Role of the Protein C System in Breast Cancer Cell Invasion and Endothelial Cell Angiogenesis

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ABSTRACT

Lea M. Beaulieu: Role of the Protein C System in Breast Cancer Cell Invasion and Endothelial Cell Angiogenesis
(Under the Direction of Frank C. Church)

Cell migration is involved in multiple processes including development, angiogenesis, and cancer invasion. Various receptors, signaling pathways, and extracellular proteases all work in concert to promote cell motility. Stimuli cause cells to move, including soluble chemoattractant and extracellular matrix-bound molecules. Two examples of cell migration, cancer cell invasion and endothelial cell angiogenesis, are explored further in this Dissertation.

Serine proteases have key roles in coagulation. Some proteases have non-hemostatic functions related to regulation of inflammation, apoptosis, and cell migration. Activated protein C (APC), anticoagulant serine protease, is also involved in regulating such cellular functions. However, it is unclear which receptors, signaling pathways, and/or extracellular proteases are involved in the mechanism utilized by APC to regulate cell motility. I tested a hypothesis that APC increased cell migration of breast cancer and endothelial cells through similar interdependent extracellular and intracellular processes. I studied the mechanism of how APC promoted cell motility using MDA-MB-231 breast cancer cells in chemotaxis and invasion assays and using human umbilical vein endothelial cells in angiogenesis assays.

APC increased breast cancer and endothelial cell migration. For breast cancer cells, increase in migration was concentration dependent. Endothelial cells responded to an optimal APC concentration, closer to physiological protein C levels. Only active APC increased

cancer cell migration and endothelial cell tube formation. APC-increased cell motility was also dependent on binding endothelial protein C receptor, and activating protease activated receptor-1 and epidermal growth factor receptor (EGFR). Through these receptors, APC activates phosphatidylinositol 3 kinase (PI3K) and mitogen activated protein kinase pathways to increase cell migration. Extracellularly, APC does not interact with urokinase and plasminogen activator inhibitor-1. Instead, APC-promoted invasion involves degradation of the extracellular matrix (ECM) by matrix metalloprotease-2 (MMP-2) and -9. Inhibition of any component of this mechanism completely abrogates the effects of APC. In *ex vivo* aortic ring assays, active APC increased the formation of endothelial sprouts around the aortic section periphery. Similar to cancer cells, APC increased sprout formation through activation of EGFR and PI3K, along with degradation of the ECM through MMPs. Collectively, APC increases breast cancer and endothelial cell migration through similar interdependent extracellular and intracellular pathways.

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I truly appreciate all that everyone has done over the years. I finally did it.

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IIa	Thrombin
α_2AP	α_2-antiplasmin
α_1-PI	α_1-protease inhibitor
α_1-PI$_{Pittsburgh}$	α_1-protease inhibitor Pittsburgh variant
α_1AT	α_1-antitrypsin
ADAM	A disintegrin and metalloprotease
Akt	Serine/threonin kinase; also known as protein kinase B
AnII	Annexin II
AP-1	Activator protein-1
APC	Activated protein C
AT	Antithrombin
Bax	BCL2-associated X protein
BBE	Bovine brain extract
Bcl-2 (A1)	B cell lymphomal leukemia-2 protein (A1 subunit)
bFGF	Basic fibroblast growth factor
BMAL	Aryl hydrocarbon receptor nuclear translocator-like
BSA	Bovine serum albumin
c JUN	Jun oncogene involved in transcription
CLOCK	Clock homolog transcription factor
CRY	Cryptochrome
DEGR-APC	5-Dimethylaminonaphthalene-1-sulfonyl-glutamylgylcylarginyl chloromethyl ketone

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco’s Phosphate Buffered Saline
EBM	Endothelial cell basal media
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Nitric oxide synthase 3 (endothelial cell)
EPCR	Endothelial cell protein C receptor
ERK1/2	Extracellular signal-regulated kinase 1/2 (mitogen-activated protein kinase 1)
FA	Focal adhesion
FBS	Fetal bovine serum
fV_{Leiden}	Factor V Leiden
Gα	G α subunit
Gβ/γ	G β/γ subunit
GAG	Glycosaminoglycan
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
Grb2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
Gu Helicase	Nucleolar RNA helicase Gu (2)
HB-EGF	Heparin bound-epidermal growth factor

HCII	Heparin cofactor II
HDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein
HIT	Heparin induced thrombocytopenia
HUVEC	Human umbilical vein endothelial cell
IκB	Inhibitor of kappa B
IACUC	Institutional Animal Care and Use Committee
IAP B	Inhibitor of apoptosis protein B
ICAM-1	Intercellular adhesion molecule 1
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-6, -1β, -8	Interleukin-6, -1β, -8
IRS-1	Insulin receptor substrate-1
JNK	c Jun NH_2-terminal kinase pathway
LMWH	Low molecular weight heparin
LRP	Lipoprotein receptor-related protein
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MCP-1	Monocyte chemoattractant protein-1
MEK1/2	Mitogen-activated protein kinase kinase 1/2
MEKK1/3	Mitogen-Activated Protein Kinase Kinase Kinase 1/3
MetS	Metabolic Syndrome
MKK4/7	Mitogen activated protein kinase kinase 4/7

MMP-2, -9	Matrix metalloprotease-2, -9
mRNA	Messenger ribonucleic acid
MT1-MMP	Membrane-type 1 matrix metalloproteinase; also known as MMP-14
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,3-diphenyl-2H-tetrazolium bromide
MEM	Minimum essential media
NFκB (2)	Nuclear factor κ B (2 subunit)
NFAT	Nuclear factor of activated T-cells
NMDA	N-methyl-D-aspartate
NO_2^-/NO_3^-	Nitrite/Nitrate ions
NOS	Nitric oxide synthase
p 21	Cyclin-dependent kinase inhibitor 1A
p 38	Mitogen-activated protein kinase 14
p 53	Tumor protein p53 (Li-Fraumeni syndrome)
PAI-1	Plasminogen activator inhibitor-1
$PAI\text{-}1^{-/-}$	Plasminogen activator inhibitor knockout
PAK	p21-activated kinase
PAR-1, 3, 4	Protease activated receptor-1, -3, -4
PBS	Phosphate buffered saline
PC	Protein C
$PC^{-/-}$	Protein C knockout
PCI/PAI-3	Protein C inhibitor/ plasminogen activator inhibitor-3
PCNA	Proliferating cell nuclear antigen

PDGF	Platelet-derived growth factor
PDK1	Pyruvate dehydrogenase kinase 1
PH	Plextrin homology domain
PI3K	Phosphatidylinositol 3 kinase
PIP2	Phosphatidylinositol-4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKC	Protein kinase C
PL	Phospholipids
PLCγ	Phospholipase C γ
PROWESS	Protein C worldwide evaluation of severe sepsis
Raf	v-Raf-1 murine leukemia viral oncogene
RAP	Receptor-associated protein
Ras	Neuroblastoma RAS viral (v-ras) oncogene
RSL	Reactive site loop
SAPK	Stress-activated protein kinase
Serpin	Serine protease inhibitor
SFM	Serum-free media
SH2	Src homology domain
SNAC-heparin	Sodium N-(8-[2-hydroxybenzoyl] amino) caprylate bound heparin
Sos	Son of sevenless; functions as a guanine exchange factor
STAT6	Signal transducer and activator of transcription 6
TACE	TNF-α converting enzyme

TAFI	Thrombin activatable fibrinolysis inhibitor
TAT	Thrombin-antithrombin complex
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF-β	Transforming growth factor-β
TM	Thrombomodulin
TNF-α (-induced A20)	Tumor necrosis factor-α (-induced A20)
tPA	Tissue-type plasminogen activator
UFH	Unfractionated heparin
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VN	Vitronectin
vWF	von Willebrand Factor
wt	Wildtype
ZPI	Protein Z-dependent protein inhibitor

Chapter 1

Literature Review

A. Introduction

Cell migration is a complex series of coordinated events that allow a cell to move in response to external stimuli, such as chemokines, and internal processes, such as signaling pathways and actin polymerization. Cell migration is divided into 4 types: chemotaxis, chemokinesis, haptotaxis, and invasion. Chemotaxis is the directed movement of cells in response to a soluble gradient of chemoattractants [1-3]. Chemokinesis is the random movement of cells in the absence of any chemoattractant gradient [1]. Haptotaxis is the movement of cells towards an insoluble factor [2, 3]. Invasion is the movement of cells through an extracellular matrix towards a soluble chemoattractant or insoluble factor [4, 5]. Cell migration is involved in multiple processes including development, inflammation, repair, and pathological processes, such as cancer and atherosclerosis. Many factors that contribute to cell migration during repair, including mediators of inflammation and angiogenesis, can also contribute to uncontrolled cell migration involved in cancer invasion and metastasis. One such factor is thrombin (IIa), a serine protease involved in blood coagulation. IIa promotes inflammation by activating protease activated receptor-1 (PAR-1) on endothelial cells [6]. IIa also promotes the migration of the endothelial cells to form new blood vessels, a process called angiogenesis, to repair a wound [7]. However, through PAR-1, IIa can also promote breast cancer cell invasion through Matrigel [8].

The following sections further examine two processes, cancer metastasis and angiogenesis, where cell migration plays a central role and which will be further studied in this thesis.

B. Cellular Migration and Cancer Metastasis

Cancer is the second leading cause of death in the U.S. according to the Centers for Disease Control. There will be an estimated 1,444,920 new cases of cancer in 2007; of those, 678,060 will be diagnosed in women, with an estimated 178,480 new cases affecting the breast [9]. The 5 year survival rate for localized breast cancer, in which the cancer has not spread to the lymph nodes or regions outside of the breast, is 98% [9]. If the cancer has spread regionally, the survival rate decreases to 83% [9]. If the tumor has spread and formed distant metastases, the survival rate decreases even more to 26% [9]. Breast cancer is second in cancer deaths among women, predicted to account for an estimated 40,460 deaths in 2007 [9].

Cancer metastasis involves the spread of malignant tumor cells from one site to a distant location in the body. It is a multi-step process that begins with tumor angiogenesis, the vascularization of a tumor mass. A tumor can grow to be 1-2 mm in thickness and still rely on diffusion of oxygen and nutrients from nearby blood vessels [10, 11]. Any growth beyond this diameter is limited by hypoxia-induced apoptosis. Vascularization of the tumor allows for growth and the spread of the tumor cells to distant organ sites. Angiogenesis is induced by the tumor cells releasing angiogenic factors, specifically vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [10-12]. Endothelial cells that are activated by growth factors release enzymes that degrade the surrounding

extracellular matrix (ECM), such as matrix metalloproteases (MMPs). MMPs are a family of zinc-dependent proteases, each with a specific substrate, important for extracellular matrix degradation and tissue remodeling. The combined activity of VEGF and bFGF derived from the tumor cells and MMPs derived from the endothelial cells allows for capillary sprouting from existing blood vessels. Ultimately, endothelial cells migrate towards the angiogenic growth factors, proliferate, and form tumor vessels, to allow for gas exchange and nutrient transfer to the surrounding tumor. Growth factors released by endothelial cells to promote proliferation, such as platelet-derived growth factor (PDGF) [10-12] and insulin-like growth factor [10], also promote tumor cell proliferation. Proliferating tumor cells at a primary site have the potential to migrate and invade the surrounding tissue. Tumor cells will likely invade capillaries, venules, and lymph nodes because of (a) the lack of surrounding dense connective tissue and smooth muscle cells and (b) the "relaxed" intercellular junctions [10]. For some tumors, the overall crowding caused by the growth of the tumor forces cells through the intercellular spaces of the vessels [10]. Tumor cells continue to synthesize hydrolytic enzymes (including heparanases) and other proteases that degrade the surrounding extracellular matrix, such as MMPs and plasminogen activators – urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), which facilitate the ability of the tumor cells to invade surrounding tissue [10] [11, 12]. Normal cells express numerous protease inhibitors that oppose the degradation initiated by these proteolytic enzymes. Tumor cells over express ECM proteolytic enzymes and do not respond to negative feedback pathways set up to control ECM degradation [10]. Following this formation of tumor vessels, tumor cells invade through the ECM and enter into circulation. In the circulation, emboli can potentially form when platelets aggregate with tumor cells, which result in the initiation of

the coagulation pathway and fibrin deposition around the tumors cells. The presence of fibrin promotes the release of plasminogen activators to degrade the ECM as well as the fibrin network itself [10]. The proteolytic activity allows individual tumor cells or clumps of tumor cells to be shed. These shed tumor cells can circulate throughout the body and adhere to endothelial cells in distant organs, where they can form metastic tumors. Of the millions of tumor cells that go into circulation, less than 0.1% survive to form metastases [10].

A generally excepted theory of how tumor cells form metastases involves (1) the nonspecific interactions of the tumors cells with coagulation factors or (2) tumor cells becoming stuck in the capillary beds of organs [13]. Alternatively, it is also hypothesized that the glycosylation and integrin profile of the endothelium will change in response to the tumor cells in circulation and promote the formation of metastases [13]. From clinical observations, it has been believed that specific tumor cells will only form metastatic tumors in certain tissues. Endothelial cells from capillaries in different organs have a distinct surface protein expression profile [13]. The tumor cell surface has an altered carbohydrate composition with an elevated proportion of higher molecular weight glycans. These changes in surface carbohydrates serve as a signal for deposition of tumor cells in specific organs [10, 13]. When the metastatic tumor cells become lodged in the capillary bed of another organ, the tumor cells will adhere to the endothelium, diapadese between endothelial cells and into the surrounding ECM, invade into the tissue, proliferate, and form another tumor.

