PROCOAGULANT EFFECTS OF LUNG CANCER CHEMOTHERAPY AGENTS

PROCOAGULANT EFFECTS OF PLATINUM-BASED LUNG CANCER CHEMOTHERAPY AGENTS

By:

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ABSTRACT

Chemotherapy-associated thrombosis is a common complication in cancer patients. Cancer patients have a 5- to 7-fold increased risk for a thrombotic event compared to healthy individuals. While the overall risk for a thrombotic event in lung cancer patients is approximately 1.4%, the rates of thrombosis vary depending on the stage of the disease and the chemotherapeutic agents used. Activation of coagulation after initiation of chemotherapy has been reported in clinical studies. However, the mechanisms by which lung cancer chemotherapy agents modulate coagulation in lung cancer patients are not completely understood. The focus of this thesis is to investigate the mechanisms by which chemotherapy agents cisplatin, carboplatin, gemcitabine, and paclitaxel (in platinum-based combinations) induce procoagulant effects utilizing *in vitro* and *in vivo* approaches.

First, we investigated the mechanisms by which lung cancer chemotherapy modulates cell-surface tissue factor (TF) activity on endothelial cells (HUVEC), monocytes, and non-small cell lung carcinoma (NSCLC) A549 cells. We observed that treatment of all three cell lines with platinum-based lung cancer chemotherapy increased cell surface TF activity. We found that the increased TF activity on chemotherapy-treated monocytes was due to increased phosphatidylserine (PS) exposure, whereas the increased TF activity on HUVEC and A549 cells was due to protein disulfide isomerase (PDI)-mediated decryption of TF. These studies demonstrate that lung cancer chemotherapy agents can

exert procoagulant effects by increasing PS exposure and by inducing TF decryption on healthy and tumour cells.

Next, we determined the effects of lung cancer chemotherapy on the generation of microparticles (MP) and the impact of MPs on thrombin generation. Our *in vitro* and *in vivo* studies demonstrate that lung cancer chemotherapy agents increase the generation of TF- and PS-positive MPs from tumour cells and that the MPs contribute to thrombin generation in a FVII-dependent manner. We also investigated the role of cell-free DNA (CFDNA) in mediating procoagulant effects induced by lung cancer chemotherapy agents. We found that lung cancer chemotherapy agents induce CFDNA release from healthy host neutrophils and that this leads to additional generation of thrombin by the intrinsic pathway of coagulation.

Lastly, CFDNA levels have been shown to increase in cancer models through formation of neutrophil extracellular traps (NETs). Formation of NETs by NETosis, a process by which neutrophils release extracellular web-like structures composed of DNA, histones, and granular proteins, is dependent on histone citrullination by protein arginine deaminase-4 (PAD-4). In addition, PAD4 inhibition prevents NET formation. Therefore, we wanted to demonstrate that the neutrophil-derived CFDNA release induced by lung cancer chemotherapy is PAD4-dependent. Chemotherapy treatment of PAD4 knockout mice failed to increase CFDNA levels. Furthermore, chemotherapy-treatment did not increase

thrombin generation in PAD4 knockout mice. This suggests that chemotherapy-induced CFDNA release occurs through NETosis.

In conclusion, lung cancer chemotherapy leads to increased thrombin generation which occurs through increased TF decryption, MP generation, and CFDNA release. Therefore, lung cancer chemotherapy results in simultaneous activation of the extrinsic and intrinsic pathways of coagulation. These studies provide novel insight into the mechanisms of lung cancer chemotherapy-associated thrombosis.

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LIST OF ABREVIATIONS

II – prothrombin

IIa – thrombin

ADP – adenosine diphosphate

ALK – anaplastic lymphoma kinase

AT – antithrombin

aPTT – activated partial thromboplastin time

ASCO – American Society of Clinical Oncology

$CaCl_2$ – calcium chloride

CCO – Cancer Care Ontario

CFDNA – cell-free deoxyribonucleic acid

DNA – deoxyribonucleic acid

DVT – deep-vein thrombosis

EGFR – epidermal growth factor receptor

EPCR – endothelial protein C receptor

ER – endoplasmic reticulum

F – factor

Gla – γ-carboxyglutamic acid

HUVEC – human umbilical vein endothelial cells

LPS - lipopolysaccharide

MP – microparticle

NET – neutrophil extracellular trap

NSCLC – non-small cell lung carcinoma

PAD – peptidylargenine deaminase

PE – pulmonary embolism

PC – protein C

PDI – protein disulphide isomerase

PS – phosphatidylserine

PT – prothrombin time

RNA – ribonucleic acid

TAT – thrombin anti-thrombin

TF – tissue factor

TFPI – tissue factor pathway inhibitor

TM – thrombomodulin

TNF – tumour necrosis factor

VEGF – vascular endothelial growth factor

VTE – venous thromboembolism

vWF – von Willebrand factor

DECLARATION OF ACADEMIC ACHIEVEMENT

Zakhar Lysov contributed to the design of studies, performed experiments, analyzed, and interpreted the data for chapters 3, 4, and 5. Zakhar Lysov wrote all manuscript chapters.

Dr. Patricia Liaw contributed to the conception and design of studies, obtained financial support for the research, contributed to the writing of manuscript chapter 3, 4, and 5, as well as reviewed all chapters in this thesis.

Dr. Dhruva J. Dwivedi and Travis Gould assisted with experiments and critically reviewed manuscript chapters 3 and 4..

Dr. Sara Kuruvilla and Dr. Andrew Arnold assisted with review of chapter 3.

Chapter 1: General Introduction

1.1 Hemostasis and Thrombosis

Hemostasis is a finely tuned, ongoing process that ensures adequate blood flow and prevents excess bleeding through activation of the coagulation system (Mackman et al. 2007). In a healthy system, the coagulation and fibrinolytic systems balance clot formation and clot lysis in order to maintain blood in a fluid state (Mackman et al. 2007). Upon vascular damage, activation of the coagulation system results in a formation of a platelet plug as well as a number of enzymatic reactions between coagulation factors ultimately leading to a formation of a fibrin clot (Mann et al. 2003, Mackman et al. 2007). When the hemostatic balance is lost, inadequate or excess clotting can lead to hemorrhage or thrombosis, respectively. The coagulation pathway can be subdivided into the extrinsic (tissue factor) pathway, intrinsic (contact) pathway, and the common pathway (Figure 1.1).

1.2 Platelets and Platelet Plug Formation

Vascular damage results in vasoconstriction (Abraham et al. 2007) as well as adhesion and activation of platelets through exposure of subendothelial collagen and von Willebrand factor (vWF) (Dahlback 2005). Upon activation, platelets release the contents of their alpha and dense granules leading to shape change and platelet aggregation (discussed in further detail below) (Jennings 2009). This leads to the formation of a protective primary hemostatic plug.

Platelets are discoid shaped cells that are 0.5-3 μm in diameter and have a circulating life span of 8-10 days in humans (Thon et al. 2010). Platelets are produced by fragmentation of megakaryocytes within the bone marrow and are released into the circulation. The major function of platelets is to rapidly limit blood loss at the site of vascular injury (Broos et al. 2011). Injury to the vascular wall results in exposure of adhesive macromolecules located in the subendothelial extracellular matrix (ECM) including collagen and vWF (Broos et al. 2011). Circulating platelets bind directly to VFW and collagen in a process termed adhesion. Interactions between platelets, vWF, and subendothelial ECM regulate the formation of a platelet plug though subsequent platelet activation and aggregation (Farndale et al. 2003).

Platelet adhesion is initiated by a tethering interaction between platelet surface glycoproteins and the newly exposed ECM (Broos et al. 2011). Tethering of flowing platelets to the ECM at low shear rates, such as those found within veins, occurs primarily through binding of platelet surface receptor GPVI (GPVI) or α2β (Gα2β1) to collagen (Nieswandt et al. 2003). At high shear rates, such as those found in the microvasculature, tethering is primarily mediated by interactions between platelet surface receptor glycoprotein Ibα (GPIbα) and vWF (Nishio et al. 2004). While the GPIbα-vWF interaction is not sufficient for stable platelet adhesion, the binding of GPIbα to vWF effectively slows the flowing platelets in areas of high shear flow while keeping them in close proximity to collagen allowing for activation to occur (Ruggeri et al. 2006).

Platelet activation involves a number of stepwise processes whereby platelets undergo a shape change, release the contents of their alpha and dense granules, which in turn will amplify platelet activation. Following adhesion, occupancy of GPIb by vWF (Du 2007) or GPVI/α2β1 by collagen (Watson et al. 2010) results in intracellular signal transduction via tyrosine kinases leading to increased cytosolic Ca^{2+} levels and subsequently, platelet activation (Broos et al. 2011). Alternatively, platelets can be activated by thrombin via the PAR1 receptor (Broos et al. 2011). Upon activation, platelets will secrete the contents of their alpha granules such as VWF, FV, platelet factor 4, and fibrinogen and the contents of their dense granules such as adenosine diphosphate (ADP), polyphosphates, and serotonin (Ren et al. 2008, Hu et al. 2010). Secretion of alpha and dense granule contents results in autocrine and paracrine signaling effects leading to amplification of platelet activation, platelet shape change, and additional recruitment of nearby platelets (Ren et al. 2008).

Lastly, platelet aggregation relies on activation of integrin αIIbβ3. As a result of platelet activation, elevations in cytosolic Ca^{2+} levels induce a conformational change of integrin αIIbβ3 which leads to an unmasking of a fibrinogen binding site (Shattil et al. 2010). Once activated, integrin αIIbβ3 binds circulating fibrinogen which acts as a bridge between neighbouring platelets. This leads to further aggregation of platelets and formation of the platelet plug at the site of vascular injury.

1.3 Extrinsic Pathway of Coagulation

Tissue factor is the initiator of the extrinsic pathway of coagulation. Tissue factor is a 47 kDa transmembrane glycoprotein that is synthesized as a 295 amino acid polypeptide which includes a signal sequence (Morrissey et al. 1987). Once TF undergoes post-translational modification, TF consists of 3 domains: a 219 amino acid extracellular domain which binds to FVIIa, a 23 amino acid transmembrane domain, and a 21 amino acid cytoplasmic domain which is involved in signal transduction (Morrissey et al. 1987, Chen et al. 2006). The extracellular domain of TF contains four cysteines, which are able to undergo disulphide bond formation to regulate TF procoagulant activity (Ruf et al. 2010).

Under physiologic conditions, TF is absent on endothelial cells but is constitutively expressed on cells in the sub-endothelial layer and acts as a high affinity receptor for circulating activated factor VII (FVIIa) (Mackman 2004, Mandal et al. 2006, Monroe et al. 2007, Bluff et al. 2008). FVIIa is a 50 kDa glycoprotein produced by the liver that contains an amino terminal γ-carboxyglutamic acid (Gla) domain required for binding to PS-bearing cell surfaces in a Ca^{2+}-dependent manner (Dennis et al. 2000). FVII circulates in plasma at a concentration of ~ 0.01 µM (Butenas et al. 1996). Upon exposure to blood, TF forms a complex with FVII and FVIIa forming the TF-FVIIa complex, also known as the extrinsic tenase complex (Milsom et al. 2008). The TF-VIIa complex in the presence of a negatively charged phospholipid surface and Ca^{2+} converts factors IX (FIX) and X (FX) into their active forms FIXa and FXa,

respectively (Dahlback 2008). In the absence of phospholipids and Ca^{2+}, FVIIa is unable to activate FIX (Osterud et al. 1977). Upon the formation of the extrinsic tenase complex, FVIIa catalytic activity towards FX is enhanced by approximately 1000–fold (Mackman 2009).

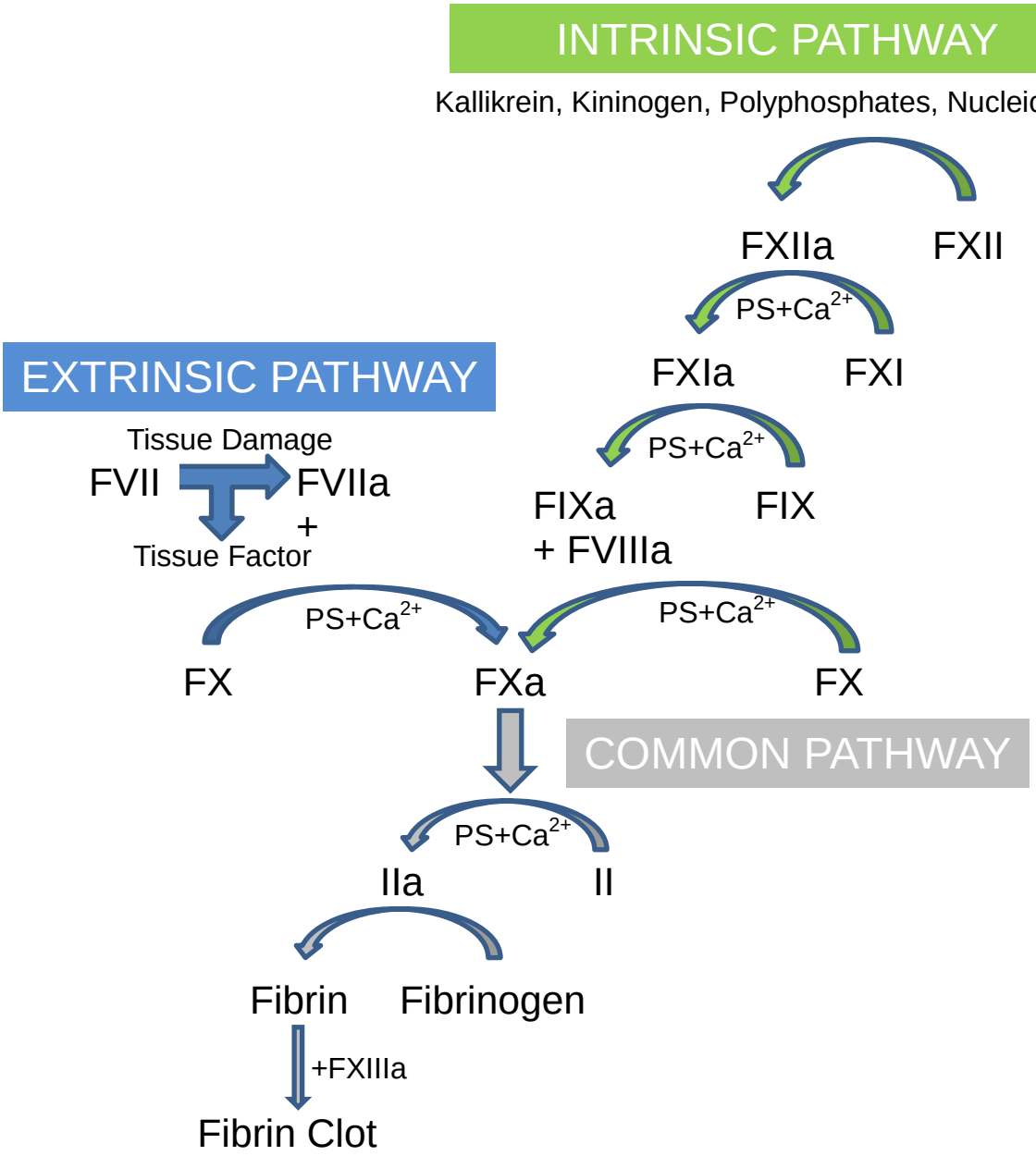
FXa generated by the TF-FVIIa complex binds to FVa, and in the presence of Ca^{2+} and a negatively charged phospholipid surface forms the prothrombinase complex. The prothrombinase complex is essential for cleaving the zymogen prothrombin (FII) into thrombin. Thrombin exerts procoagulant effects through activation of FV, FVIII, FXI, and platelets as well as through cleavage of soluble fibrinogen into insoluble fibrin monomers necessary to form a stable fibrin clot (Covic et al. 2000, Milsom et al. 2008).

TF antigen and activity levels vary throughout different tissues of the human body. TF is present in the vascular adventitia, connective tissue capsules surrounding organs, astrocytes in the brain, and endometrial lining (Drake et al. 1989, Fleck et al. 1990, Eddleston et al. 1993, Lockwood et al. 1993, Flossel et al. 1994). Although expression of TF can be induced on the surface of monocytes and endothelial cells by vascular endothelial growth factor or LPS, intravascular TF is absent under physiologic conditions to prevent aberrant activation of coagulation (Osterud et al. 2000). Since unperturbed endothelium and intravascular blood cells are negative for TF, it was deemed that TF on extravascular cells forms a “hemostatic envelope” to achieve rapid activation of the coagulation system in the event of vascular damage (Drake et al. 1989).

The physiological importance of TF can be observed in TF deficient mice. Animals lacking TF die in utero due to vascular abnormalities that impair yolk sac blood vessel development and result in excess bleeding (Toomey et al. 1996, Aasrum et al. 2002). In contrast to TF deficient mice, FVII deficient mice survive to term. However, ~70% of FVII deficient mice die within 24 hours due to inta-abdominal bleeding with the remaining mice experiencing fatal intracranial bleeding before 24 days of age (Rosen et al. 1997). In addition to its role in coagulation, TF also participates in cell signaling. The TF/FVIIa complex can directly cleave protease activated receptor 2 (PAR2), contributing to non-hemostatic roles in cancer and inflammation (Pawlinski et al. 2004, Versteeg et al. 2004).

Figure 1.1 - The coagulation cascade.

Initiation of the coagulation cascade occurs through the extrinsic pathway upon exposure of TF to blood. TF, in complex with FVIIa, converts FIX and FX to FIXa and FXa, respectively. Alternatively, coagulation can be initiated through the intrinsic pathway by activation of FXII to FXIIa by kallikrein, kininogen, polyphosphates, or nucleic acids. FXIIa activates FXI, which in turn activates FIX. The intrinsic and extrinsic pathways converge onto the common pathway to convert FX to FXa which cleaves prothrombin (II) into thrombin (IIa). Thrombin is then able to cleave soluble fibrinogen into insoluble fibrin monomers to form a fibrin clot. In addition, thrombin participates in an amplification of coagulation via activation of FV, FVIII, and FXI (Smith 2009). Adapted from (Lazzaro et al. 2012).


INTRINSIC PATHWAY
Kallikrein, Kininogen, Polyphosphates, Nucleic Acids
FXIIa
FXII
PS+Ca2+
FXIa
FXI
EXTRINSIC PATHWAY
Tissue Damage
FVII
FVIIa
+
Tissue Factor
PS+Ca2+
FIXa
+ FVIIIa
FIX
PS+Ca2+
PS+Ca2+
FX
FXa
FX
COMMON PATHWAY
PS+Ca2+
IIa
II
Fibrin
Fibrinogen
+FXIIIa
Fibrin Clot

1.4 Tissue Factor Decryption

Changes in cell surface TF antigen levels do not always correlate with changes in TF procoagulant activity levels. As a result of this dissociation between antigen and activity levels, two distinct populations of TF have been identified. Cell surface TF that possesses procoagulant activity is termed active/decrypted TF, whereas TF that does not possess procoagulant activity is termed inactive/cryptic TF (Bach 2006). TF decryption has been attributed to mechanisms modulated by protein disulphide isomerase (PDI) activity, phosphatidylserine exposure, and TF self-association (Bach 2006, Chen et al. 2006, Monroe et al. 2007).

1.4.1 Protein Disulphide Isomerase

PDI is a member of the thioredoxin family of dithiol/disulphide oxidoreductases and is essential for disulphide bond formation and isomerization (Wilkinson et al. 2004). PDI is primarily retained within the endoplasmic reticulum (ER) and catalyzes protein disulphide formation as well as rearrangement of disulphide bonds such as incorrectly paired cysteines or disulphide bonds that were formed in incorrect temporal order (Schwaller et al. 2003). In addition, PDI acts as a chaperone to prevent aggregation of unfolded/misfolded proteins within the ER which aids in reducing ER stress (Wilkinson et al. 2004).

1.4.2 Protein Disulphide Isomerase and Tissue Factor Decryption

Although located primarily within the ER, PDI has also been shown to be expressed on the surface of endothelial cells where it associates with cell surface TF (Ahamed et al. 2006). PDI is postulated to play a role in TF decryption through the formation of a disulphide bond between Cys186 and Cys209 found in the extracellular domain of TF (Chen et al. 2006, Pendurthi et al. 2007). Studies supporting PDI-mediated TF decryption demonstrate that cryptic TF contains unpaired Cys186 and Cys209 residues (Chen et al. 2006). Furthermore, mutation of either Cys186 or Cys209 residues severely impairs TF procoagulant activity (Rehemtulla et al. 1991). Lastly, TF activity is increased in a time- and dose-dependent manner by thiol oxidizing agents (Chen et al. 2006, Pendurthi et al. 2007).

1.4.3 Surface Exposure of Phosphatidylserine

PS is a negatively charged phospholipid that is primarily confined within the inner leaflet of the cell membrane. Extracellular PS is an essential factor for recognition and clearance of apoptotic cells by phagocytic cells which utilize cell-surface PS to identify and engulf cells undergoing apoptosis (Miyanishi et al. 2007, Park et al. 2009). Asymmetrical distribution of PS is maintained by three types of lipid transporters/translocases: flippases, floppases, and scramblases (Leventis et al. 2010). Flippases are phospholipid translocases responsible for trafficking PS from the extracellular leaflet of a lipid membrane to the cytosolic side (Leventis et al. 2010). In contrast, floppases counteract the action of

flippases by translocating PS from the cytosolic to the extracellular side of the membrane (Leventis et al. 2010). Unlike the unidirectional effect of flippases and floppases, scramblases are able to translocate PS bidirectionally in an attempt to minimize PS asymmetry (Sahu et al. 2007). Therefore, the sequestering of PS to the inner leaflet of the cell membrane is a continuous balance between the actions of three lipid transporters.

1.4.4 Phosphatidylserine and Tissue Factor Decryption

A number of studies have demonstrated a correlation between increased PS exposure and increased TF activity (Lentz 2003). PS exposure and TF activity are both Ca^{2+} dependent processes. Calcium ionophore treatment of cells results in an influx of extracellular Ca^{2+} across the cell membrane triggering a disruption of PS asymmetry at the same magnitude of Ca^{2+} influx that causes TF activity to be increased (Bach 2006). This effect is reportedly due to inhibition of flippase as a result of large increases in cytosolic Ca^{2+} levels causing translocation of PS to the outer leaflet of the cell membrane (Zwaal et al. 2005). Furthermore, the binding of Annexin V to PS on calcium ionophore-stimulated monocytes attenuates calcium-ionophore induced TF activity (Wolberg et al. 1999).

A potential mechanism of PS-mediated TF decryption involves a direct interaction between PS and TF leading to a PS-induced change in TF tertiary structure. This is believed to occur through interaction between PS and Lys165/166 residues found in the extracellular domain of TF (Bach 2006). A

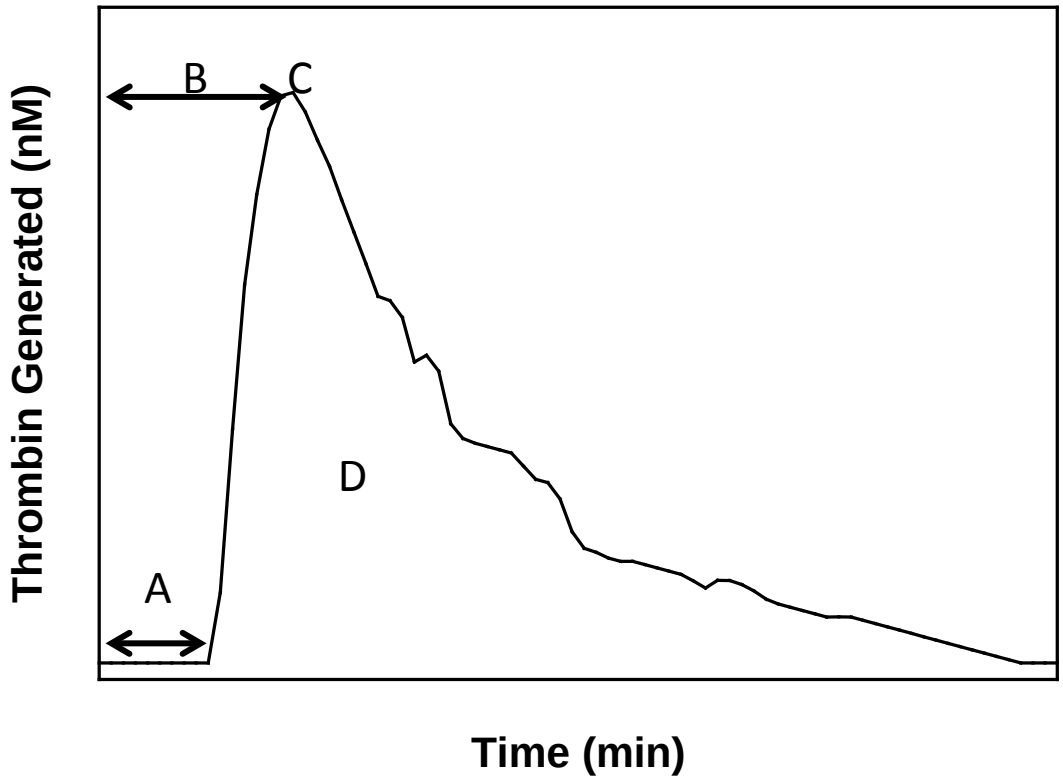
modification of either Lys165 or Lys166 residues reduces the *in vitro* effect of PS and subsequently TF procoagulant activity, but does not affect TF/FVIIa binding (Roy et al. 1991). Thus, PS-induced changes in TF tertiary structure allows for an optimal orientation of the active site of the FVIIa in the extrinsic tenase complex, thereby leading to more efficient cleavage of FX (Bach 2006).

1.4.5 Tissue factor self-association model

In addition to PS and PDI, TF encryption/decryption has been postulated to be dependent on TF self-association. This model of TF encryption/decryption proposes that non-coagulant (cryptic pools) TF is present in homodimer form, whereas procoagulant (decrypted) TF is present as monomers (Roy et al. 1991). This model is supported by studies demonstrating chemical cross linking between non-coagulant TF monomers (Roy et al. 1991). However, the cross linking between TF monomers can be inhibited with calcium ionophore treatment (Bach et al. 1997). Use of calcium ionophore can induce changes in PS exposure on the cell surface which is also correlated with increased TF activity as previously described (Zwaal et al. 1997, Lentz 2003). Therefore, since PS exposure and TF dimerization are both affected by Ca^{2+}it is possible that the TF self-association model of TF decryption and the PS-dependent TF model of decryption are not mutually exclusive.

Figure 1.2 The Thrombogram.

The thrombogram includes a number of commonly measured variables to assess thrombin generation within a given sample/condition. Lag time refers to the time between the initiations of experiment to first measurable levels of thrombin generated. This is often followed by the maximal thrombin generated (peak thrombin) and the time from the initiation of experiment to reach peak thrombin (time to peak). Lastly, area under the curve (AUC) or endogenous thrombin potential (ETP) refer to the total amount of thrombin generated in the sample.



A – Lag Time
B – Time to Peak Thrombin
C – Peak Thrombin Generated
D – Endogenous Thrombin Potential

1.5 Intrinsic Pathway of Coagulation

Initiation of coagulation can also occur through the intrinsic (contact) pathway through activation of FXII by polyanionic activators (Gailani et al. 2007). The binding of FXII to polyanions results in auto activation of FXII to FXIIa (Colman et al. 1997). Once FXIIa is generated, it is able to cleave prekallikrein to kallikrein. Generation of kallikrein provides a positive feedback loop which supports additional FXIIa generation (Colman et al. 1997). In addition to prekallikrein, FXIIa is able to cleave FXI into active FXIa. However, FXI can also be activated by thrombin independent of FXIIa (Choi et al. 2011). In turn, FXIa converts FIX to FIXa which in the presence of its cofactor FVIIIa, PS, and Ca^{2+} forms the intrinsic tenase complex (Monroe et al. 2006).

