Figuieres,M., Resina S., Lavigne C., Barlovatz-Meimon G., Lebleu B., and Thierry A.R. (2007). Inhibition of PAI-1 expression in breast cancer carcinoma cells by siRNA at nanomolar range. Biochimie *89,* 1228-1233.

Mewhort-Buist,T.A., Liaw P.C., Patel S., Atkinson H.M., Berry L.R., and Chan A.K. (2008). Treatment of endothelium with the chemotherapy agent vincristine affects activated protein C generation to a greater degree in newborn plasma than in adult plasma. Thromb. Res. *122,* 418-426.

Meyer,T., Robles-Carrillo L., Robson T., Langer F., Desai H., Davila M., Amaya M., Francis J.L., and Amirkhosravi A. (2009). Bevacizumab immune complexes activate platelets and induce thrombosis in FCGR2A transgenic mice. J. Thromb. Haemost. *7,* 171-181.

Michota,F. (2005). Venous thromboembolism: epidemiology, characteristics, and consequences. Clin. Cornerstone. *7,* 8-15.

Minotti,G., Menna P., Salvatorelli E., Cairo G., and Gianni L. (2004). Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol. Rev. *56,* 185-229.

Misiti,F., Giardina B., Mordente A., and Clementi M.E. (2003). The secondary alcohol and aglycone metabolites of doxorubicin alter metabolism of human erythrocytes. Braz. J Med Biol. Res. *36,* 1643-1651.

Misonou,Y., Asahi M., Yokoe S., Miyoshi E., and Taniguchi N. (2006). Acrolein produces nitric oxide through the elevation of intracellular calcium levels to induce apoptosis in human umbilical vein endothelial cells: implications for smoke angiopathy. Nitric. Oxide. *14,* 180-187.

Misonou,Y., Takahashi M., Park Y.S., Asahi M., Miyamoto Y., Sakiyama H., Cheng X., and Taniguchi N. (2005). Acrolein induces Hsp72 via both PKCdelta/JNK and calcium signaling pathways in human umbilical vein endothelial cells. Free Radic. Res. *39,* 507-512.

Mohri,M., Sugimoto E., Sata M., and Asano T. (1999). The inhibitory effect of recombinant human soluble thrombomodulin on initiation and extension of coagulation--a comparison with other anticoagulants. Thromb. Haemost. *82,* 1687-1693.

Monkovic,D.D., Tracy P.B. (1990). Functional characterization of human platelet-released factor V and its activation by factor Xa and thrombin. J. Biol. Chem. *265,* 17132-17140.

Moore,K.L., Andreoli S.P., Esmon N.L., Esmon C.T., and Bang N.U. (1987). Endotoxin enhances tissue factor and suppresses thrombomodulin expression of human vascular endothelium in vitro. J. Clin. Invest *79,* 124-130.

Moore,K.L., Esmon C.T., and Esmon N.L. (1989). Tumor necrosis factor leads to the internalization and degradation of thrombomodulin from the surface of bovine aortic endothelial cells in culture. Blood *73,* 159-165.

Morris,A.D., Morris R.D., Wilson J.F., White J., Steinberg S., Okunieff P., Arriagada R., Le M.G., Blichert-Toft M., and van Dongen J.A. (1997). Breast-conserving therapy vs mastectomy in early-stage breast cancer: a meta-analysis of 10-year survival. Cancer J. Sci. Am. *3,* 6-12.

Morrissey,J.H., Macik B.G., Neuenschwander P.F., and Comp P.C. (1993). Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. Blood *81,* 734-744.

Mukherjee,S.D., Swystun L.L., Mackman N., Wang J.G., Pond G., Levine M.N., and Liaw P.C. (2010). Impact of chemotherapy on thrombin generation and on the protein C pathway in breast cancer patients. Pathophysiol. Haemost. Thromb. *37,* 88-97.

Muller,I., Niethammer D., and Bruchelt G. (1998). Anthracycline-derived chemotherapeutics in apoptosis and free radical cytotoxicity (Review). Int. J. Mol. Med. *1,* 491-494.

Naito,K., Fujikawa K. (1991). Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces. J. Biol. Chem. *266,* 7353-7358.

Nawroth,P.P., Handley D.A., Esmon C.T., and Stern D.M. (1986). Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. Proc. Natl. Acad. Sci. U. S. A *83,* 3460-3464.

Nazaroff,W.W., Singer B.C. (2004). Inhalation of hazardous air pollutants from environmental tobacco smoke in US residences. J. Expo. Anal. Environ. Epidemiol. *14 Suppl 1,* S71-S77.

Nemerson,Y., Repke D. (1985). Tissue factor accelerates the activation of coagulation factor VII: the role of a bifunctional coagulation cofactor. Thromb. Res. *40,* 351-358.