C. Cellular Migration and Angiogenesis

During the process of tissue repair, many of the same events mentioned in tumor angiogenesis and invasion occur but with a higher degree of regulation. The purpose of tissue

repair is to remove the damaged tissue, regenerate this tissue or form scar tissue, and restore blood flow. This process is particularly important for recovery after a myocardial infarction or a stroke, the number one and three cause of death in the U.S., respectively, according to the Centers for Disease Control. Of the 79,400,000 Americans, who suffer from 1 or more cardiovascular diseases, 7,900,000 will be affected with a myocardial infarction while 5,600,000 will be affected by a stroke [14].

During myocardial infarction and stroke, blood flow is restricted and/or completely blocked, limiting gas exchange and nutrients from the surrounding tissue. Cell death occurs over time as a result along with reversible and irreversible damages to the heart, as is the case during a myocardial infarction, or to the brain, which occurs during a stroke. The time until repair of this affected area is crucial to the recovery of the patient. Because of the accumulation of blood in the vessels, coagulation and inflammation can be initiated. During inflammation, macrophages will clear the damaged tissue, fibroblasts and vascular endothelial cells proliferate to form granulation tissue, consisting of connective tissue and leaky blood vessels. Angiogenesis occurs in the damaged area, in which preexisting blood vessels produce capillary sprouts that develop into new vessels. Migration of endothelial cells during angiogenesis is regulated by integrins, cell surface receptors that mediate attachment, by proteins that destabilize cell-extracellular matrix interactions, such as SPARC and thrombospondin 1, and by extracellular matrix proteases. The matrix proteases that facilitate migration are controlled by inhibitors, plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitors of metalloproteases, and by negative feedback pathways, such as matrix cleavage products that can signal for the termination of proliferation and migration. There is a proliferating zone of endothelial cells behind the migrating front of cells. VEGF binds to

receptors on the endothelial cells and promotes proliferation, tube formation, and migration. Its expression is stimulated by cytokines and growth factors, such as transforming growth factor-β (TGF-β) and PDGF, and hypoxia. Cells then undergo maturation, where they stop proliferating and start forming capillary tubes. Pericytes and smooth muscle cells are recruited to the developing vessel to form a support system around the endothelial cells. Nutrients and oxygen are now able to diffuse further into the tissue and support growth and proliferation [11, 15].

My thesis will be focused on two types of cell migration, chemotaxis and invasion, which occur during cancer and angiogenesis. I hypothesize that a factor, such as APC, could increase chemotaxis and invasion of cancer cells and endothelial cells through similar pathways. The following sections go into detail of these pathways involved in cell migration.

D. Cell Signaling Pathways Involved in Cell Migration

The Mitogen-Activated Protein Kinase (MAPK) and Phosphatidylinositol 3 Kinase (PI3K) pathways are known to be key regulators of cell migration. These pathways can be activated by receptor tyrosine-kinases and their associated co-receptors, serine/threonine-kinase receptors, and G-protein coupled receptors (GPCR). For the receptors with kinase activity, activation involves dimerization and autophosphorylation of specific tyrosine, threonine, and serine residues on the cytoplasmic tail of the receptor [16]. Proteins that contain SH2 or PTB domains bind to these specific phosphorylated residues. Both phospholipase C γ (PLCγ) and Grb2 contain SH2 domains. PLCγ is phosphorylated by the receptor and is able to interact with phosphatidylinositol-3,4,5-triphosphate (PIP3) on the membrane. Cleavage of this membrane lipid associated protein results in the generation of

inositol-1,4,5-triphosphate and diacylglycerol [16, 17], which activates Ca^{2+} release and protein kinase C (PKC), respectively. PKC can activate the MAPK pathway. Another way the MAPK pathway can be activated is through Grb2. Adaptor proteins bound by the receptors induce the coupling of Grb2 with the guanine nucleotide exchange factor, Sos. Sos becomes localized to the inner leaflet of the plasma membrane by receptor-associated Grb2, allowing it to activate the GTP-ase Ras [16, 17]. Ras will go on to activate the ERK pathway.

To activate the PI3K pathway, adaptor proteins, such as IRS-1, bind to phosphorylated residues on the cytoplasmic tail of activated receptor and subsequently recruit PI3K to the membrane. PI3K will phosphorylate phosphatidylinositol-4,5-biphosphate (PIP2) in the plasma membrane to PIP3. This modified membrane lipid binds proteins that contain a plextrin homology (PH) domain. Two important PH domain containing proteins are PDK1 and its downstream target, Akt. PDK1 bound to PIP3 in the membrane can phosphorylate Akt. There are 3 isoforms of Akt – Akt1, Akt2, and Akt3. Akt activation leads to the regulation of various other pathways involved in cell survival, cell growth, and cell migration [18].

PI3K activity itself facilitates leading edge formation through its introduction of PIP3 into the cell membrane which recruits PH domain containing proteins involved in actin polymerization [19]. Akt will phosphorylate and inactivate Bad, a pro-apoptotic factors, and and the Forkhead family of transcription factors, which upregulates pro-apoptotic factors. Akt inactivation of GSK3 prevents the phophorylation of β-catenin and results in the increase in cell cycle progression. Additionally, Akt phosphorylates mTOR, which increases mRNA translation and cell cycle progression [18]. Although a minor role, Akt can also regulate the ERK pathway [16, 20]. Akt regulates cell migration through two transcriptional factors,

NFAT and AP-1. NFAT is associated with increased cellular invasion. Akt activation leads to the downregulation of this transcriptional factor through ubiquitination by HDM2, resulting in the loss of cell invasion. Akt1 down regulation results in MAPK activation and subsequent upregulation of the transcriptional factor, AP-1, of the expression of ECM degradation proteases [20]. PI3K can directly lead to the activation of the MAPK pathway through p21-activated kinase (PAK). PDK1 will phosphorylate PAK, which can activate the MAPK pathway [21]. Even though the activation of the ERK pathway by PI3K pathway is not a major function of this pathway, I believe that it is a key interaction in the mechanism utilized by APC to increase cell migration. Additionally, APC has been implicated to reduce apoptosis in endothelial and nerve cells, which could be potentially mediated through the PI3K pathway. Finally, APC has been shown to utilize the PI3K pathway to induce proliferation of endothelial cells. Therefore, APC could be activating the PI3K pathway to increase cell migration.

There are three MAPK pathways: the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, c-Jun NH_2-terminal kinase pathway, also known as the stress-activated protein kinase (JNK/SAPK) pathway, and the p38 pathway. MAPKs (i.e. ERK1/2) are phosphorylated at conserved tyrosine and threonine residues by MAPKKs (i.e. MEK1/2), which results in the nuclear translocation and phosphorylation of various transcription factors. MAPKKs are activated by the phosphorylation of 2 serine residues by MAPKKKs (i.e. Raf) [22]. ERK1/2 is mainly activated through Ras-Raf-MEK1/2 or PKC-Raf-MEK1/2 pathways. C-Jun of the JNK pathway is activated through MEKK1/3-MKK4/7-JNK1/2/3. p38α,β,γ,δ are activated by MAPKKK-MKK3/6 [22]. The p38 and JNK pathways have been typically associated with cellular stress [22]. However, both of these pathways have been

shown to affect migration as well. The JNK pathway has been shown to have a role in cell survival and cell migration [23-25], and is activated by EGFR [26] and GPCRs [27]. The JNK pathway has also been implicated in regulating cytoskeleton formation and focal adhesion (FAs) in certain cell types [28, 29]. The p38 pathway is also involved in cytoskeleton reorganization and regulation of FAs [28]. The ERK1/2 pathway has been the most extensively studied of the MAPK pathways, with regard to cell migration. Activation of this pathway leads to FA and focal adhesion kinase turnover, the formation of membrane protrusions at the leading edge of the cell, and cytoskeleton reorganization [22, 28]. ERK1/2 is also involved in promoting the expression of proteases that will degrade the ECM, such as MMPs, including MMP-9, and uPA, through activation of transcriptional factor, AP-1 [20, 22]. Because the JNK and p38 pathways are typically associated with cell stress, I believe that the ERK pathway is the important pathway in the mechanism utilized by APC to induce cell migration. In addition, this pathway has been implicated in other studies focused on the proliferative effects of APC.

For GPCRs, activation of the receptor typically involves the binding to extracellular ligands. One type of GPCR, PARs, will be cleaved at specific residues on the NH_2-terminal region with the ligand remaining tethered to the receptor. Activation leads to a conformation change in the GPCR, which allows for the removal of GDP and binding of GTP to G-protein α subunit (Gα). The Gα bound to GTP will disassociate from the receptor and the β/γ subunits (Gβ/γ). Both the GTP bound Gα and the Gβ/γ can modulate various signaling pathways [30, 31]. $G\alpha_i$ can activate Ras, which will activate the MAPK pathway. $G\alpha_q$ can also activate PLC, which will also activate the MAPK pathway. Gβ/γ subunits can activate

PI3K isozyme γ, which will phosphorylate MEK1/2, a MAPKK in the MAPK pathway [32]. PI3K isozyme γ can also activate Akt [33].

It has been experimentally shown that GPCRs can transactivate epidermal growth factor receptor (EGFR) and tumor necrosis factor-α (TNF-α)-converting enzyme (TACE). Activation of EGFR occurs when the Gα or β/γ activate Src [34, 35]. Src phosphorylates the cytoplasmic end of EGFR at Tyr 845 [36], which leads to the activation of the MAPK pathway and amplification of the PI3K pathway. TACE, a member of a disintegrin and metalloprotease (ADAM; known as ADAM17) family, is activated also by the Gα and/or β/γ. TACE will cleave transmembrane bound ligands, such as of heparin bound-EGF [37-39]. EGF will bind to EGFR and result in the activation of signaling pathways [40-42].

Both GPCR, specifically PAR-1, and EGFR are important for APC-induced cell migration. I believe that APC utilizes both receptors to amplify the signal to increase cell migration in both cancer cells and endothelial cells through the PI3K and MAPK, specifically ERK, pathways. Both receptors have been implicated in the other non-hemostatic effects of APC, including its anti-inflammatory and anti-apoptic roles. It is possible that APC could also use these receptors to activate pathways and amplify the signal in the cell to increase cell migration.

E. Hemostatic Function of the Protein C Pathway

As mentioned before, components of the coagulation pathway interact with tumor cells both in circulation and in the organs. The blood vessels that form during tumor angiogenesis and tissue repair are leaky, allowing for interaction of cell surface components with coagulation proteins. Leaky vessels result in activation of the extrinsic pathway of

coagulation. This pathway brings about IIa generation and fibrin deposition. Degradation of the fibrin clot is mediated through uPA, tPA, and plasmin, which can also activate MMPs. IIa will bind to thrombomodulin (TM) and activate PC. APC can now interact with endothelial cells and tumor cells. The goal of this project was to characterize the mechanism of the APC-mediated effects on invasion and chemotaxis of breast cancer cells and endothelial cells. The final sections describe in detail the biological roles of the Protein C Pathway.

When the blood vessel wall is injured, endothelial cells are torn away from the vessel surface and the underlying tissue factor (TF) bearing cells, such as smooth muscle cells, are exposed to the blood plasma. Factor VII circulating in plasma binds to TF and becomes activated by proteases at the site of injury. Factor VIIa-TF complex will activate factors X and IX. Factor Xa and factor Va still on the cell surface will produce enough IIa to activate platelets through PAR-1 and -4 and activate factor VIII. During platelet activation, platelets localize to the site of injury and adhere to each other and the damaged vessel wall. They release α-granules containing factor V, which is activated by IIa and any previously activated factor Va. IIa will also cleave von Willebrand factor/factor VIII bound to the platelet surface to release factor VIII and activate it. Factors IXa when bound to factor VIIIa on the platelet surface will activate factor X. Factor Xa will then complex with factor Va and the zymogen prothrombinase to produce enough IIa to form a fibrin clot. IIa will cleave fibrinogen to fibrin. Fibrin polymerizes and forms the clot [43-45]. Actions are taken to control the size of the fibrin clot to guarantee proper blood flow through the area. The protein C pathway works to control IIa formation in the area surrounding the clot. Zymogen protein C circulating in the plasma is localized to the endothelium surface by endothelial cell protein C receptor (EPCR). Protein C is brought to a nearby receptor complex of IIa bound to thrombomodulin (TM) and

is activated. In the presence of protein S, APC inactivates factors Va and VIIIa that are beyond the area of the clot, preventing any unnecessary activation of IIa [44, 46].

Because of this anti-coagulant function of APC, it was believed that it would prevent the formation of thrombi in the micro-circulation of organs during sepsis. However, it was shown in human clinical trials that APC could do much more.