Activation of FXII by substances such as kaolin and silica *in vitro* is well known (Renne et al. 2012). These substances are commonly used as activators for the activated partial thromboplastin time (aPTT), a global coagulation assay used to identify contact system defects (Renne et al. 2012). FXII activation can also be achieved with naturally occurring activators of the intrinsic pathway of coagulation such as platelet polyphosphates, DNA, and RNA (Smith et al. 2006, Kannemeier et al. 2007, Muller et al. 2009). Interestingly, individuals with deficiencies of contact FXII, kallikrein, and high-molecular-weight kininogen (HK) do not present with impaired hemostasis (Muller et al. 2011). These findings are supported by animal studies demonstrating that animals deficient in FXI, FXII, kallikrein, or HK do not experience abnormal bleeding (Renne et al. 2005, Wang

et al. 2005, Revenko et al. 2011). However, the intrinsic pathway has been shown to play a role in pathologic thrombosis associated with medical devices such as indwelling catheters (Yau et al. 2014). Furthermore, animals lacking FXI or FXII as well as animals that were treated with a FXI inhibiting antibody were resistant to experimentally-induced thrombosis (Renne et al. 2005, Tucker et al. 2009, Cheng et al. 2010).

1.6 Common Pathway of Coagulation

The generation of FXa leads to the convergence of the intrinsic and extrinsic pathways to the common pathway of coagulation (Monroe et al. 2006). The resulting prothrombinase complex, consisting of FXa, FVa, PS, and Ca^{2+}, is able to cleave prothrombin to generate thrombin (Riewald et al. 2003) . The resulting thrombin is then able to initiate formation of a fibrin clot.

1.7 Thrombin Generation Assay.

Generation of thrombin can be measured in a plasma sample with the use of a continuous thrombin generation assay (TGA), a global coagulation assay capable of measuring both thrombin generation and thrombin inhibition (Lance 2015). The generation of thrombin can be quantified by measuring cleavage of a thrombin-specific substrate which can be plotted and examined over time through thrombograms (Figure 1.2). Thrombograms can be subdivided into 4 main components: lag time, time to peak thrombin, peak thrombin generated, and the area under the curve (AUC), also known as the endogenous thrombin potential

(ETP). The TGA is initiated with Ca^{2+} in the presence of either relipidated TF or a negatively charged surface such as kaolin to initiate the extrinsic or intrinsic pathways of coagulation, respectively. Upon initiation, a measurable (minutes) lag phase occurs during which small amounts of thrombin are generated but are not detectable by the assay. Formation of measurable amounts of thrombin can be observed as a rapid increase in the thrombin generation curve. Once equilibrium between thrombin generation and thrombin inhibition are reached, a peak in thrombin generation is reached. This can be quantified as amount of thrombin generated (peak thrombin, nM) or the time required to achieve peak thrombin (time to peak thrombin). Lastly, the total amount of thrombin generated throughout the course of the assay can be quantified by AUC or ETP.

1.8 Fibrin Clot Formation

Formation of a stable thrombus in response to vascular injury is essential for hemostasis and to allow for wound healing to occur. However, clot formation must also be tightly regulated in order to prevent pathological thrombus formation. Furthermore, once wound healing is complete, resolution of the formed clot is necessary to re-establish adequate blood flow.

Fibrinogen, a 340 kDa soluble plasma protein, is comprised of one central globular E-domain flanked by two globular D-domains (Walker et al. 1999). Cleavage of fibrinogen by thrombin results in release of fibrinopeptides A and B, located in the E-domain, leading to an exposure of polymerization sites (Nesheim 2003). The newly exposed polymerization sites within the E-domain allow for

spontaneous interfibrin polymerization between the E-domain of one fibrin monomer and the D-domains of additional nearby fibrin monomers forming protofibrils (Nesheim 2003). The protofibrils also associate laterally with nearby protofibrils in order to form thicker fibers and protofibril matrix. The resulting protofibril matrix is stabilized by FXIIIa through formation of cross-links between adjacent D-domains (Nesheim 2003).

1.9 Fibrinolysis

Resolution of the fibrin clot is mediated by the proteolytic cleavage of the fibrin matrix by the serine protease plasmin. Plasminogen is the zymogen precursor to plasmin and is activated by plasminogen activators tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) (Baruah et al. 2006). Both tPA and uPA are synthesized and secreted by endothelial cells with a short half-life of approximately 5 and 2 minutes, respectively (Baruah et al. 2006). Endothelial cells release tPA in response to a number of stimuli such as physical activity, thrombin, and shear stress (Oliver et al. 2005). Plasminogen activation to plasmin by tPA is enhanced 1000-fold in the presence of fibrin, which functions as a co-factor by providing a surface for simultaneous binding and localization of plasminogen and tPA (Cesarman-Maus et al. 2005). In contrast, fibrin does not augment uPA-mediated cleavage of plasminogen to plasmin (Cesarman-Maus et al. 2005) and uPA functions primarily in tissue remodeling (Li et al. 2003). Once active, plasmin cleaves its

major substrate, fibrin, converting the insoluble fibrin clot into soluble fibrin degradation products (FDP).

Fibrinolysis must be tightly regulated in order to prevent premature thrombus dissolution (Carpenter et al. 2008). Fibrinolysis can be modulated by serine protease inhibitors (SERPINs) such as plasminogen activator inhibitor-1 (PAI-1) and α_2-antiplasmin (α_2PI), or by activation of the zymogen thrombin activatable fibrinolysis inhibitor (TAFI) into TAFIa by thrombin in complex with TM (Bajzar 2000). PAI-1 and α_2PI are physiologic inhibitors of tPA and plasmin, respectively (Schaller et al. 2011). While α_2PI is synthesized in the liver, a number of cell types are capable of secreting PAI-1 such as hepatocytes, platelets, endothelial cells, and smooth muscle cells (Dellas et al. 2005, Nakatsuka et al. 2006, Schaller et al. 2011). Deficiencies in tPA or α_2PI lead to a loss of regulation of fibrinolysis and subsequently bleeding disorders (Binder et al. 2002, Carpenter et al. 2008). TAFI is produced by the liver and also by megakaryocytes (Foley et al. 2013). Regulation of fibrinolysis by TAFIa is achieved by removal of carboxyterminal lysine residues from fibrin. Removal of carboxyterminal lysine residues reduces the localization of plasminogen and tPA on fibrin thus effectively reducing plasminogen activation by tPA (Bouma et al. 2006).

1.10 Inhibition of Coagulation

In a healthy system, coagulation is tightly regulated as uncontrolled coagulation may potentially lead to lethal consequences. Regulation of this

system is done in main part by four key players: antithrombin (AT), tissue factor pathway inhibitor (TFPI), protein C (PC), and heparin cofactor II (HCII). These molecules act on different stages of the coagulation cascade in a negative feedback manner.

1.10.1 Protein C

Protein C is a circulating zymogen that once cleaved into activated protein C (APC) has anticoagulant and anti-inflammatory activities (Levi et al. 2003). Protein C activation occurs by thrombin cleavage although this reaction occurs at an inadequate rate to be physiologically relevant . Once thrombin is generated, it binds to its receptor thrombomodulin (TM) on the surface of endothelial cells to form the thrombin-TM complex. The thrombin-TM complex enhances activation of PC by approximately 1000-fold (Esmon 2006). Protein C activation is further enhanced by approximately 20-fold by the endothelial protein C receptor (EPCR), an endothelial receptor that binds circulating protein C and presents it to the thrombin-TM complex (Taylor et al. 2001). APC inhibits coagulation by degrading FVa (Egan et al. 1997) and FVIIIa (Regan et al. 1994). Mice lacking PC are able to survive to term although die shortly thereafter (within 24 hours) due to coagulopathy (Jalbert et al. 1998). In contrast, disruption of EPCR or TM causes death in utero due to placental thrombosis (Gu et al. 2002). Ablation of TM or EPCR causes death at approximately day 10 of embryonic development as a result of placental thrombosis (Gu et al. 2002).

1.10.2 Tissue Factor Pathway Inhibitor

TFPI is a Kunitz-type protease inhibitor that is synthesized primarily by endothelium of the microvasculature (Ahnstrom et al. 2012, Ellery et al. 2014). *In vivo,* TFPI is distributed in three pools. Approximately 85% of TFPI concentrations is bound to endothelial cells, ~10% is found circulating in plasma either as free or lipoprotein-bound, and ~ 3% is found in platelets (Kemme et al. 2002). TFPI acts as a regulator of coagulation by forming a quaternary complex with TF-FVIIa-FXa which inhibits FXa (Baugh et al. 1998). Since TFPI binding and activity is FXa-dependent, the role of TFPI is not to stop coagulation, but rather to act as part of a negative feedback loop in the presence of FXa to prevent excess formation of thrombin (Gomez et al. 2005, Ndonwi et al. 2010).

1.10.3 Antithrombin

AT is a SERPIN that inhibits a number of coagulation enzymes (Rau et al. 2007). It acts primarily on thrombin and FXa and to a lesser extent on FIXa, FXIa, and FXIIa (Hirsh et al. 2001). In the absence of heparin or heparan sulfate, the rate of thrombin inhibition by AT is very slow. However, the presence of heparin (Hirsh et al. 2001) or heparan sulfate (Lindahl 2007) increases the rate of thrombin inhibition by approximately 4000-fold (Olson et al. 1992).

Increased inhibition rate in the presence of heparin depends in large part on a specific pentasacharide sequence (Hirsh et al. 2001, Gomez et al. 2005). The

interaction between AT and the pentasaccharide sequence induces a conformational change in AT that increases binding and inhibition of FXa (Gomez et al. 2005). To achieve full inhibition of thrombin, an additional 13 saccharide units are required to bind thrombin and AT simultaneously (Hirsh et al. 2001). A complete absence of AT causes death in utero due to extensive fibrin deposition in the myocardium and liver (Ishiguro et al. 2000). Individuals with congenital deficiencies in AT have increased rates of thrombosis (Khor et al. 2010)

1.10.4 Heparin Cofactor II

Similar to AT, HCII is a serpin that inhibits thrombin. HCII is a very slow inhibitor of thrombin and requires activation to reach physiologically relevant rates of inhibition (Pike et al. 2005). Activation of HCII by heparin, heparan sulfate, or dermatan sulfate can enhance HCII activity by 1000-fold (Huntington 2013). Despite measurable thrombin-HCII complex levels in human plasma, HCII does not appear to play a major role in hemostasis under normal conditions.

Studies exploring the role of HCII have produced conflicting results on the viability of animals lacking HCII. One study demonstrated that mice lacking HCII (homozygous knockout) survive to term and present with normal hemostasis under unchallenged conditions (He et al. 2002). When challenged with endothelial injury, decreased time to occlusion was observed in HCII null mice compared to the wild type counterparts (He et al. 2002). In contrast, Aihara et al. demonstrate that HCII knockout mice die in utero (E6.5-E8.5) (Aihara et al.

2007). The authors speculate that the differences in fetal development may be due to strain-specific genetic differences (Aihara et al. 2007).

In humans, individuals with decreased levels of HCII do not have increased rates of thrombosis compared to individuals with normal HCII levels (Tollefsen 2002). Although HCII deficiency does not manifest in increased rates of thrombosis in an unchallenged system, it has been suggested that HCII plays a protective role in the vasculature in response to vascular injury (Tollefsen 1997, Tollefsen 2002). The beneficial effect of HCII is supported by studies that report a protective role of HCII against arterial stenosis and carotid atherosclerosis (Aihara et al. 2004, Schillinger et al. 2004), whereas decreased HCII levels were indicative of endothelial dysfunction (Huang et al. 2007).

1.11 Cancer overview

Thrombosis is a well-recognized complication in cancer patients that may manifest through upregulation of procoagulant and/or downregulation of anticoagulant pathways. Cancer is a complex, multistep process that is characterized by genetic alterations that induce progressive changes of healthy cells into malignant counterparts (Hanahan et al. 2000). These genetic alterations result in gain-of-function of oncogenes or loss-of-function in tumour suppression genes (Hanahan et al. 2000). In Canada, approximately 191,000 new cancer cases were diagnosed in 2014, accompanied with 76,000 deaths (Canadian Cancer Society's Advisory Committee for Canadian Cancer 2014).

1.10.1 Lung cancer and incidence rates

Lung cancer is the second leading type of cancer affecting men and women equally (14% vs. 13%, respectively) (Siegel et al. 2014). Amongst Canadians, nearly 30% of all deaths annually are attributed to cancer with lung cancer being the leading cause of cancer-related death (Canadian Cancer Society's Advisory Committee for Canadian Cancer 2014). Diagnosis of lung cancer is classified as either small cell or non-small cell lung carcinoma (NSCLC) and is further subdivided into 4 stages (stage I-IV) (Pisters et al. 2007). Approximately 85% of all new lung cancer cases are diagnosed as NSCLCs (Pisters et al. 2007). While chemotherapy is not recommended following complete resection of stage IA and IB tumours, adjuvant chemotherapy is recommended for stage II-IV solid lung tumours (Pisters et al. 2007).

1.11.2 Cancer and thrombosis: Pathogenic Mechanisms

The association between cancer and increased thrombotic events is well recognized. Cancer patients have a ~ 5- to 7-fold elevated risk for venous thromboembolism (VTE) compared to healthy individuals (Blom et al. 2005, Sud et al. 2009). In fact, 1 in 7 cancer patients die from pulmonary embolism, making thrombosis the second leading cause of death amongst cancer patients (Khorana et al. 2007).

Cancer patients may present with numerous abnormalities predisposing them to increased risk of VTE. Rudolf Virchow initially described three broad categories (stasis of the blood, hypercoagulability, and vascular damage) that

contribute to thrombosis. Subsequently, the interplay between the three categories has been termed “Virchow’s Triad” (Kumar et al. 2010). A number of abnormalities observed in cancer patients can be traced back to the components of Virchow’s triad providing an explanation for the hypercoagulable state (Lip et al. 2002). Blood stasis may occur through decreased patient mobility or blood vessel compression by a growing tumour, hypercoagulability through direct tumour cell contribution (cell surface TF or MP shedding), and vascular damage through the number of therapies (surgery, chemotherapy, or catheter) that may be used for treatment (Lip et al. 2002). Taken together, the simultaneous presence of the procoagulant abnormalities may result in thrombus formation.

The hypercoagulable state in cancer patients is multifactorial. Contributing factors include tumour production of procoagulant factors, venous compression, immobilization, and anticancer therapies such as surgery, chemotherapy, radiation therapy, and use of indwelling catheters (Caine et al. 2002). Incidence of thrombosis in cancer patients also varies depending on the type of malignancy (Blom et al. 2005). For example, patients with brain, pancreatic, and lung cancer have an approximate 3.2%, 2.3%, and 1.4% overall rate of thrombosis respectively within the first 6 months of diagnosis (Blom et al. 2006). It has been demonstrated that cancer cells express increased TF antigen levels (Ueno et al. 2000) and cancer procoagulant (CP) on the cell membrane (Mielicki 2001). Expression of TF has been reported on numerous types of cancer cells including pancreatic, lung, and colorectal cancer cells. Furthermore, cancer-cell TF

expression has been identified as a predictive factor of VTE development in some cancers (Uno et al. 2007) .

1.12 Chemotherapy

1.12.1 Association of chemotherapy and thrombosis

As mentioned above, use of chemotherapeutic regimens increases the thrombotic risk in cancer patients. Platinum-based (cisplatin or carboplatin) combination chemotherapy is recommended over non-platinum based therapies for first line treatment of NSCLC (Azzoli et al. 2010). Cisplatin and carboplatin are administered in combination with gemcitabine and paclitaxel, respectively, and have been studied extensively for their anti-tumor efficacy (Scagliotti et al. 2002, Sandler et al. 2006). Varying rates of thrombosis have been reported depending on the type of cancer present and the chemotherapy regimen administered with brain, lung, and pancreatic cancer patients experiencing the highest rates of VTE (4.7%, 5.1%, and 8.1% respectively) (Khorana et al. 2007).

Interestingly, vascular events (DVT and PE) begin to occur within 30 days of initiation of chemotherapy treatment (Numico et al. 2005). In lung cancer patients undergoing chemotherapy, the incidence rate of thrombosis was reported to be as high as 27.9%, with thrombotic events occurring within 1 month of therapy initiation (Blom et al. 2004) and elevated plasma levels of thrombin-antithrombin (TAT) complex are observed as early as 1 hour after chemotherapy administration (Weitz et al. 2002).

1.12.2 Mechanisms of action of classical chemotherapy agents

A hallmark of a cancer cell is rapid and uncontrolled cell division (Hanahan et al. 2000). Therefore, the principle of many classical chemotherapeutic agents is to target the cell division process of rapidly dividing cells while leaving quiescent cells unharmed (Makin 2002). This is most commonly achieved by utilizing chemotherapeutic agents capable of inducing DNA “damage”, leading to cell cycle arrest and subsequently, apoptosis.

1.12.3 Agents used for treatment of lung cancer

Recommendations for lung cancer chemotherapy use vary depending on the stage of disease. American Society of Clinical Oncology (ASCO) and Cancer Care Ontario (CCO) recommend that adjuvant cisplatin-based chemotherapy regimens be used for treatment of lung cancer patients with stage IIA-IIIA progression of disease (Pisters et al. 2007). If disease progression reaches stage IV, platinum-based over non platinum combinations are recommended for first line chemotherapy with either cisplatin or carboplatin being acceptable choices as the platinum agent (Azzoli et al. 2011). Chemotherapeutic agents used in our studies in platinum-based combinations include gemcitabine and paclitaxel. Although not used in the present studies, a number of other agents have been utilized for targeted therapy such as nucleotide synthase and vascular endothelial growth factor (VEGF) inhibitors, pemetrexed and bevacizumab, respectively (Scagliotti et al. 2008). Furthermore, use of an epidermal growth factor receptor (EGFR) inhibitor, such as cetuximab, as well as EGFR tyrosine

kinase inhibitors (erlotinib, gefitinib, afatinib) is recommended for use in patients with identified EGFR mutations. Likewise, for individuals with anaplastic lymphoma kinase (ALK) mutations, ALK inhibiting agents such as crizotinib or ceritinib remain the optimal agents for treatment (Schiller et al. 2002, Azzoli et al. 2011, Masters et al. 2015).

1.12.4 Cisplatin

Cisplatin is a potent cytotoxic anti-tumour agent displaying efficacy against a number of cancer types (Siddik 2003). Following injection, cisplatin must undergo intracellular hydrolysis steps in order to achieve the necessary antiproliferative cytotoxic effects (el-Khateeb et al. 1999, Kelland 2000). The cytotoxicity of cisplatin is achieved by formation of platinum-DNA adducts, causing DNA inter- and intrastrand crosslinks. Formation of platinum adducts leads to cell cycle arrest, and apoptosis (Siddik 2003). Cisplatin-based combination chemotherapy is recommended for use in patients with stage II-III lung cancer (Pisters et al. 2007), whereas cisplatin or carboplatin are acceptable for use in stage IV lung cancer (Azzoli et al. 2009).

1.12.5 Carboplatin

Carboplatin is a derivative of cisplatin and thus has a similar mechanism of action of binding DNA, inhibiting DNA replication, and inducing cell death (Amptoulach et al. 2011). Similarly, carboplatin must undergo intracellular hydrolysis steps prior to forming DNA inter-and intrastrand crosslinks

(Amptoulach et al. 2011). Unlike its precursor cisplatin, carboplatin has less potency but is coupled with fewer side effects, both of which have been attributed to lower rates of platinum-DNA adduct formation (Knox et al. 1986). Due to its lower side effects, carboplatin can be administered at higher doses. Use of carboplatin-based combination chemotherapy is recommended for patients with stage IV lung cancer (Kelland 2000, Azzoli et al. 2009).

1.12.6 Gemcitabine

The anti-tumour efficacy of gemcitabine has been demonstrated in a number of *in vitro* and *in vivo* studies. Gemcitabine is indicated for use as a single agent in the treatment of pancreatic cancer (Burris et al. 1997) and as part of combination chemotherapy in lung, bladder, and breast cancers (Sandler et al. 2000, von der Maase et al. 2000). Gemcitabine is administered as a prodrug that must undergo a number of intracellular phosphorylation steps until the active drug metabolite is achieved (Heinemann et al. 1988). The active drug exerts anti-proliferative effects by inhibiting DNA polymerase as well as through incorporation into DNA leading to termination of chain elongation (Gandhi et al. 1990, Huang et al. 1991). Gemcitabine has been suggested as an ideal candidate for combination chemotherapy and consequently has been widely used to achieve synergistic antiproliferative effects with other neoplastic agents such as cisplatin (Mini et al. 2006).

Table 1.1 Summary of studied chemotherapy agents used for treatment of NSCLC.

Drug	Method of Entry	Drug Target	Maximum Plasma Concentration	Mechanism of Action	References
Cisplatin	Copper Transporter 1 (CTR1)	DNA	20 µM	DNA Adduct	(Go et al. 1999) (Kroep et al. 2006)
Carboplatin	Copper Transporter 1 (CTR1)	DNA	40 µM	DNA Adduct	(Go et al. 1999) (Kern et al. 2001)
Gemcitabine	Nucleoside Transporter	DNA, DNA polymerase	60 µM	DNA Adduct	(Plunkett et al. 1995) (Kroep et al. 2006)
Paclitaxel	Passive Diffusion	β-tubulin	4 µM	Microtubule Stabilization	(Horwitz 1992) (Kroep et al. 2006)

1.12.7 Paclitaxel

Initially isolated from the bark of a western yew tree (Wani et al. 1971), paclitaxel and the related taxanes were identified as promising agents for cancer therapy due to their ability to inhibit proliferation. Paclitaxel exerts antitumour activity through stabilization of tubulin (microtubules) leading to inhibition of microtubule disassembly (Schiff et al. 1979). Microtubules are cytoskeletal polymers which play a role in cell shape, transport, motility, and division (Nogales 2000). Microtubule stabilization is achieved by preferential binding of paclitaxel to β-tubulin (Nogales et al. 1995) which leads to mitotic arrest and induces apoptosis in proliferating cells (Schiff et al. 1980). Taxanes were found to have antitumour activity against numerous tumour types including breast, ovarian, brain, and lung cancers (Marupudi et al. 2007).

1.12.8 Procoagulant effects of lung cancer chemotherapy – clinical studies

Several clinical studies have identified perturbations of the hemostatic balance in lung cancer patients receiving chemotherapy. The incidence rates of thrombotic events reported throughout the literature vary greatly depending largely on (i) stage of disease progression and (ii) chemotherapeutic agent(s) used for treatment.

Clinical studies that investigate hemostatic markers, such as levels of TATs, D-Dimer, prothrombin time (PT), and activated partial prothrombin time (aPTT) in lung cancer patients have shed light on the procoagulant effects of lung cancer chemotherapy. Increased D-Dimer levels in lung cancer patients are

attributed to disease progression rather than chemotherapy treatment whereas PT and aPTT values in chemotherapy treated lung cancer patients fall within the normal reference range (Altiay et al. 2007, Tas et al. 2013).

In contrast, lung cancer patients have elevated TAT levels compared to healthy controls (Gabazza et al. 1992, Seitz et al. 1997, Kazmierczak et al. 2005). However, direct effects of chemotherapy can be elucidated only from studies that report the pre- and post-therapy TAT levels. Weitz et al. demonstrate that post-chemotherapy TAT levels were increased compared to pre-chemotherapy levels in lung cancer patients within 1 hour of receiving treatment (Weitz et al. 2002). During follow up, it was observed that TAT levels remained elevated for up to 48 hours post-chemotherapy demonstrating early and sustained activation of the coagulation system (Weitz et al. 2002). This supports clinical studies where more than a third (36%) of patients treated with cisplatin/gemcitabine combination therapy experienced a thrombotic event within the first 30 days of therapy initiation (Numico et al. 2005). Therefore, lung cancer chemotherapy agents exert procoagulant effects and increase the risk for thrombotic events early in therapy.

1.12.9 Procoagulant effects of lung cancer chemotherapy - in vitro studies

Although clinical studies have identified procoagulant consequences associated with lung cancer chemotherapy use, the specific mechanisms by which these chemotherapeutic agents induce procoagulant effects are not completely understood. While chemotherapeutic agents primarily target rapidly

dividing tumour cells, they may also exert cytotoxic effects on relatively quiescent host cells. Patients receiving cisplatin-based chemotherapy expressed elevated circulating levels of von Willebrand factor which suggests chemotherapy-induced damage to the vascular endothelial layer (Dieckmann et al. 2011).

Studies investigating procoagulant effects of chemotherapy have focused largely on HUVEC and specific cancer cells of interest, with some studies reporting conflicting findings. Cisplatin-based therapy exerts procoagulant effects on HUVEC through up regulation of TF activity in a dose-dependent manner (Ma et al. 2005). Furthermore, cisplatin contributes to the prothrombotic state through activation of platelets (increased platelet aggregation and thromboxane release) (Togna et al. 2000) and peripheral blood monocytes (increased TNF secretion) (Gan et al. 1992). Taken together, we can conclude that while chemotherapy agents are meant to target primarily dividing cells, these agents also exert procoagulant effects on normal host cells.

However, conflicting findings have been reported on the effect of paclitaxel on host cells, particularly HUVEC cell surface TF antigen and activity. In one study, TF activity and antigen were reported to increase on endothelial cells as a result of paclitaxel treatment (Wang et al. 2009) whereas another study reported decreased TF antigen and activity on LPS-stimulated HUVEC and monocytes (Napoleone et al. 2009). Therefore, the mechanism(s) by which lung cancer chemotherapy agents induce procoagulant effects remain unknown. Because platinum-based combination therapy is recommended for lung cancer therapy, it

is important to understand how these combinations induce procoagulant effects on host and tumour cells and will be addressed in Research Objective #1.

1.12.10 Procoagulant effects of lung cancer chemotherapy – animal studies

There is a lack of studies that have investigated the prothrombotic effects of lung cancer chemotherapy agents *in vivo*. Cisplatin injection in mice has been demonstrated to induce nephrotoxicity and increased macrophage infiltration (Lu et al. 2008). Circulating vWF levels were elevated 2 days post cisplatin injection which may reflect endothelial cell damage (Lu et al. 2008). Cisplatin-induced endothelial cell injury has been demonstrated to manifest as thrombotic microangiopathy in humans (Lu et al. 2008), although animal studies looking specifically at cisplatin-induced thrombosis have not been reported. Carboplatin, a derivative of cisplatin, has been shown to induce thrombocytopenia in mice within 8 days following a single dose injection (Ulich et al. 1995). Taken together, these studies suggest that chemotherapeutic agents may induce procoagulant effects through multiple ways including tissue damage and platelet activation.

1.13 Microparticles

Tumor cell derived microparticles (MPs) possess procoagulant activity (Yu et al. 2004) and elevated levels of MPs have been reported in the presence of cancer (Kim et al. 2003). MPs are small (0.5-1μM), cell-derived membrane vesicles that are released during cell activation or apoptosis (Bidot et al. 2008). Composition of MPs often reflects that of the originating cell (Martinez et al.

2005). As a result, MPs can express transmembrane proteins and receptors, as well as cytosolic components of the parent cell such as enzymes, mRNA, and DNA (VanWijk et al. 2003). MPs have been shown to originate from a number of cell types including platelets, leukocytes, and endothelial cells (Tramontano et al. , Mesri et al. 1999, Dignat-George et al. 2004, Flaumenhaft 2006). In healthy individuals, it has been suggested that >80% of circulating MPs are of platelet origin (Flaumenhaft et al. 2009). Although present in blood of healthy individuals at very low concentrations, levels of circulating MPs are increased in the presence of disease such as diabetes, sepsis, and cancer (Kim et al. 2003, Morel et al. 2006, Burnier et al. 2009).