Nishibe,T., Parry G., Ishida A., Aziz S., Murray J., Patel Y., Rahman S., Strand K., Saito K., Saito Y., Hammond W.P., Savidge G.F., Mackman N., and Wijelath E.S. (2001). Oncostatin M promotes biphasic tissue factor expression in smooth muscle cells: evidence for Erk-1/2 activation. Blood *97,* 692-699.

Numico,G., Garrone O., Dongiovanni V., Silvestris N., Colantonio I., Di Costanzo G., Granetto C., Occelli M., Fea E., Heouaine A., Gasco M., and Merlano M. (2005). Prospective evaluation of major vascular events in patients with nonsmall cell lung carcinoma treated with cisplatin and gemcitabine. Cancer *103,* 994-999.

O'Connor,P.M., Wassermann K., Sarang M., Magrath I., Bohr V.A., and Kohn K.W. (1991). Relationship between DNA cross-links, cell cycle, and apoptosis in Burkitt's lymphoma cell lines differing in sensitivity to nitrogen mustard. Cancer Res. *51,* 6550-6557.

O'Toole,T.E., Zheng Y.T., Hellmann J., Conklin D.J., Barski O., and Bhatnagar A. (2009). Acrolein activates matrix metalloproteinases by increasing reactive oxygen species in macrophages. Toxicol. Appl. Pharmacol. *236,* 194-201.

Ofosu,F.A., Modi G.J., Smith L.M., Cerskus A.L., Hirsh J., and Blajchman M.A. (1984). Heparan sulfate and dermatan sulfate inhibit the generation of thrombin activity in plasma by complementary pathways. Blood *64,* 742-747.

Oh,H., Siano B., and Diamond S. (2008). Neutrophil isolation protocol. J. Vis. Exp. *17,* 1-2.

Osterud,B., Bjorklid E. (2006). Sources of tissue factor. Semin. Thromb. Hemost. *32,* 11-23.

Osterud,B., Bouma B.N., and Griffin J.H. (1978). Human blood coagulation factor IX. Purification, properties, and mechanism of activation by activated factor XI. J. Biol. Chem. *253,* 5946-5951.

Osterud,B., Rapaport S.I. (1977). Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. Proc. Natl. Acad. Sci. U. S. A *74,* 5260-5264.

Otten,H.M., Mathijssen J., ten C.H., Soesan M., Inghels M., Richel D.J., and Prins M.H. (2004). Symptomatic venous thromboembolism in cancer patients treated with chemotherapy: an underestimated phenomenon. Arch. Intern. Med. *164,* 190-194.

Owens,A.P., III, Mackman N. (2010). Tissue factor and thrombosis: The clot starts here. Thromb. Haemost. *104,* 432-439.

Owens,A.P., III, Mackman N. (2011). Microparticles in hemostasis and thrombosis. Circ. Res. *108,* 1284-1297.

Paredes,N., Xu L., Berry L.R., and Chan A.K. (2003). The effects of chemotherapeutic agents on the regulation of thrombin on cell surfaces. Br. J Haematol. *120,* 315-324.

Parry,G.C., Erlich J.H., Carmeliet P., Luther T., and Mackman N. (1998). Low levels of tissue factor are compatible with development and hemostasis in mice. J. Clin. Invest *101,* 560-569.

Pathak,A., Zhao R., Monroe D.M., Roberts H.R., Sheridan B.C., Selzman C.H., and Stouffer G.A. (2006). Thrombin generation in vascular tissue. J Thromb. Haemost. *4,* 60-67.

Patnaik,M.M., Moll S. (2008). Inherited antithrombin deficiency: a review. Haemophilia. *14,* 1229-1239.

Pauer,H.U., Renne T., Hemmerlein B., Legler T., Fritzlar S., Adham I., Muller-Esterl W., Emons G., Sancken U., Engel W., and Burfeind P. (2004). Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation in vivo. Thromb. Haemost. *92,* 503-508.

Pawlinski,R., Mackman N. (2010a). Cellular sources of tissue factor in endotoxemia and sepsis. Thromb. Res. *125 Suppl 1,* S70-S73.

Pawlinski,R., Wang J.G., Owens A.P., III, Williams J., Antoniak S., Tencati M., Luther T., Rowley J.W., Low E.N., Weyrich A.S., and Mackman N. (2010b). Hematopoietic and nonhematopoietic cell tissue factor activates the coagulation cascade in endotoxemic mice. Blood *116,* 806-814.

Pendurthi,U.R., Ghosh S., Mandal S.K., and Rao L.V. (2007). Tissue factor activation: is disulfide bond switching a regulatory mechanism? Blood *110,* 3900-3908.

Penn,M.S., Patel C.V., Cui M.Z., DiCorleto P.E., and Chisolm G.M. (1999). LDL increases inactive tissue factor on vascular smooth muscle cell surfaces: hydrogen peroxide activates latent cell surface tissue factor. Circulation *99,* 1753-1759.