F. Non-Hemostatic Function of Activated Protein C

Investigation into the non-hemostatic functions of APC has shown that it not only has an anticoagulant role but also has anti-inflammatory, anti-apoptotic, pro-migratory, and pro-angiogenic roles. In a clinical trial, the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS), patients diagnosed with sepsis with acute organ dysfunction were treated with recombinant human APC for 96 hours and monitored for 28 days. Treatment with APC reduced the death rate by 19.4%. Because of the anticoagulant role of APC, it was initially thought APC was reducing the formation of thrombi in the microcirculation of organs, which would lead to organ failure. However, it was also shown APC inhibition of IIa formation prevented IIa from activating inflammatory pathways. APC also inhibited production of inflammatory cytokines by monocytes and the binding of immune cells to selectins on endothelial cells [47, 48]. It was previously shown that young patients with purpura fulminans-associated meningococcemia treated with APC had a decreased death rate and reduced number of amputations [49]. From these studies, it is concluded that APC is not only anti-coagulant but can affect inflammation and other cellular processes.

Animal studies have further examined the anti-inflammatory effects of APC. In asthma studies, mice treated with an inhalation of APC exhibited a reduction in T helper 2 cytokines expression, IgE, and eosinophils. APC also reduced the binding of co-factors to STAT6 and NFκB in the lung, further reducing the inflammatory response [50]. Using APC as a treatment in an endotoxin-induced hypotension model resulted in a reduction in iNOS mRNA and production of NO_2^-/NO_3^-. This reduction leads to a decrease in TNF-α expression in the lung [51]. *In vivo* wound healing models have also shown a reduction in the inflammation in the affected area with APC treatment [52]. Knockout animal models along with the use of blocking antibodies have been used to further define the role of APC in modulating inflammation. Baboons were infected with a sublethal level of *E. coli* were also given antibodies that blocked the binding of APC/PC to EPCR. Blocking this receptor led to an increase in thrombus formation and hemorrhage particularly in adrenal and renal cortex as well as a reduction in neutrophil infiltration. It was concluded that APC/PC binding to EPCR was responsible for its ability to regulate coagulation and inflammation [53]. EPCR-deficient mice and mice injected with blocking antibodies to PAR-1 cleavage site were used in a murine ischemic stroke model. APC treatment reduced neutrophil infiltration into the affected area, which did not occur in the $EPCR^{-/-}$ mice and mice were treated with blocking antibodies to PAR-1 [54]. It was concluded that APC bound to EPCR was able to cleave PAR-1 at the amino terminal extracellular domain, RSFLLRN, the same site as IIa [6, 54-57]. Yet, IIa cleavage of PAR-1 has pro-inflammatory, pro-coagulant, and pro-adhesive effects [6, 55, 56]. These studies identify how important it is for APC to interact with EPCR and PAR-1 in order to affect inflammation. APC does not have any effect while in solution.

Work has also been done to identify how APC reduces inflammation. *In vitro,* eosinophils and lymphocytes migration towards chemoattractants was inhibited in the presence of APC through its binding to EPCR [58] and EGFR [58, 59]. Gene array studies done on endothelial cells showed an alteration in gene expression patterns with APC treatment [60]. With regards to inflammation, APC treatment reduced the expression of NFκB2 in endothelial cells pretreated with or without TNF-α [60]. However, in another gene array study using endothelial cells, APC was shown to induce MCP-1 through PAR-1 activation [61]. MCP-1, known to be a pro-inflammatory cytokine, has also been shown to be protective during sepsis by reducing the expression of inflammatory cytokines [62]. APC was shown to not be signaling through EPCR, as mutations in the palmitoylation sites on the cytoplasmic tail had no effect on activation of the Egr-1 promoter shown using a luciferase activity assay [61].

In vitro and *in vivo* studies have also studied the role of APC as a modulator of apoptosis. *In vitro* work looked to identify how APC affects cells undergoing apoptosis and the receptors APC binds to alter apoptosis. Using various cell lines, APC treatment reduced the number of cells undergoing hypoxia-, staurosporine-, and N-methyl-D-aspartate (NMDA)-induced apoptosis by altering the expression and activation of various pro- and anti-apoptotic proteases, including p53, caspases, and Bcl-2. Blocking antibodies to the APC/PC binding site of EPCR [54, 63], the N-terminal cleavage site of PAR-1[54, 63, 64], and the N-terminus of PAR-3 in mice [64] independently reversed the effects of APC on apoptosis. It was further shown that the active form of APC was required to reduce apoptosis [54, 63, 64]. APC treatment reduced the expression and protein levels of p53 [54, 64], the protein levels of Bax [54, 64], a pro-apoptotic factor, active caspase 3 levels [54, 63-65], and

active caspase 8 [64, 65]. APC also increased the expression [60] and protein level [54, 64] of Bcl-2, an inhibitor of caspase directed apoptosis. Gene array analysis of endothelial cells treated with APC showed an increase in the expression of anti-apoptotic associated genes, including Bcl-2 A1, IAP, and cell cycle related gene, Gu helicase [60]. When these cells were pretreated with TNF-α, APC further enhanced the expression of anti-apoptotic genes Bcl-2 A1, IAP B, TNF-α-induced A20, IκB, and the cell cycle related gene Gu helicase [60]. Finally, APC increased the expression of the cell survival associated gene, PCNA, in endothelial cells pretreated with TNF-α [60]. These studies have identified the receptors that APC is interacting with to affect inflammation along with the genes/proteins that APC is regulating. However, there has been no work done to identify the signaling pathways activated by APC to affect these genes/proteins. Identifying these pathways would help us understand how APC signaling, resulting in anti-inflammatory effects, varies from IIa signaling, resulting in pro-inflammatory effects. From all of this work, I can focus on three potential receptors, EPCR, PAR-1, and EGFR, which APC could be interacting with to increase cell migration.

In vivo work has confirmed *in vitro* findings that APC has anti-apoptotic effects. In a murine ischemic stroke model and tPA induced cerebral ischemia, APC has been shown to reduce the motor neurological score and the volume of brain injury in a dose dependent manner [54, 65, 66]. Treatment with higher doses of APC also decreased fibrin deposition [65] and neutrophil infiltration [54, 66], while increasing the post-ishemic cerebral blood flow in the murine brains affected by focal ischemic stroke [54, 65, 66]. Additionally, APC reduced the expression of ICAM-1 of endothelial cells in the area affected, thereby reducing the adhesion of neutrophils to the endothelium [66]. Apoptosis was shown to be reduced with

APC treatment through a reduction of both caspase 3 and 8 activities [65]. The anti-inflammatory, anti-apoptotic, neuroprotective effects of APC were shown to be mediated through EPCR and PAR-1 [54]. In mice injected with NMDA to induce apoptosis, APC reduced the affected area in a dose dependent manner, which was reversed by blocking antibodies to PAR-1 and PAR-3 [64]. The most important information from these *in vivo* studies that I can utilize for my thesis is the potential receptors APC could be interacting with on the cell surface. APC can improve recovery from a stroke, which included the increase in post-ischemic cerebral blood flow. This increase could occur through APC anti-coagulant role and/or through its ability to increase cell migration in angiogenesis.

APC has also been shown to have a role in promoting cell migration of various cell types. APC was shown to increase invasion of two different cancer cell lines through Matrigel by complexing with PAI-1 to allow for uPA to activate other ECM proteases [67]. uPA binds to urokinase plasminogen activator receptor (uPAR) on the cell surface and activates plasminogen to plasmin. uPA has been shown to not only activate MMP-2 but also regulate MMP-9 expression [68-70]. Plasmin activates MMP-9 [71] and MMP-2 [72]. However, this mechanism is controversial, since the physiological relevance of APC inhibition by PAI-1 is still not well understood. Through zymography, APC was shown to directly activate MMP-2 from the intermediate to fully active form [73-76], independent of membrane-type 1-matrix metalloprotease (MT1-MMP) [74, 75]. It has also been shown that APC [74, 75] [76] and APC generated on the cell surface in the presence of IIa bound to TM can activate MMP-2 [73]. The question still remains whether or not APC can activate MMPs bound to the cell surface in solution. Keratinocytes used in an *in vitro* wound healing assay showed that APC increased migration by increasing the expression and activation of MMP-2

and increased proliferation by increasing expression of interleukin-6 and -8 (IL-6 and -8), two inducers of keratinocyte proliferation [77]. APC also decreased NFκB, which at high levels will inhibit keratinocyte migration and initiate cell death during Ca^{2+}-induced differentiation [77]. In another study using keratinocytes, it was shown that APC induced further expression of EPCR, PAR-1, and MMP-2 along with activation of ERK and p38 [78]. Blocking the binding of APC to EPCR and PAR-1 prevented the APC-induced proliferation, increased expression of MMP-2, and activation of ERK and p38 [78]. In fibroblasts and endothelial cells, APC was shown to increase MMP-2, VEGF, and MCP-1, all of which are pro-angiogenic factors [52]. In a concentration dependent manner, APC increased HUVEC proliferation through activation of ERK1/2 and MEK1/2. Signaling was shown to be dependent on binding to EPCR, with PAR-1 activation only necessary for initial activation of the MAPK pathway [79]. This has been the only study to state that one receptor is more important than another. Previous work has stated APC does not signal through EPCR [61]. Therefore, further work is needed to understand how APC interacts with the receptors. Endothelial nitric oxide synthase (eNOS) phosphorylation through PI3K was also induced by APC [79]. Through eNOS activation, APC is able to increase angiogenesis in mouse cornea angiogenesis assay [79]. Using an *in vivo* rat skin healing model, APC increased angiogenesis through MMP-2 activity and reduced inflammation through a reduction in neutrophil infiltration [52]. Each study identifies various pathways that are activated by APC to effect cell migration. I can focus in on EPCR, PAR-1, and EGFR as potential receptors, the MAPK and PI3K pathways, and MMPs and the Plasminogen Activation System as potential extracellular proteases.

G. Regulation of APC and the Protein C Pathway by Serpins

Serine protease inhibitors (serpins) regulate the activation of PC as well as its activity to control the activity of APC. Protein C inhibitor [PCI; also known as plasminogen activator inhibitor-1(PAI-3)] will inhibit APC in the presence of heparin [80-85] and will prevents protein C activation by binding to IIa bound to TM [80, 82, 85, 86]. To control cleavage of the fibrin clot, PCI also inhibits both uPA and tPA [80] [82, 87, 88]. PCI can also inhibit proteases within the coagulation cascade, such as IIa, factors Xa and XIa, and kallikrein [80, 82] making it a regulator of IIa production. The question remains if PCI can also inhibit APC and its effects on cell migration, particularly if APC is bound to EPCR and interacting with PAR-1 and EGFR.

APC can also be inhibited by PAI-1. PAI-1 is a vitronectin (VN)-binding serpin involved in the fibrinolytic regulation of the coagulation pathway. PAI-1 is produced by endothelial cells and other epithelial tissues. It has a very short half-life in circulation, approximately 1 hour. Binding to VN increases the half-life of PAI-1 to 4-6 hours [89-91] and catalyzes its inhibitory reactions with proteases such as APC [92]. Upon depostition of fibrin, tPA will cleave and activate plasminogen to plasmin. Plasmin will degrade the fibrin clot, controlling the size of the clot in the blood vessel and remove the clot once the vessel wall is repaired. To control the degradation of the clot by plasmin to allow for repair and prevent hemorrhage, PAI-1 will inhibit tPA [93] to control the activation of plasminogen. PAI-1 will also inhibit IIa [94, 95], APC [92], and uPA [96, 97]. Whether or not PAI-1 and APC interact has been controversial. Previously, it had been shown that APC and PAI-1 form a complex, but that APC could cleave PAI-1, and inactivate the serpin [98] [99-104] [105]. Kobayashi, *et al.* showed that APC would form a complex with and be inhibited by PAI-1 in

a tumor cell setting, allowing for uPA activity and an increase tumor cell invasion [67]. Recent work has shown that, in fact, PAI-1 inhibits APC and the rate of inhibition increased in the presence of vitronectin approximately 300-fold [92]. If APC is bound to the cell through EPCR, it is unknown whether or not PAI-1 can inhibit APC and regulate its non-hemostatis function.

The non-fibrinolytic functions of PAI-1 have been extensively studied in vascular disease and cancer. PAI-1 has been linked to obesity and diabetes [106]. Visceral adipose tissue produces high levels of PAI-1. Synthesis is upregulated by insulin, glucocorticoids, angiotensin II, fatty acids, TNF-α, and TGF-β [107]. Clinical data has shown that high levels of PAI-1 are associated with a bad prognosis following a myocardial infarction [108, 109]. Research looking into the effects of PAI-1 in the development of atherosclerosis is controversial. Some work has shown PAI-1 to be protective by limiting plaque formation and matrix remodeling in the vessel wall [110, 111], while others have shown that PAI-1 inhibition of the fibrinolytic system allows formation of fibrin thrombi [112, 113]. PAI-1 has also been shown to inhibit smooth muscle cell migration [114, 115]. In hypertension, PAI-1 has been associated with increase in collagen deposition when NOS is inhibited [116]. Inflammatory cytokines, IL-6 and IL-1β, induce PAI-1 expression, seen in hypertensive patients [117]. Finally, PAI-1 has also been associated with kidney disease due to increased fibrin deposition [118, 119]. However, the opposite effect has been seen in PAI-1 deficiency mice, where a lack of PAI-1 was associated with severe glomerular injury [120].