1.13.1 Microparticle generation and release

Calcium-dependent membrane remodeling leading to externalization of PS (discussed in section 1.4.3), and Ca^{2+}-dependent cytoskeleton proteolysis is a common underlying feature of MP generation and release (Zwaal et al. 2005, Freyssinet et al. 2010). The physiological importance of phospholipid asymmetry loss and the resulting MP generation/release is evidenced in patients with Scott syndrome. Scott syndrome is a rare bleeding disorder which is characterized by reduced floppase activity, diminished externalization of PS, and impaired MP generation/release (Zwaal et al. 2004). The physiological consequence of Scott syndrome is impaired activation of coagulation leading to limited thrombin generation (Zwaal et al. 2004).

1.13.2 Microparticles and Cancer

A number of studies have demonstrated that tumor cell derived MPs possess procoagulant activity that is TF-dependent (Dvorak et al. 1981, Dvorak et al. 1983, Yu et al. 2004, Davila et al. 2008). Clinical studies have also reported an association between plasma levels of TF^+ MPs, cancer, and VTE (Zwicker et al. 2009). Furthermore, a 2-fold increase in TF^+ MPs was observed in patients with colorectal cancer compared to healthy controls (Hron et al. 2007). Although colorectal and pancreatic cancers are strongly associated with increased plasma levels of TF^+ MPs, increased levels of TF^+ MPs have also been reported in lung cancer patients (Del Conde et al. 2007, Langer et al. 2008, Fleitas et al. 2012). While these studies had low patient numbers, these findings are supported by *in vitro* findings that demonstrate increased total number of MP release, as well as TF^+ MP shedding from lung cancer cells (Thomas et al. 2009, Wysoczynski et al. 2009). In addition, infusion of MPs isolated from lung cancer cells into mice leads to accelerated thrombus formation (Thomas et al. 2009). While constitutive expression of TF on tumour cells and shedding of TF^+ MP from tumour cells has been reported, the effects of platinum-based chemotherapy use on procoagulant MP generation have not been studied and is the focus of study objective #2.

1.13.3 Effects of Chemotherapy on Microparticles

The effect of chemotherapy on microparticle levels and MP procoagulant activity varies depending on i) the tumour type and ii) chemotherapy agents or combinations used for treatment. Increased MP levels are reported in cancer patients compared to healthy controls prior to initiating therapy in cancers such as pancreatic and colorectal (Zwicker et al. 2009). However, there are limited reports on the effect of chemotherapy on MP generation.

Studies investigating the correlation between MP levels, MP activity, and cancer have produced varying results depending on the type of cancer/chemotherapy studied. In patients with multiple myeloma, MP associated TF activity was reduced following chemotherapy (Auwerda et al. 2011). In patients with pancreatic cancer, investigators did not see a difference between pre- and post-chemotherapy levels of MPs (Khorana et al. 2008). Other studies report a correlation between MP levels and procoagulant activity in cancer patients who present with VTE compared to cancer patients without VTE or healthy controls (Tesselaar et al. 2009). However, since some studies do not measure or make a direct comparison between pre- and post-chemotherapy, the effects of cancer specific chemotherapy on generation of procoagulant MPs remain unknown (Tesselaar et al. 2009).

1.14 Chemotherapy-induced cell-free DNA release

1.14.1 Cell-free DNA

DNA can be expelled extracellularly as a result of cells undergoing stimulation, apoptosis, or necrosis (van der Vaart et al. 2008). Cell-free DNA (CFDNA) encompasses nucleic acids that may be associated with circulating proteins, lipids, or microparticles (El-Hefnawy et al. 2004, Gormally et al. 2007).

Low levels of CFDNA can be detected in healthy individuals (Tamkovich et al. 2006) . Elevated levels of CFDNA are observed in a number of disease states including coronary artery disease, sepsis, and cancer where thrombosis is a common complication (Preissner 2007, Dwivedi et al. 2012, Borissoff et al. 2013). However, the mechanism(s) of DNA release into the circulation remain unclear. *In vitro*, CFDNA release can occur due to necrosis or apoptosis (Choi et al. 2005). Furthermore, CFDNA release has been demonstrated on neutrophils (Brinkmann et al. 2004, Urban et al. 2006), macrophages (Choi et al. 2005), eosinophils (Yousefi et al. 2008), and tumour cells (Diehl et al. 2008). More recently, it has been demonstrated that CFDNA release occurs primarily from neutrophils through NETosis, a newly described form of cell death that differs from necrosis or apoptosis (Kaplan et al. 2012, Yipp et al. 2013). While CFDNA is a component of NETs, which play a role in innate immunity, they can also exert procoagulant effects (Yipp et al. 2013).

1.14.2 Formation of neutrophil extracellular traps

NETosis can be induced through neutrophil activation by various stimuli such as interleukin-8, lipopolysaccharide (LPS), or tumour necrosis factor (TNF) (Kaplan et al. 2012, Tadie et al. 2013, Maugeri et al. 2014). Overall, NET formation can be described as disintegration of nuclear and plasma membranes and protrusion of web-like structures containing DNA, histones, and cytoplasmic proteins (Yipp et al. 2013).

NET formation has been shown to be dependent on reactive oxygen species (ROS) as neutrophils from patients harboring NADPH oxidase mutations fail to form NETs (Papayannopoulos et al. 2010). Following ROS generation, histone arginine residues are converted to citrulline which leads to chromatin decondensation and NET formation (Wang et al. 2004, Wang et al. 2009).

1.14.3 Procoagulant Effects of NETs

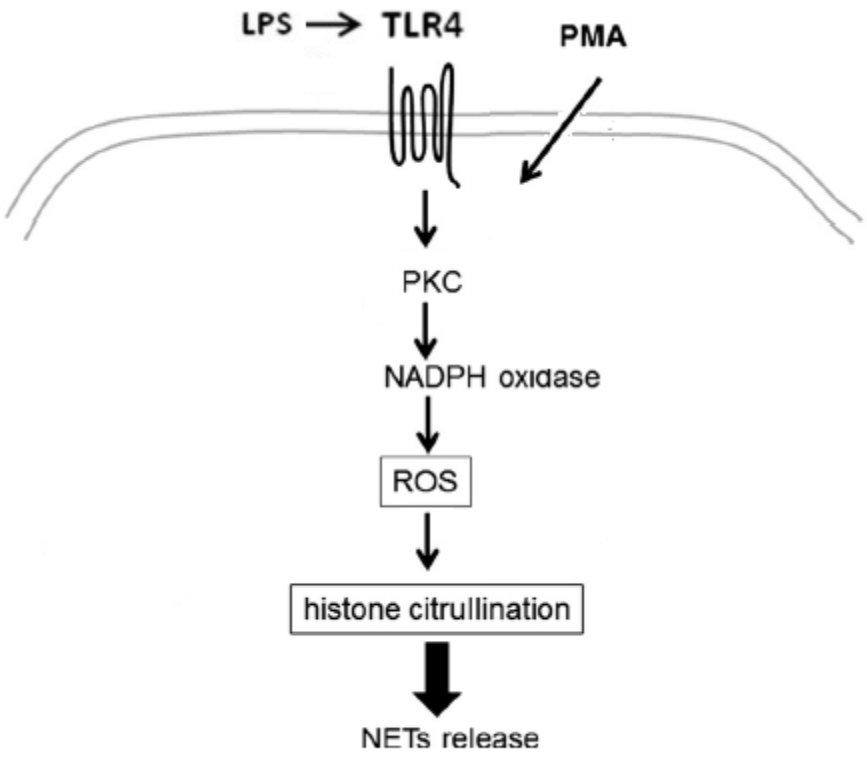
The structural components of NETs (DNA, histones, and granular proteins such as neutrophil elastase) have been implicated in thrombosis (Yipp et al. 2013). DNA induces activation of the contact pathway whereas histones activate platelets in a toll-like receptor dependent manner (Fuchs et al. 2010, Semeraro et al. 2011, Swystun et al. 2011). Several studies have demonstrated a procoagulant role of DNA and NETs in thrombosis. CFDNA has been shown to promote clotting in plasma and whole blood in a FXII-dependent manner (Kannemeier et al. 2007, Swystun et al. 2011). Supporting animal studies have demonstrated elevated levels of circulating DNA in baboon and rodent models of

DVT (Fuchs et al. 2010, Brill et al. 2012), and treatment of mice with DNase attenuates DVT (Brill et al. 2012). In addition to increased thrombin generation, CFDNA also impairs fibrinolysis and delays clot lysis times (Longstaff et al. 2013, Gould et al. 2015, Varju et al. 2015). Therefore, CFDNA may be a therapeutic target to inhibit thrombosis.

Histones have also been shown to induce prothrombotic effects independently of CFDNA. Histones induce platelet aggregation as well as increase thrombin generation in a TLR2- and TLR4-dependent manner (Semeraro et al. 2011). Furthermore, histones dose-dependently increase PS exposure on the surface of red blood cells leading to accelerated fibrin formation (Semeraro et al. 2014). In addition, histones promote prothrombin autoactivation as well as impair protein C activation (Ammollo et al. 2011, Barranco-Medina et al. 2013). These studies identify histones as additional therapeutic targets for management of thrombosis.

Figure 1.3 Schematic Diagram of Extracellular Trap Formation.

A number of stimuli can trigger the process of neutrophil extracellular trap (NET) formation. Treatment of neutrophils with PMA or LPS leads to phosphorylation of NADPH oxidase by protein kinase C (PKC). Increased NADPH oxidase activity results in formation of reactive oxygen species (ROS) and PAD4-dependent histone citrullination. This in turn leads to chromatin decondensation, disintegration of the nuclear membrane, and formation of NETs. Adapted from (Itakura et al. 2013).


LPS
TLR4
PMA
PKC
NADPH oxidase
ROS
histone citrullination
NETs release

1.14.4 Peptidylargenine Deaminases

Peptidylarginine deaminases (PADs) are a family of enzymes that catalyze the conversion of arginine residues to citruline residues on proteins in a Ca^{2+}-dependent manner (Vossenaar et al. 2003). This conversion results in a loss of charge of the target protein and the process is referred to as peptidylargenine deamination or citrullination (Hensen et al. 2014). In humans, five isotypes of PAD have been found (PAD1-4 and PAD6) with tissue specific expression of the isotypes (Vossenaar et al. 2004, Foulquier et al. 2007, van Beers et al. 2013).

Since PAD function is Ca^{2+}-dependent, the activity of PADs is relatively low under normal physiologic conditions when cytosolic and nucleoplasmic Ca^{2+} concentrations are low. Upon cellular stimulation/activation and subsequent influx of extracellular Ca^{2+} or release from intracellular stores, Ca^{2+} binding to PAD induces a conformational change of the PAD enzyme leading to citrullination of the target proteins (Hensen et al. 2014). As a result of their wide distribution, PADs are involved in numerous processes such as hair growth, myelin formation, and NETosis (Li et al. 2010, Demers et al. 2012, Wang et al. 2013).

In addition to numerous physiologic roles, the role of PADs in protein citrullination has been implicated in a number of diseases such as cancer, multiple sclerosis, rheumatoid arthritis, and thrombosis (Demers et al. 2012, Hensen et al. 2014). One of the research goals in this thesis was to investigate the chemotherapy-induced effects on CFDNA release and the subsequent

procoagulant consequences. Of the existing isotypes, PAD4 is found primarily in white blood cells, is the only isotype that resides within the nucleus, and inhibition of PAD4 prevents histone H3 citrullination and NET formation (Lewis et al. 2015). Mice lacking PAD4 are unable to decondense chromatin or form NETs and are protected from thrombosis (Martinod et al. 2013). Therefore, the focus of the research was primarily on PAD4 as its function has been linked to thrombosis (Martinod et al. 2013, Yipp et al. 2013).

Chapter 2: Hypothesis and Aims

2.1 Aim and Hypothesis

Rates of VTE are increased in lung cancer patients receiving chemotherapy. Studies have shown that lung cancer chemotherapy agents can induce procoagulant effects through increased endothelial cell-surface TF antigen levels, increased endothelial cell surface TF activity, and endothelial cell damage. However, the mechanisms by which lung cancer agents induce procoagulant effects on other host cells (monocytes and neutrophils, and lung tumour-cells) remain unknown. Therefore, the aim of the studies within this thesis is to identify the mechanisms by which platinum-based lung cancer chemotherapy modulates hemostatic pathways and increases thrombotic risk.

We hypothesize that platinum-based lung cancer chemotherapy agents increase thrombotic risk by i) increasing cell-surface TF activity, ii) causing the generation and release of procoagulant MPs, and iii) inducing CFDNA release.

To test the hypothesis, we studied the effects of lung cancer chemotherapy agents on cell surface TF antigen and activity. Furthermore, we determined the effects of these agents on MP generation and CFDNA release *in vitro* and *in vivo* and investigated their role in thrombin generation. In addition, we investigated the role of PAD4 in the procoagulant effects induced by lung cancer chemotherapy agents.

2.2 Objectives

The following were the specific objectives:

1. To investigate the procoagulant effects of lung cancer chemotherapy agents on endothelial cells, monocytes, and lung cancer cells.

2. To study the effects of lung cancer chemotherapy on the generation of procoagulant MPs *in vitro* and *in vivo.*

3. To determine the effects of lung cancer chemotherapy on release of CFDNA and thrombin generation *in vitro* and *in vivo.*

4. To investigate the role of PAD4 in chemotherapy-induced release of CFDNA.

Chapter 3: Lung cancer chemotherapy agents increase procoagulant activity via protein disulfide isomerase-dependent tissue factor decryption.

Foreword

These studies investigate the mechanisms by which lung cancer chemotherapy agents (cisplatin, carboplatin, paclitaxel, and gemcitabine) induce procoagulant effects on endothelial cells (HUVEC), monocytes, and NSCLC A549 cells. The results demonstrate that lung cancer chemotherapy agents can induce procoagulant effects within 24 hours of treatment through PDI-mediated TF decryption on the cell-surface of HUVEC and A549 cells leading to increased FXa generation.

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3.1 Abstract

Background: Lung cancer patients undergoing chemotherapy have an elevated risk for thrombosis. However, the mechanisms by which chemotherapy agents increase the risk for thrombosis remains unclear. *Objectives:* To determine the mechanism(s) by which lung cancer chemotherapy agents cisplatin, carboplatin, gemcitabine, and paclitaxel elicit increased tissue factor (TF) activity on endothelial cells, A549 cells, and monocytes. *Methods:* TF activity, TF antigen, and PS exposure were measured on chemotherapy-treated human umbilical vein endothelial cells (HUVEC), A549 cells, and monocytes. Cell surface protein disulfide isomerase (PDI) and cell surface free-thiol levels were measured on HUVEC and A549 non-small cell lung carcinoma cells. Results*:* Treatment of HUVECs, A549 cells, and monocytes with lung cancer chemotherapy significantly increased cell surface TF activity. However, elevated TF antigen levels were observed only on cisplatin- and gemcitabine-treated monocytes. Cell surface levels of PS were increased on HUVEC and monocytes treated with cisplatin/gemcitabine combination therapy. Chemotherapy also resulted in increased cell surface levels of PDI and reduced cell surface free thiol levels. Glutathione treatment and PDI inhibition, but not PS inhibition attenuated TF activity. Furthermore, increased TF activity was reversed by reducing cysteines with dithiothreitol. *Conclusions:* These studies are the first to demonstrate that lung cancer chemotherapy agents increase procoagulant activity on endothelial

cells and A549 cells by TF decryption through a disulfide bond formation in a PDI-dependent mechanism.

3.2 Introduction:

Cancer patients have a ~7-fold elevated risk for venous thromboembolism (VTE) compared to healthy individuals (Blom et al. 2005). 14% of cancer related deaths can be attributed to pulmonary embolism, making it the second leading cause of mortality in these patients (Ambrus et al. 1975, Shen et al. 1980, Rickles et al. 1983). In addition to the procoagulant properties of malignant cells (Sawada et al. 1999, Rickles et al. 2001), interventions such as surgery, indwelling catheters, and anti-cancer therapies can shift the hemostatic balance towards a more procoagulant state. The incidence of thrombosis in cancer patients also varies depending on the type of malignancy (Levitan et al. 1999, Blom et al. 2005).

Lung cancer is the second leading type of cancer with an estimated death rate of 26% and 29% for female and male patients, respectively (Jemal et al. 2010). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases with increased rates of thrombosis associated with disease progression. The American Society of Clinical Oncology (ASCO) recommends platinum-based (cisplatin or carboplatin) combination chemotherapy over non-platinum based therapies for first line treatment of NSCLC (Azzoli et al. 2010). Cisplatin and carboplatin are commonly administered in combination with gemcitabine and paclitaxel, respectively, and have been studied extensively for their anti-tumor efficacy (Sandler et al. 1999, Scagliotti et al. 2002, Sandler et al. 2006).

VTE is a frequent complication of lung cancer. A retrospective study of 493 NSCLC patients reported a 14% incidence of thrombotic events during the course of the disease (Tagalakis et al. 2007). However, it is important to note that the majority of patients in this study presented with stage IIIB or greater NSCLC which accounted for 82% of all thrombotic events. In lung cancer patients, varying rates of thrombosis have been reported during chemotherapy depending on the combination of agents employed. For example, a 3% rate of thrombosis was reported in stage III-IV lung cancer patients who received carboplatin and paclitaxel-based therapy (Johnson et al. 2004), whereas 22% of stage III-IV lung cancer patients who received cisplatin and gemcitabine-based therapy experienced a thrombotic event within 1 year of starting therapy (Numico et al. 2005).

Previous *in vitro* studies have demonstrated that cisplatin and gemcitabine increase tissue factor (TF) activity on endothelial cells in a dose-dependent manner(Ma et al. 2005). Furthermore, use of cisplatin-based therapy is associated with an increase in circulating levels of von Willebrand Factor (VWF) antigen which may reflect damage to vascular endothelial cells (Dieckmann et al.). However, studies investigating the procoagulant effects of paclitaxel on endothelial cells have produced conflicting results. Wang et al. (Wang et al. 2009) reported an increase in TF activity and antigen levels on paclitaxel treated aortic endothelial cells while Napoleone et al. (Napoleone et al. 2009) reported that paclitaxel decreased TF antigen and activity on LPS-stimulated HUVECs,

and monocytes. Thus, the molecular mechanisms by which lung cancer drugs increase VTE remain unclear. The overall goal of this study is to reveal potential mechanisms by which anti-lung cancer agents increase the risk of thrombosis.

3.3 Materials and Methods

3.3.1 Materials

Human umbilical vein endothelial cells (HUVECs) and endothelial growth medium (EGM-2) were purchased from Lonza (Basel, Switzerland). A549 non-small cell lung cancer cells were from ATCC (Manassas, VA, USA). RPMI 1640 growth medium, trypsin-EDTA, and penicillin-streptomycin were from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum, dithiothreitol (DTT), and paclitaxel were from Sigma-Aldrich (St Louis, MO, USA). Cisplatin was from Mayne Pharma (Quebec, Canada) and carboplatin was from TevaCanada (Ontario, Canada). Gemcitabine (Eli Lilly, Ontario, Canada) was diluted in 0.9% sodium chloride and aliquots were stored at -80 ^{o}C. Anti-human TF monoclonal antibody (FITC) was from Affinity Biologicals (Ancaster, ON, Canada). Anti-protein disulfide isomerase (PDI) antibody (RL90) was from AbCam, (San Diego, CA, USA). Annexin V, annexin V-FITC, annexin V binding buffer, and TF inhibitory antibody (HTF-1) were from BD Biosciences (San Jose, CA, USA). Factors VIIa, FXa, and FX were from Haematologic Technologies (Essex Junction, VT, USA). Chromogenic substrate S2765 was purchased from DiaPharma (West Chester, OH, USA). Magnetic–activated cell sorting (MACS) columns and CD-14 microbeads were from MiltenyiBiotec (Auburn, CA, USA).

3.3.2 Cell Culture and Treatment

HUVECs were cultured in 2% gelatin-coated plates on passages 1–4 in EGM-2 growth medium at 37^{o}C and 5% CO2. A549 cells were cultured in RPMI-1640 supplemented with 10% heat inactivated FBS and 100 U/ml penicillin-streptomycin. Upon reaching 80-90 % confluency, the cells were treated with specified concentrations of chemotherapy agents diluted in cell culture media.

3.3.3 Isolation of human peripheral blood monocytes

Monocytes were isolated from healthy individuals using MACS with CD-14 magnetic beads as previously described (Stephenson et al. 2006). The isolation and use of monocytes from donors was voluntary, imposed minimal risk to the donor, and was approved by the Research Ethics Board of Hamilton Health Sciences (REB# 12-712-T). Isolated monocytes were resuspended in RPMI-1640 supplemented with 10% heat inactivated FBS and 100 U/ml penicillin-streptomycin at a density of 1 x 10^6 cells/mL and treated with chemotherapy agents diluted in RPMI-1640 to specified concentrations.

3.3.4 Concentrations of Chemotherapeutic Agents

Concentrations of chemotherapy agents used in our *in vitro* studies were based on maximum plasma concentrations (C_{max}) observed in chemotherapy patients. Cisplatin, carboplatin, gemcitabine, and paclitaxel were reported to have a C_{max}

of approximately 20 µM, 40 µM, 60 µM, and 4 µM respectively (Kern et al. 2001, Kroep et al. 2006).

3.3.5 Cell surface TF activity

HUVECs, A549 cells, and monocytes were plated in 24-well dishes and treated for 24 hours with chemotherapy agents. Cells were washed twice with TBS and incubated with TBS containing 5nM FVIIa, 150nM FX and 5mM $CaCl_2$ for 30 minutes at 37^{o}C. FXa generation was assessed by cleavage of chromogenic substrate (S-2765) (0.2 mM final). The reaction was terminated with 50% acetic acid and endpoint absorbance was measured at 405 nm.

3.3.6 Flow cytometric measurement of cell surface PDI, TF expression, and phosphatidylserine exposure

HUVECs and A549 cells were cultured on 6-well plates and treated with chemotherapy agents for 24 hours upon reaching 80-90% confluency. Cells were washed in PBS and incubated with 2 µg/mL anti-human TF-FITC antibody for 30 min in the dark at room temperature. Incubation with annexin V-FITC was performed in Annexin V binding buffer according to supplier's recommendation. Cell surface PDI levels were measured using a mouse anti-human PDI (RL90) and Alexa Fluor 488 goat anti-mouse IgG antibodies. Untreated cells were permeabilized with 0.1% Triton X-100 for 30 minutes prior to incubation with RL90 or IgG isotype antibodies to be used as positive and negative controls respectively. Cell-bound fluorescence was determined with a FACSCaliber flow cytometer (Becton Dickinson, San Jose, CA, USA). Monocytes were pre-

incubated with ChromPure Human IgG (200 µg/mL) on ice for 1 hour prior to labeling with a FITC conjugated antibody.

3.3.7 Measurement of cell surface free thiol levels

HUVEC and A549 cells were cultured in 6-well dishes as described above. Upon confluence, cells were treated with combination chemotherapy either in the presence or absence of RL90 for 24 hours. Cell surface free thiol levels were measured on treated cells using Measure-IT thiol assay kit (Life Technologies) according to manufacturer's instructions.

3.3.8 Statistical analysis

Statistical analysis was performed using one way ANOVA, Tukey's pair wise comparisons, or t-tests on SigmaPlot software version 11.2. All results are representative of at least 3 replicates for each experiment.

3.4 Results

3.4.1 Effects of platinum-based chemotherapy on TF activity

To better understand the mechanisms by which lung cancer chemotherapy agents can modulate procoagulant pathways, we measured the effects of platinum-based therapy on cell surface TF activity of HUVECs, A549 cells, and monocytes. Cells were treated with single agent (data not shown) and combination chemotherapy for 24 hours and TF activity was assessed by FXa generation. Platinum-based chemotherapy agents were used in combinations according to ASCO recommendations (Azzoli et al. 2010) using C_{max} dosing

observed in lung cancer patients (Kern et al. 2001, Kroep et al. 2006). We observed a significant increase in TF activity on HUVEC (Fig. 3.1A) and A549 cells (Fig. 3.1B) treated with cisplatin/gemcitabine and carboplatin/paclitaxel combination therapies. In addition, we observed increased TF activity on monocytes treated with cisplatin/gemcitabine combination but not carboplatin/paclitaxel combination (Fig. 3.1C).

To verify that FXa generation in this assay was TF-dependent, we performed the assay in the absence of FVIIa (data not shown), or in the presence of a TF-inhibiting antibody (HTF-1). In both instances we observed a complete inhibition of FXa generation on HUVEC, A549 cells, and monocytes treated with combination therapy (Figs. 3.1A-C).

3.4.2 Effects of platinum-based chemotherapy on TF antigen levels

The increase in TF activity observed on chemotherapy-treated HUVEC, A549 cells, and monocytes could be attributed to increased levels of cell surface TF antigen or due to decryption (activation) of TF. Current theories of TF decryption include phosphatidylserine (PS) exposure and PDI mediated formation of the Cys186-Cys209 disulfide bond in the extracellular domain of TF. We measured cell surface TF antigen levels by flow cytometry and observed no changes in cell surface levels of TF antigen on HUVEC and A549 cells in all chemotherapy treatment conditions (Fig. 3.2A). In contrast, we observed a significant increase in cell surface TF antigen levels on monocytes treated with cisplatin alone and cisplatin/gemcitabine combination treatment (Fig. 3.2B),but

not carboplatin or paclitaxel (Fig. 3.2C). TNF was used as positive control for TF antigen expression on HUVEC and A549 cells (Mechtcheriakova et al. 2001). LPS was used as a positive control for TF antigen expression on monocytes (Meszaros et al. 1994).

Figure 3.1 Effects of chemotherapy on cell surface TF procoagulant activity.