Pepler L. Regulation of thrombin generation by vascular endothelial cells 2009.

Petersen,L.C., Bjorn S.E., Olsen O.H., Nordfang O., Norris F., and Norris K. (1996). Inhibitory properties of separate recombinant Kunitz-type-protease-inhibitor domains from tissue-factor-pathway inhibitor. Eur. J. Biochem. *235,* 310-316.

Petropoulou,A.D., Gerotziafas G.T., Samama M.M., Hatmi M., Rendu F., and Elalamy I. (2008). In vitro study of the hypercoagulable state in multiple myeloma patients treated or not with thalidomide. Thromb. Res. *121,* 493-497.

Pineo,G.F., Brain M.C., Gallus A.S., Hirsh J., Hatton M.W., and Regoeczi E. (1974). Tumors, mucus production, and hypercoagulability. Ann. N. Y. Acad. Sci. *230,* 262-270.

Plescia,J., Altieri D.C. (1996). Activation of Mac-1 (CD11b/CD18)-bound factor X by released cathepsin G defines an alternative pathway of leucocyte initiation of coagulation. Biochem. J *319 (Pt 3),* 873-879.

Plutzky,J., Hoskins J.A., Long G.L., and Crabtree G.R. (1986). Evolution and organization of the human protein C gene. Proc. Natl. Acad. Sci. U. S. A *83,* 546-550.

Poggi,A., Kornblihtt L., Delaini F., Colombo T., Mussoni L., Reyers I., and Donati M.B. (1979). Delayed hypercoagulability after a single dose of adriamycin to normal rats. Thromb. Res. *16,* 639-650.

Popescu,N.I., Lupu C., and Lupu F. (2010). Role of PDI in regulating tissue factor: FVIIa activity. Thromb. Res. *125 Suppl 1,* S38-S41.

Pozza,L.M., Austin R.C. (2005). Getting a GRP on tissue factor activation. Arterioscler. Thromb. Vasc. Biol. *25,* 1529-1531.

Prandoni,P., Falanga A., and Piccioli A. (2005). Cancer and venous thromboembolism. Lancet Oncol. *6,* 401-410.

Previtali,E., Bucciarelli P., Passamonti S.M., and Martinelli I. (2011). Risk factors for venous and arterial thrombosis. Blood Transfus. *9,* 120-138.

Qu,D., Wang Y., Esmon N.L., and Esmon C.T. (2007). Regulated endothelial protein C receptor shedding is mediated by tumor necrosis factor-alpha converting enzyme/ADAM17. J. Thromb. Haemost. *5,* 395-402.

Rabbani,A., Finn R.M., Thambirajah A.A., and Ausio J. (2004). Binding of antitumor antibiotic daunomycin to histones in chromatin and in solution. Biochemistry *43,* 16497-16504.

Rajkumar,S.V., Blood E., Vesole D., Fonseca R., and Greipp P.R. (2006). Phase III clinical trial of thalidomide plus dexamethasone compared with dexamethasone alone in newly diagnosed multiple myeloma: a clinical trial coordinated by the Eastern Cooperative Oncology Group. J Clin. Oncol. *24,* 431-436.

Rand,J.H., Wu X.X., Quinn A.S., and Taatjes D.J. (2008). Resistance to annexin A5 anticoagulant activity: a thrombogenic mechanism for the antiphospholipid syndrome. Lupus *17,* 922-930.

Rao,L.V., Nordfang O., Hoang A.D., and Pendurthi U.R. (1995). Mechanism of antithrombin III inhibition of factor VIIa/tissue factor activity on cell surfaces. Comparison with tissue factor pathway inhibitor/factor Xa-induced inhibition of factor VIIa/tissue factor activity. Blood *85,* 121-129.

Rao,L.V., Pendurthi U.R. (2005). Tissue factor-factor VIIa signaling. Arterioscler. Thromb. Vasc. Biol. *25,* 47-56.

Regan,L.M., Lamphear B.J., Huggins C.F., Walker F.J., and Fay P.J. (1994). Factor IXa protects factor VIIIa from activated protein C. Factor IXa inhibits activated protein C-catalyzed cleavage of factor VIIIa at Arg562. J. Biol. Chem. *269,* 9445-9452.

Regan,L.M., Mollica J.S., Rezaie A.R., and Esmon C.T. (1997). The interaction between the endothelial cell protein C receptor and protein C is dictated by the gamma-carboxyglutamic acid domain of protein C. J. Biol. Chem. *272*, 26279-26284.

Reigner,B., Watanabe T., Schuller J., Lucraft H., Sasaki Y., Bridgewater J., Saeki T., McAleer J., Kuranami M., Poole C., Kimura M., Monkhouse J., Yorulmaz C., Weidekamm E., and Grange S. (2003). Pharmacokinetics of capecitabine (Xeloda) in Japanese and Caucasian patients with breast cancer. Cancer Chemother. Pharmacol. *52*, 193-201.