The same contradictory results have been seen with PAI-1 and cancer. PAI-1 and uPA expression in breast cancer tissue is elevated and associated with a poor prognosis and an increased risk of relapse [121, 122]. *In vitro* work has shown PAI-1 disrupting the binding

of uPAR-VN-integrins and uPA-uPAR-integrins, detaching the cell and facilitating invasion [123, 124] [125, 126]. *In vivo*, mouse models for cancer have shown that PAI-1 either has no effect on tumor growth or metastasis [127] [128], or its expression reduces growth and metastasis by inhibiting uPA and extracellular matrix degradation and angiogenesis [129-132]. Further, other mice models have shown that PAI-1 is associated with increased proliferation and vascularity in tumors implanted in knock out mice [133]. It is hypothesized that the surrounding environment stimulates PAI-1 production, which in turn will increase cell motility, decrease cell adhesion, and promote chemotherapeutic resistance.

Finally, APC can be regulated by α_1-antitrypsin (α_1-AT; also known as α_1-protease inhibitor), a serpin that primarily inhibits neutrophil elastase [134]. α_1-AT inhibits APC in a heparin-independent manner, with a very slow second-order rate constant of 10 $M^{-1}s^{-1}$ [135]. However, this serpin is hypothesized to not be a relavent inhibitor of the non-hemostatic functions of APC because of the slow inhibition rate. In the reactive center loop of α_1-AT, the P1 residue that interacts with the serine protease is a methionine at position 358. A known variant of this residue from Met to Arg creates a mutant inhibitor with the ability to interact and inhibit numerous coagulation serine proteases involved in coagulation. Individuals with this mutation, called α_1-AT Pittsburgh, have a fatal bleeding disorder [136]. As a result, α_1-AT becomes a potent inhibitor of IIa, independent of heparin [136], kallikrein, factor XIIa fragment [137], factor XIa, and APC, again, also independent of heparin [138, 139]. Because of the greater association rate of α_1-AT Pittsburgh variant for IIa than APC, the half like of IIa in the plasma of α_1-AT Pittsburgh patients is 0.1s [138]. Therefore, patients suffer form hemorrhaging rather than from thrombosis. It has been shown that the plasma concentration of PC is decreased in these patients as well [140] because of an

increase in association with the variant serpin over its association with its physiological inhibitor, PCI. The variant form of this serpin would probably be an excellent inhibitor of APC and its non-hemostatic functions. The question that still remains is whether or not it can inhibit APC when bound to the cell surface.

H. Conclusion

Cell migration has a key role in multiple processes, including cancer metastasis and angiogenesis. Many factors are involved to not only promote the movement of cells, but to control this movement. Understanding how these factors work in concert to allow for normal cell migration will help us understand how additional factors can promote pathological cell migration. The goal of this dissertation, entitled the "Role of the Protein C System in Breast Cancer Cell Invasion and Endothelial Cell Angiogenesis", is to identify how the Protein C Pathway, specifically APC, known traditionally for its anticoagulant role, is able to promote cell migration in both pathological and normal processes. Previous work has proposed several mechanisms that are involved in APC-induced cell migration. The work proposed in this dissertation looks to clarify and add to these proposed mechanisms. Additionally, this work will show how in one setting of normal cell migration, such as angiogenesis, APC can promote this processes and increase wound healing in stroke and cardiovascular diseases to the benefit of the patient, while in another setting of pathological cell migration, such as cancer metastasis, APC can advance the disease and be detrimental to the patient, all mediated through a similar mechanism.

Chapter 2 of this dissertation is a review of hemostasis, thrombosis, and fibrinolysis, focusing on the role of serpins and the state-of-the-art research being done to advance the

field. Chapter 3 is a research article describing the effects of APC on breast cancer cell migration, mediated through EPCR and PAR-1. Chapter 4 is a submitted publication characterizing the intracellular signaling events and extracellular processes activated by APC to induce breast cancer cell migration. Chapter 5 focuses on the vascular aspect of this dissertation. It describes the effects of APC on endothelial cell migration, tube formation, and aortic ring sprouting, mediated through EGFR, PI3K, and MMPs. Chapter 6 is a hypothesis paper that focuses on the relationship of PAI-1, the Metabolic Syndrome, and breast cancer. It reviews ongoing work and presents data that supports the connection of breast cancer to the Metabolic Syndrome through the common link, PAI-1. Finally, Chapter 7 summarizes the findings presented in this dissertation and provides an overview of the future direction of this work. The research in this dissertation contributes to our overall knowledge of APC and the Protein C Pathway in a non-traditional setting apart from hemostasis, and it provides mechanistic insight into the processes used by APC to promote cell migration.

Chapter 2

Serpins in Thrombosis, Hemostasis and Fibrinolysis

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[‡] These authors contributed equally to this review. (2007) ***J. Thromb. Haemost.*** 5: in press.

Summary

Hemostasis and fibrinolysis, the biological processes that maintain proper blood flow, are the consequence of a complex series of cascading enzymatic reactions. Serine proteases involved in these processes are regulated by feedback loops, local cofactor molecules, and serine protease inhibitors (serpins). The delicate balance between proteolytic and inhibitory reactions in hemostasis and fibrinolysis, described by the Coagulation, Protein C and Fibrinolytic Pathways, can be disrupted resulting in the pathological conditions of thrombosis or abnormal bleeding. Medicine capitalizes on the importance of serpins, using therapeutics to manipulate the serpin-protease reactions for the treatment and prevention of thrombosis and hemorrhage. Therefore, investigation of serpins, their cofactors, and their structure-function relationships is imperative for the development of state-of-the-art pharmaceuticals for the selective fine-tuning of hemostasis and fibrinolysis. This review describes key serpins important in the regulation of these pathways: antithrombin, heparin cofactor II, protein Z-dependent protease inhibitor, α_1-protease inhibitor, protein C inhibitor, α_2-antiplasmin and plasminogen activator inhibitor-1. We focus on the biological function, the important

structural elements, their known non-hemostatic roles, the pathologies related to deficiencies or dysfunction, and the therapeutic roles of specific serpins.

Introduction

Blood flow is maintained by the proper balance of hemostasis and fibrinolysis, an interdependent network of physiologic processes and succession of proteolytic reactions. Hemostasis, the physiologic cessation of bleeding, involves the interaction of vasoconstriction, platelet aggregation and coagulation. The end result of coagulation is the deposition of cross-linked fibrin polymers to form blood clots. Both the Protein C and the Fibrinolytic Pathways are activated by the Coagulation Pathway and serve to restrict excessive clot formation or thrombosis. The enzymatic reactions that propel these pathways are dominated by serine proteases and are subject to control by serpins and their local cofactors. Dysfunction, deficiencies or over-expression of serpins can cause either abnormal bleeding or thrombosis. Investigations into the structure and related activities of serpins, their target proteases and cofactors has provided valuable information regarding both serpin-related disease states and potential mechanisms by which medicine can manipulate serpin-protease interactions for the treatment and prevention of thrombosis and bleeding.

Hemostasis

Coagulation Pathway

The factors of the Coagulation Pathway generally circulate in an inactive state until they are activated through proteolysis by an upstream factor. While the end goal of coagulation is fibrin polymerization, the most crucial feature of the Coagulation Pathway is

the generation of thrombin (Figure 2-1). Thrombin is responsible for cleaving fibrinogen to fibrin, activating fXIII to fXIIIa (which cross-links fibrin), activating platelets, and positively feeding back into the cycle by activating upstream factors [141].

Thrombin generation is initiated when damage to a vessel wall exposes the blood to tissue factor (TF) in the subendothelium [142]. TF is also expressed by activated platelets and leukocytes [143]. Therefore, coagulation can also be initiated by inflammation. TF forms a complex with fVIIa and activates fX. Together, fVa and fXa form the prothrombinase complex which then cleaves a small amount of prothrombin (fII) to thrombin (fIIa). This small amount of thrombin activates platelets, fV, fVIII and fXI, feeding back into the cycle to increase thrombin formation. Factor IXa, previously activated by either TF-VIIa or by fXIa on the platelet surface, and fVIIIa in the presence of calcium, complex on the platelet surface to form the platelet tenase complex. Platelet tenase activates more fX, which with fVa, generates a "thrombin burst" (Figure 2-1). It is this burst of thrombin rather than the initial thrombin activation that is crucial for the formation of a stable hemostatic plug [142].

In addition to its role in hemostasis, thrombin regulates many pro-inflammatory processes including leukocyte adhesion molecule expression on the endothelium, platelet activation, leukocyte chemotaxis and endothelial cell production of pro-thrombotic factors [144]. Thrombin is also a potent growth factor, initiating endothelial, fibroblast and smooth muscle cell proliferation and up-regulating other cytokines and growth factors [145]. These activities have been attributed to proteolytic cleavage of insulin-like growth factor binding proteins [146] and protease activated receptors -1, -3, and -4 (PAR-1, -3, -4) [56] on cell surfaces, and account for thrombin's central role in atherosclerotic lesion formation [147].

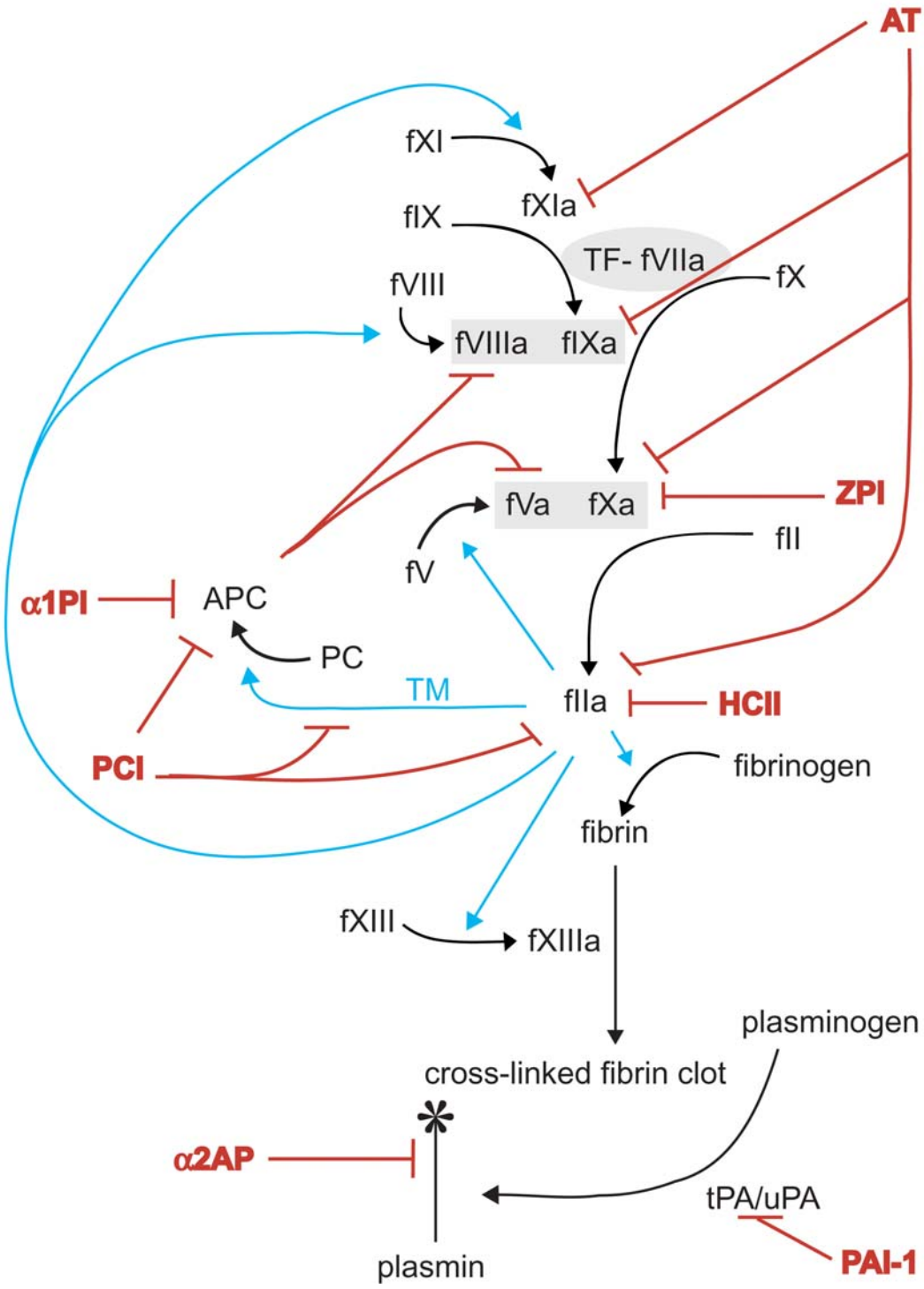

Figure 2-1. ***Serpin Regulation of Coagulation, Protein C and Fibrinolytic Pathways.*** Serpins and inhibitory functions are shown in red, thrombin activity is shown in cyan. Prothrombinase and tenase complexes are shown in grey boxes. Coagulation is initiated by the exposure of tissue factor to fVIIa shown in grey oval. The symbol ✻ indicates degradation. Necessary cofactors, Ca^{++}, phospholipids, proteins S and Z, vitronectin and GAGs are not shown to maintain the simplicity of the schematic.

Coagulation is regulated predominantly by antithrombin (AT) [148], tissue factor pathway inhibitor (TFPI) [149], the Protein C Pathway [150] and to a lesser extent heparin cofactor II (HCII) [151] and protein Z-dependent protease inhibitor (ZPI) [152]. Protein C inhibitor (PCI) and plasminogen activator inhibitor (PAI-1) may also contribute by inhibiting thrombin [153] van [94]. TFPI is not a member of the serpin family and so will not be discussed in this paper.