Cell surface TF activity was measured by FXa cleavage of a chromogenic substrate on **A)** HUVEC, **B)** A549 cells, and **C)** monocytes treated with combination chemotherapy. Treatments were performed using combination therapies for 24 hours. Values are expressed as mean $\pm$ SE of 3 separate experiments. HTF-1: Anti-human tissue factor antibody, Car: Carboplatin, Tax: Paclitaxel, Cis: Cisplatin, Gem: Gemcitabine. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N.S. – Not Significant

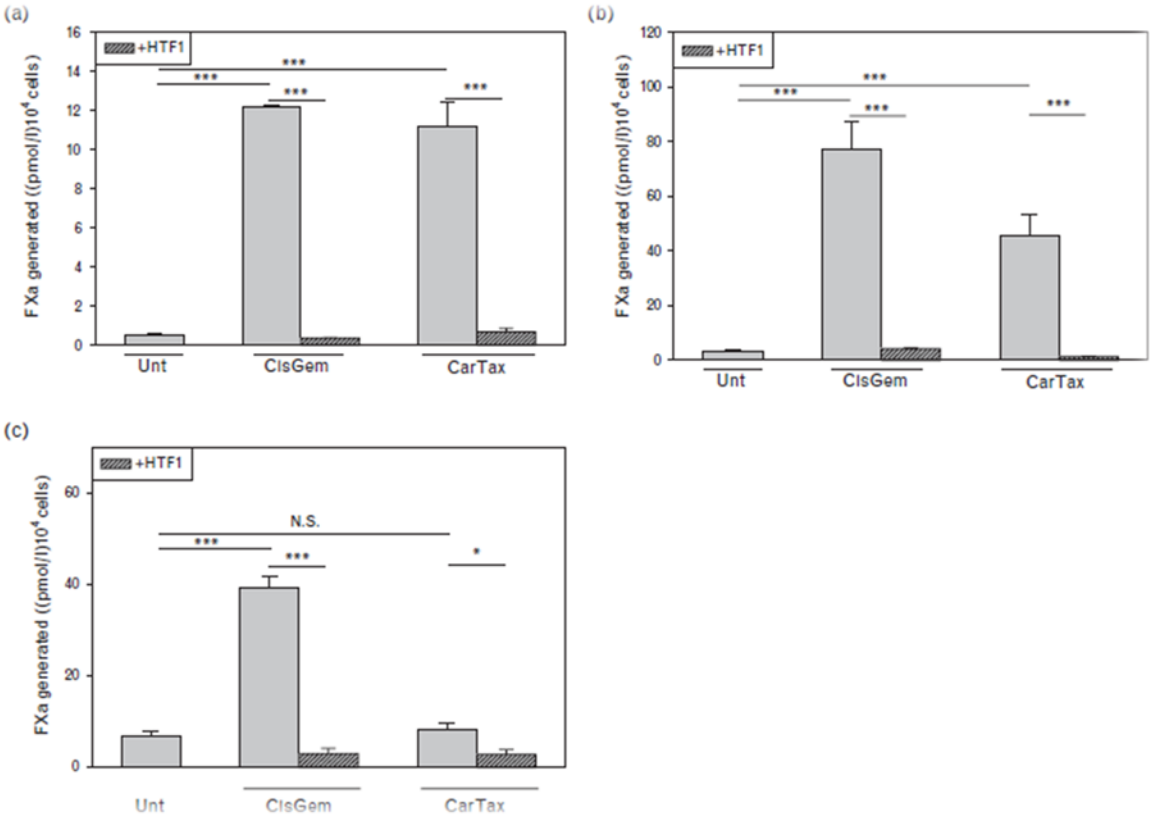
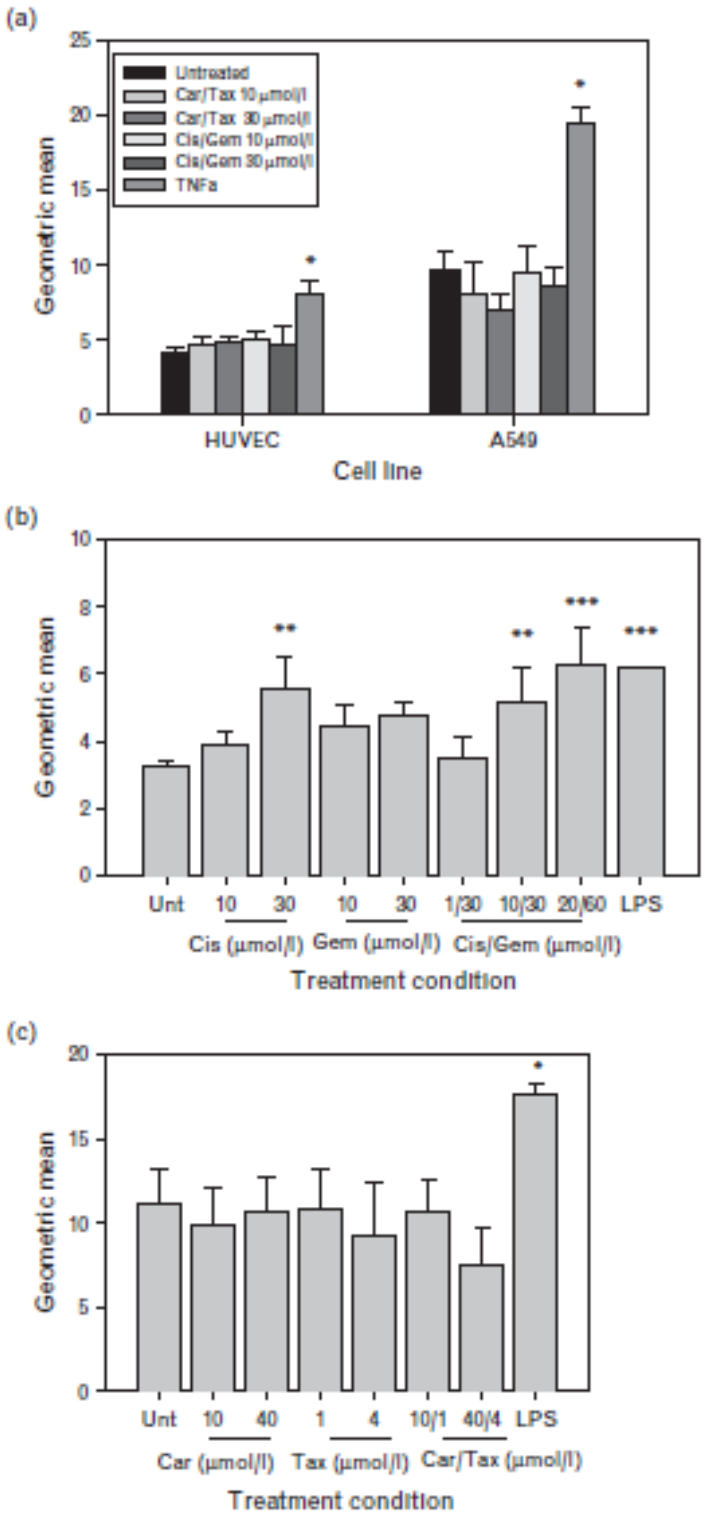

(a)
+HTF1
FXa generated ((pmol/l)10^4 cells)
Unt
CisGem
CarTax
(b)
+HTF1
FXa generated ((pmol/l)10^4 cells)
Unt
CisGem
CarTax
(c)
+HTF1
FXa generated ((pmol/l)10^4 cells)
N.S.
Unt
CisGem
CarTax

Figure 3.2 – Effects of chemotherapy on cell surface TF antigen levels.

Cell surface TF antigen levels on **A)** HUVEC and A549 cells treated with carboplatin/paclitaxel as well as cisplatin/ gemcitabine combination therapies, **B)** monocytes treated with cisplatin and gemcitabine and **C)** monocytes treated with carboplatin and paclitaxel. Values are expressed as mean $\pm$ SE of 3 separate experiments. Car: Carboplatin, Tax: Paclitaxel, Cis: Cisplatin, Gem: Gemcitabine, LPS: Lipopolysaccharide. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.


(a)
Untreated
Car/Tax 10 µmol/l
Car/Tax 30 µmol/l
Cis/Gem 10 µmol/l
Cis/Gem 30 µmol/l
TNFa
Geometric mean
HUVEC
A549
Cell line
(b)
Geometric mean
Unt
10
30
10
30
1/30
10/30
20/60
LPS
Cis (µmol/l)
Gem (µmol/l)
Cis/Gem (µmol/l)
Treatment condition
(c)
Geometric mean
Unt
10
40
1
4
10/1
40/4
LPS
Car (µmol/l)
Tax (µmol/l)
Car/Tax (µmol/l)
Treatment condition

3.4.3 Effects of platinum-based chemotherapy on phosphatidylserine exposure

An increased concentration of phosphatidylserine in the outer plasma membrane of cells has been proposed to induce a structural change in TF that exposes the FX and FIX binding sites on TF (Bach 2006). To determine the effect of chemotherapy on extracellular phosphatidylserine exposure, we measured annexin-V binding to chemotherapy treated cells using flow cytometry. We observed a dose-dependent increase in phosphatidylserine on the surface of HUVEC treated with cisplatin/gemcitabine but not carboplatin/paclitaxel combinations (Figs. 3.3A and 3.3B). Conversely, treatment of A549 cells with cisplatin/gemcitabine or carboplatin/paclitaxel combination therapies (Fig. 3.3C) did not modulate cell surface phosphatidylserine levels. Hydrogen peroxide (H_2O_2) was used as a positive control as it has been shown to induce apoptosis and increase cell surface phosphatidylserine exposure (Swystun et al. 2011, Dreschers et al. 2013).

Treatment of monocytes with cisplatin and gemcitabine as single agent or in combination did not significantly increase cell surface levels of phosphatidylserine (Fig. 3.3D). Interestingly, while carboplatin alone did not increase phosphatidylserine exposure on monocytes, paclitaxel alone and the addition of paclitaxel to carboplatin treatment (carboplatin/paclitaxel combination) significantly reduced cell surface phosphatidylserine levels (Fig. 3.3D).

3.4.4 TF decryption by platinum based chemotherapy agents

Since lung cancer chemotherapy agents increase TF activity but not antigen levels, we next investigated the mechanism(s) by which these agents may de-encrypt TF. The association between phosphatidylserine and TF procoagulant activity has been shown in a number of studies which demonstrate parallel increases in phosphatidylserine exposure and TF procoagulant activity (Zwaal et al. 1997, Lentz 2003). Phosphatidylserine was blocked using purified recombinant annexin-V in binding buffer at 25µg/mL for the duration of chemotherapy exposure. Although cisplatin/gemcitabine combination treatment significantly increased cell surface TF activity and phosphatidylserine exposure on HUVEC, TF activity was not affected by annexin-V binding (Fig. 3.4A), suggesting the chemotherapy-induced TF activity on cisplatin- and gemcitabine-treated cells is not phosphatidylserine-dependent. In contrast to HUVEC, inhibition of phosphatidylserine on chemotherapy treated monocytes did not achieve significant attenuation of cell surface FXa generation (Fig.3.4B).

PDI has been suggested to play a role in TF de-encryption (Rao et al. 2012). PDI may facilitate TF de-encryption through the formation of the Cys186-Cys209 disulfide bond in the extracellular domain of TF (Pendurthi et al. 2007, Versteeg et al. 2007). We measured baseline and post-chemotherapy cell surface PDI levels on chemotherapy treated HUVEC (Fig. 3.4C) and A549 cells (Fig. 3.4D) and observed a significant increase in cell surface PDI levels on

carboplatin/paclitaxel treated HUVEC. To determine if PDI activity was contributing to TF decryption, we pretreated HUVECs and A549 cells

Figure 3.3 – Effects of chemotherapy on cell surface phosphatidylserine exposure.

Phosphatidylserine exposure on chemotherapy treated HUVEC **(A and B)**, A549 cells **(C),** and monocytes **(D)**. Values are expressed as mean ± SE of 3 separate experiments. Car: Carboplatin, Tax: Paclitaxel, Cis: Cisplatin, Gem: Gemcitabine, H_2O_2: Hydrogen Peroxide, TNF: Tumor Necrosis Factor-alpha. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

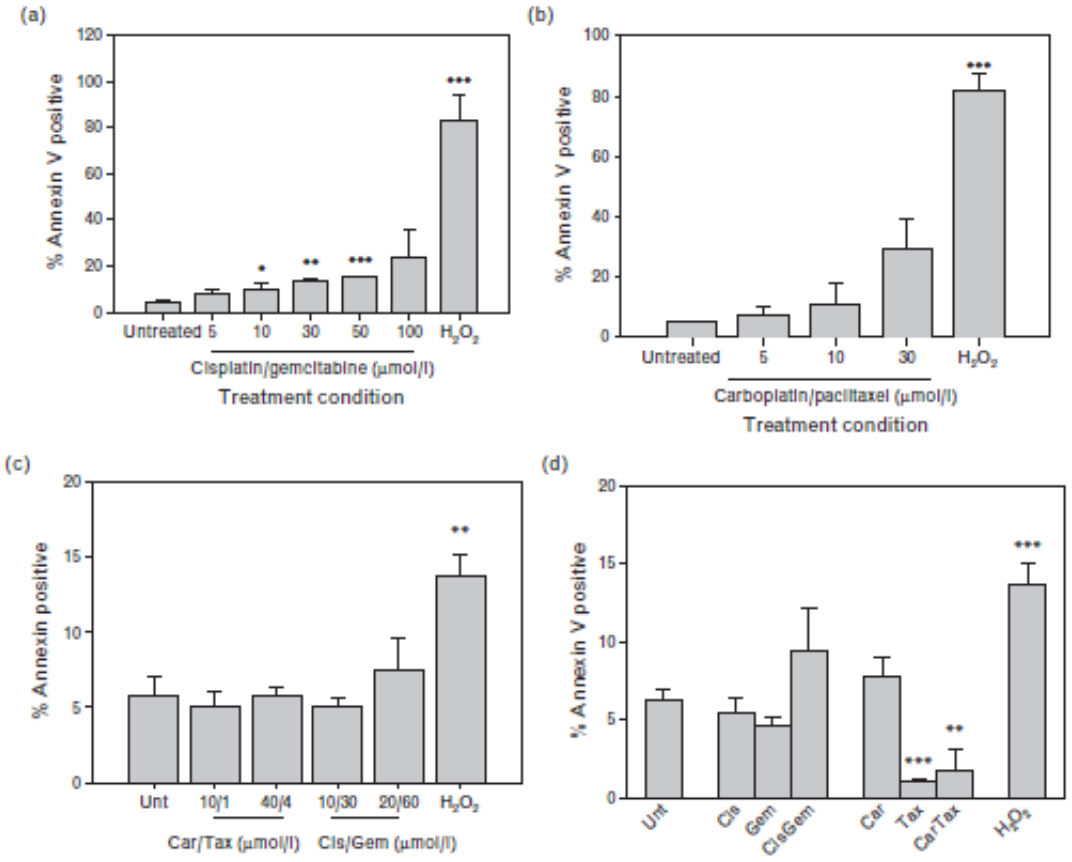
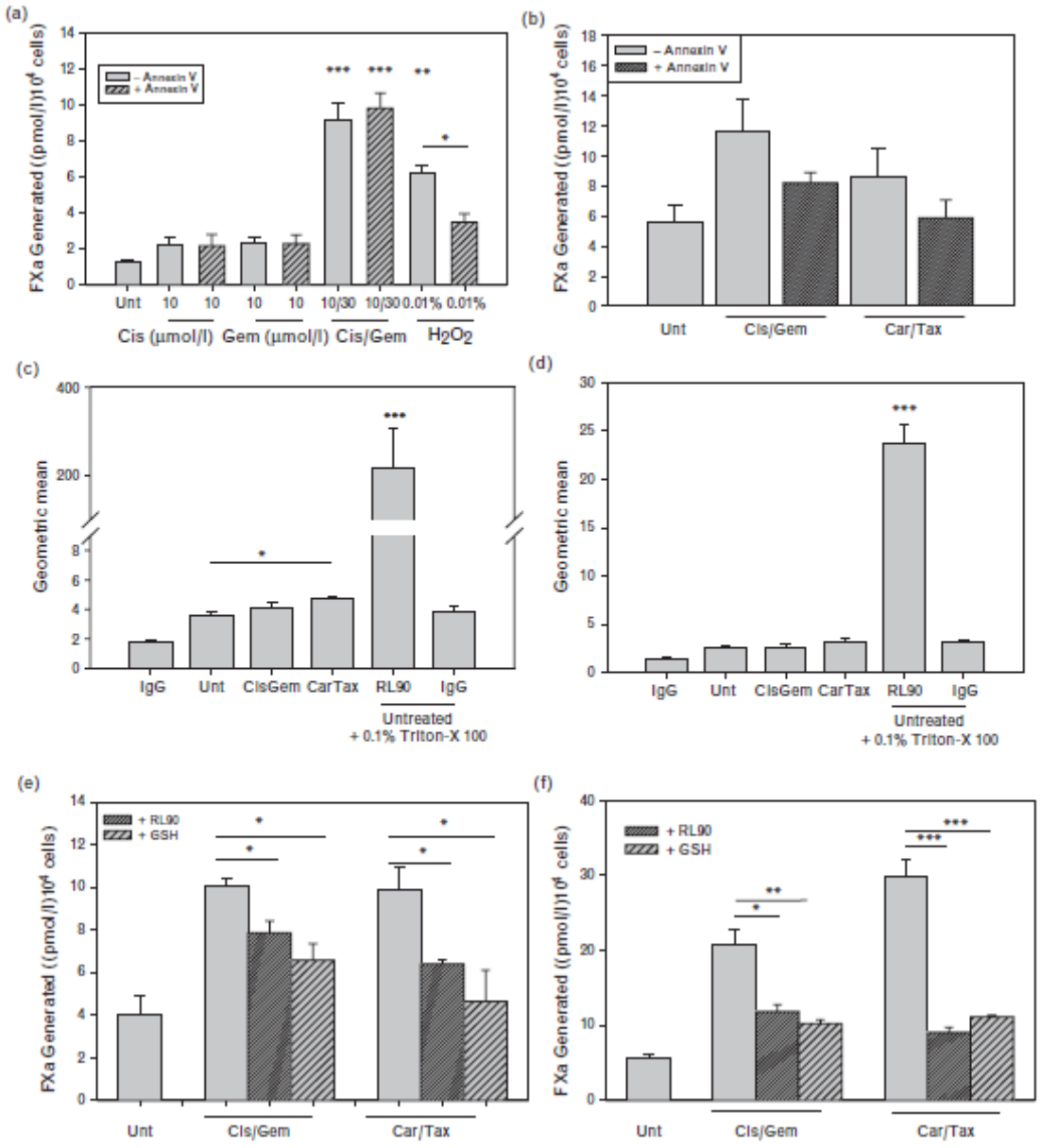

(a)
% Annexin V positive
Untreated
5
10
30
50
100
H2O2
Cisplatin/gemcitabine (µmol/l)
Treatment condition
(b)
% Annexin V positive
Untreated
5
10
30
H2O2
Carboplatin/paclitaxel (µmol/l)
Treatment condition
(c)
% Annexin positive
Unt
10/1
40/4
10/30
20/60
H2O2
Car/Tax (µmol/l)
Cis/Gem (µmol/l)
(d)
% Annexin V positive
Unt
Cis
Gem
CisGem
Car
Tax
CarTax
H2O2

Figure 3.4 – Effects of chemotherapy on TF decryption mechanisms.

Cell surface TF activity on chemotherapy treated **A)** HUVEC and **B)** Monocytes in the presence of Annexin-V. Cell surface levels of PDI on chemotherapy-treated **C)** HUVEC and **D)** A549 cells in the presence of RL-90 (PDI inhibiting antibody). Cell surface TF activity on chemotherapy-treated **E)** HUVEC and **F)** A549 cells in the presence or absence of RL90 and glutathione. Car: Carboplatin, Tax: Paclitaxel, Cis: Cisplatin, Gem: Gemcitabine, H_2O_2: Hydrogen Peroxide, IgG: IgG Isotype control antibody, GSH: Glutathione, RL90: PDI inhibiting antibody. Values are expressed as mean $\pm$ SE of 3 separate experiments unless otherwise noted. Significant compared to the untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.


(a)
FXa Generated ((pmol/l)10⁴ cells)
– Annexin V
+ Annexin V
***
***
**
*
Unt
10
10
10
10
10/30
10/30
0.01%
0.01%
Cis (μmol/l)
Gem (μmol/l)
Cis/Gem
H2O2
(b)
FXa Generated ((pmol/l)10⁴ cells)
– Annexin V
+ Annexin V
Unt
Cis/Gem
Car/Tax
(c)
Geometric mean
***
*
IgG
Unt
CisGem
CarTax
RL90
IgG
Untreated
+ 0.1% Triton-X 100
(d)
Geometric mean
***
IgG
Unt
CisGem
CarTax
RL90
IgG
Untreated
+ 0.1% Triton-X 100
(e)
FXa Generated ((pmol/l)10⁴ cells)
+ RL90
+ GSH
*
*
*
*
Unt
Cis/Gem
Car/Tax
(f)
FXa Generated ((pmol/l)10⁴ cells)
+ RL90
+ GSH
**
*
***
***
Unt
Cis/Gem
Car/Tax

for 1 hour with the PDI inhibitory antibody (RL-90), prior to treatment with platinum-based chemotherapy, and measured TF activity. Our results show that inhibition of PDI significantly reduced FXa generation on HUVEC and A549 cell surface (Figs. 3.4E and 3.4F respectively)

As PDI may function to facilitate TF disulfide bond isomerization, we then examined the effects of glutathione and dithiolthreitol (DTT) on chemotherapy-associated TF de-encryption. We found that pretreatment of cells for 3 hours with 10 mM glutathione prior to (Figs. 3.4E and 3.4F), or 0.01 mM DTT for 30 minutes at 37^{o}C post chemotherapy exposure resulted in significantly reduced FXa generation on HUVEC and A549 cell surface (Figs.3.5A and 3.5B respectively), confirming the involvement of disulfide bond formation in chemotherapy-associated TF decryption.

The formation of the disulphide bond is postulated to take place between free thiol groups of Cys186 and Cys209 in TF extracellular domain (Pendurthi et al. 2007, Versteeg et al. 2007). We observed that upon treatment with lung cancer chemotherapy agents, cell surface free thiol levels were significantly reduced on chemotherapy treated HUVEC (Fig. 3.6A) and A549 cells (Fig. 3.6B). Treatment of HUVEC and A549 cells with the PDI-inhibitory antibody RL90 or DTT inhibited the chemotherapy-mediated decrease in cell surface free-thiols. This suggests that chemotherapy treatment of HUVEC and A549 cells results in TF decryption by disulfide bond formation in a PDI-dependent manner.

Figure 3.5 – Effects of DTT on TF activity of chemotherapy treated cells.

Cell surface TF activity on chemotherapy treated **A)** HUVEC (n=4) and **B)** A549 cells (n=4) in the presence of 0.01 mM DTT. Values are expressed as mean ± SE. Car: Carboplatin, Tax: Paclitaxel, Cis: Cisplatin, Gem: Gemcitabine, DTT: Dithiothreitol. Significant differences between untreated and chemotherapy treated conditions are denoted by: * $P < 0.05$, *** $P < 0.001$.

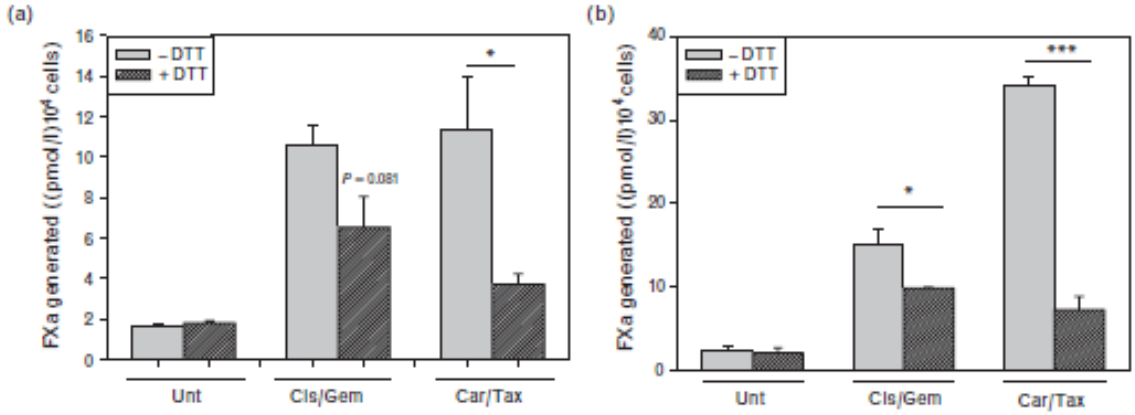
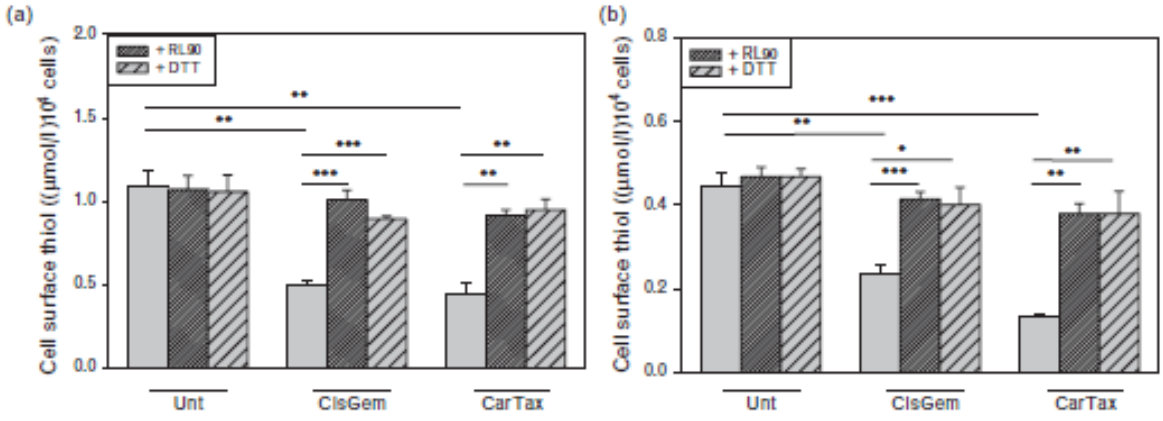

(a)
FXa generated ((pmol/l)10⁴ cells)
– DTT
+ DTT
P = 0.081
*
Unt
Cis/Gem
Car/Tax
(b)
FXa generated ((pmol/l)10⁴ cells)
– DTT
+ DTT
*
***
Unt
Cis/Gem
Car/Tax

Figure 3.6 – Cell surface thiol levels on chemotherapy treated cells.

Cell surface thiol levels on chemotherapy treated **A)** HUVEC and **B)** A549 cells. Values are expressed as mean ± SE. Car: Carboplatin, Tax: Paclitaxel, Cis: Cisplatin, Gem: Gemcitabine, DTT: Dithiothreitol, RL90: PDI inhibiting antibody. Significant differences between untreated and chemotherapy treated conditions are denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.


(a)
(b)
+ RL90
+ DTT
Cell surface thiol ((µmol/l)/10⁴ cells)
Unt
CisGem
CarTax

3.5 Discussion

While the association between thrombosis and chemotherapy is well recognized, the mechanism(s) by which chemotherapy can increase the risk for thrombosis remain largely unknown. A study of patients with stage IIIB or IV non-small cell lung cancer receiving combination chemotherapy demonstrated that elevations in thrombin-antithrombin (TAT) levels persisted for up to 48 hours (Weitz et al. 2002). Our studies provide experimental evidence and insight into the potential mechanisms by which lung cancer chemotherapy agents can modulate procoagulant pathways. Our results of increased cell surface TF activity as measured by FXa generation on HUVEC treated with cisplatin are consistent with studies that have demonstrated increased TF activity on endothelial cells treated with these chemotherapy agents (Ma et al. 2005). In contrast to Ma et al. (Ma et al. 2005), we did not observe increased TF antigen levels on chemotherapy treated HUVEC. Our findings of increased TF activity on paclitaxel treated HUVEC are also contradictory to that of Napoleone et. al (Napoleone et al. 2009) who reported a decrease in TF activity on paclitaxel treated endothelial cells using a one stage clotting assay performed in human plasma (Napoleone et al. 1997). We hypothesize that the conflicting results may be due to utilization of different cell lines between the studies. Ma et. al reported increased TF antigen levels on human microvascular endothelial cells (HMVECs) treated with chemotherapy, whereas our studies used HUVEC (macrovascular endothelial cells). A number of differences have previously been demonstrated

between micro- and macrovascular cells including variability in TF expression (Palomo et al.). In addition, thrombotic events in cancer patients are most frequently diagnosed as deep vein thrombosis (DVT), a condition affecting the deep veins (macrovasculature) of the limbs (Kyrle et al. 2005).

This study is the first to examine the ability of lung cancer chemotherapy agents to induce procoagulant phenotypes on monocytes and A549 cells. Our studies reveal that although cell surface TF activity is increased on all cell types, TF antigen increase is only observed on monocytes. The absence of FXa generation in FVIIa-deficient buffer indicates that FX activation is FVIIa-dependent. The absence of FXa generation in the presence of a TF neutralizing antibody further confirms that chemotherapy-induced TF activity is TF-dependent. Taken together, data obtained from these experiments suggest that increased TF activity without a corresponding increase in cell surface TF antigen on treated HUVEC and A549 cells is likely the result of TF decryption.