Reitsma,P.H. (1996). Protein C deficiency: summary of the 1995 database update. Nucleic Acids Res. *24*, 157-159.

Rella,C., Coviello M., Giotta F., Maiello E., Colavito P., Colangelo D., Quaranta M., Colucci G., and Schittulli F. (1996). A prothrombotic state in breast cancer patients treated with adjuvant chemotherapy. Breast Cancer Res. Treat. *40*, 151-159.

Renne,T., Pozgajova M., Gruner S., Schuh K., Pauer H.U., Burfeind P., Gailani D., and Nieswandt B. (2005). Defective thrombus formation in mice lacking coagulation factor XII. J. Exp. Med. *202*, 271-281.

Rickles,F.R., Edwards R.L. (1983a). Activation of blood coagulation in cancer: Trousseau's syndrome revisited. Blood *62*, 14-31.

Rickles,F.R., Edwards R.L., Barb C., and Cronlund M. (1983b). Abnormalities of blood coagulation in patients with cancer. Fibrinopeptide A generation and tumor growth. Cancer *51*, 301-307.

Rickles,F.R., Hair G.A., Zeff R.A., Lee E., and Bona R.D. (1995). Tissue factor expression in human leukocytes and tumor cells. Thromb. Haemost. *74*, 391-395.

Ritchie,H., Robbie L.A., Kinghorn S., Exley R., and Booth N.A. (1999). Monocyte plasminogen activator inhibitor 2 (PAI-2) inhibits u-PA-mediated fibrin clot lysis and is cross-linked to fibrin. Thromb. Haemost. *81*, 96-103.

Robert,J., Vrignaud P., Nguyen-Ngoc T., Iliadis A., Mauriac L., and Hurteloup P. (1985). Comparative pharmacokinetics and metabolism of doxorubicin and epirubicin in patients with metastatic breast cancer. Cancer Treat. Rep. *69*, 633-640.

Rodgers,G.M., Shuman M.A. (1983). Prothrombin is activated on vascular endothelial cells by factor Xa and calcium. Proc. Natl. Acad. Sci. U. S. A *80*, 7001-7005.

Roemisch,J., Gray E., Hoffmann J.N., and Wiedermann C.J. (2002). Antithrombin: a new look at the actions of a serine protease inhibitor. Blood Coagul. Fibrinolysis *13*, 657-670.

Rogers,J.S., Murgo A.J., Fontana J.A., and Raich P.C. (1988). Chemotherapy for breast cancer decreases plasma protein C and protein S. J Clin. Oncol. *6,* 276-281.

Rosen,E.D., Gailani D., and Castellino F.J. (2002). FXI is essential for thrombus formation following FeCl3-induced injury of the carotid artery in the mouse. Thromb. Haemost. *87,* 774-776.

Rosendaal,F.R. (2005). Venous thrombosis: the role of genes, environment, and behavior. Hematology. Am. Soc. Hematol. Educ. Program. 1-12.

Rosing,J., Tans G., Govers-Riemslag J.W., Zwaal R.F., and Hemker H.C. (1980). The role of phospholipids and factor Va in the prothrombinase complex. J. Biol. Chem. *255,* 274-283.

Rubin,R.N., Kies M.S., and Posch J.J., Jr. (1980). Measurements of antithrombin III in solid tumor patients with and without hepatic metastases. Thromb. Res. *18,* 353-360.

Rudra,P.K., Krokan H.E. (1999). Acrolein cytotoxicity and glutathione depletion in n-3 fatty acid sensitive- and resistant human tumor cells. Anticancer Res. *19,* 461-469.

Ruf,W., Versteeg H.H. (2010). Tissue factor mutated at the allosteric Cys186-Cys209 disulfide bond is severely impaired in decrypted procoagulant activity. Blood *116,* 500-501.

Ruggeri,Z.M., De M.L., Gatti L., Bader R., and Montgomery R.R. (1983). Platelets have more than one binding site for von Willebrand factor. J. Clin. Invest *72,* 1-12.

Rugo,H.S. (2004). Bevacizumab in the treatment of breast cancer: rationale and current data. Oncologist. *9 Suppl 1,* 43-49.

Sagripanti,A., Carpi A., Baicchi U., and Grassi B. (1993). Plasmatic parameters of fibrin formation and degradation in cancer patients: correlation between fibrinopeptide A and D-dimer. Biomed. Pharmacother. *47,* 235-239.

Sakata,K., Kashiwagi K., Sharmin S., Ueda S., Irie Y., Murotani N., and Igarashi K. (2003). Increase in putrescine, amine oxidase, and acrolein in plasma of renal failure patients. Biochem. Biophys. Res. Commun. *305,* 143-149.