Protein C Pathway

The Protein C Pathway works in hemostasis to control thrombin formation in the area surrounding the clot [46]. The zymogen protein C (PC) is localized to the endothelium by endothelial cell protein C receptor (EPCR) [154]. Thrombin, generated via the Coagulation Pathway, is localized to the endothelium by binding to the integral membrane protein, thrombomodulin (TM). TM occupies exosite I on thrombin which is needed for fibrinogen binding and cleavage, thus reducing thrombin's procoagulant activities [155]. However, TM bound thrombin is able to cleave PC to activated protein C (APC), a serine protease, on the endothelial cell surface [156]. In the presence of protein S, APC inactivates fVa and fVIIIa [157] (Figure 2-1). This limits further thrombin generation on the clot periphery where the endothelium is not damaged [158].

The Protein C Pathway is also associated with non-hemostatic functions. APC has been shown to be an anti-inflammatory protein [50, 59] and modulates gene expression [60]. It also enhances vascular permeability by signaling through both PAR-1 and sphingosine 1-phosphate receptor-1 [159]. Using focal ischemic stroke animal models, APC treatment restored blood flow, reduced infarct volume and inflammation [160]. These neuroprotective

effects of APC were shown to be mediated through EPCR, PAR-1 [54], and PAR-3 [64]. In the PROWESS Study, patients diagnosed with severe sepsis were treated with recombinant human APC, resulting in a mortality reduction of 19.4% [48].

The proteolytic activity of APC is regulated predominantly by protein C inhibitor (PCI) [148]. Additionally, plasminogen activator inhibitor-1 (PAI-1) [92] and α_1-protease inhibitor (α_1PI) [161] have been shown to inhibit APC, although their role in the hemostasis is not well understood.

Fibrinolysis

Fibrinolytic Pathway

Fibrinolysis is the physiologic breakdown of fibrin to limit and resolve blood clots [162]. Fibrin is degraded primarily by the serine protease, plasmin, which circulates as a zymogen, plasminogen. In an auto-regulatory manner, fibrin serves as both the cofactor for the activation of plasminogen and the substrate for plasmin (Figure 2-1). In the presence of fibrin, tissue plasminogen activator (tPA) cleaves plasminogen to plasmin which proteolyzes the fibrin. Because it is a necessary cofactor for the reaction, the degradation of fibrin limits further activation of plasminogen [163-165]. The serine protease, tPA, is synthesized and released by endothelial cells [162]. In addition to binding fibrin, tPA binds Annexin II (AnII) and other receptors on endothelial cell and platelet surfaces [166]. Thus, plasmin generation and fibrinolysis are restricted to the site of thrombus formation.

In addition to its role in fibrinolysis, plasmin has other physiologic functions as evidenced by its ability to degrade components of the extracellular matrix [167] and activate matrix metalloproteases 2 and 9 [168, 169]. Plasminogen can also be converted to plasmin

by the serine protease, urokinase plasminogen activator (uPA) [167]. Urokinase-catalyzed events are localized on the cell surface through the uPA receptor (uPAR). Complex formation and subsequent reactions are thought to be more important during pericellular proteolysis, cell adhesion and migration than it is for vascular fibrinolysis [162]. These additional functions contribute to the role of the Fibrinolytic Pathway in cancer [167, 170, 171].

Fibrinolysis is controlled predominantly by α_2-antiplasmin (α_2AP) [172], PAI-1 [163, 173] and thrombin activatable fibrinolysis inhibitor (TAFI) [80, 174]. PCI can inhibit tPA and uPA [80, 175], but its role in fibrinolysis is unclear. TAFI is not a member of the serpin family and so will not be discussed in this paper.

Serpin Overview

Serpins

Serpins are a superfamily of proteins classified into 16 clades (A-P). The systematic name of each serpin is, SERPINXy where X is the clade and y is the number within the clade [176]. Serpins have been identified in the genomes of organisms representing all of the branches of life (Bacteria, Archaea, Eukarya and Viruses), and the genome of humans contains ~36 serpins [177]. While serpins are named for their ability to inhibit *serine* proteases (of the chymotrypsin family) (Table 2-1), some are capable of cross-class inhibition of proteases from the subtilisin, papain and caspase families. In addition, some serpins utterly lack protease inhibitory activity and serve other roles, such as hormone transporters, molecular chaperones or catalysts for DNA condensation. Serpins are typically composed of ~400 amino acids, but can have large N-, C-terminal or internal insertion loops [176]. Serpins can

also be post-translationally modified by glycosylation, sulfation, phosphorylation and oxidation to alter their function. In spite of a low overall primary sequence identity for the family, serpins share a highly conserved three-dimensional fold comprised of a bundle of 9 α-helices (A-I) and a β-sandwich composed of three β-sheets (A-C) (Figure 2-2A). It is useful to view a serpin in the 'classic orientation' to illustrate the important structural features (Figure 2-2A, left panel). In this view the main β-sheet A is facing and the reactive site loop (RSL) is on top. The RSL is typically composed of 20 amino acids running from P17 at the N-terminus (at the C-terminal end of strand 5A) to P3′ at the C-terminal end (using the nomenclature of Schechter and Berger, where residues are numbered from the scissile P1-P1′ bond). In the normal native state of a serpin, β-sheet A is composed of five strands and the RSL (bridging the C-terminus of strand 5A to the N-terminus of strand 1C) is exposed. This state is, however, not the most stable. An astounding increase in thermodynamic stability (best estimate -32 kcal/mol) [192] can be achieved through the incorporation of the RSL into β-sheet A, triggered either through extension of strand 1C (to form the so-called 'latent' state), or through proteolytic nicking anywhere near the scissile bond (the cleaved state). The metastability of the native serpin is critical for its unusual mechanism of protease inhibition [193]).

The Serpin Mechanism of Protease Inhibition

The serpin mechanism of protease inhibition has been worked out over the last 20 years through a series of biochemical, fluorescence and structural studies. A minimalist kinetic scheme is composed of two steps: the formation of the encounter complex (also known as the Michaelis complex) where the sequence of the RSL is recognized by the protease as a substrate; and, the formation of a final covalent complex where the protease is

trapped in an inactive state (Figure 2-2B). The rates of formation and dissociation of the reversible Michaelis complex, along with colocalization in tissues, determines the specificity of the serpin-protease interaction [90, 194]. While the obligate RSL-active site contacts contribute significantly to the formation of the Michaelis complexes, exosite interactions may also be involved. As with actual substrates of serine proteases, this step is followed by the nucleophilic attack of the peptide bond between the P1-P1′ residues by the catalytic Ser195 of the protease. This ultimately results in the formation of a covalent ester bond between the P1 residue and Ser195 of the protease (acyl-enzyme intermediate), and then separation of the P′ residues from the active site of the protease. At this stage the serpin rapidly adopts its lowest energy conformation through the incorporation of the N-terminal portion of the RSL into β-sheet A. The tethered protease is thus flung from the top to the bottom of the serpin (~70Å), and the resulting pulling force exerted on the catalytic loop results in a conformational distortion of the protease [195]. The acyl-enzyme intermediate is thus trapped, with deacylation prevented largely due to the destruction of the oxyanion hole. Two structures of final complexes have been solved by X-ray crystallography [196, 197], with one showing an additional distortion of ~37% of the protease structure [197]. This mechanism is particularly well suited to tightly regulated processes such as hemostasis and fibrinolysis because inhibition is irreversible, and the conformational changes in the serpin and the protease alter cofactor interactions. An example of the physiologic relevance of the conformational change in the protease component of the complex is the complete destruction of thrombin's exosite I in complex with serpins [198]. Thus, when PCI inhibits thrombin bound to thrombomodulin the interaction with thrombomodulin is broken, allowing the serpin-protease complex to diffuse away so that another thrombin molecule can bind [86].

Table 2-1. Second Order Rate Constants of Protease Inhibition by Serpins in the Presence and Absence of Cofactors.[a]

SERPIN	SYSTEMATIC NAME	TARGET PROTEASE	COFACTOR	SECOND ORDER RATE k_2 (M^{-1} s^{-1})	CITATION
AT	SERPINC1	Thrombin	--	7.5×10^3, 1×10^4	[178], [179]
			UFH	2×10^7, 4.7×10^7	[178],[180]
			LMWH	5.3×10^6	[180]
			pentasaccharide	2×10^4	[179]
		fXa	--	2.5×10^3, 6×10^3	[181]
			UFH	5×10^6, 6.6×10^6	[179], [180]
			LMWH	1.3×10^6	[180]
			pentasaccharide	7.5×10^5	[179]
		fIXa	--	1.3×10^2, 5×10^2	[179], [182]
			UFH	8×10^6, 1.75×10^6	[179], [182]
			LMWH	3.7×10^5	[182]
			pentasaccharide	3×10^4	[179]
HCII	SERPIND1	Thrombin	--	6×10^2	[183]
			UFH	5×10^6	[183]
			LMWH	$\sim 5 \times 10^6$	[184]
			Dermatan sulfate	1×10^7	[183]
			hexasaccharide	2×10^4	[185]
ZPI	SERPINA10	fXa	-- , Ca^{++}, PL	2.3×10^3	[186]
			protein Z, Ca^{++}, PL	6.1×10^5	[186]
		fIXa	--	2×10^5	[86]; [187]
			UFH	4×10^5	[86]; [187]
PCI	SERPINA5	Thrombin	--	1.7×10^4	[86]
			UFH	$\sim 2 \times 10^5$	[86]
			thrombomodulin	2.4×10^6	[86]
		APC	--	3×10^2	[188]
			UFH	5×10^4	[188]
			UFH, Ca^{++}	2.9×10^5	[83]
		tPA (2-chain)	--	8×10^2	[80]
			UFH	3×10^4	[80]
α_1*PI*	SERPINA1	Thrombin	--	4.8×10^1	[189]
		APC	--	4×10^1	[84]
$\alpha_1 PI_{Pittsburgh}$		Thrombin		4.8×10^5	[190]
		APC		7×10^4	[190]
α_2*AP*	SERPINF2	Plasmin		2×10^7	[172]; [191]
PAI-1	SERPINE1	Thrombin	--	7.9×10^2	[94]
			UFH	1.6×10^5	[95]
			vitronectin	1.9×10^5	[95]
		APC	--	5.7×10^2	[92]
			vitronectin	1.8×10^5	[92]
		tPA (1- , 2-chain)	--	4×10^7, 1.5×10^8	[97]

[a]The rates constants indicated here are from selected references and may vary slightly under different experimental conditions. UHF = Unfractionated heparin, LMWH = low molecular weight heparin, PL = phospholipids.

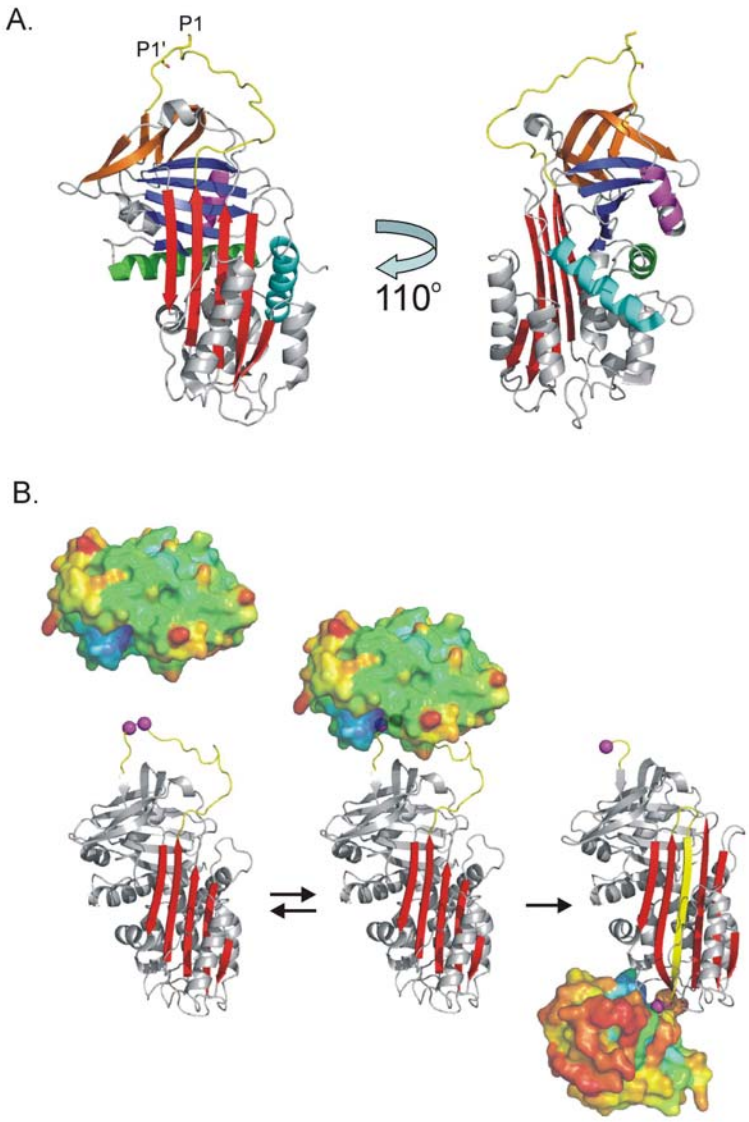

Figure 2-2. ***Serpin Structure and Mechanism of Protease Inhibition.*** A. The shared serpin fold is illustrated by the structure of the prototypical native serpin α_1PI. The 'classic' orientation shown on the left places the RSL (yellow) on top and the main β-sheet A (red) to the front. Sheets B and C are blue and orange, respectively, and helices A, D and H are colored green, cyan and magenta. The accessibility of the RSL is illustrated by rotating the molecule by 110° to the left along the long axis. It shows how the P1-P1′ (rods) scissile bond is exposed for proteolytic attack. Also clearer in this orientation are helices D and H which are the heparin binding helices. B. The serpin mechanism of protease inhibition is minimally expressed as a two step process. In the first step native serpin (ribbon with the P1 and P1′ residues as magenta balls, below) interacts reversibly with a protease (surface representation, colored according to temperature factors from blue to red) to form the Michaelis complex (middle). After formation of the acyl-enzyme intermediate the protease is flung to the opposite pole of the serpin and its catalytic architecture is destroyed, and consequently there is a loss of ordered structure (notice the smaller size and increase in temperature factors).