A number of studies have examined the role of phosphatidylserine exposure and PDI activity in TF decryption (Zwaal et al. 1997, Lentz 2003, Pendurthi et al. 2007, Versteeg et al. 2007, Rao et al. 2012). Although phosphatidylserine levels were significantly increased on HUVEC treated with cisplatin/gemcitabine combination therapy, inhibition of cell surface phosphatidylserine did not attenuate TF activity (Fig. 3.4A), suggesting that the chemotherapy-induced increase in TF activity is due to TF decryption that is not phosphatidylserine dependent. Furthermore, we did not observe significant

increases in PS exposure on chemotherapy-treated monocytes and inhibition of PS did not attenuate TF activity. However, the increased TF activity on CisGem- but not CarTax-treated monocytes was observed in parallel with increased cell surface TF antigen in respective treatment conditions. Therefore, chemotherapy-induced TF activity on monocytes can be attributed to parallel increases of cell surface TF antigen levels.

In contrast, our inhibition experiments suggest a PDI-mediated method of TF decryption, as inhibition of cell surface PDI with a neutralizing antibody significantly attenuated TF procoagulant activity presumably through prevention of Cys186-Cys209 disulfide bond formation (Krudysz-Amblo et al. 2013). Cryptic TF has been shown to contain unpaired cysteine thiols which were decreased upon TF activation and formation of the disulfide bond (Chen et al. 2006). We observed that treatment of HUVEC and A549 cells with platinum-based chemotherapy decreased free thiols, and the prevention of this with the use of a PDI inhibiting antibody, support our hypothesis that lung cancer chemotherapy agents induce TF decryption through the formation of Cys186-Cys209 disulfide bond. While it has been shown that inhibition of PDI can lead to changes in phosphatidylserine exposure resulting in modulation of TF procoagulant activity (Popescu et al.), the increased TF activity in our studies was largely observed in the absence of a corresponding increase of phosphatidylserine levels. Furthermore, inhibition of PDI or treatment with DTT did not alter cell surface phosphatidylserine levels as was confirmed by flow cytometry (data not shown).

It has been previously reported that constitutive glutathionylation of Cys209 in the extracellular domain of TF maintains TF in a low procoagulant (cryptic) state by preventing the formation of Cys186-Cys209 disulfide bond (Reinhardt et al. 2008). We employed glutathione to determine if glutathionylation prior to chemotherapy exposure would result in reduced TF activity. We observed that pre-treatment of HUVEC and A549 cells with glutathione significantly attenuated TF activity to similar levels achieved with PDI inhibition on both cell lines treated with combination therapies. This further supports a PDI-dependent mechanism of TF decryption which can be prevented by glutathionylation potentially through impairment of Cys186-Cys209 disulfide bond formation.

DTT has been previously shown to reduce redox active cysteines and attenuate TF activity on lysed cells (Pendurthi et al. 2007). To confirm that the resulting increase in TF activity was in fact due to the formation of a disulfide bond in the extracellular domain of TF, we employed low dose (0.01 mM) DTT to reverse the chemotherapy-induced Cys186-Cys209 disulfide formation as higher doses (2 mM) of DTT have been previously shown to induce reactive oxygen species formation and apoptosis (Tartier et al. 2000). TF activity on HUVEC and A549 cells treated with DTT post-chemotherapy (Fig. 5) resulted in a significant decrease in FXa generation at a magnitude similar to what was observed with PDI inhibition and glutathione treatment. The decreased FXa generation on cells treated with DTT was observed in parallel with increased cell surface thiol levels further suggesting a PDI-dependent mechanism of TF decryption. Although

further studies are needed to identify the direct effect of lung cancer chemotherapy agents on PDI levels and activity, our findings suggest that the increased procoagulant TF activity observed on cells treated with platinum based lung cancer chemotherapy is due to PDI-mediated formation of the Cys186-Cys209 disulfide bond.

In addition to differences in TF antigen levels on monocytes compared to HUVEC and A549 cells (Fig 3.2A and 3.2B), we also observed varying levels of PS on HUVEC depending on which platinum-based chemotherapy regimen (cisplatin or carboplatin) was utilized (Fig. 3.3A and 3.3B). Although all cells were treated with a platinum-based (cisplatin or carboplatin) regimen, we observed different procoagulant effects between these two platinum agents. While both cisplatin and carboplatin contain a platinum backbone, cisplatin is a predecessor of carboplatin with a 100-fold faster rate of intracellular aquation (activation) and significantly higher side effects clinically (Calvert et al. 1982, Perez 1998, Go et al. 1999). Furthermore, cell lines used in this study have varying cell cycle/doubling times and varying sensitivity towards platinum agents have been reported in different mammalian cell lines (Eichholtz-Wirth et al. 1986). Taken together, we hypothesize that these different variables contribute to the differences observed in this study between cell lines and platinum-based chemotherapy regimens.

Lung cancer chemotherapy agents have distinct mechanisms of action. Gemcitabine and platinum compounds (cisplatin and carboplatin) prevent proper

DNA replication whereas paclitaxel stabilizes the intracellular microtubule environment preventing cell division (Calvert et al. 1982, Horwitz 1992, Plunkett et al. 1995, Jamieson et al. 1999). Despite the different mechanisms of action, inhibition of PDI was able to attenuate chemotherapy-induced TF activity on cells treated with these agents. Platinum compounds, gemcitabine, and paclitaxel have been shown to increase reactive oxygen species (ROS) generation (Lin et al. 2000, Preston et al. 2009, Donadelli et al. 2011) and induce or enhance endoplasmic reticulum (ER) stress (Nawrocki et al. 2005, Swanton et al. 2007, Donadelli et al. 2011). We hypothesize that the increased ROS generation and ER stress induced by lung cancer chemotherapy agents may promote increased expression and/or trafficking of PDI to the cell surface where it is able to mediate TF decryption through Cys186-Cys209 disulfide bond formation thereby enhancing cell surface TF activity.

Collectively, our studies show that lung cancer chemotherapy agents significantly modulate the coagulation system by inducing procoagulant effects on healthy host tissues as well as malignant non-small cell lung cancer cells by increasing TF procoagulant activity, phosphatidylserine exposure, and PDI antigen and activity levels. This suggests that the administration of platinum-based chemotherapy to lung cancer patients can induce activation of coagulation localized to the site of the tumor as well as systemically due to activation of monocytes and endothelial cells. Our studies are the first to investigate the procoagulant effects of platinum-based chemotherapy on monocytes and non-

small cell lung carcinoma cells. Furthermore, these results help elucidate mechanisms by which chemotherapy agents can increase coagulation activation in lung cancer patients receiving chemotherapy and may prove to be useful in development of prophylactic anticoagulant strategies for lung cancer patients receiving chemotherapy.

Chapter 4: Procoagulant Effects of Lung Cancer Chemotherapy: Impact on Microparticles and Cell-Free DNA.

Foreword

These studies investigate the mechanisms by which platinum-based lung cancer chemotherapy induce generation of procoagulant MPs from endothelial cells (HUVEC), monocytes, and NSCLC A549 and A427 cells. The results demonstrate that lung cancer chemotherapy agents can induce the generation of procoagulant MPs, bearing both TF and PS, originating from NSCLC cells. This work also shows that platinum-based chemotherapy induces CFDNA release from host neutrophils. In addition, we demonstrate that platinum-based chemotherapy leads to simultaneous activation of extrinsic and intrinsic pathways of coagulation in tumour-bearing mice.

This work was submitted to the journal of Blood Coagulation and Fibrinolysis. The authors are the following: Zakhar Lysov, Dhruva J. Dwivedi, Travis Gould, and Patricia C. Liaw. The corresponding author is Dr. Liaw. Majority of experiments in the manuscript were performed by Zakhar Lysov. Travis Gould and Dhruva Dwivedi contributed/assisted with the experiments. The manuscript was written by Zakhar Lysov and Dr. Liaw. Dr. Liaw contributed to experimental design, writing, and editing the manuscript.

4.1 Abstract

Introduction: Lung cancer is the second leading type of cancer, with venous thromboembolism (VTE) being the second leading cause of death. Studies have shown increased levels of microparticles (MPs) and cell-free DNA (CFDNA) in cancer patients, which can activate coagulation through extrinsic and intrinsic pathways, respectively. However, the impact of lung cancer chemotherapy on MP and/or CFDNA generation is not completely understood.

Objective: To study the effects of platinum-based chemotherapeutic agents on generation of procoagulant MPs and CFDNA *in vitro* and *in vivo*

Methods: MPs were isolated from chemotherapy-treated monocytes, HUVEC, or cancer cells. Tissue factor (TF) and phosphatidylserine (PS) levels were characterized and thrombin/FXa generation assays were used to determine MP procoagulant activity. CFDNA levels were isolated from cell supernatants and plasma. A murine xenograft model of human lung carcinoma was used to study the procoagulant effects of TF^+ MPs and CFDNA *in vivo*.

Results: *In vitro,* platinum-based chemotherapy induced TF^+/PS^+ MP shedding from A549 and A427 lung cancers cells which enhanced thrombin generation in plasma in a FVII-dependent manner. CFDNA levels were increased in supernatants of chemotherapy-treated neutrophils and plasma of chemotherapy-treated mice. TF^+ MPs were elevated in plasma of chemotherapy-treated tumour-bearing mice. Plasma CFDNA levels are increased in chemotherapy treated

tumour-free mice and correlate with increased thrombin generation. In tumour-bearing mice, chemotherapy increases plasma levels of CFDNA and TF^+/PS^+ MPs.

Conclusion: Platinum-based chemotherapy induces the shedding of TF^+/PS^+ MPs from tumour cells and the release of CFDNA from host neutrophils.

4.2 Introduction

VTE remains the second leading cause of death in cancer patients (Khorana et al. 2007). The rates of venous thromboembolism (VTE) vary depending on the type of malignancy as well as the medical interventions used (Levitan et al. 1999, Blom et al. 2005). Lung cancer is the second leading type of cancer with approximately 80% of all newly diagnosed cases being non-small cell lung carcinomas (NSCLC) (Jemal et al. 2010). While VTE is reported in 1.4% of the healthy population, lung cancer patients experience the third highest incidence of VTE (13.9%) amongst cancer patients (Khorana et al. 2013) with the highest rates VTE reported in cancer patients receiving chemotherapy (Khorana et al. 2007). Of these thrombotic events, 18-36% occurs within the first month of initiating chemotherapy (Numico et al. 2005, Khorana et al. 2013).

Although the association between cancer, chemotherapy, and thrombosis is well recognized, the molecular mechanisms by which chemotherapeutic agents contribute to thrombosis are multifactorial and not fully understood. The procoagulant activity of cancer cells (pancreatic, breast, and colorectal) has been shown to be linked with the cell surface expression of tissue factor (TF) (Welsh et al. 2012, Date et al. 2013). We have recently demonstrated that chemotherapy agents can induce procoagulant TF activity on the cell surface of lung cancer cells via TF decryption (Lysov et al. 2015). However, other potential mechanisms by which cancer and/or chemotherapy induce a hypercoagulable state include platelet activation (van Es et al. 2014), increasing levels of circulating TF- and/or

phosphatidylserine (PS) bearing microparticles (MPs) (Zwicker 2008, Aharon et al. 2009, Thaler et al. 2014), and increasing levels of procoagulant cell-free DNA (CFDNA) (Swystun et al. 2011).

MPs are cell-derived membrane vesicles (0.1-1μm), which are released during cell activation or apoptosis (Burnier et al. 2009). MPs originate from a number of cells including platelets, leukocytes, and endothelial cells (Tramontano et al. , Mesri et al. 1999, Dignat-George et al. 2004, Janowska-Wieczorek et al. 2005, Flaumenhaft 2006). Although present in the blood of healthy individuals at very low concentrations, elevated levels of circulating MPs have been reported in the presence of disease states such as diabetes, sepsis, and cancer (Kim et al. 2003, Morel et al. 2006, Burnier et al. 2009). Studies have demonstrated that tumor cell derived MPs possess procoagulant activity that is TF-dependent (Yu et al. 2004) with clinical studies demonstrating an association between plasma levels of tissue factor positive (TF^+) MPs, cancer, and VTE (Zwicker et al. 2009).

Studies have shown that lung cancer cells constitutively shed TF^+ MPs and infusion of lung-cancer cell-derived MPs into mice accelerated thrombus formation (Thomas et al. 2009). However, limited findings with conflicting results have been reported on MPs in lung cancer patients. Some studies report no differences in plasma levels of TF^+ MPs between lung cancer patients and healthy controls (Zwicker et al. 2009) whereas others demonstrate increased levels of MPs (Fleitas et al. 2012) and increased TF MP activity in lung cancer

patients compared to healthy individuals (Manly et al. 2010). In addition, while some studies report increased levels of MPs in NSCLC patients (Kanazawa et al. 2003), TF antigen levels and the procoagulant effects of these MPs were not determined (Kanazawa et al. 2003). Lastly, Tilley et al demonstrate increased TF activity in plasma of lung cancer patients (Tilley et al. 2008). However, the plasma samples contained a platelet/MP mix and thus the authors were not able to discern the contribution of MPs alone to the observed TF activity (Tilley et al. 2008). Thus, the procoagulant contribution of MPs and the effect of platinum-based lung cancer chemotherapy on MP generation and coagulation remains poorly understood.

Another potential mediator of coagulation in cancer patients is cell-free DNA (CFDNA). Most studies have investigated circulating DNA in the context of cancer screening, tumour burden, and metastasis (Schwarzenbach et al. 2011). Quantitative and tumour-specific alterations in CFDNA have been used as a “liquid biopsy” to improve diagnosis and to monitor the response to anticancer therapies (Schwarzenbach et al. 2011). In lung cancer patients, plasma levels of DNA are elevated compared to healthy controls and are further elevated post chemotherapy (Gautschi et al. 2004, Lee et al. 2011). Recently, it has been demonstrated that chemotherapy can increase CFDNA in cancer patients with procoagulant consequences (Fuchs et al. 2010, Swystun et al. 2011, Demers et al. 2012). CFDNA has been shown to activate the intrinsic pathway of

coagulation as well as impair fibrinolysis (Fuchs et al. 2010, Swystun et al. 2011, Longstaff et al. 2013).

In this study, we examined the effects of platinum-based (cisplatin/gemcitabine or carboplatin/paclitaxel) chemotherapy regimens on MP generation and CFDNA release utilizing *in vitro* and *in vivo* approaches. We hypothesize that platinum-based chemotherapy regimens enhance the generation of tumour-derived TF^+ MPs and host-derived CFDNA, which result in activation of the extrinsic and intrinsic coagulation pathways, respectively.

4.3 Materials and Methods

4.3.1 Materials

Human umbilical vein endothelial cells (HUVECs) and endothelial growth medium (EGM-2) bullet kits were purchased from Lonza (Basel, Switzerland). A549 and A427 non-small cell lung cancer cells were from ATCC (Manassas, VA, USA). RPMI 1640 growth medium, trypsin-EDTA, and penicillin-streptomycin were from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum and paclitaxel were from Sigma-Aldrich (St Louis, MO, USA). Cisplatin was from MaynePharma (Quebec, Canada) and carboplatin was from Teva Canada (Ontario, Canada). Gemcitabine (Eli Lilly, Ontario, Canada) was diluted in 0.9% sodium chloride and aliquots stored at -80 ^{o}C. Anti-human TF monoclonal antibody (FITC) was from Affinity Biologicals (Ancaster, ON, Canada). Annexin V, annexin V-FITC, annexin V binding buffer, and TF inhibitory antibody (HTF-1) were from BD Biosciences (San Jose, CA, USA). Magnetic-activated cell sorting (MACS) columns and CD-14 microbeads were from Miltenyi Biotec (Auburn, CA, USA). Technothrombin (1 mM Z-Gly-Gly-Arg-AMC and 15 mM $CaCl_2$) was purchased from Bachem (Switzerland).

4.3.2 Isolation of human peripheral blood monocytes

Monocytes were isolated from healthy individuals using MACS with CD-14 magnetic beads as previously described (Stephenson et al. 2006). Isolated monocytes were resuspended in RPMI-1640 supplemented with 10% heat

inactivated FBS and 100 U/ml penicillin-streptomycin at a density of 1 x 10^6 cells/mL and treated with chemotherapy agents diluted in RPMI-1640 to specified concentrations.

4.3.3 Isolation of human neutrophils

Neutrophils were collected by venipuncture from drug- and caffeine-free volunteers into 10 U/mL heparin. Whole blood was layered onto Lympholyte Poly cell separation media (Cedarlane, Burlington, ON) and centrifuged at 500 x g for 40 minutes. The neutrophil layer was isolated and residual red blood cells (RBC) lysed using RBC lysis buffer (Roche Applied Science, Basel, Switzerland). Neutrophils were then re-suspended in HBSS containing $CaCl_2$, $MgCl_2$ (Gibco Invitrogen, Carlsbad, CA), and 2% human serum albumin (Canadian Blood Services, Ottawa, ON). Viabiltiy of >80% was assessed by flow cytometry using propidium iodide uptake.

4.3.4 Tissue culture and microparticle isolation

HUVECs were cultured in 2% gelatin-coated plates on passages 1–4 in EGM-2 growth medium at 37^{o}C and 5% CO_2. A549 cells were cultured in RPMI-1640 supplemented with 10% heat inactivated FBS and 100 U/ml penicillin-streptomycin. Upon reaching 80-90 % confluency, the cells were treated with chemotherapy agents for 24 hours diluted in cell culture media. Chemotherapy agent concentrations were based on maximal plasma concentrations observed in cancer patients as previously reported (Lysov et al. 2015).

Cell supernatant was collected and microparticles were isolated. MPs were prepared by centrifugation at 1500 x g for 10 minutes to remove large cell debris; the supernatant was centrifuged again at 20 000 x g for 30 minutes. The supernatant was then discarded and pelleted MPs were washed in PBS prior to performing experiments. All experiments were performed on freshly isolated microparticles.

4.3.5 Microparticle Tissue Factor and Phosphatidylserine Levels

Microparticles isolated from supernatant of chemotherapy treated cells were incubated with human IgG (10 µg/mL) for 30 minutes prior to addition of anti-human TF antibody (FITC) (2 µg/ml) or Annexin V-FITC (25 µg/mL) for 30 minutes in the dark. The microparticles were then centrifuged at 20 000 x g, resuspended in fresh PBS, and analyzed using flow cytometry. Forward scatter gating parameters were established by using 1 µm beads. MPs were considered positive for TF (TF^+) or phosphatidylserine (PS^+) as any fluorescent event less than 1 µm in size.

4.3.6 Microparticle Thrombin Generation

Microparticle based thrombin generation assays were performed using pooled, citrated, platelet-poor plasma using a calibrated thrombin generation assay (Technothrombin TGA). Microparticles were isolated from chemotherapy treated cells as described above. Isolated microparticles were then labeled with annexin V-FITC and counted using AccuCheck Counting Beads. Each thrombin

generation was based on $1x10^4$ microparticles from each treatment condition. Samples were incubated at 37^oC for 5 minutes and fluorogenic substrate (Technothrombin) containing 15 mM $CaCl_2$ was added. Fluorescent measurements were taken at 1 minute intervals for 60-90 minutes.

4.3.7 Tissue culture and xenograft cell injection.

All animal studies were approved by the McMaster University Research Ethics Board (AUP 12-03-07). Eight to 10 week old severe combined immunodeficient (Fox Chase SCID) mice were used to establish a NSCLC xenograft. The injection site (hind flank) was prepared by shaving and sterilization with alcohol prior to injection. A549 cells were cultured as previously described. Once confluent, cells were harvested using trypsin-EDTA. The cells were washed twice with sterile PBS and resuspended in sterile 0.9% saline. Approximately 5×10^6 cells were injected using 28G insulin syringes in 200 µL volumes. All mouse preparation and cell injection was performed under isoflurane anesthesia. Tumours were allowed to grow until they reach an approximate size of 10mm x 10mm at which point mice received intraperitoneal injections of lung cancer chemotherapy agents in combination (Cis/Gem or Car/Tax) or saline. Drug concentrations used for injections of cisplatin, paclitaxel, carboplatin, and gemcitabine were 1, 6, 60, and 80 mg/kg, respectively. Mice were sacrificed 24 hours post chemotherapy or saline injection and blood was collected into 0.105 M sodium citrate via inferior vena cava. Plasma was then stored at -80^o C for further analysis.

4.3.8 DNA isolation

DNA isolation from murine plasma was performed using QiAmp DNA isolation kit as per manufacturer's instructions. Briefly, plasma was thawed at room temperature and spun at 10,000 x g for 10 minutes to remove cells and debris. DNA was purified using a Qiamp DNA blood mini kit (Qiagen, Mississauga Ontario) according to the provided protocol. DNA was isolated from 100 uL of plasma and eluted into 100 uL buffer AE. Purified DNA was quantified using BioPhotometer Plus spectrophotometer (Eppendorf, Mississauga ON).

4.3.9 Thrombin generation in SCID murine plasma

Blood was collected as described above and spun twice at 5000 x g to obtain platelet poor plasma (PPP) and an additional spin (20,000 x g for 30 min) to obtain MP-free plasma. SCID murine plasma was supplemented with PCPS (30 µM) prior to addition of calcium and fluorogenic substrate. DNase (20 µM) treatments were performed for 8 hours at 37^{o}C.

4.3.10 Statistical analysis:

Statistical analysis was performed using one way ANOVA with Tukey's pair wise comparisons, or t-tests on SigmaPlot software version 11.2 (Systat Software, San Jose, California, USA). All results are representative of a minimum of three replicates for each experiment.

4.4 Results

4.4.1 Effects of chemotherapy on phosphatidylserine (PS^+) and TF^+ MP generation

To determine if platinum-based lung cancer chemotherapy regimens promote generation of MPs expressing procoagulant phospholipids, PS levels were measured on MPs isolated from supernatants of chemotherapy treated HUVEC, monocytes, and human lung adenocarcinoma epithelial cell lines (A549 and A427 cells). We found that treatment of HUVEC with combination chemotherapy did not result in increased generation of PS^+ MPs, as measured by FITC-annexin V binding (Figure 4.1A). However, treatment of monocytes, A549, or A427 cells with either Cis/Gem or Car/Tax combination therapies resulted in a significant increase in PS^+ MPs (Figure 4.1C, E, and G). These results demonstrate that platinum-based chemotherapy can induce MP generation from host cells (monocytes) as well as multiple lung cancer cell lines.

We also investigated the effects of chemotherapy agents on generation of TF^+ MPs from HUVEC, monocytes, A549, and A427 cells. Treatment of HUVEC (Figure 4.1B) and monocytes (Figure 4.1D) with combination chemotherapy did not result in generation of TF^+ MPs whereas treatment of A549 and A427 cells with either combination therapy (Cis/Gem or Car/Tax) promoted increased generation of TF^+ MPs (Figure 4.1F and H). These findings demonstrate that while platinum-based lung cancer therapy regimens induce PS^+ MPs from

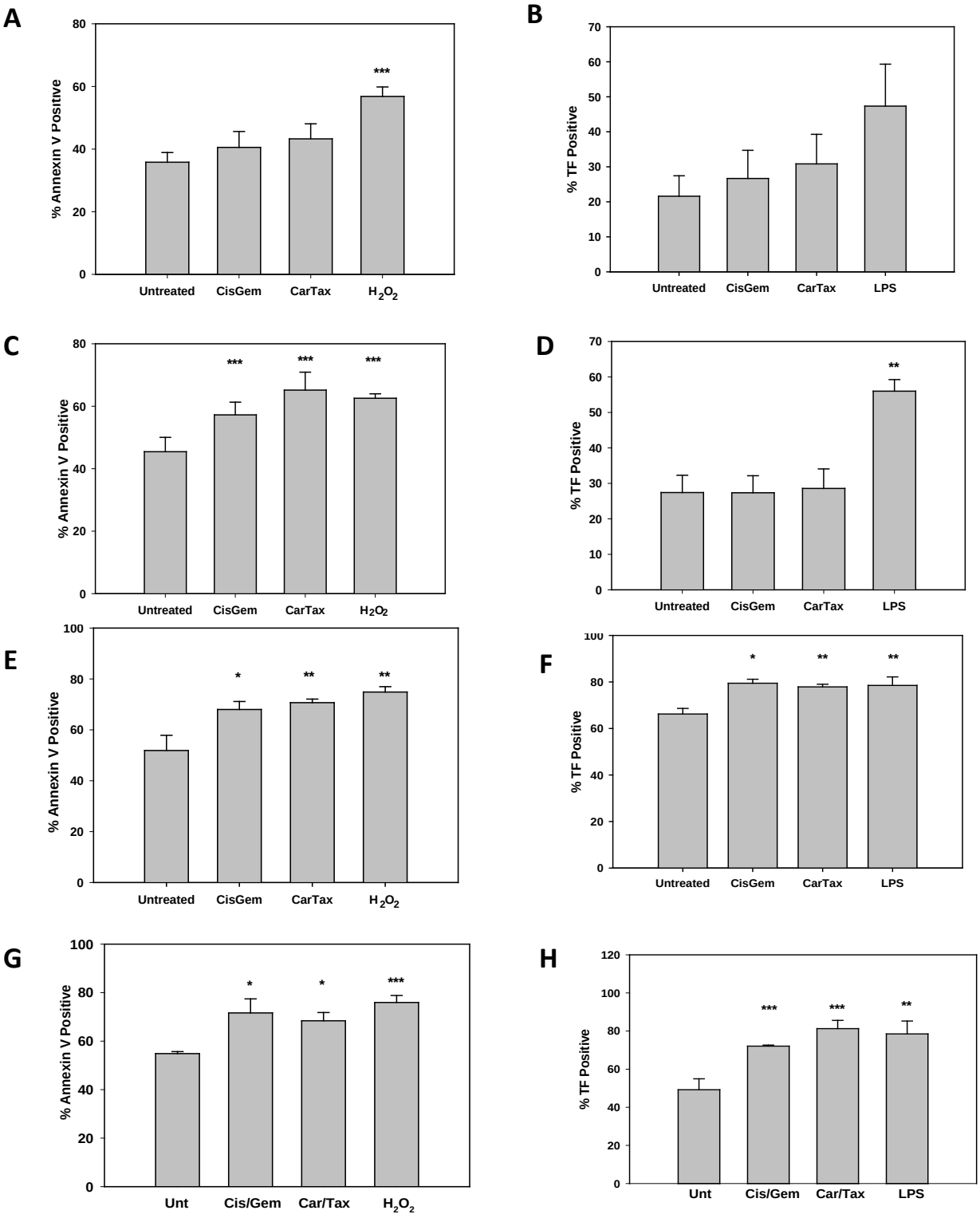
NSCLC cells and monocytes, only chemotherapy-treated NSCLC cells release TF^+/PS^+ MPs.

4.4.2 Effects of chemotherapy-induced MPs on thrombin generation

The release of TF^+/PS^+ MPs into the circulation leads to direct interaction with coagulation factors and serves as a platform for the tenase and prothrombinase complex assembly (Davizon et al. 2010). We measured the procoagulant effects of MPs generated from chemotherapy-treated cells using a calibrated automated thrombin generation assay performed in platelet poor plasma (PPP). An equal number of PS^+ MPs (1×10^4) obtained from supernatants of chemotherapy-treated HUVEC, monocytes, and A549 cells were used for each thrombin generation condition. Thrombin generation was initiated with 15 mM $CaCl_2$. Thrombin generation was not altered in the presence of MPs isolated from chemotherapy-treated monocytes or HUVEC (data not shown). Since MPs from chemotherapy–treated A549 cells and A427 cells expressed similar PS and TF levels, the following studies were performed using MPs from chemotherapy-treated A549 cells. In contrast to HUVEC and monocytes, addition of $TF^{+/}PS^+$.

Figure 4.1. Chemotherapy-induced generation of microparticles.