Sallah,S., Wan J.Y., and Nguyen N.P. (2002). Venous thrombosis in patients with solid tumors: determination of frequency and characteristics. Thromb. Haemost. *87,* 575-579.

Saphner,T., Tormey D.C., and Gray R. (1991). Venous and arterial thrombosis in patients who received adjuvant therapy for breast cancer. J. Clin. Oncol. *9,* 286-294.

Saposnik,B., Reny J.L., Gaussem P., Emmerich J., Aiach M., and Gandrille S. (2004). A haplotype of the EPCR gene is associated with increased plasma levels of sEPCR and is a candidate risk factor for thrombosis. Blood *103,* 1311-1318.

Satoh,K., Yamada S., Koike Y., Igarashi Y., Toyokuni S., Kumano T., Takahata T., Hayakari M., Tsuchida S., and Uchida K. (1999). A 1-hour enzyme-linked immunosorbent assay for quantitation of acrolein- and hydroxynonenal-modified proteins by epitope-bound casein matrix method. Anal. Biochem. *270,* 323-328.

Saukkonen,K., Lakkisto P., Pettila V., Varpula M., Karlsson S., Ruokonen E., and Pulkki K. (2008). Cell-free plasma DNA as a predictor of outcome in severe sepsis and septic shock. Clin. Chem. *54,* 1000-1007.

Schmaier,A.H. (2008). The elusive physiologic role of Factor XII. J. Clin. Invest *118,* 3006-3009.

Schwarzenbach,H., Hoon D.S., and Pantel K. (2011). Cell-free nucleic acids as biomarkers in cancer patients. Nat. Rev. Cancer *11,* 426-437.

Semeraro,F., Ammollo C.T., Morrissey J.H., Dale G.L., Friese P., Esmon N.L., and Esmon C.T. (2011). Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. Blood *118,* 1952-1961.

Semeraro,N., Triggiani R., Montemurro P., Cavallo L.G., and Colucci M. (1993). Enhanced endothelial tissue factor but normal thrombomodulin in endotoxin-treated rabbits. Thromb. Res. *71,* 479-486.

Shantsila,E., Lip G.Y. (2009). The role of monocytes in thrombotic disorders. Insights from tissue factor, monocyte-platelet aggregates and novel mechanisms. Thromb. Haemost. *102,* 916-924.

Shen,V.S., Pollak E.W. (1980). Fatal pulmonary embolism in cancer patients: is heparin prophylaxis justified? South. Med. J. *73,* 841-843.

Shi,J., Pipe S.W., Rasmussen J.T., Heegaard C.W., and Gilbert G.E. (2008). Lactadherin blocks thrombosis and hemostasis in vivo: correlation with platelet phosphatidylserine exposure. J. Thromb. Haemost. *6,* 1167-1174.

Shifman,M.A., Pizzo S.V. (1982). The in vivo metabolism of antithrombin III and antithrombin III complexes. J. Biol. Chem. *257,* 3243-3248.

Singletary,S.E. (2003). Rating the risk factors for breast cancer. Ann. Surg. *237,* 474-482.

Singletary,S.E., Allred C., Ashley P., Bassett L.W., Berry D., Bland K.I., Borgen P.I., Clark G., Edge S.B., Hayes D.F., Hughes L.L., Hutter R.V., Morrow M., Page D.L., Recht A., Theriault R.L., Thor A., Weaver D.L., Wieand H.S., and Greene F.L. (2002).

Revision of the American Joint Committee on Cancer staging system for breast cancer. J. Clin. Oncol. *20,* 3628-3636.

Smith,I., Procter M., Gelber R.D., Guillaume S., Feyereislova A., Dowsett M., Goldhirsch A., Untch M., Mariani G., Baselga J., Kaufmann M., Cameron D., Bell R., Bergh J., Coleman R., Wardley A., Harbeck N., Lopez R.I., Mallmann P., Gelmon K., Wilcken N., Wist E., Sanchez R.P., and Piccart-Gebhart M.J. (2007). 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. Lancet *369,* 29-36.

Smith,S.A., Mutch N.J., Baskar D., Rohloff P., Docampo R., and Morrissey J.H. (2006). Polyphosphate modulates blood coagulation and fibrinolysis. Proc. Natl. Acad. Sci. U. S. A *103,* 903-908.

Sorensen,H.T., Mellemkjaer L., Olsen J.H., and Baron J.A. (2000). Prognosis of cancers associated with venous thromboembolism. N. Engl. J. Med. *343,* 1846-1850.

Stahli,B.E., Camici G.G., Steffel J., Akhmedov A., Shojaati K., Graber M., Luscher T.F., and Tanner F.C. (2006). Paclitaxel enhances thrombin-induced endothelial tissue factor expression via c-Jun terminal NH2 kinase activation. Circ. Res. *99,* 149-155.