Cofactor Interactions

Because serpin specificity is determined largely by the rate of formation of the Michaelis complex, cofactors which bind to serpins (and sometimes the protease) can radically alter specificity [90]. Table 2-1 presents serpin second order rates of protease inhibition in the presence and absence of relevant cofactors. The best understood cofactor for serpins is the glycosaminoglycan (GAG), heparin. It binds to and activates most of the serpins involved in hemostasis and thrombosis [199]. Acceleration of protease inhibition is generally conferred through a template effect where the protease and the serpin bind to the same heparin chain. The hypothesis is that this co-occupation will limit the diffusional freedom from three to one dimension to increase the likelihood (rate) of encounter. In addition, heparin also provides a bridge between the serpin and the protease to help stabilize the Michaelis complex. However, heparin and other GAGs are also capable in some cases of altering the conformation of the serpin to permit more rapid complexation with proteases. The best characterized examples are AT and HCII, whose activation by heparin is the basis of its therapeutic anticoagulant effect. In the next sections we describe each of the serpins involved in hemostasis and fibrinolysis, their targets, the role of cofactors and available structural data.

Serpins in Hemostasis and Fibrinolysis

Antithrombin – SERPINC1

Antithrombin (AT) is a 58 kDa, 432 amino acid glycoprotein [200], synthesized in the liver, circulating at approximately 150 μg/mL with a half-life of ~3 days [201]. It is the most important physiologic inhibitor of the coagulation pathway [202]. As its name implies,

antithrombin inhibits thrombin. Additionally, AT is capable of inhibiting all of the other proteolytic coagulation factors (e.g. factors IXa, Xa, and XIa). The predominance of its anticoagulant activity, however is focused on the regulation of fXa, fIXa and thrombin. Measurement of thrombin-AT (TAT) complex is used as a marker of hemostatic activation and helps diagnose thrombotic events [203]. Thrombin bound to fibrin, clot-bound thrombin, is protected from inhibition by AT [204]. This may explain the occurrence of rethrombosis after fibrinolytic therapy as clot-bound thrombin is released from the dissolving hemostatic plug [205].

The anticoagulant activity of AT is dependent on its cofactor, heparin. Consisting of variably sulfated repeating dissacharide units, heparin can have a molecular weight ranging from 3 to 40 kDa [206-208]. A unique pentasaccharide sequence in heparin is responsible for the high affinity binding to AT [209]. *In vivo* forms of heparin relevant to AT include heparan sulfate found on the endothelium, and heparin released from endothelium-associated mast cell granules. The interaction of AT with heparan sulfate on the endothelium and subendothelium localizes AT activity to the vessel wall and maintains its normal, nonthrombogenic nature [201]. AT is expressed as both an α-form and a β-form. α-AT represents 90% of AT and is glycosylated at all four positions. While comprising only 10% of AT, β-AT, which is not glycosylated at one position (N135), has a higher affinity for heparin and is thought to exert an overall larger anticoagulant effect [210].

Heparin utilizes two distinct mechanisms for accelerating protease inhibition by AT. AT undergoes a well characterized conformational change upon heparin binding, which expels the N-terminus of the RSL from β-sheet A (Figure 2-3A). This 'liberation' of the RSL is sufficient to confer the majoritiy of the acceleration of fIXa and fXa inhibition, but

thrombin inhibition is not appreciably affected. Recently, the structures of the AT-heparin-protease Michaelis complexes have been solved [196, 211, 212] revealing the interactions behind the allosteric and template mechanisms (Figure 2-3B).

In addition to its anticoagulant activity, AT has been shown to have anti-inflammatory and anti-angiogenic functions. These properties are independent of AT's inhibitory activity. AT regulates inflammation by signalling through heparan sulfate on endothelial and leukocyte cell surfaces [213]. Latent and cleaved AT exerts anti-angiogenic effects [214] by binding cell surface heparan sulfate. This blocks fibroblast growth factor-2 and vascular endothelial cell growth factor from forming pro-angiogenic ternary signalling complexes with their protein receptors and the heparan sulfate co-receptors [215].

Antithrombin in Disease-- Inherited and acquired AT deficiency predisposes individuals to different degrees of thrombotic disease. The severity of thromophilia can be exacerbated by other risk factors for thrombosis. Inherited AT is classified as type I or type II. Type I deficiencies, which generally confer a higher thrombotic risk, are caused by geneticmutations that impair the synthesis and secretion of AT. Type II deficiencies are caused by genetic mutations that functionally imparied AT. Variations in the degree of thrombophilia in inherited AT deficiencies can be attributed to homozygosity versus heterozygosity and where the mutation lies in the AT structure [216]. An up-to-date database of AT mutations can be found online at http:// www1.imperial.ac.uk/medicine/about/divisions/is/haemo/coag/antithrombin [217]. Some research suggests that certain mutations predispose AT to convert to its latent form, which preferentially dimerizes with native β-AT. Dimerization reduces the presence of highly active AT monomers, thus increasing thrombogenicity [218]. Concern has been raised that therapeutic preparations of AT-

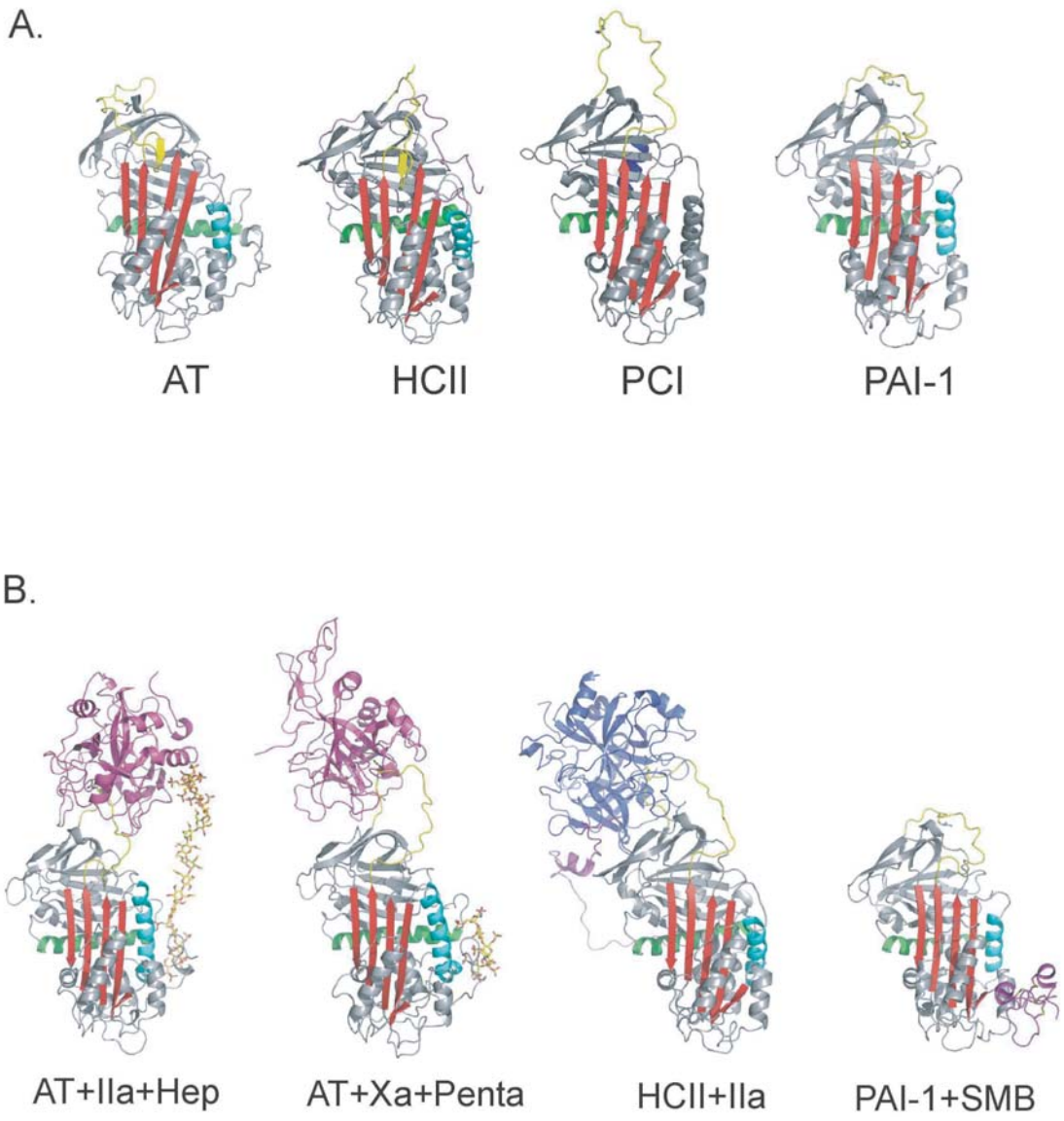

Figure 2-3. ***Native and Complexed Serpin Structures.*** A. The native structures of important hemostatic and fibrinolytic serpins are shown as ribbon diagrams, colored essentially as in Fig. 2. The monomeric structure of AT is shown in the left panel, and is similar to that of HCII with the partial insertion of the N-terminal portion of the RSL. A modeled position for the N-terminal tail of HCII is shown in magenta although its true position is not known. For AT, HCII and PAI-1 the heparin binding helix (helix D) is shown in cyan, but for PCI heparin binds to helix H (blue). The increased size and flexibility of the RSL of PCI is also evident from this depiction. B. Some important serpin complexes are shown. Using S195A proteases it was possible to obtain the structures of the AT Michaelis complexes with thrombin (magenta) and fXa (magenta) with their activating synthetic heparins (SR123781 and fondaparinux, rods). Similarly, the HCII-thrombin (blue) complex was also solved. The somatomedin domain of VN (magenta) binds to s1A and helix E to prevent the latent transition through expansion of sheet A.

concentrates (contain >10% latent AT) might have thrombotic effects. However, a recent study demonstrated that the addition of latent AT alone does not decrease the activity of AT in plasma [219]. Other mutated forms of AT do not show impaired activity or decreased AT levels in the standard hospital laboratory assays despite associated thrombophilia. In particular, the antithrombin Cambridge II (A384S) variant was found to be undetected by some protocols, but estimated to be the most frequent cause of antithrombin deficiency in Caucasian populations [220]. These results suggest a need for alternative methods for detection of AT deficiencies [221].

Antithrombin related treatements for coagulation disorders-- Therapeutic unfractionated heparin (UFH), derived from porcine mucosa, is one of the most commonly used anticoagulant agents administered for treatment and prophylaxis of thrombotic events. Additionally, UFH is used to coat blood collection tubes and surgical devices to prevent clotting on their surfaces. UFH's primary mechanism of action is to accelerate AT's inhibition of thrombin, fXa and fIXa. UFH also accelerates thrombin inhibition by other circulating serpins. It has a very short half-life and optimal dosing of heparin is notoriously difficult to achieve, therefore requiring frequent monitoring [222]. Still in trials, an orally available form of heparin, sodium N-(8-[2-hydroxybenzoyl] amino) caprylate bound heparin or SNAC-heparin, has dose-dependent antithrombotic effects, and has an efficacy comparable to low-molecular weight heparin in reducing venous thrombosis in patients undergoing hip replacement surgery [223]. Contraintuitively, heparin can cause a dangerous thrombotic condition called heparin induced thrombocytopenia (HIT). In this autoimmune reaction antibodies develop against platelets [224]. Currently in development stages,

synthetic oligosaccharide heparin mimetics show thrombin and fXa inhibition comparable to UFH without inducing HIT and with far fewer side effects [225].

Low molecular weight heparin (LMWH) is a fractionated preparation of heparin between 1 and 10 kDa with an enriched population of high affinity pentassacharide sequences. Because of the smaller average size, LMWH acts predominantly by inducing conformational change in AT, the mechanism which activates fXa. It has a longer half-life than UFH and does not need coagulation monitoring. LMWH also has significantly reduced risk of HIT. Multiple LMWH variants are available or in clinical trials [226].