Annexin V and tissue factor positive MPs isolated from chemotherapy-treated HUVEC (A and B, n=4), monocytes (C and D, n=4), A549 cells (E and F, n=6), and A427 cells (G and H, n=3). Values are expressed as mean $\pm$ SE. Hydrogen peroxide (H_2O_2) and lipopolysaccharide (LPS) used as positive controls for PS exposure and TF antigen, respectively. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.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Cis/Gem
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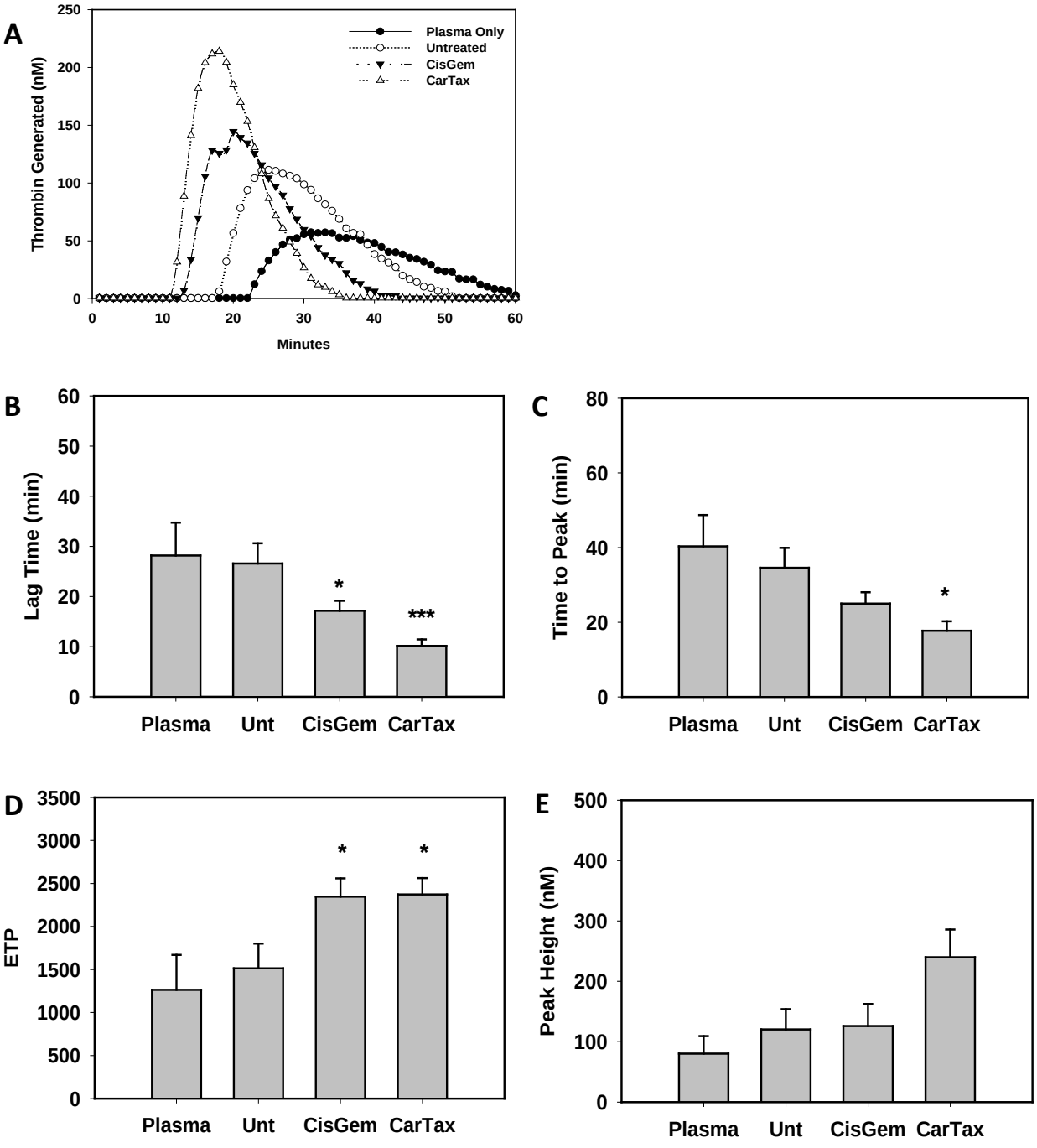
LPS

Figure 4.2. Effects of MPs from chemotherapy-treated A549 cells on thrombin generation.

Thrombin generation was performed in recalcified plasma on $1x10^4$ MPs isolated from the supernatant of chemotherapy treated A549 cells. (A) Representative thrombin generation curves for MPs from A549 cells treated with combination chemotherapy. (B) Lag time, (C) peak thrombin generated, (D) ETP, and (E) peak thrombin generated analysis. Values are expressed as mean $\pm$ SE, n=5-7 per group. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, *** $P < 0.001$.


A
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Peak Height (nM)
Plasma
Unt
CisGem
CarTax

MPs from chemotherapy-treated A549 cells to plasma significantly enhanced thrombin generation (Figure 4.2A) by reducing the lag time (Figure 4.2B) and time to peak thrombin (Figure 4.2C) and by increasing the endogenous thrombin potential (ETP) (Figure 4.2D).

To determine if the increased thrombin generation in the presence of MPs isolated from chemotherapy-treated A549 cells was TF-dependent, we repeated the thrombin generation assay in factor VII deficient (FVIId) plasma and FVIId plasma reconstituted with FVII. Our results demonstrate that thrombin generation (as measured by the AUC) was reduced in FVIId plasma compared to PPP (Table 4.1). Furthermore, when FVIId plasma is supplemented with FVII, AUC is restored to levels similar to that of PPP (Table 4.1). This suggests that MPs derived from chemotherapy-treated A549 cells enhance thrombin generation in a FVII-dependent manner.

4.4.3 Effects of cancer and chemotherapy on the generation of procoagulant MPs *in vivo*

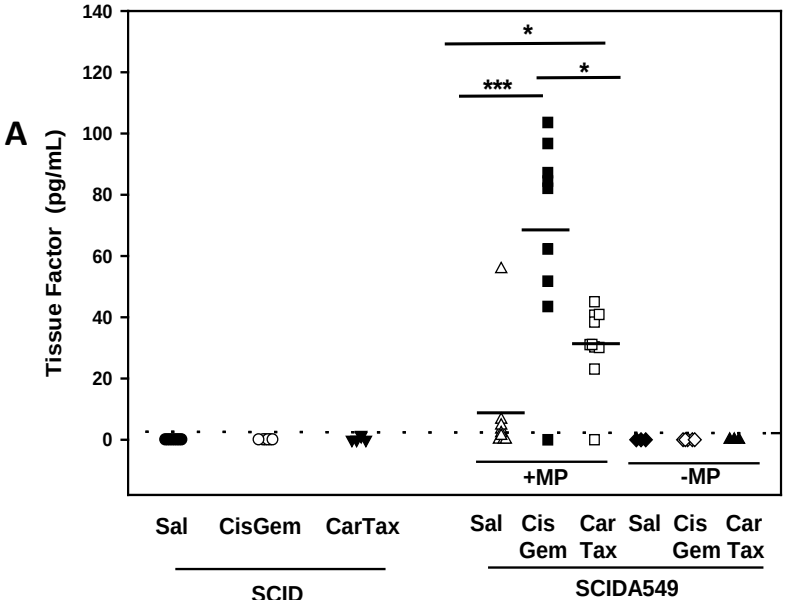
We next utilized a lung cancer xenograft model to study the effects of chemotherapy-induced MP generation *in vivo*. First, we set out to quantify plasma levels of TF^+ MPs in the plasma of tumour–bearing (SCIDA549) and tumour-free (SCID) mice. We observed very low (<2 pg/ml) levels of TF^+ MP in saline-treated tumour-bearing SCID mice (SCIDA549 Saline) and undetectable levels of MPs in tumour-free SCID mice (SCID). In response to Car/Tax (SCIDA549 CarTax) and Cis/Gem (SCIDA549 CisGem), levels of TF^+ MPs were

increased by 4- and 8-fold in tumour-bearing mice, respectively (Figure 4.3A). Furthermore, removal of MPs from the plasma of saline- or chemotherapy-treated tumour-bearing mice by centrifugation resulted in unmeasurable levels of plasma TF antigen (Figure 4.3A).

To determine if the resulting MPs are procoagulant we performed a TF activity assay which measures FXa generation in the presence of MPs isolated from tumour-free and tumour-bearing mouse plasma as previously described (Khorana et al. 2008). We observed increased FXa generation in the presence of MPs from saline-treated tumour-bearing mice compared to saline-treated tumour-free mice (Figure 4.3B). FXa generation was further enhanced in the presence of MPs of chemotherapy-treated tumour-bearing mice but not chemotherapy-treated tumour-free mice (Figure 4.3B). We confirmed that the increased FXa generation is TF-dependent as FXa generation was abolished in the presence of a TF inhibiting antibody (HTF-1, 4 ug/ml). These results suggest that chemotherapy induces the release of procoagulant MPs from tumours.

Figure 4.3. Effects of chemotherapy on generation of procoagulant MPs *in vivo*.

Plasma from tumour bearing (SCID-A549) and tumour free (SCID) mice was used to determine circulating levels of TF^+ MPs (n=5-10) (A). Procoagulant activity of MPs was determined by performing FXa generation assay (B) in the presence of MPs isolated from murine plasma (n=3-4). A TF inhibiting antibody (HTF-1) was used to inhibit TF. Values are expressed as mean ± SE. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, *** $P < 0.001$.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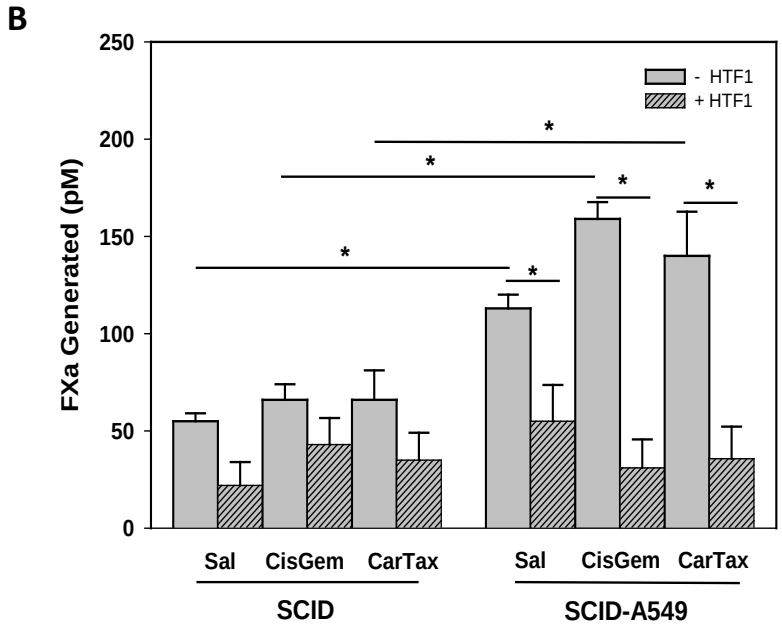
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SCID
SCID-A549

Table 4.1 Effects of A549 cell-derived MPs on thrombin generation in platelet-poor plasma or in FVII deficient platelet-poor plasma

Summary of coagulation parameters in the presence of A549 cell-derived MPs in platelet poor plasma (PPP) or factor VII deficient (FVIId) plasma. Results reflect mean ± SE. N= 3 separate experiments per group. AUC indicates area under the curve.

* P < 0.05 compared to PPP of same treatment group
† P < 0.05 compared to FVIId plasma of same treatment group
§ P < 0.05 compared to PPP containing MPs from untreated A549 cells

Treatment	ETP		
	PPP	FVIId	FVIId+FVII
Untreated	1435 ± 328	0 ± 0*	3610 ± 685†
CisGem	2454 ±219§	119 ± 74*	4003 ± 226†
CarTax	2420 ±217§	238 ± 133*	4610 ± 240†

4.4.5 Effects of cancer and chemotherapy on CFDNA levels

To explore the possibility that CFDNA also contributes to a hypercoagulable state, we measured the levels of CFDNA in the mice. As shown in Figure 4.4A, tumour-free mice treated with CisGem exhibit elevated levels of CFDNA compared to tumour-free mice treated with saline. While the presence of a tumour alone does not result in increased levels of CFDNA (SCID saline vs. SCIDA549 Saline), treatment of tumour-bearing mice with either platinum-based combination therapy significantly increased levels of CFDNA compared to saline treated tumour-bearing mice (Figure 4.4A). To determine the source of CFDNA *in vitro,* we treated HUVEC, A549 cells, monocytes, and neutrophils with chemotherapy agents. We observed that only chemotherapy-treated neutrophils released DNA into the conditioned medium (Figure 4.4B).

4.4.6 Relative procoagulant contribution of MPs and CFDNA *in vivo*

Thrombin generation assays were used to study the relative procoagulant contribution of MPs and CFDNA as a result of platinum-based chemotherapy *in vivo*. To determine if the increase in plasma levels of TF-bearing MPs correlates with an increase in procoagulant activity we performed thrombin generation assays in the presence or absence of MPs. Representative thrombograms for tumour-bearing mice treated with saline (Figure 4.5A), CisGem (Figure 4.5B), and CarTax (Figure 4.5C) are shown. ETP analysis demonstrates that MP removal reduced thrombin generation in plasma of tumour-bearing but not

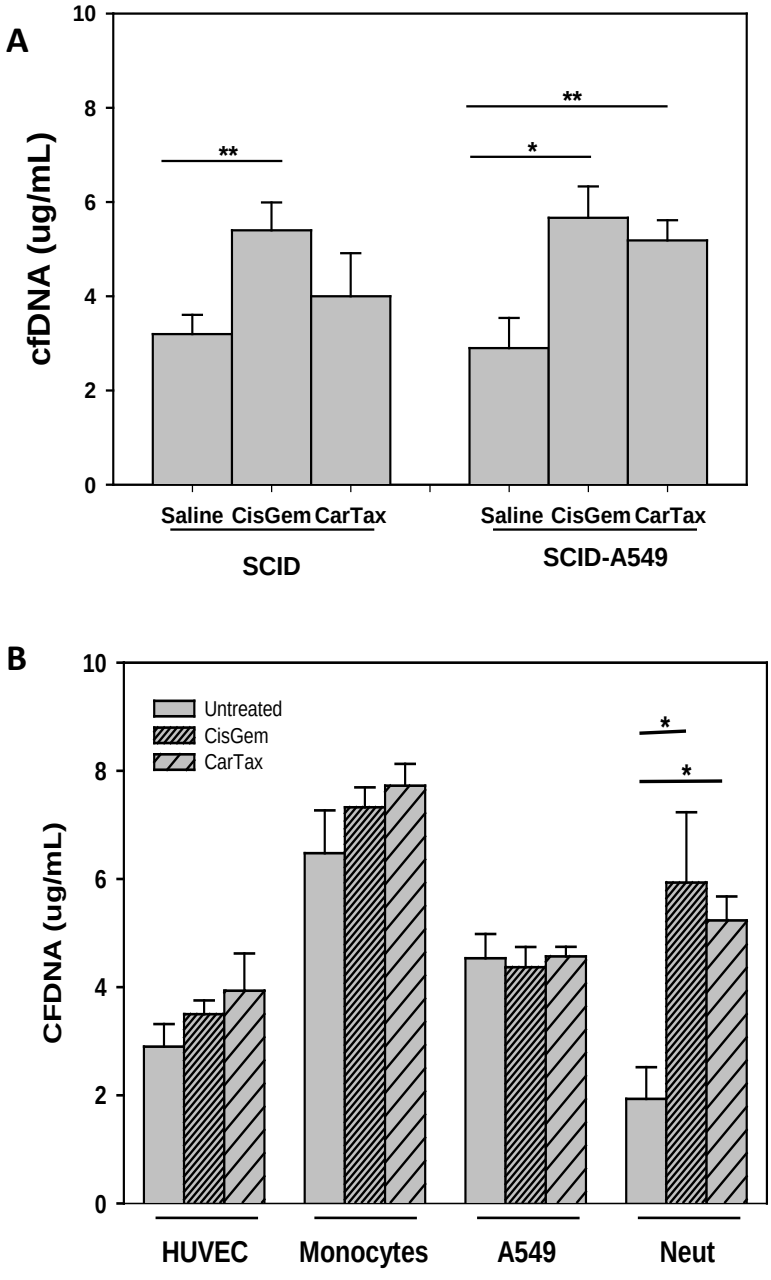
tumour-free mice. These results support our hypothesis that lung cancer chemotherapy results in increased levels of TF^+ procoagulant MPs that originate from the tumour.

To confirm that the procoagulant effects are mediated, in part, by CFDNA in our model, mice received intraperitoneal injections of DNase (100 ug) 1 hour prior to blood collection as previously described (Demers et al. 2012). In contrast to MP removal, DNase injections reduced thrombin generation in both tumour-free (Figure 4.5D) and tumour-bearing mice (Figure 4.5E). Addition of DNAse (20 uM) directly to plasma for 8 hours prior to measuring thrombin generation resulted in similar findings (data not shown). Treatment of plasma with RNase in the presence or absence of MPs did not affect thrombin generation (data not shown). When MP removal was combined with DNase treatment (-MP +DNase) in plasma from tumour-bearing mice, we observed a further reduction in ETP compared to MP removal (-MP –Dnase) or DNase treatment (+MP +DNase) alone (Figure 5E).

To confirm that in the absence of MPs the residual thrombin generation was due to activation of the intrinsic pathway by CFDNA, we removed MPs by centrifugation and repeated our thrombin generation assays in the presence or

Figure 4.4. Effects of chemotherapy on CFDNA release.

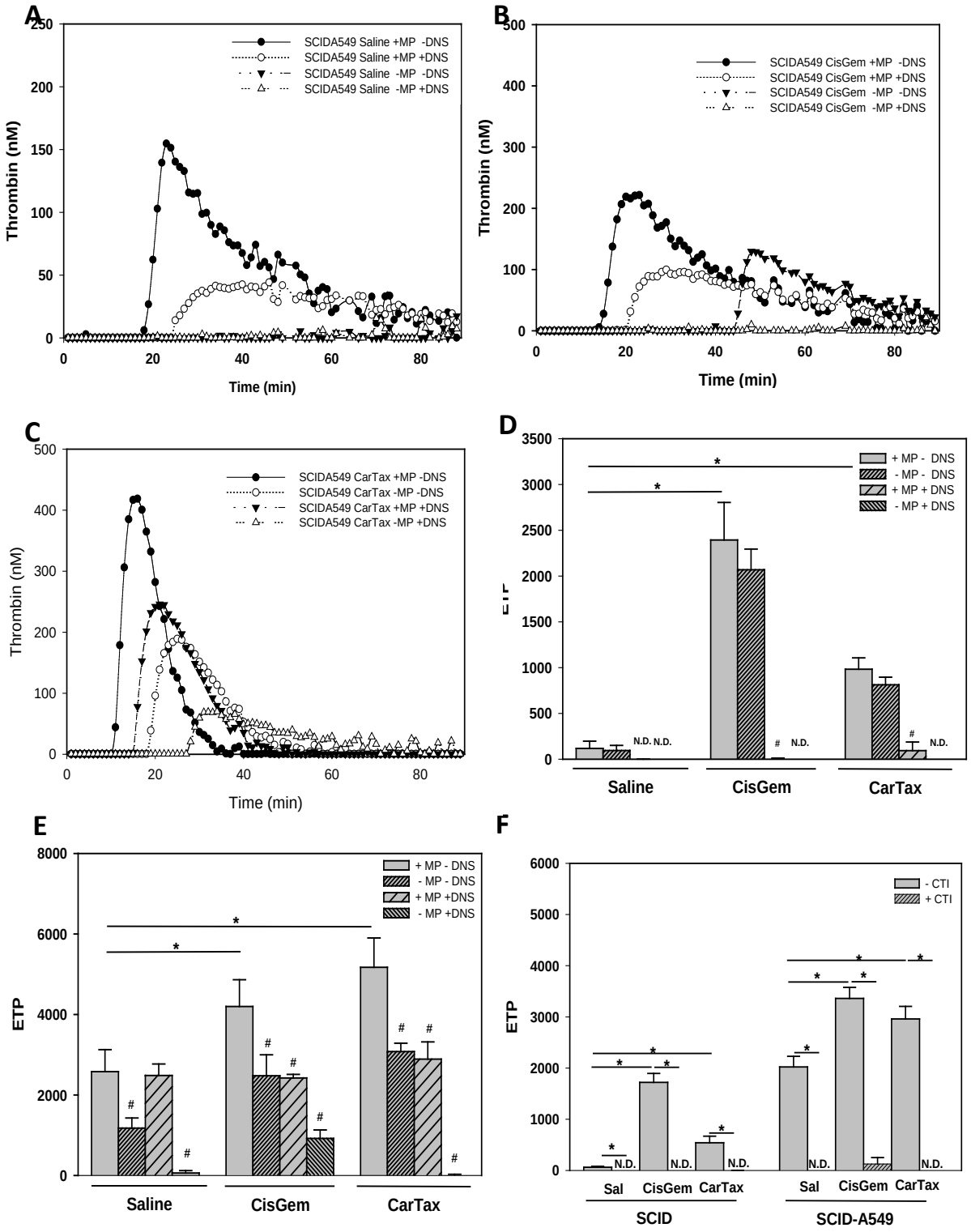
CFDNA was isolated and quantified from (A) plasma of tumour bearing (SCID-A549) and tumour free (SCID) chemotherapy treated mice (n=6-13) or (B) supernatant from chemotherapy treated cells. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$.


A
cfDNA (ug/mL)
10
8
6
4
2
0
**
**
*
Saline CisGem CarTax
Saline CisGem CarTax
SCID
SCID-A549
B
CFDNA (ug/mL)
Untreated
CisGem
CarTax
*
*
HUVEC
Monocytes
A549
Neut

absence of corn trypsin inhibitor (CTI) (Figure 4.5F). We observed that in the absence of MPs, CTI abolished thrombin generation in all treatment conditions (Figure 4.5F). These findings demonstrate that there is simultaneous activation of the extrinsic and intrinsic coagulation pathway by tumour-derived TF-bearing MPs and CFDNA, respectively, in tumour bearing mice treated with platinum-based chemotherapy.

Figure 4.5. Effects of chemotherapy on thrombin generation in tumour-free and tumour-bearing mice.

Representative thrombograms from mice treated with (A) Saline, (B) CisGem, and (C) CarTax in the presence or absence of MPs or DNase. Endogenous thrombin potential (ETP) in plasma of tumour-free (D) and tumour-bearing mice (E), or (F) ETP in plasma from tumour-free and tumour-bearing mice supplemented with corn trypsin inhibitor (CTI). Values are expressed as mean $\pm$ SE. Significant difference is denoted by: * $P < 0.05$ compared to specified condition or # $P < 0.05$ compared to (+MP-DNS) of same treatment group.


A
SCIDA549 Saline +MP -DNS
SCIDA549 Saline +MP +DNS
SCIDA549 Saline -MP -DNS
SCIDA549 Saline -MP +DNS
Thrombin (nM)
Time (min)
B
SCIDA549 CisGem +MP -DNS
SCIDA549 CisGem +MP +DNS
SCIDA549 CisGem -MP -DNS
SCIDA549 CisGem -MP +DNS
Thrombin (nM)
Time (min)
C
SCIDA549 CarTax +MP -DNS
SCIDA549 CarTax -MP -DNS
SCIDA549 CarTax +MP +DNS
SCIDA549 CarTax -MP +DNS
Thrombin (nM)
Time (min)
D
+ MP - DNS
- MP - DNS
+ MP + DNS
- MP + DNS
N.D.
Saline
CisGem
CarTax
E
+ MP - DNS
- MP - DNS
+ MP +DNS
- MP +DNS
ETP
Saline
CisGem
CarTax
F
- CTI
+ CTI
ETP
N.D.
Sal
CisGem
CarTax
SCID
SCID-A549

4.5 Discussion

In the present study, we found that platinum-based lung cancer combination chemotherapy induces the generation of PS^+/TF^+ MPs from A549 and A427 human lung-cancer cells. The resulting PS^+/TF^+ MPs enhance thrombin generation in plasma in a TF/FVII-dependent manner. We also found that platinum-based chemotherapy increased the release of DNA from neutrophils. In a lung cancer xenograft model, platinum-based chemotherapy agents induce procoagulant effects through TF^+ MP generation and CFDNA release. Removal of MPs and CFDNA from the plasma of chemotherapy-treated tumour-bearing mice attenuated thrombin generation. Together, these findings indicate that tumour-derived MPs and host-derived CFDNA contribute to the procoagulant effects of platinum-based lung cancer chemotherapy.

While platinum-based lung cancer chemotherapy increased thrombin generation in the absence of a tumour, the presence of a tumour alone is enough to augment coagulation as evidenced an approximate 20-fold increase in ETP (Figure 4.5D and 4.5E). Increased levels of TF^+ MPs, but not CFDNA, in the plasma of saline-treated tumour-bearing mice suggests that in the absence of chemotherapy tumours induce procoagulant effects *in vivo* through generation of tumour-derived TF^+ MPs. We confirm that tumours contribute to a hypercoagulable state in the absence of chemotherapy through generation of procoagulant TF^+ MPs. Furthermore, we demonstrate that the addition of platinum-based chemotherapy further elevates levels of tumour-derived

procoagulant MPs which in turn increases TF-dependent procoagulant activity. The addition of a chemotherapy regimen in the presence of a tumour further enhances thrombin generation. Chemotherapy treatment results in increased levels of TF^+ MPs and CFDNA in tumour-bearing mice (Figure 4.3A and 4.4A). The removal of MPs from plasma of chemotherapy-treated tumour-bearing mice attenuated thrombin generation demonstrating that chemotherapy induces generation of tumour-derived procoagulant MPs.

Although levels of TF^+ MPs and CFDNA were elevated by both platinum-based chemotherapy combinations, the greatest increase in TF^+ MPs and CFDNA observed in our studies was with the use of Cis/Gem combination. Clinically, the use of Cis/Gem is associated with greater incidence rates of thrombotic events (6-22%) compared to Car/Tax, where thrombotic events were either not observed/reported, or reported at a much lower frequency (1.1%) (Belani et al. 2005, Numico et al. 2005, Sandler et al. 2006, Reck et al. 2009, Scagliotti et al. 2010). Therefore, the increased incidence rate of thrombosis observed with the use of Cis/Gem may be attributed to the increased generation of procoagulant MPs and CFDNA.

CFDNA may induce procoagulant effects in lung cancer patients through activation of the intrinsic pathway of coagulation. Our *in vitro* studies demonstrate that neutrophils are the major source of CFDNA in response to chemotherapy (Figure 4.4B) and treatment of tumour-free mice with DNase significantly reduce

ETP (Figure 4.5D) suggesting that CFDNA is the primary driver of coagulation in chemotherapy-treated tumour-free mice. However, in tumour-bearing mice, MPs and CFDNA both drive thrombin generation as evidenced by an additive reduction of thrombin generated with concurrent MP removal and DNase treatment.

Our group has previously demonstrated that breast cancer chemotherapy agents induce formation of neutrophil extracellular traps (NETs), web-like structures composed of DNA, histones, and antimicrobial proteins released by neutrophils in response to microbial or inflammatory stimuli (Swystun et al. 2011, Yipp et al. 2013). NET formation requires the production of reactive oxygen species (ROS) as patients with NADPH oxidase mutations that disrupt ROS production fail to form NETs (Bianchi et al. 2011). All agents used in this study have the capacity to induce ROS generation (Lin et al. 2000, Maehara et al. 2004, Bragado et al. 2007, Cheng et al. 2008). However, cisplatin has been shown to induce ROS generation at 7.5-fold lower concentrations (20 uM) compared to carboplatin (150 uM) (Marullo et al. 2013). The reduced ability of carboplatin to induce ROS generation compared to cisplatin may explain the increased CFDNA levels observed in our tumour-free mice treated with Cis/Gem but not Car/Tax (Figure 4.4A).