Stephenson,D.A., Toltl L.J., Beaudin S., and Liaw P.C. (2006). Modulation of monocyte function by activated protein C, a natural anticoagulant. J. Immunol. *177,* 2115-2122.

Sun,N.C., McAfee W.M., Hum G.J., and Weiner J.M. (1979). Hemostatic abnormalities in malignancy, a prospective study of one hundred eight patients. Part I. Coagulation studies. Am. J. Clin. Pathol. *71,* 10-16.

Sun,W.Y., Witte D.P., Degen J.L., Colbert M.C., Burkart M.C., Holmback K., Xiao Q., Bugge T.H., and Degen S.J. (1998). Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc. Natl. Acad. Sci. U. S. A *95,* 7597-7602.

Sweeney,J.D., Killion K.M., Pruet C.F., and Spaulding M.B. (1990). von Willebrand factor in head and neck cancer. Cancer *66,* 2387-2389.

Swystun,L.L., Mukherjee S., Levine M., and Liaw P.C. (2011). The chemotherapy metabolite acrolein upregulates thrombin generation and impairs the protein C anticoagulant pathway in animal-based and cell-based models. J. Thromb. Haemost. *9,* 767-775.

Swystun,L.L., Shin L.Y., Beaudin S., and Liaw P.C. (2009). Chemotherapeutic agents doxorubicin and epirubicin induce a procoagulant phenotype on endothelial cells and blood monocytes. J Thromb. Haemost. *7,* 619-626.

Taylor,F.B., Jr., Peer G.T., Lockhart M.S., Ferrell G., and Esmon C.T. (2001). Endothelial cell protein C receptor plays an important role in protein C activation in vivo. Blood *97,* 1685-1688.

Tesselaar,M.E., Romijn F.P., van d.L., I, Prins F.A., Bertina R.M., and Osanto S. (2007). Microparticle-associated tissue factor activity: a link between cancer and thrombosis? J. Thromb. Haemost. *5,* 520-527.

Thomas,G.M., Panicot-Dubois L., Lacroix R., gnat-George F., Lombardo D., and Dubois C. (2009). Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. J. Exp. Med. *206,* 1913-1927.

Thompson,R.E., Mandle R., Jr., and Kaplan A.P. (1977). Association of factor XI and high molecular weight kininogen in human plasma. J. Clin. Invest *60,* 1376-1380.

Toomey,J.R., Kratzer K.E., Lasky N.M., Stanton J.J., and Broze G.J., Jr. (1996). Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood *88,* 1583-1587.

Totan,M., Dagdemir A., Ak A.R., Albayrak D., and Kucukoduk S. (2001). Effects of high-dose methotrexate on the hemostatic system in childhood acute lymphoblastic leukemia. Med. Pediatr. Oncol. *36,* 429-433.

Tracy,P.B., Mann K.G. (1983). Prothrombinase complex assembly on the platelet surface is mediated through the 74,000-dalton component of factor Va. Proc. Natl. Acad. Sci. U. S. A *80,* 2380-2384.

Valsami,S., Ruf W., Leikauf M.S., Madon J., Kaech A., and Asmis L.M. (2011). Immunomodulatory drugs increase endothelial tissue factor expression in vitro. Thromb. Res. *127,* 264-271.

Van der Wilt,C.L., Van Groeningen C.J., Pinedo H.M., Smid K., Hoekman K., Meijer S., and Peters G.J. (1997). 5-fluorouracil/leucovorin-induced inhibition of thymidylate synthase in normal tissues of mouse and man. J. Cancer Res. Clin. Oncol. *123,* 595-601.

VanDeWater,L., Tracy P.B., Aronson D., Mann K.G., and Dvorak H.F. (1985). Tumor cell generation of thrombin via functional prothrombinase assembly. Cancer Res. *45,* 5521-5525.

Venugopal,S.K., Devaraj S., and Jialal I. (2003). C-reactive protein decreases prostacyclin release from human aortic endothelial cells. Circulation *108,* 1676-1678.

Venugopal,S.K., Devaraj S., Yuhanna I., Shaul P., and Jialal I. (2002). Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. Circulation *106,* 1439-1441.

Verhoven,B., Schlegel R.A., and Williamson P. (1995). Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. J. Exp. Med. *182,* 1597-1601.

Versteeg,H.H., Ruf W. (2007). Tissue factor coagulant function is enhanced by protein-disulfide isomerase independent of oxidoreductase activity. J Biol. Chem. *282,* 25416-25424.

Vicente,V., Gonzalez-Conejero R., Rivera J., and Corral J. (1999). The prothrombin gene variant 20210A in venous and arterial thromboembolism. Haematologica *84,* 356-362.

Vigano,D.S., Comp P.C., Esmon C.T., and D'Angelo A. (1986). Relationship between protein C antigen and anticoagulant activity during oral anticoagulation and in selected disease states. J. Clin. Invest *77,* 416-425.