Fondiparinux and idraparinux are synthetic pentassacharide sequences derived from heparin, which activate AT to specifically inhibit fXa. Because of this the single target, the side effect of over-anticoagulation, bleeding, is reduced. Both have longer half-lives than LMWH. Additionally, neither preparation causes HIT [226, 227].

Thrombotic events due to antithrombin deficiency are treated with AT purified from human plasma. A covalent AT-heparin complex is currently under preliminary investigations for possible use as a novel anticoagulant because unlike AT or heparin alone, it is able to inhibit clot-bound thrombin [228].

Heparin Cofactor II – SERPIND1

Heparin cofactor II (HCII) is a 66.5 kDa, 480 amino acid glycoprotein, synthesized in the liver, circulating at ~80 μg/mL with a half-life of 2-3 days. HCII inhibits thrombin in the presence of many polyanionic molecules including the GAGs heparin and dermatan sulfate [151]. A unique hexasaccharide sequence within dermatan sulfate has been determined to be responsible for its high affinity binding to HCII [229]. Dermatan sulfate does not accelerate

any other serpin activity. HCII has a unique N-terminal extension of ~80 residues that contains two acidic regions, critical for its GAG-associated anti-thrombin activity [230]. HCII inhibits thrombin and clot-bound thrombin, but not other coagulation proteases [151]. Evidence suggests that HCII contributes 20-30% to thrombin inhibition in coagulation. Neither humans nor mice deficient in HCII exhibit thrombophilia under normal conditions [231]. However, HCII homozygous deficient mice form occlusive thrombi faster than wild-type mice after photochemical vascular endothelial cell injury to the carotid artery [232]. Recent data suggests that the primary physiologic function of HCII is to inhibit thrombin's non-hemostatic roles such as in the development of atherosclerosis. Elevated levels of HCII are shown to protect against atherosclerosis and restenosis [233-235].

The structure of native HCII was solved in 2002 and revealed a surprising resemblance to native antithrombin [230], with the N-terminus of the RSL inserted into β-sheet A (Fig. 2-3). As HCII also shares a similar heparin binding site along helix D [148, 236], it was proposed that HCII underwent a similar conformational change upon heparin binding [230]. Recently, it was shown that the smallest heparin length capable of tight binding to HCII was 14 monosaccharide unit chains, and that the majority of the acceleration effect was due to this allosteric change in HCII conformation [184]. Disappointingly, however, the native structure could not resolve the position of the N-terminal extension. Several mutagenesis studies concluded that the acidic tail is binding to the basic heparin binding site in native HCII [236], but it is still unclear how the tail interacts with the body of HCII in the native state. From the structure of HCII bound to S195A thrombin [230] it was clear how the tail confers specificity to thrombin. The tail binds to exosite I of thrombin in a manner similar to the hirudin, primarily through hydrophobic contacts. The tail was found

sandwiched between thrombin and the body of HCII, essentially providing a shared exosite (Fig. 2-3B). A complex allosteric mechanism has been proposed based on these structures, supporting biochemical studies and analogy to AT [194, 199].

HCII related treatements for coagulation disorders-- As alternatives to heparin-based treatments, dermatan sulfate derivatives and other polyanionic molecules that act to accelerate HCII's antithrombotic activity are being investigated. They are of particular interest for use in HIT, AT deficiency and for the inhibition of clot-bound thrombin. Two types of fractionated dermatan sulfate enriched for the hexasaccharide sequence (Intimatan™ and Desmin™) have been tested in humans [237, 238]. Intimatan is beginning Phase I trials. Other HCII agonists in laboratory investigations include over-sulfated dermatan sulfate [239], fucosylated chondroitin sulfate [240] and fucoidan [241].

Protein Z-dependent Protease Inhibitor– SERPINA10

Protein Z-dependent protease inhibitor (ZPI) is a 72 kDa, 444 amino acid glycoprotein, synthesized in the liver, circulating at ~1.5 μg/mL. In the presence of protein Z, phospholipids and calcium, ZPI rapidly inhibits fXa. In the absence of cofactors, ZPI also inhibits factor XIa, which can be accelerated two-fold by heparin. It is thought that the major physiologic function of ZPI is to attenuate the coagulation response prior to the formation of the prothrombinase complex [86, 187]. In humans, mutations in ZPI are associated with increased risk of venous thrombosis [242-244]. Additionally, reductions in protein Z plasma concentrations result in an aggravated thromboembolic risk in humans and mice with fV_{Leiden} [245].

Protein C Inhibitor – SERPINA5

PCI is 57 kDa, 387 amino acid glycoprotein [246], synthesized in the liver, circulating at ~5 μg/mL. It is also found in other bodily fluids including urine, saliva, amniotic fluid, milk, tears and seminal fluid [88, 247]. PCI is a heparin-binding serpin that inhibits many proteases including APC [82], IIa, IIa bound to TM [86] tPA and uPA [88]. PCI may have contrary anticoagulant and procoagulant functions depending on the target protease and the presence of specific cofactors. In the presence of heparin, PCI is anticoagulant, inhibiting the proteolytic cleavage of fibrinogen by thrombin. However, in the presence of TM, PCI is procoagulant, inhibiting the activation of PC by thrombin [148].

The structures of RSL-cleaved PCI [246] and of native PCI (PDB # 2HI9 and # 2OL2) (Fig. 3A) have now been solved, revealing a typical serpin structure with some notable differences. The RSL of PCI is unusually long and flexible, accounting for its broad protease specificity, and its heparin-binding site is found along the highly basic helix H. The effect of heparin on PCI activity can either be to accelerate protease inhibition (e.g. APC) [248] or to abrogate it (tissue kallikrein) [249]. The position of the heparin-binding site close to the protease docking site may help explain this property.

Protein C Inhibitor in Disease-- PCI is not synthesized by the liver in mice, and thus PCI is unlikely to play a role in hemostasis or fibrinolysis in the mouse [250]. Transgenic mice that over-express human PCI (hPCI) provide evidence of PCI's ability to inhibit thrombin, APC and tPA. These mice do not exhibit symptoms of pulmonary hypertension induced by monocrotaline. TAT complexes are reduced compared to their wild-type counterparts suggesting that PCI competes with AT to inhibit thrombin. Additionally, a decrease in free tPA and subsequent reduction in fibrinolysis is seen. Finally, when APC

was administered for endotoxemia, hPCI-expressing transgenic mice demonstrated a reduction in the anticoagulant and anti-inflammatory effects of the treatment [251]. Male homozygous PCI-knockout mice were infertile due to abnormal spermatogenesis caused by loss of the Sertoli cell barrier [252] due unregulated proteolytic activity.

In humans, APC-PCI complex is indicative of atherosclerosis and aortic aneurysms [253], an early indicator of myocardial infarction [254] and predicts poor patient outcome after aortic surgery [255]. Additionally APC-PCI complex is increased (4-fold) in patients with fV_{Leiden} who have suffered a previous venous thrombosis [256]. PCI alone has been shown to be elevated in survivors of acute coronary events [257].

α_1-Protease Inhibitor – SERPINA1

α_1-protease inhibitor (α_1PI) – historically known as α_1-antitrypsin – is a 51 kDa, 394 amino acid glycoprotein, synthesized in the liver, circulating at ~1.3 mg/mL with a half-life of 4.5 days (structure shown in Fig. 2). Its physiologic target is neutrophil elastase [134], however, it has also been shown to inhibit APC in a heparin-independent manner [161]. In pediatric ischemic stroke patients, α_1PI levels were significantly increased independent of other pro-thrombotic factors. Authors suggest this pathology is due to APC inhibition [258].

α_1PI is not thought to contribute significantly to coagulation. However, a variant of the protein ($\alpha_1PI_{Pittsburgh}$) with a reactive site mutation (M358R) can cause a fatal bleeding disorder [136]. The Met → Arg polymorphism creates a potent inhibitor of several coagulation serine proteases, especially thrombin and APC, that is not dependent on heparin or other cofactors [259]. Therapies utilizing recombinant $\alpha_1PI_{Pittsburgh}$ were considered but abandoned due to side effects of promiscuous protease inhibition [138, 140].

α_2-antiplasmin – SERPINF2

α_2-antiplasmin (α_2AP) is a 63 kDa, 452 amino acid glycoprotein, synthesized in the liver, circulating at ~70 μg/ml with a half-life of 2.6 days [172, 260, 261]. α_2AP is the primary physiological inhibitor of plasmin, but has also been reported to inhibit other enzymes such as trypsin, elastase, and APC. Homozygous deficiency of α_2AP results in uncontrolled fibrinolysis and subsequent severe hemorrhagic tendencies [260, 262]. While α_2AP has all of the key structural features of the serpin family, it uniquely has both N- and C-terminal extensions of 42 and 55 residues, respectively [172, 263]. In thrombus formation, the N-terminal region of α_2AP is cross-linked to fibrin by fXIIIa, and the C-terminal Lys binds to the Lys-binding site of plasmin. The rate of fibrinolysis is proportional to cross-linked α_2AP.

Plasminogen Activator-1 – SERPINE1

PAI-1 is a 50 kDa, 379 amino acid glycoprotein, synthesized in endothelial cells, platelets and other mesenchymal cells surrounding the vasculature [264-266]. This serpin is relatively unstable with a half-life of 1-2 hours in circulation [267]. However, PAI-1 is found bound to the extracellular matrix protein, vitronectin (VN) [268, 269]. The PAI-1-VN complex has an enhanced half-life of 4-6 hours [267]. PAI-1 regulates both tPA and uPA and is considered the main physiological inhibitor of plasminogen activation [162, 163, 173, 270]. As platelets are activated following vessel injury, they release PAI-1 to protect the developing thrombus from premature fibrinolysis. Later in the coagulation process, tPA and

plasminogen/plasmin are bound to fibrin within the thrombus, which protects tPA from inhibition by PAI-1, resulting in plasmin generation and fibrinolysis [162, 163, 173, 270].

Several structures of PAI-1 have been solved, but, due to its rapid conversion to the latent form, all structures of native PAI-1 are of a stabilized quadruple mutant [271-273]. Although the structure shows a native state similar to α_1PI, not AT and HCII, some mutagenesis studies suggest an equilibrium for wild-type PAI-1 where the native state is in equilibrium between α_1PI-like and AT-like states [99, 274]. The structure of the stabilized mutant bound to the somatomedin domain of VN revealed the mechanism of stabilization of the native state through a blocking of the expansion of β-sheet A [269] (Fig. 3B).

PAI-1 can also inhibit APC [92] and thrombin [94, 95] in the presence of VN and/or heparin. It is not known to what extent these activities contribute to coagulation. Previously, it has been shown that APC cleaves PAI-1, inactivating the serpin [99, 105]. Recently, it has been shown that PAI-1 inhibits APC and the rate of inhibition increases in the presence of vitronectin ~300-fold [92].

Plasminogen Activator-1 in Disease-- Studies show that PAI-1 levels are sensitive to many different pathophysiological factors and increased synthesis of PAI-1 contributes to numerous cardiovascular disease states. In metabolic syndrome, both glucose and insulin increase PAI-1 synthesis in vascular endothelial and smooth muscle cells [275]. Controlling hyperglycemia in type 2 diabetes results in a decrease in PAI-1 levels. One of the clinical benefits of "statins" may be due to their decrease of PAI-1 expression and simultaneous increase of tPA expression, altering the balance of the Fibrinolytic Pathway [276, 277]. Circadian clock proteins, including CLOCK, BMAL, and CRY, regulate PAI-1 gene expression which may explain the increased risk of adverse cardiovascular events in the

morning [278]. Inhibition of nitric oxide synthase induces PAI-1 expression, which contributes to the development of perivascular fibrosis [116]. Increased PAI-1 levels are associated with coronary artery disease and myocardial infarction. However, studies examining the association of cardiovascular disease with a polymorphism within the PAI-1 promoter region (4G/5G) which increases the expression PAI-1 are controversial [279]. Stents with rapamycin and paclitaxel are used in interventional cardiology due to the antiproliferative effects of these drugs [280, 281]. These stents have been shown to be associated with an increased risk of thrombosis and it is speculated that this may be due to an up-regulation of PAI-1 by rapamycin and paclitaxel [282]. There is also strong evidence for a role of PAI-1 in cancer metastasis independent of its protease inhibitory activity [171, 283, 284].

Effectors of Plasminogen Activator Inhibitor-1 Activity and Synthesis-- Physiological levels of PAI-1 provide crucial regulation of fibrinolysis, yet excess levels contribute to disease. Numerous factors have been found that up-regulate PAI-1 expression and secretion, including inflammatory cytokines, angiotensin II, aldosterone, transforming growth factor-β, and very-low density lipoproteins [173, 285, 286]. Monoclonal antibodies have been prepared against PAI-1, which express inhibitory activity by (i) preventing the formation of the encounter complex between PAI-1 and tPA/uPA, (ii) increasing PAI's susceptibility to cleavage by target proteases, and (iii) promoting the tendency of PAI-1 to become latent and inactive [287]. Sequence-specific catalytic DNA enzyme, short-interfering RNA structures, and antisense technology have all been used to down-regulate PAI-1 levels [288]. Negatively charged organochemical compounds have been found to bind to a hydrophobic site on PAI-1 and induce polymerization and inactivation. Finally, several small molecules

(XR5118, ZK4044, and PAI-039) have been developed to inhibit PAI-1 activity by either reduction of accessibility to the RSL, by promoting a latent-like state or by favoring a substrate-like conformation [289-291] [292-297].