Although our *in vitro* studies demonstrate that Car/Tax does not induce the release of CFDNA from A549 cells (Figure 4.4B), administration of Car/Tax to

tumour-bearing mice results in an increase in plasma levels of CFDNA. This suggests that the tumour creates an environment that promotes CFDNA release. Recently, it has been shown that tumours create an environment that allows for neutrophils to be “primed”, by tumour-secreted granulocyte colony-stimulating factor (G-CSF), to release CFDNA (Demers et al. 2012). The authors demonstrate that G-CSF primed neutrophils were more sensitive to a “second hit”, such as low grade infection, resulting in increased CFDNA levels (Demers et al. 2012). Although the tumour-bearing mice in our studies were not exposed to a low grade infection, chemotherapy may act as an alternative “second hit” leading to greater CFDNA release.

In conclusion, the findings of this study demonstrate that chemotherapy-treated lung cancer cells and host neutrophils release PS^+/TF^+ MPs and CFDNA, respectively. Both chemotherapy-induced MPs and CFDNA may contribute to the hypercoagulable state in patients undergoing platinum-based chemotherapy treatment. Characterization of lung cancer chemotherapy-induced MP generation and CFDNA release may suggest targets for prophylaxis in lung cancer patients and assist in identification of patients who have the greatest risk for chemotherapy-associated VTE.

4.6 Acknowledgments

Z. Lysov, D.J. Dwivedi, and T.J. Gould performed the experiments. Z. Lysov and P.C. Liaw wrote the paper. D.J. Dwivedi, T.J. Gould, and P.C. Liaw edited the paper. Z. Lysov and P.C. Liaw designed the research study.

Chapter 5: The impact of platinum-based chemotherapy on PAD4-dependent CFDNA release.

Foreword

These studies investigate the role of PAD4 in lung cancer chemotherapy-induced CFDNA release. The results demonstrate that lung cancer chemotherapy agents can induce CFDNA release which has been previously shown to originate from neutrophils. We demonstrate that in wild type mice, chemotherapy treatment results in elevated levels of CFDNA and increased thrombin generation. In contrast, CFDNA levels and thrombin generation remains low in chemotherapy treated PAD4 knock out mice.

This work has not been submitted for publication. The authors are the following: Zakhar Lysov, Dhruva J. Dwivedi, and Patricia C. Liaw. The corresponding author is Dr. Liaw. Most of the experiments in the manuscript were performed by Zakhar Lysov. Dhruva Dwivedi contributed/assisted with the experiments. The manuscript was written by Zakhar Lysov and Dr. Liaw. Dr. Liaw contributed to the experimental design, writing, and editing of the manuscript.

5.1 Abstract

Introduction: Cancer associated thrombosis is a well-recognized complication in cancer patients receiving chemotherapy. In cancer, neutrophils may release neutrophil extracellular traps (NETs) which are composed of DNA and histones. NETs have been shown to contribute to cancer-associated thrombosis through activation of the contact pathway. Release of NETs (NETosis), is a peptidylargenine deaminase 4 (PAD4)-dependent process that occurs upon neutrophil stimulation. While neutrophil activation is essential for host immune defense, the aberrant effect of lung cancer chemotherapy on neutrophil activation, NETosis, and coagulation is not well understood.

Materials and Methods: Eight to ten week old PAD4 $^{+/+}$ and PAD4 $^{-/-}$mice were treated with platinum-based chemotherapy. Blood was collected via inferior vena cava 24-hours post chemotherapy treatment. Neutrophil and CFDNA levels were quantified. Thrombin generations were performed in platelet-poor plasma and endogenous thrombin potential was quantified.

Results: Platinum-based chemotherapy did not affect neutrophil counts at 24-hours. However, treatment with platinum-based chemotherapy resulted in increased CFDNA levels and endogenous thrombin potential (ETP) in PAD4 $^{+/+}$ mice but not PAD4 $^{-/-}$mice. Treatment with DNase decreased ETP in PAD4 $^{+/+}$ mice whereas no effect was observed in PAD4 $^{-/-}$mice.

Conclusion: Platinum-based chemotherapy elicits procoagulant effects through CFDNA release in a PAD4-dependent manner. These findings suggest that platinum-based chemotherapy-induced CFDNA contributes to chemotherapy-associated thrombotic risk through increased thrombin generation.

5.2 Introduction

Thrombosis remains the second leading cause of death in cancer patients (Khorana et al. 2007). The association between cancer and thrombosis is well recognized and has been attributed to procoagulant properties of cancer cells and medical interventions such as surgery or chemotherapy (Rickles et al. 2001, Swystun et al. 2009). In addition, cell-free DNA (CFDNA) has also been reported to contribute to thrombotic risk in studies investigating chancer- and/or chemotherapy-associated thrombosis (Swystun et al. 2011, Demers et al. 2012). Cancer patients exhibit elevated levels of CFDNA compared to healthy controls and the source of CFDNA in cancer patients may be host-or tumour cell derived (Jahr et al. 2001, Gould et al. 2015). Furthermore, chemotherapy may modulate CFDNA levels. Breast cancer patients receiving chemotherapy exhibited elevated levels of CFDNA 24-hours post chemotherapy which also correlated with increased thrombin-antithrombin (TAT) complex levels (Swystun et al. 2011). However, the effect of chemotherapy on CFDNA levels in other cancers remains unknown.

Release of neutrophil extracellular traps (NETS), web-like structures composed of DNA, histones, and proteases has been identified as a mechanism for bacterial entrapment and killing (Brinkmann et al. 2004). The process of NET release (NETosis) is a stepwise process that first requires neutrophil activation either by bacteria or non-physiologic activators such as phorbol-12-myristate-13-acetate (PMA) (Fuchs et al. 2007). Following activation, there is degradation of

the nuclear membrane and histone citrullination leading to chromatin decondensation (Wang et al. 2009). Histone citrullination is mediated by peptidylargenine deaminase 4 (PAD4), an enzyme that converts arginine to citrulline (Li et al. 2010). However, NETs may also exert harmful effects to the host by promoting coagulation (Fuchs et al. 2010, Brill et al. 2012), and the presence of DNA and histones has been reported in thrombi (Fuchs et al. 2010).

Recently it has been demonstrated that cancer cells can secrete granulocyte colony stimulating factor (G-CSF) which in turn “primes” neutrophils for NETosis (Demers et al. 2012). The authors report increased plasma G-CSF levels in mice bearing leukemia, breast, or Lewis Lung carcinomas which correlated with an increase in neutrophil counts (Demers et al. 2012). The resulting neutrophils were more susceptible to NETosis upon exposure to a second “hit” such as low grade infection. While the previous study focused on murine cancers, human host (HUVEC) and tumour cells (NSCLC A549 cells) also secrete G-CSF when stimulated with LPS, TNF, or IL-1β (Lenhoff et al. 1996, Koyama et al. 1998). Furthermore, we have previously shown that platinum-based lung cancer chemotherapy increases CFDNA levels in both lung cancer tumour-bearing and tumour-free mice causing increased thrombin generation (Lysov et.al 2015 under revision). Therefore we wanted to determine if CFDNA levels were elevated in mice treated with platinum-based lung cancer chemotherapy due to increased NET formation. To explore the possibility that

chemotherapy induces PAD4-dependent NETosis we utilized PAD4 $^{-/-}$ mice to study the role of PAD4 in chemotherapy-associated thrombosis.

5.3 Materials and Methods

5.3.1 Materials

QIAamp DNA Blood Minikit was purchased from Qiagen Inc. (Valencia, CA). Paclitaxel was from Sigma-Aldrich (St Louis, MO, USA). Cisplatin was from MaynePharma (Quebec, Canada) and carboplatin was from Teva Canada (Ontario, Canada). Gemcitabine (Eli Lilly, Ontario, Canada) was diluted in 0.9% sodium chloride and aliquots stored at -80 ^{o}C. Technothrombin (1 mM Z-Gly-Gly-Arg-AMC and 15 mM $CaCl_2$)was purchased from Bachem (Switzerland).

5.3.2 Chemotherapy treatment

Animal studies were approved by the McMaster University Research Ethics Board (AUP 12-03-07). Eight to 10 week old mice received intraperitoneal injections of saline or lung cancer chemotherapy agents in combination (Cis/Gem or Car/Tax). Concentrations of chemotherapeutic agents used for injections for gemcitabine, carboplatin, paclitaxel, and cisplatin were 80, 60, 6, and 1 mg/kg, respectively. Mice received an additional intraperioteal injection of either DNAse (100 ug) or saline 1 hour prior to blood collection as previously described (Demers et al. 2012).

5.3.3 Murine hematology profile

Mice were sacrificed 24 hours post chemotherapy injection and inferior vena cava was used for blood collection into 0.105 M sodium citrate. Hemavet 950FS

hematology cell analyzer (Drew Scientific, Waterbury CT) was used to obtain a complete blood count. Plasma was then isolated and stored at -80^{o} C for further analysis.

5.3.4 DNA isolation

DNA was extracted from murine plasma using the QIAamp DNA Blood Minikit (Qiagen Inc., Valencia, CA) as per manufacturer's instructions. Briefly, plasma was thawed at room temperature and spun at 10,000 x g for 10 minutes to remove cells and debris. DNA was isolated from 100 uL of plasma and eluted into 100 uL buffer AE. Purified DNA was quantified using BioPhotometer Plus spectrophotometer (Eppendorf, Mississauga ON).

5.3.5 Thrombin generation in murine plasma

Blood was collected as described above and spun twice at 5000 x g to obtain platelet poor plasma (PPP). Plasma was supplemented with PCPS (30 uM), incubated for 5 minutes at 37^{o}C, and thrombin generation was initiated using 15 mM$CaCl_2$ and thrombin fluorogenic substrate.

5.3.6 Statistical analysis:

Statistical analysis was performed using one way ANOVA with Tukey's pair wise comparisons, or t-tests on SigmaPlot software version 11.2 (Systat Software,

San Jose, California, USA). All results represent a minimum of 3 mice per treatment condition.

5.4 Results:

5.4.1 Effects of chemotherapy on murine neutrophil counts.

Chemotherapy-induced neutropenia is a common complication of chemotherapy use in cancer patients (Crawford et al. 2004). To determine if the platinum-based chemotherapy used in our studies reduced neutrophil counts, we measured the absolute number of neutrophils in mice 24-hours post saline- or chemotherapy treatment. We observed that neutrophil number is not affected by platinum-based chemotherapy at 24 hours-post treatment in either PAD4 $^{+/+}$ or PAD4 $^{-/-}$mice (Figure 5.1A and B, respectively).

5.4.2 Effects of chemotherapy on plasma cell-free DNA levels.

Cancer patients exhibit increased levels of CFDNA compared to healthy controls (Gould et al. 2015). To determine if platinum-based lung cancer chemotherapy contributes to CFDNA release in a PAD4-dependent manner we measured plasma CFDNA levels in PAD4$^{+/+}$ and PAD4$^{-/-}$mice treated with saline or platinum-based combination chemotherapy. We observed increased CFDNA levels in PAD4$^{+/+}$mice treated with platinum-based chemotherapy (Cis/Gem, Figure 5.2A). In contrast, CFDNA levels remained low in PAD4$^{-/-}$mice treated with platinum-based chemotherapy suggesting that chemotherapy-induced CFDNA release is PAD4-dependent.

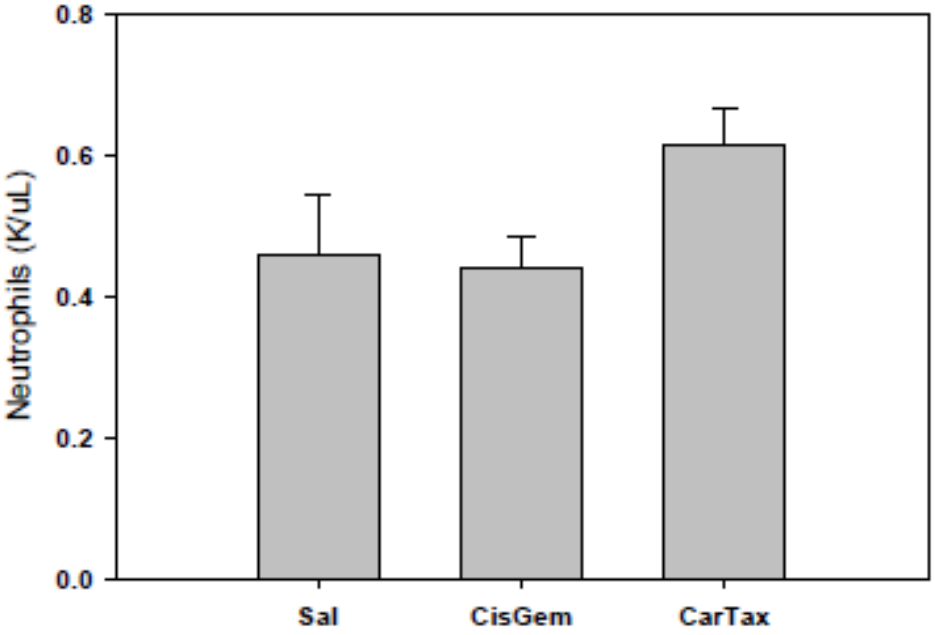
5.4.3 Procoagulant effects of platinum-based chemotherapy

Thrombin generation assays were used to determine the procoagulant effects of platinum-based chemotherapy in $PAD4^{+/+}$ and $PAD4^{-/-}$ mice (Figure 5.3). We observed increased endogenous thrombin potential (ETP) in chemotherapy-treated $PAD4^{+/+}$ mice compared to saline-treated $PAD4^{+/+}$ mice (Figure 5.3). The ETP was reduced in $PAD4^{+/+}$mice that received DNase treatment post chemotherapy. However, DNase treatment did not reduce ETP in saline- or chemotherapy-treated $PAD4^{-/-}$mice. These findings demonstrate that platinum-based chemotherapy increases thrombin generation in $PAD4^{+/+}$ mice as a result of increased CFDNA levels, whereas thrombin generation was not affected by platinum-based chemotherapy or DNase treatment in mice lacking PAD4.

Figure 5.1: Effects of chemotherapy on murine neutrophil counts.

Blood was collected from mice 24-hours post saline or chemotherapy treatment. Neutrophil counts were obtained from blood of **(A)** $PAD4^{+/+}$ mice (n=5-8) or **(B)** $PAD4^{-/-}$ mice (n=3-5). K/uL denotes 1000 neutrophils per uL of blood.

A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0.6
0.4
0.2
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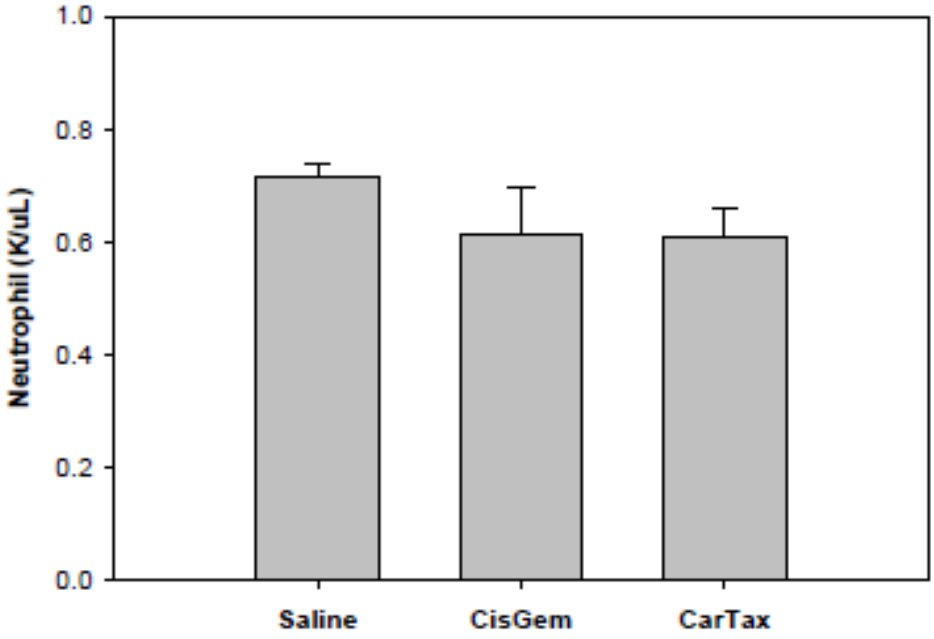
Neutrophils (K/uL)
Sal
CisGem
CarTax

B


1.0
0.8
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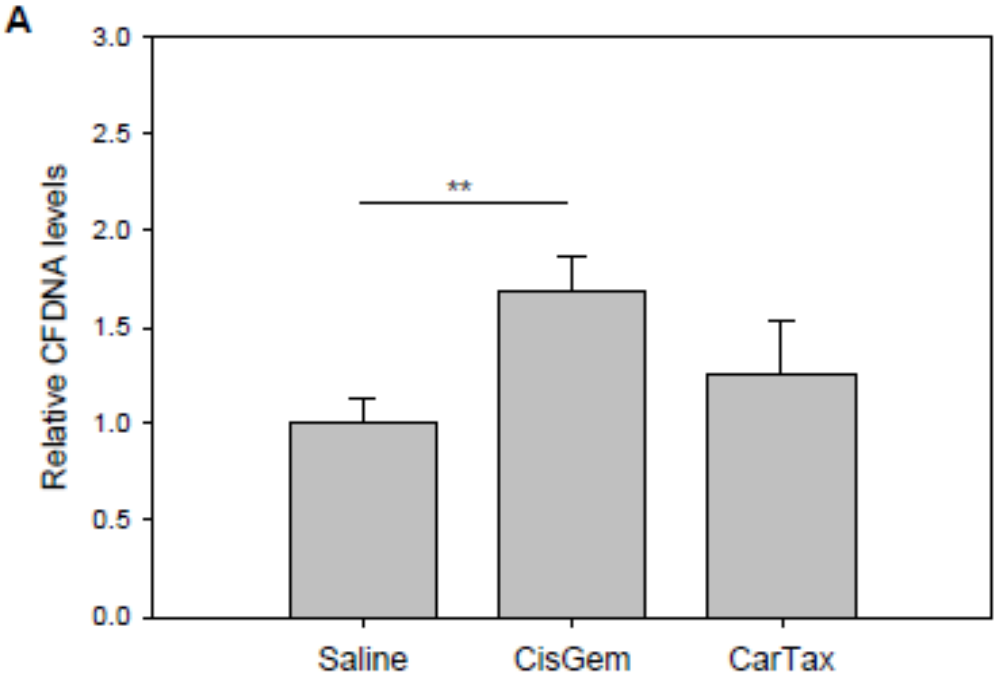
Neutrophil (K/uL)
Saline
CisGem
CarTax

Figure 5.2: Effects of chemotherapy on murine cell-free DNA levels. CFDNA was isolated and quantified from plasma of saline- or chemotherapy-treated **(A)** $PAD4^{+/+}$ mice (n=6-8) or **(B)** $PAD4^{-/-}$ mice (n=4-5). Significant difference compared to saline-treated mice is denoted by: ** $P < 0.01$. K/uL denotes 1000 neutrophils/uL of blood.


A
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Relative CFDNA levels
**
Saline
CisGem
CarTax

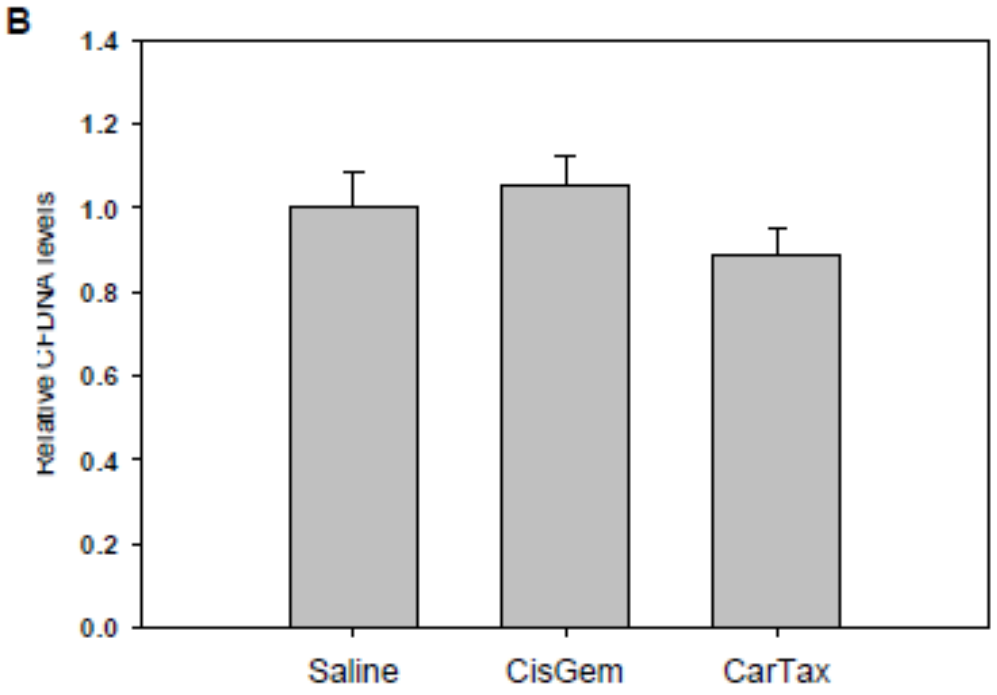
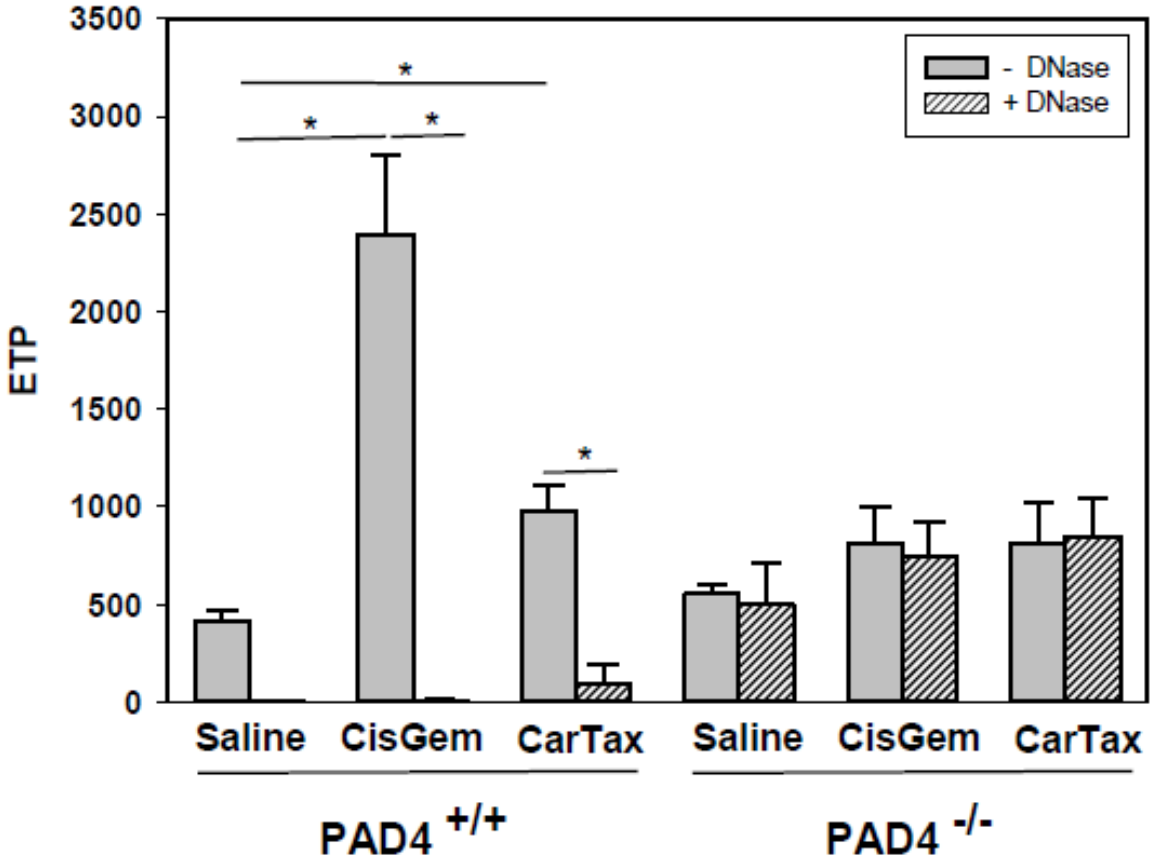

B
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Saline
CisGem
CarTax

Figure 5.3 Procoagulant effects of platinum-based chemotherapy in murine plasma.

Thrombin generations were performed in plasma obtained from saline or chemotherapy-treated $PAD4^{+/+}$ mice and $PAD4^{-/-}$ mice (n=3-5). Intraperitoneal DNase injections were administered 1-hour prior to blood collection. ETP: Endogenous thrombin potential. Significant difference is denoted by: * $P < 0.05$.


3500
3000
2500
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ETP
- DNase
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Saline
CisGem
CarTax
Saline
CisGem
CarTax
PAD4 +/+
PAD4 -/-

5.5 Discussion

In the present study we set out to determine the role of PAD4 in chemotherapy-associated coagulation and CFDNA release. We found that platinum-based lung cancer chemotherapy induced CFDNA release which resulted in increased thrombin generation in $PAD4^{+/+}$ mice. Moreover, the increase in CFDNA and thrombin generation was PAD4-dependent as chemotherapy had no effect on either CFDNA or thrombin generation levels in $PAD4^{-/-}$mice. Together these findings suggest that platinum-based chemotherapy is able to induce procoagulant effects through NETosis in healthy mice within 24 hours of treatment initiation.

Cancer patients exhibit elevated levels of CFDNA (Gould et al. 2015). Furthermore, significant increases in CFDNA levels can be observed within 24-hours of chemotherapy initiation and correlate with increased TAT complex levels (Swystun et al. 2011). However, the origin of CFDNA in cancer patients is unknown and tumour-derived CFDNA can account for 3-93% of total CFDNA (Jahr et al. 2001). Since a tumour xenograft model was not used in these studies the increased CFDNA is not tumour-derived but rather is released from host cells. Of the host cells, neutrophils have been previously identified as the source of chemotherapy-induced CFDNA release (Swystun et al. 2011). We have also shown that platinum-based chemotherapy induces CFDNA release from healthy donor neutrophils and not lung cancer cells or other host cells such as endothelial cells or monocytes (Lysov 2015).

Chemotherapy-induced CFDNA release from neutrophils has been shown to occur via NETosis and not apoptosis or necrosis (Swystun et al. 2011). NETosis is PAD4-dependent as neutrophil histones must undergo citrullination by PAD4 in order for chromatin decondensation to occur (Wang et al. 2009). We observed CFDNA levels increase in PAD4 $^{+/+}$ but not PAD4 $^{-/-}$ mice treated with chemotherapy. This suggests that similar to breast cancer chemotherapy, platinum-based chemotherapy-induced CFDNA release is also through NETosis. Furthermore, DNase treatment reduced thrombin generation in plasma of chemotherapy-treated PAD4 $^{+/+}$ but not PAD4 $^{-/-}$ mice demonstrating that platinum-based chemotherapy induces procoagulant effects through CFDNA release in a PAD4-dependent manner. Interestingly DNase treatment reduced ETP in saline-treated PAD4 $^{+/+}$ mice but not saline-treated PAD4 $^{-/-}$ mice (Figure 5.3), suggesting that baseline thrombin generation in PAD4 $^{-/-}$ mice may be due to increased circulating TF, such as TF-bearing exosomes, which may remain in the plasma sample as centrifugation speeds utilized for MP removal are not sufficient for exosome removal (Lobb et al. 2015).