Vogel,V.G., Costantino J.P., Wickerham D.L., Cronin W.M., Cecchini R.S., Atkins J.N., Bevers T.B., Fehrenbacher L., Pajon E.R., Jr., Wade J.L., III, Robidoux A., Margolese R.G., James J., Lippman S.M., Runowicz C.D., Ganz P.A., Reis S.E., Caskill-Stevens W., Ford L.G., Jordan V.C., and Wolmark N. (2006). Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA *295,* 2727-2741.

von Tempelhoff,G.F., Dietrich M., Hommel G., and Heilmann L. (1996). Blood coagulation during adjuvant epirubicin/cyclophosphamide chemotherapy in patients with primary operable breast cancer. J. Clin. Oncol. *14,* 2560-2568.

von Tempelhoff,G.F., Dietrich M., Niemann F., Schneider D., Hommel G., and Heilmann L. (1997). Blood coagulation and thrombosis in patients with ovarian malignancy. Thromb. Haemost. *77,* 456-461.

Vormittag,R., Simanek R., Ay C., Dunkler D., Quehenberger P., Marosi C., Zielinski C., and Pabinger I. (2009). High factor VIII levels independently predict venous thromboembolism in cancer patients: the cancer and thrombosis study. Arterioscler. Thromb. Vasc. Biol. *29,* 2176-2181.

Wagner,D.D., Burger P.C. (2003). Platelets in inflammation and thrombosis. Arterioscler. Thromb. Vasc. Biol. *23,* 2131-2137.

Wahrenbrock,M., Borsig L., Le D., Varki N., and Varki A. (2003). Selectin-mucin interactions as a probable molecular explanation for the association of Trousseau syndrome with mucinous adenocarcinomas. J. Clin. Invest *112,* 853-862.

Wakita,K., Stearns-Kurosawa D.J., and Marumoto Y. (1994). The effect of calcium ionophore A23187 on tissue factor activity and mRNA in endothelial cells. Thromb. Res. *74,* 95-103.

Walker,F.J. (1990). Protein C deficiency in liver disease. Ann. Clin. Lab Sci. *20,* 106-112.

Walker,F.J. (1980). Regulation of activated protein C by a new protein. A possible function for bovine protein S. J. Biol. Chem. *255,* 5521-5524.

Wall,J.G., Weiss R.B., Norton L., Perloff M., Rice M.A., Korzun A.H., and Wood W.C. (1989). Arterial thrombosis associated with adjuvant chemotherapy for breast carcinoma: a Cancer and Leukemia Group B Study. Am. J. Med. *87,* 501-504.

Walsh,J., Wheeler H.R., and Geczy C.L. (1992). Modulation of tissue factor on human monocytes by cisplatin and adriamycin. Br. J Haematol. *81,* 480-488.

Wang,J.G., Manly D., Kirchhofer D., Pawlinski R., and Mackman N. (2009a). Levels of microparticle tissue factor activity correlate with coagulation activation in endotoxemic mice. J. Thromb. Haemost. *7,* 1092-1098.

Wang,L., Miller C., Swarthout R.F., Rao M., Mackman N., and Taubman M.B. (2009b). Vascular smooth muscle-derived tissue factor is critical for arterial thrombosis after ferric chloride-induced injury. Blood *113,* 705-713.

Wang,W., Boffa M.B., Bajzar L., Walker J.B., and Nesheim M.E. (1998). A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor. J. Biol. Chem. *273,* 27176-27181.

Weiler,H., Lindner V., Kerlin B., Isermann B.H., Hendrickson S.B., Cooley B.C., Meh D.A., Mosesson M.W., Shworak N.W., Post M.J., Conway E.M., Ulfman L.H., von Andrian U.H., and Weitz J.I. (2001). Characterization of a mouse model for thrombomodulin deficiency. Arterioscler. Thromb. Vasc. Biol. *21,* 1531-1537.

Weiler-Guettler,H., Christie P.D., Beeler D.L., Healy A.M., Hancock W.W., Rayburn H., Edelberg J.M., and Rosenberg R.D. (1998). A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J. Clin. Invest *101,* 1983-1991.

Weisel,J.W., Francis C.W., Nagaswami C., and Marder V.J. (1993). Determination of the topology of factor XIIIa-induced fibrin gamma-chain cross-links by electron microscopy of ligated fragments. J. Biol. Chem. *268,* 26618-26624.

Weiss,H.J., Turitto V.T., Baumgartner H.R., Nemerson Y., and Hoffmann T. (1989). Evidence for the presence of tissue factor activity on subendothelium. Blood *73,* 968-975.

Weiss,R.B., Tormey D.C., Holland J.F., and Weinberg V.E. (1981). Venous thrombosis during multimodal treatment of primary breast carcinoma. Cancer Treat. Rep. *65,* 677-679.

Weitz,I.C., Israel V.K., Waisman J.R., Presant C.A., Rochanda L., and Liebman H.A. (2002). Chemotherapy-induced activation of hemostasis: effect of a low molecular weight heparin (dalteparin sodium) on plasma markers of hemostatic activation. Thromb. Haemost. *88,* 213-220.

Wheeler,H.R., Geczy C.L. (1990). Induction of macrophage procoagulant expression by cisplatin, daunorubicin and doxorubicin. Int. J Cancer *46,* 626-632.

White,R.H., Chew H., and Wun T. (2007). Targeting patients for anticoagulant prophylaxis trials in patients with cancer: who is at highest risk? Thromb. Res. *120 Suppl 2,* S29-S40.

WHO. World Health Organization International Agency for Research on Cancer World Cancer Report. 2003.

Wolberg,A.S., Campbell R.A. (2008). Thrombin generation, fibrin clot formation and hemostasis. Transfus. Apher. Sci. *38,* 15-23.

Wolberg,A.S., Monroe D.M., Roberts H.R., and Hoffman M.R. (1999). Tissue factor de-encryption: ionophore treatment induces changes in tissue factor activity by phosphatidylserine-dependent and -independent mechanisms. Blood Coagul. Fibrinolysis *10,* 201-210.

Woodley-Cook,J., Shin L.Y., Swystun L., Caruso S., Beaudin S., and Liaw P.C. (2006). Effects of the chemotherapeutic agent doxorubicin on the protein C anticoagulant pathway. Mol. Cancer Ther. *5,* 3303-3311.

Woodruff,R.S., Sullenger B., and Becker R.C. (2011). The many faces of the contact pathway and their role in thrombosis. J. Thromb. Thrombolysis. *32,* 9-20.

Xu,J., Qu D., Esmon N.L., and Esmon C.T. (2000). Metalloproteolytic release of endothelial cell protein C receptor. J. Biol. Chem. *275,* 6038-6044.

Xu,J., Zhang X., Pelayo R., Monestier M., Ammollo C.T., Semeraro F., Taylor F.B., Esmon N.L., Lupu F., and Esmon C.T. (2009). Extracellular histones are major mediators of death in sepsis. Nat. Med *15,* 1318-1321.

Xue,J., Wu Q., Westfield L.A., Tuley E.A., Lu D., Zhang Q., Shim K., Zheng X., and Sadler J.E. (1998). Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc. Natl. Acad. Sci. U. S. A *95,* 7603-7607.

Yegneswaran,S., Smirnov M.D., Safa O., Esmon N.L., Esmon C.T., and Johnson A.E. (1999). Relocating the active site of activated protein C eliminates the need for its protein S cofactor. A fluorescence resonance energy transfer study. J. Biol. Chem. *274,* 5462-5468.

Yu,J., May L., Milsom C., Anderson G.M., Weitz J.I., Luyendyk J.P., Broze G., Mackman N., and Rak J. (2008). Contribution of host-derived tissue factor to tumor neovascularization. Arterioscler. Thromb. Vasc. Biol. *28,* 1975-1981.

Yu,J.L., May L., Lhotak V., Shahrzad S., Shirasawa S., Weitz J.I., Coomber B.L., Mackman N., and Rak J.W. (2005). Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis. Blood *105,* 1734-1741.

Zangari,M., Anaissie E., Barlogie B., Badros A., Desikan R., Gopal A.V., Morris C., Toor A., Siegel E., Fink L., and Tricot G. (2001). Increased risk of deep-vein thrombosis in patients with multiple myeloma receiving thalidomide and chemotherapy. Blood *98,* 1614-1615.

Zangari,M., Siegel E., Barlogie B., Anaissie E., Saghafifar F., Fassas A., Morris C., Fink L., and Tricot G. (2002). Thrombogenic activity of doxorubicin in myeloma patients receiving thalidomide: implications for therapy. Blood *100,* 1168-1171.

Zeng,L., Yan Z., Ding S., Xu K., and Wang L. (2008). Endothelial injury, an intriguing effect of methotrexate and cyclophosphamide during hematopoietic stem cell transplantation in mice. Transplant. Proc. *40,* 2670-2673.

Zhou,J., Shi J., Hou J., Cao F., Zhang Y., Rasmussen J.T., Heegaard C.W., and Gilbert G.E. (2010). Phosphatidylserine exposure and procoagulant activity in acute promyelocytic leukemia. J. Thromb. Haemost. *8,* 773-782.

Zhu,J., Guo W.M., Yao Y.Y., Zhao W.L., Pan L., Cai X., Ju B., Sun G.L., Wang H.L., Chen S.J., Chen G.Q., Caen J., Chen Z., and Wang Z.Y. (1999). Tissue factors on acute promyelocytic leukemia and endothelial cells are differently regulated by retinoic acid, arsenic trioxide and chemotherapeutic agents. Leukemia *13,* 1062-1070.