Numerous stimuli can lead to formation of NETs (Fuchs et al. 2007). Priming of neutrophils by cancers has been demonstrated to result in sensitized neutrophils leading to greater NET formation upon secondary stimulation such as low grade infection (Demers et al. 2012). While we did not utilize a low grade infection model, we demonstrate that PAD4 $^{+/+}$ mice treated with platinum-based chemotherapy exhibited elevated CFDNA and ETP levels compared to saline-

treated PAD4 $^{+/+}$ mice (Figures 5.2 and 5.3). Therefore, we hypothesize that lung cancer chemotherapy can act as the secondary stimuli (in lieu of infection), leading to greater NET formation and increasing the risk for a thrombotic event in cancer patients.

In conclusion, the findings of this study suggest that platinum-based chemotherapy exerts procoagulant effects by inducing CFDNA release from neutrophils via NETosis. Since PAD4$^{-/-}$mice were protected against chemotherapy-induced procoagulant effects, these findings may lead to better prophylactic targeting of patients undergoing platinum-based cancer therapy.

Chapter 6: General Discussion

Cancer-associated thrombosis is a well-recognized complication in patients with cancer. Cancer patients experience a 4.1-fold increased risk of VTE (DVT and PE) compared to the healthy population (Heit et al. 2000). The cause of VTE in cancer patients is multi-multifactorial and is influenced by factors including the type of cancer and stage of disease progression. The risk for VTE increases ~ 6.5-fold in patients upon initiation of treatment of chemotherapy compared to healthy individuals (Heit et al. 2000, Otten et al. 2004, Blom et al. 2005). As a result, approximately 10% of all cancer patients will present with VTE during the course of the disease (Ambrus et al. 1975, Blom et al. 2005). However, the mechanism(s) behind chemotherapy-associated thrombosis are not fully understood.

The studies described in this thesis were conducted to gain a better understanding of the mechanism(s) by which chemotherapeutic agents used for the treatment of lung cancer induce procoagulant effects. We hypothesized that procoagulant pathways are upregulated by lung cancer chemotherapy agents on both tumour and healthy host cells. The four main objectives of this thesis were: i) to determine the procoagulant effects of lung cancer chemotherapy agents on endothelial cells, blood monocytes, and NSCLC cells, ii) to examine the effects of lung cancer chemotherapy cells on MP generation and assess the role of chemotherapy-induced MP on thrombin generation, iii) to investigate the effects of lung cancer chemotherapy on CFDNA release and assess the contribution of

chemotherapy-induced CFDNA on thrombin generation, and iv) to investigate the role of PAD4 in chemotherapy-induced CFDNA release.

We tested the hypothesis by treating HUVEC, monocytes, A549 cells, as well as mice with lung cancer chemotherapy agents in platinum-based combinations (Cisplatin/Gemcitabine or Carboplatin/Paclitaxel). Procoagulant TF and PS were measured on cells as well as on MPs. We determined the contribution of cell- and MP-surface TF and PS on hemostatic pathways by performing FXa and thrombin generation assays. Furthermore, the relevance of our *in vitro* work was demonstrated using chemotherapy treatment in tumour-free and tumour-bearing mice.

6.1 Effects of lung cancer chemotherapy agents on the TF pathway

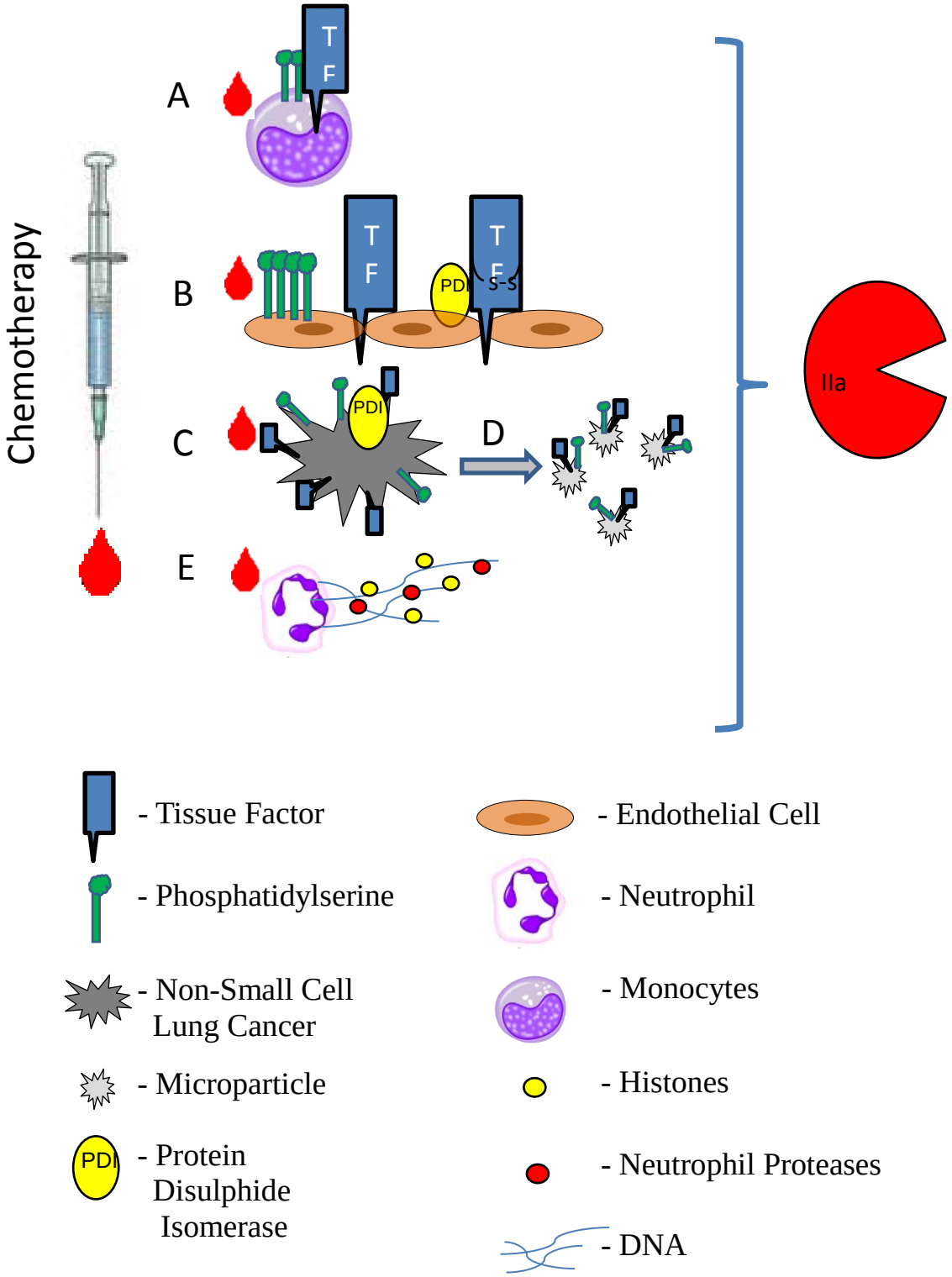
Exposure of TF to blood leads to the initiation of coagulation and may result in cancer-associated thrombosis in cancer patients. Aberrant expression of TF on cell surface of cancer cells has been reported in a variety of cancers including lung cancer cells (Sawada et al. 1999, Goldin-Lang et al. 2008). In addition, lung cancer patients have been shown to have higher levels of TF on monocytes compared to healthy controls (Lwaleed et al. 2000). Since thrombotic rates increase in cancer patients receiving chemotherapy, the focus of chapter 1 was to investigate the effects of lung cancer chemotherapeutic agents cisplatin, carboplatin, gemcitabine, and paclitaxel on TF antigen and activity on endothelial cells, monocytes, and NSCLC A549 cells.

Our studies show that treatment of endothelial cells, monocytes, and A549 cells with platinum-based combinations of lung cancer chemotherapy agents (Cisplatin/Gemcitabine and Carboplatin/Paclitaxel) results in increased cell-surface TF activity as measured by FXa generation. The increase in FXa generation was TF-dependent as inhibition of TF with an inhibiting antibody (HTF-1) reduced the amount of FXa generated to negligible levels. However, flow cytometry studies determined that chemotherapy treatment did not affect cell-surface TF antigen levels. Our results are in partial agreement with previously published studies demonstrating increased TF activity on cisplatin-treated endothelial cells. However, we did not observe an increase in TF antigen on chemotherapy-treated endothelial cells as previously reported (Ma et al. 2005). This demonstrates that lung cancer chemotherapy agents induce varying procoagulant effects on macrovascular endothelial cells used in our studies or microvascular endothelial cells used in previous reports.

Chemotherapy-induced increase in TF activity may be attributed to increased TF antigen and/or modification (decryption) of existing TF. Since cell-surface TF antigen levels were not increased on chemotherapy-treated cells in our studies, we explored mechanisms of TF decryption. Regulation of TF activity and increased decryption may be attributed to increased exposure of PS and/or PDI-mediated formation of a disulfide bond (Bach 2006). While previous studies reporting increased TF activity on endothelial cells treated with platinum-based chemotherapy did not explore mechanisms of TF decryption or PS exposure, an

increase in the number of apoptotic cells was observed (Ma et al. 2005). Exposure of PS on cell surfaces is a hallmark of apoptotic cells (Marino et al. 2013). Therefore, increased PS exposure on cells treated with platinum-based chemotherapy could lead to increased TF activity. To determine the contribution of PS to TF activity in our studies, we blocked cell-surface PS with annexin V prior to measuring FXa generation. Annexin V was able to attenuate FXa generation on chemotherapy-treated monocytes but not HUVEC.

Figure 6.1: Procoagulant effects of platinum-based chemotherapy. Platinum based chemotherapy causes **(A)** PS-dependent TF decryption on monocyte cell surface, and PDI-dependent TF decryption on the surface of **(B)** endothelial cells and **(C)** non-small cell lung carcinoma cells. In addition, platinum-based chemotherapy induces **(D)** tumour-derived generation of procoagulant MPs that express PS and TF as well as **(E)** release of cell-free DNA leading to increased thrombin generation.


Chemotherapy
A
B
C
D
E
T
F
PDI
S-S
IIa
- Tissue Factor
- Phosphatidylserine
- Non-Small Cell Lung Cancer
- Microparticle
- Protein Disulphide Isomerase
- Endothelial Cell
- Neutrophil
- Monocytes
- Histones
- Neutrophil Proteases
- DNA

This suggests that the increased FXa generation on chemotherapy-treated monocytes was at least partially due to increased PS (Figure 6.1 A).

Since Annexin V did not attenuate TF activity on HUVEC or A549 cells, we explored the role of PDI in chemotherapy-induced TF decryption. In contrast to monocytes, inhibition of PDI on HUVEC and A549 cells significantly reduced FXa generation. A reduction in cell-surface free thiol levels on chemotherapy-treated cells also suggested the formation of disulphide bonds. Therefore, we measured free-thiol levels in the presence or absence of a PDI inhibiting antibody. In addition to reduced FXa, inhibition of PDI also restored free-thiol levels confirming that chemotherapy-induced TF activity on HUVEC and A549 cells was through PDI mediated formation of disulfide bonds (Figure 6.1 B and C).

Cisplatin can induce procoagulant activity on the cell surfaces of multiple cancer types. However, the mechanisms by which this occurs differs depending on the cell type as cisplatin has recently been shown to induce pleiotropic effects and that these effects occur in a cell-specific manner (Jacobsen et al. 2015). Studies from our lab as well as others have shown that cisplatin induces procoagulant effects on NSCLC and germ cell tumour (GCT) cells (Lysov et al. 2014, Jacobsen et al. 2015). Despite differences in drug concentrations and time points studied, both studies demonstrate increased TF procoagulant activity on NSCLC and GCT cells treated with lung cancer chemotherapy. However, the mechanisms by which cisplatin increases cell-surface TF activity are different between the two studies. Our results demonstrate that increased TF

procoagulant activity is due to PDI-dependent decryption of TF antigen rather than upregulation of TF antigen levels (Lysov et al. 2014). In contrast, Jacobsen et al reported that increased TF procoagulant activity on cisplatin-treated GCT cells is due to increased TF antigen levels rather than decryption (Jacobsen et al. 2015). The authors attribute the differences between mechanisms of increased TF activity to be the cell-specific effects of cisplatin.

While cancer and chemotherapy associated thrombosis is well recognized, the varying effects of therapy- and cell-specific TF procoagulant activity make it challenging to identify high risk patients. Our studies are the first to explore the procoagulant effects of platinum-based chemotherapy on monocyte and A549 cells. Although platinum-based chemotherapy does not promote TF antigen generation on HUVEC or NSCLC cells, we identify the mechanisms by which these agents elicit decryption of TF in the absence of de novo synthesis.

6.2 Effects of lung cancer chemotherapy agents on MP generation

Chemotherapy may also result in MP generation due to cellular activation or induction of apoptosis in proliferating cells. Cellular activation and apoptosis can lead to MP generation from numerous cells such as platelets, endothelial cells, and tumour cells (Tramontano et al. , Mesri et al. 1999, Dignat-George et al. 2004, Martinez et al. 2005, Flaumenhaft 2006, Bidot et al. 2008). MPs often resemble the membrane and cytosolic content of their parent cells (VanWijk et al. 2003). Therefore, circulating MPs originate from multiple cellular sources and

chemotherapy induced MPs may express TF and/or PS on the membrane surface which can contribute to thrombin generation and increase prothrombotic risk in cancer patients.

We measured MP generation from chemotherapy-treated endothelial cells, monocytes, and NSCLC (A549 and A427) cells and characterized MP membrane TF antigen levels and PS exposure. We observed generation of PS^+ MPs from chemotherapy-treated monocytes and PS^+/TF^+ MPs from chemotherapy-treated lung cancer cells. These results differ from a study reporting generation of PS^+ MPs from cisplatin-treated endothelial cells (Lechner et al. 2007). However, a number of differences exist between the designs of the two studies. While the concentrations of cisplatin used in both studies were the same (20 uM), Lechner et al focused on the effects of cisplatin exclusively whereas our studies aimed to determine the effects of platinum-based chemotherapy regimens in combinations as per the ASCO guidelines for treatment of lung cancer (Pisters et al. 2007). Furthermore, the treatment duration in our studies was 24 hours at which point the cell supernatant was collected and MPs isolated. In contrast, Lechner et al extended their treatment time up to 120 hours and only observed significant increases in MP generation 48 hours post chemotherapy (Lechner et al. 2007).

To determine the procoagulant effect of chemotherapy-induced MPs, we measured thrombin generation in plasma containing MPs isolated from the various cell lines. Similar to our findings, a study by Jacobsen et al demonstrates

generation of procoagulant MPs from cisplatin-treated GCT cells (Jacobsen et al. 2015). Although the authors did not determine the levels of TF antigen or PS exposure on chemotherapy-induced MPs, they demonstrate that the MPs isolated from chemotherapy-treated GCT cells contribute to thrombin generation in a TF-dependent manner (Jacobsen et al. 2015).

In contrast to Lechner et al who reported a PS-dependent increase in thrombin generation in the presence of endothelial cell-derived PS^+ MPs, we observed increased thrombin generation in the presence of MPs isolated from chemotherapy-treated lung cancer cells (PS^+/TF^+ MPs), but not monocytes (PS^+ MPs) or HUVEC (Lechner et al. 2007). It is possible that an extension of chemotherapy exposure to 48+ hours in our studies would result in similar findings. Although prolonged exposure (48 hours) of HUVEC to platinum based chemotherapy may lead to generation of procoagulant MPs, the rapid generation of lung-cancer cell-derived MPs (24 hours) suggests that MPs originating from chemotherapy-treated tumour cells will contribute to thrombin generation earlier in the course of treatment than host-cell derived MPs.

Our *in vitro* results are confirmed by *in vivo* studies in which platinum-based chemotherapy treatment induces generation of procoagulant tumour-cell derived PS^+/TF^+ MPs (Figure 6.1 D) in a lung cancer xenograft model but not tumour-free mice. Furthermore, our results also complement clinical studies that investigate MP generation in lung cancer patients. Previous studies have reported increased levels of MPs (Kanazawa et al. 2003, Fleitas et al. 2012), or

increased MP procoagulant activity (determined by increased FXa generation) in lung cancer patients (Manly et al. 2010). Our results on the relevant procoagulant contribution of chemotherapy-induced MPs to thrombin generation also build upon previous findings examining increased levels of MPs, MP TF antigen levels, or MP-associated procoagulant activity (Kanazawa et al. 2003).

In conclusion, the findings of this study are the first to investigate the effects of platinum-based chemotherapy on the generation of procoagulant MPs. Furthermore, these results are the first to demonstrate that platinum-based chemotherapy results in activation of the extrinsic pathway of coagulation by tumour cell derived PS^+/TF^+MPs and not monocyte- or endothelial cell-derived MPs.

6.3 Effects of lung cancer chemotherapy agents on CFDNA release

Cell free nucleic acids also contribute to the prothrombotic pathophysiology of diseases such as sepsis and cancer (Preissner 2007, Borissoff et al. 2013, Gould et al. 2014, Gould et al. 2015). In addition, breast cancer chemotherapeutic agents have been shown to increase levels of CFDNA *in vitro,* and in breast cancer patients (Swystun et al. 2011). Therefore, we wanted to determine if platinum-based chemotherapy induces CFDNA release *in vitro* and *in vivo*, and if CFDNA contributes to thrombin generation in our models.

We found that CFDNA levels are increased *in vivo* using tumour-free and tumour-bearing mice in response to Cis/Gem combination chemotherapy. Furthermore, we demonstrated that neutrophils are the major source of

chemotherapy-induced CFDNA release as determined by our *in vitro* studies (Figure 6.1 E). Our results are consistent with previous studies which reported that breast cancer chemotherapy induces the release of procoagulant CFDNA (Swystun et al. 2011). In addition, we show that DNase administration attenuated thrombin generation in chemotherapy-treated mice.

Interestingly, Car/Tax combination chemotherapy increased CFDNA levels in tumour-bearing but not tumour-free mice. This suggests that the tumour creates an environment that may predispose neutrophils to CFDNA release. Recent studies have reported that cancer-associated neutrophils become "primed", by tumour-derived G-CSF, for NETosis. Following a secondary "hit" or stimulus, the primed neutrophils are more likely to undergo NETosis compared to neutrophils that were not exposed to G-CSF (Demers et al. 2012). While the authors utilize low grade infection to demonstrate the effect of a secondary stimulus on primed neutrophils, the effect of chemotherapy was not investigated. We hypothesized that chemotherapy, in lieu of low grade infection, can induce NETosis from primed neutrophils leading to greater CFDNA levels and increased thrombin generation.

PAD4-dependent histone citrullination is a key step that must occur in order to achieve chromatin decondensation and NETosis (Wang et al. 2004, Wang et al. 2009). To explore the possibility that chemotherapy-induced CFDNA release occurs through PAD4-dependent NETosis we utilized PAD4 knock-out mice in our chemotherapy studies. Using PAD4 $^{-/-}$ mice we demonstrated that

CFDNA levels and thrombin generation parameters were not increased in response to platinum-based chemotherapy.

Upon formation, NETs are quite stable *in vitro* and can persist for up to 90 hours in the absence of serum (Hakkim et al. 2010). However, clearance of CFDNA occurs rapidly in plasma as the half-life of CFDNA is approximately 16 minutes in healthy individuals (Lo et al. 1999) with total NET degradation occurring within 6 hours in the presence of serum *in vitro* (Hakkim et al. 2010). Interestingly, we observed elevated levels of CFDNA in tumour-free and tumour-bearing mice treated with lung cancer chemotherapy 24-hours post treatment. This suggests that lung cancer chemotherapy induces continuous NETosis and/or there is reduced NET degradation.

Degradation of NETs has been shown to be primarily DNaseI-mediated, as anti-DNaseI antibodies inhibit NET degradation (Hakkim et al. 2010). However, use of physiologic concentrations of DNaseI is insufficient to achieve complete NET degradation. In addition to DNaseI, active phagocytic engulfment of NETs by monocyte-derived macrophages contribute to NET degradation (Farrera et al. 2013). While the effects of lung cancer chemotherapy on DNaseI activity are unknown, studies have shown that DNase activity levels are unaffected by chemotherapy in breast cancer patients (Stoetzer et al. 2013). Therefore, it may be possible that elevations in CFDNA levels, as a result of lung cancer chemotherapy, are due to increased NETosis rather than decreased NET

degradation. However, further studies are needed to elucidate these mechanism(s).

CFDNA may also contribute to a procoagulant state through impairment of fibrinolysis (Longstaff et al. 2013, Varju et al. 2015). In septic patients elevated levels of CFDNA alter clot morphology and result in formation of clots that are resistant to lysis through the formation of a non-productive ternary complex consisting of plasmin, fibrin, and DNA (Gould et al. 2015). In the presence of stimulated neutrophils and recalcified blood, clot formation results in DNA and fibrin intercalation within the clot (Fuchs et al. 2010). Simultaneous treatment of the resulting clot with DNAse and tissue plasminogen activator (tPA) was the most effective in lysing clots suggesting that NETs may provide a scaffold for clot formation (Fuchs et al. 2010). However, the effects of lung cancer chemotherapy agents on clot morphology and fibrinolysis remain unknown.

In conclusion, our results suggest that CFDNA release induced by platinum-based chemotherapy occurs through PAD4-dependent NETosis and contributes to thrombin generation. Furthermore, this supports our hypothesis that chemotherapy may act as a secondary stimulus leading to increased CFDNA release.

6.4 Future Directions

6.4.1 Investigations of MPs and CFDNA in lung cancer patient plasma.

Current literature lacks conclusive findings on MP TF antigen/activity in lung cancer patients. Published studies investigating MP levels/procoagulant activity between lung cancer patients and healthy individuals report no difference, increased TF^+ MP levels, or increased TF^+ MP procoagulant activity (Kanazawa et al. 2003, Tilley et al. 2008, Manly et al. 2010, Fleitas et al. 2012). These conflicting results make it difficult to draw conclusions on the effect of lung cancer chemotherapy on MP generation/activity in cancer patients.

Our studies demonstrated increased levels of tumour-derived TF^+ MPs and increased MP procoagulant activity *in vitro* and *in vivo* (chapter 4). We also observed increased CFDNA levels in response to platinum-based chemotherapy. However, a limitation of chapter 4 was lack of access to lung cancer patient plasma samples. To extend these experiments, it would be interesting to investigate MP and CFDNA levels in parallel to their relative procoagulant contributions in lung cancer patients.

We have previously collaborated with Dr. Andrew Arnold (Chapter 3), an oncologist at McMaster University and Juravinski Cancer Centre. With Dr. Arnold's help, we can design and conduct a study which describes the procoagulant effects of platinum-based chemotherapy in lung cancer patients. The study would consist of patients presenting with stage III-IV lung cancer as this accounts for approximately 70% of all newly diagnosed cases (Howington et

al. 2013). However, including early stage lung cancer patients would be beneficial to determine if levels of TF^+ MPs correlate with disease progression. Plasma samples would be collected pre- and post-chemotherapy to determine the effect of lung cancer chemotherapy on MP generation and CFDNA release in lung cancer patients. MP, CFDNA, TAT levels, and thrombin generation will be determined at 1, 24, and 48 hours post-chemotherapy as clinical studies have shown an elevation of TAT levels in lung cancer patients within 1 hour post therapy and the increased TAT levels persisted for up to 48 hours (Weitz et al. 2002). A rapid and sustained increase in MP and/or CFDNA levels post chemotherapy may provide insight into rapid increases in TAT complex levels observed in lung cancer patients receiving chemotherapy.

The findings from these future studies would i) provide confirmatory results to our *in vitro* and *in vivo* work and ii) may further strengthen identification of patients for prophylactic treatment.

6.4.2 Confirm the role of platinum-based chemotherapy on NETosis

Previous reports have shown that breast cancer chemotherapy results in CFDNA release from cells, mice, and from cancer patients (Swystun et al. 2011). Increased CFDNA can increase procoagulant risk as evidenced by accelerated plasma clotting times and impairment of fibrinolysis (Swystun et al. , Kannemeier et al. 2007, Fuchs et al. 2010, Gould et al. 2015). In the presence of cancer, Demers et al. (Demers et al. 2012) report that neutrophils can be “primed” for

NETosis by tumour-derived G-CSF, which predisposes neutrophils to be more sensitive to NET formation upon a secondary stimulation such as a low grade infection. Demers et. al also demonstrate that tumour progression (breast cancer) is associated with increased levels of plasma G-CSF, neutrophils, and CFDNA (Demers et al. 2012).

Utilizing our murine xenograft model, we determined that there was a significant increase in CFDNA in tumour-bearing but not tumour-free mice treated with CarTax (Figure 4.4) suggesting that in the presence of a tumour chemotherapy may be acting as the secondary stimulus that induces NETosis. In addition to breast cancer cells, endothelial cells (HUVEC) and NSCLC A549 cells also produce G-CSF when stimulated with LPS, TNF, or IL-1β (Lenhoff et al. 1996, Koyama et al. 1998). We propose to determine if chemotherapy treatment of endothelial cells (HUVEC) and tumour (A549) cells results in increased levels of G-CSF *in vitro*. In addition, future studies utilizing a lung cancer xenograft model can be used to study the following in saline- or chemotherapy treated tumour-free and tumour-bearing mice: i) determine if plasma levels of G-CSF correlate with lung cancer tumour progression, ii) investigate if peripheral blood neutrophils isolated from tumour-bearing mice are more prone to generating NETs compared to neutrophils from tumour-free mice, and iii) confirm that chemotherapy-induced increase in CFDNA levels is due to NETosis by visualizing the presence of DNA as well as additional NET components (ie. citrullinated histones) on neutrophils isolated from tumour-free and tumour-

bearing mice. Furthermore, performing xenograft studies concurrently with PAD4$^{-/-}$ mice would allow us to confirm the role of PAD4 in chemotherapy-induced NETosis. The findings from these studies may determine the direct vs. indirect role of chemotherapy induced NET formation.

6.4.3 Determine the effects of platinum-based chemotherapy on anticoagulant pathways.

Chemotherapy may induce procoagulant effects through upregulation of procoagulant pathways and/or downregulation of anticoagulant pathways. Studies have shown that breast cancer chemotherapy can induce a procoagulant effects through increased TF activity, reduced cell-surface EPCR antigen levels, and impaired APC generation (Swystun et al. 2009, Swystun et al. 2011). Our findings in Chapter 3 focused on upregulation of procoagulant pathways and demonstrate that platinum-based lung cancer chemotherapy results in increased cell-surface TF activity. Although lung cancer chemotherapy agents have been shown to impair anticoagulant pathways through decreased TFPI activity (Ma et al. 2005), the effect of lung cancer chemotherapy agents on EPCR levels or PC activation are unknown.

While the agents used for treatment of breast cancer and lung cancer differ, some similarities exist between their cytotoxic effects. Breast cancer agent doxorubicin was shown to down regulate EPCR through ROS formation, as antioxidant treatment attenuated doxorubicin-induced EPCR down regulation (Woodley-Cook et al. 2006). Similarly, the chemotherapeutic agents used in our

studies also induce ROS generation and have the potential to modulate the protein C pathway (Lin et al. 2000, Maehara et al. 2004, Bragado et al. 2007, Cheng et al. 2008). Therefore, the effect of lung cancer on anticoagulant pathways is of interest.

To determine the effects of platinum-based chemotherapy on anticoagulant pathways we propose to utilize *in vitro* models to determine HUVEC cell-surface antigen levels of EPCR in the presence or absence of chemotherapy treatment. Furthermore, measurement of APC and soluble EPCR in supernatant of chemotherapy-treated cells would help determine if chemotherapy affects APC generation or induces receptor shedding, respectively, as has been previously shown for breast cancer chemotherapy agents (Woodley-Cook et al. 2006). In addition, immunohistochemical techniques can be utilized to visualize cell surface EPCR levels on tissues isolated from chemotherapy-treated mice. Furthermore, sEPCR and APC levels can be measured in plasma isolated from chemotherapy-treated mice.

These results would increase our understanding and provide novel insight into the multifactorial mechanisms by which lung cancer chemotherapy induce procoagulant effects.

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