Closing Statement

In this State of the Art manuscript, we have described our current knowledge of serpins that regulate hemostasis and fibrinolysis. Utilizing the "suicide substrate" mechanism unique to serpins AT, HCII, ZPI, α_1PI, PCI, α_2AP and PAI-1 provide rapid and specific inhibition of the activated serine proteases in the Coagulation, Protein C and Fibrinolytic Pathways. These pathways are not single independent systems, but they represent a dynamic balance between procoagulant, anticoagulant, profibrinolytic and antifibrinolytic states with serpins playing multiple and sometimes conflicting roles. While we have learned a considerable amount about their physiologic control, their structure, related activities and regulation by local cofactors, much is left to be understood. Of note, there is deciphering the primary physiologic roles of HCII and PCI, resolving the crystal structures of ZPI and α_2AP, and learning more about the non-hemostatic functions of AT and PAI-1. Continued research with the less-studied serpins such as ZPI and α_2AP will undoubtedly provide useful information about control of hemostasis and fibrinolysis and of serpins in general. The serpins described in this paper have a multitude of functions, which under some circumstances contribute to disease, but which often can be maniupulated for the benefit of medicine. Therefore, it is of paramount importance that we continue the investigations of serpins in thrombosis, hemostasis and fibrinolysis.

Chapter 3

Activated Protein C Promotes Breast Cancer Cell Migration Through Interactions with EPCR and PAR-1

with Lea M. Beaulieu and Frank C. Church, in (2007) ***Exp. Cell Res***. 313(4): 677-687

Summary

Activated protein C (APC) is a serine protease that regulates thrombin (IIa) production through inactivation of blood coagulation factors Va and VIIIa. APC also has non-hemostatic functions related to inflammation, proliferation, and apoptosis through various mechanisms. Using two breast cancer cell lines, MDA-MB-231 and MDA-MB-435, we investigated the role of APC in cell chemotaxis and invasion. Treatment of cells with increasing APC concentrations (1-50 mg/mL) increased invasion and chemotaxis in a concentration dependent manner. Only the active form of APC increased invasion and chemotaxis of the MDA-MB-231 cells when compared to 3 inactive APC derivatives. Using a modified "checkerboard" analysis, APC was shown to only affect migration when plated with the cells; therefore, APC is not a chemoattractant. Blocking antibodies to endothelial protein C receptor (EPCR) and protease activated receptor-1 (PAR-1) attenuated the effects of APC on chemotaxis in the MDA-MB-231 cells. Finally, treatment of the MDA-MB-231 cells with the proliferation inhibitor, Na Butyrate, showed that APC did not increase migration by increasing cell number. Therefore, APC increases invasion and chemotaxis of

cells by binding to the cell surface and activating specific signaling pathways through EPCR and PAR-1.

Introduction

Activated protein C (APC) is a liver-derived serine protease [298], circulating in the blood as a zymogen [299, 300]. When the blood vessel wall is injured, the coagulation pathway is initiated to produce thrombin (IIa). IIa will cleave fibrinogen to fibrin, which polymerizes to form a clot. Circulating zymogen protein C (PC) localizes to the endothelial cell surface at the site of injury by binding to Endothelial Protein C Receptor (EPCR) [154]. EPCR facilitates the interaction of PC with the receptor complex of IIa:thrombomodulin (TM) on the endothelial cell surface, properly aligning PC with IIa [156]. IIa cleaves PC to generate activated protein C (APC) [301, 302]. Once it releases from EPCR, APC will inactivate coagulation factors Va and VIIIa [301, 303], in the presence of a cofactor, Protein S (PS) [304, 305]. A reduction of factors Va and VIIIa around the area of the clot prevents any further generation of IIa and effectively helps regulate hemostasis [44, 46]. Therefore, a key role of the protein C system is to control fibrin clot formation from expanding into the vessel without jeopardizing hemostasis and vessel repair.

Recent work has focused on other functions of the protein C system beyond hemostasis. Animal models using blocking antibodies to EPCR showed an important regulatory role of the protein C system with inflammation and coagulation related to *E. coli* infection [53]. In the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis Study (PROWESS), patients diagnosed with sepsis and acute organ dysfunction were treated with recombinant human APC, which resulted in a mortality reduction of 19.4% [47, 48, 50] [51, 58]. It was initially believed that APC increased patient

survival through its anticoagulant properties, reducing microvasculature thrombi formation and promoting blood flow. However, through *in vitro* and *in vivo* models, APC has been shown to not only regulate coagulation in the microvasculature but also affect inflammation, apoptosis, proliferation, and angiogenesis. APC inhibits apoptosis through upregulation of anti-apoptotic Bcl-2 [54, 60, 64], and down-regulation of p53, Bax [54, 64], and caspases 3 [54, 64, 65], 8, and 9 [64, 65] all through interactions with EPCR and protease activated receptors (PAR). Using a murine focal ischemic stroke model, human and mouse APC treatment restored blood flow, reduced infarct volume and edema, eliminated neutrophil infiltration, and reduced fibrin deposition [54, 64] [65, 66, 306], all mediated through EPCR [54], PAR-1 [54, 64], and PAR-3 [64].

APC has also been shown to have a role in both cell migration and proliferation. APC/PC reduces the migration of immune cells towards chemoattractants through its binding to EPCR [58] and to epidermal growth factor receptor (EGFR) [59]. In a concentration dependent manner, APC increases human umbilical vein endothelial cells (HUVEC) proliferation through activation of the MAPK, PI3K, and eNOS pathways primarily via binding to EPCR rather than to PAR-1 [79]. In a mouse cornea angiogenesis assay, APC was shown to increase angiogenesis via the eNOS pathway [79]. APC increases proliferation and migration in keratinocytes by increasing the expression and activation of matrix metalloprotease-2 (MMP-2) [77]. Further, using a rat wound healing model, APC treatment reduced neutrophil infiltration and increased angiogenesis through MMP activation [52]. The initial studies looking at APC and cancer cell migration used ovarian cancer and choriocarcinoma cells in a transwell invasion assay [67]. These studies showed that APC formed a stable complex with PAI-1, which allowed for uPA to activate extracellular matrix

proteases and increase invasion [67]. From these results, it is hypothesized that APC has a regulatory role in migration that is mediated through various pathways both intracellularly and extracellularly.

The first goal of this study was to characterize the effects of APC on cell invasion and chemotaxis using two breast cancer cell lines, MDA-MB-231 and MDA-MB-435, with transwell assays. The second goal was to study the interaction of APC with EPCR and PAR-1, receptors involved in mediating other non-hemostatic effects of APC on expression of apoptotic genes and cell proliferation [57, 63] [54]. The following results show APC increases chemotaxis and invasion when incubated with the cancer cells, which requires active protease and is dependent on an interaction with both EPCR and PAR-1.

Materials and Methods

Cell Culture

MDA-MB-231 and MDA-MB-435, two breast cancer cell lines, were grown in Minimum Essential Media (Gibco) with 10% FBS (Gemini), 1% sodium pyruvate (Gibco), and 1% antiobiotic/antimycotic (Gibco). Both of these cell lines were obtained from the UNC Lineberger Comprehensive Cancer Center Tissue Culture Core Facility. Human Umbilical Vein Endothelial Cells (HUVEC) were grown in Endothelial Basal Media (EBM) with 2% FBS, 0.4% bovine brain extract with heparin, 0.1% hydrocortisone, 0.1% human epidermal growth factor, and 0.1% Gentamicin/Amphotericin B-1000 (Cambrex). HUVEC were obtained from Cambrex. All cell lines were maintained at 37^{o}C and 5% CO_2.

Transwell Invasion and Chemotaxis Assay

Invasion and chemotaxis [307, 308] were assayed using a transwell system in which cells are plated onto a porous membrane insert (pore diameter of 8 μm; BD Biosciences) and migrate through the pores to the underside towards a chemotactic agent placed in the well below. The inserts were either uncoated, indicative of chemotaxis, or coated with 1.67 μg/mL of Matrigel (BD Biosciences) per insert, indicative of invasion. 50,000 cells were plated per insert in serum free media (SFM) with 0.1% BSA, 1% sodium pyruvate, and 1% antiobiotic/antimycotic. The chemotactic agent for the breast cancer cells in the well below was media containing 10% FBS, 1% sodium pyruvate, and 1% antibiotic/antimycotic.

Cells were plated with increasing concentrations of recombinant human APC (also known as Xigris®; 1-50 μg/mL; Eli Lilly and Co.), human 5-dimethylaminonaphthalene-1-sulfonyl-glutamylglycylarginyl chloromethyl ketone (DEGR)-APC (10 μg/mL; Haematologic Technology), human PC (10 μg/mL; Haematologic Technology), S195A recombinant human APC (10 μg/mL; provided by Dr. Alireza R. Rezaie, St. Louis University, St Louis, MO), 5nM human α-IIa (Haematologic Technology), and 50 nM hirudin (Centerchem). Cells were pretreated at room temperature for 15 minutes with mouse serum IgG (4 or 30 μg/mL; Sigma), JNK 1494 anti-EPCR antibody (4 μg/mL; provided by Dr. Charles T. Esmon, OMRF, Oklahoma, OK), wede15 (20 μg/mL; Immunotech) and atap2 (10 μg/mL; Zymed) (wede15 and atap2 are anti-PAR-1 antibodies) prior to treatment with APC or α-IIa. Cells were also pretreated for 1 h at room temperature with 10 mM Na Butyrate (Sigma) prior to treatment with APC. The MDA-MB-231 breast cancer cells were incubated at 37°C, 5% CO_2 for 12 h for the chemotaxis assay and 24 h for the invasion assay. The MDA-MB-435 breast cancer cells were incubated for 24 h for the chemotaxis assay and 48 h for the invasion assay.

The MDA-MB-231 cells were also used in modified "checkerboard" invasion and chemotaxis assays in order to determine if APC could also serve as a chemotactic agent. APC (10 μg/mL) in either SFM with 0.1% BSA or in media containing 10% FBS was plated with the cells onto Matrigel coated or uncoated inserts. These conditions were alternated with APC (10 μg/mL) in either SFM with 0.1% BSA or media containing 10% FBS placed in the well as the chemotactic agent. "Checkerboard" invasion and chemotaxis assays were incubated for 24 h.

After incubation, cells that did not migrate through to the underside of the membrane were removed with a cotton-tipped applicator. The cells that migrated through to the underside of the membrane were fixed in 100% methanol, washed in 1X PBS, and stained with a nuclear fluorescence dye, Hoechst (1:20000 in 1X PBS; Molecular Probes). Membranes were then cut out from the inserts and mounted on glass sides in a 50% glycerol solution. Using a fluorescent microscope, the total number of cells were counted in 4-400X fields, averaged, and compared to no treatment [307, 308]. Each individual experiment was done in duplicate and data shown represents at least 3 experiments.

APC Activity Assay

APC activity was assessed by measuring the cleavage of an APC specific chromogenic substrate. Excess conditioned media from setting up the transwell invasion and chemotaxis assays was centrifuged for 5 minutes at 500 x g to remove cells. Fifty μL of the conditioned media was added to the APC substrate, Pefachrome Pca (final concentration of 0.3 mM; Centerchem) in a 96-well plate coated with 0.2% BSA. Change in absorbance was read at 405 nm for 2 minutes. At the end of each transwell assay, 50 μL conditioned media

from the insert was added to substrate and the change in absorbance was measured. Each condition was done in triplicate and averaged.

Generation of APC on a Cell Monolayer

Assays were based on experiments done previously [156, 309-311], with modifications. Briefly, either HUVEC or MDA-MB-231 cells were grown to confluency in a 96-well plate. Cells were washed with 1X PBS and serum starved overnight. Cells were washed two times with Hanks' Balanced Salt Solution (HBSS) without phenol red. Either JNK 1494 anti-EPCR antibody (2-20 μg/mL) or mouse serum IgG (20 μg/mL) was added and incubated at room temperature for 15 minutes. Zymogen PC (100 nM) was added to the wells and incubated for an additional 15 minutes at room temperature. Finally, 2 nM α-IIa was added to each well, giving a final volume of 170 μL. The reaction was incubated for 5 minutes to 24 h. At each timepoint, 20 μL from each well was added to 5 nM hirudin (Haematologic Technologies), a specific IIa inhibitor, in a separate 96-well plate. Pefachrome Pca (0.15 mM) was added to each well and read at 405 nm for 30 minutes. Each condition was done in triplicate.

Western Blots

Cells were grown to form a confluent monolayer and washed with 1X PBS. For 24 h, cells were treated with serum free media and collected. Cell lysate or conditioned media protein concentrations were determined with a dye-binding assay (Biorad), using BSA as a standard. 25 μg of total protein for the cell lysates or 10 μg of total protein for the conditioned media were loaded per sample, run reduced on a 12% polyacrylamide gel, and

transferred to PVDF (Millipore). Membranes were probed with a mouse monoclonal anti-EPCR antibody (1:1000; JNK 1494), mouse monoclonal anti-PAR-1 (1:1000; Immunotech), rabbit polyclonal anti-Erk2 (1:1000; Santa Cruz Biotechnology), and a rabbit polyclonal anti-PAI-1 antibody (1:1000; Molecular Innovations).

Immunofluorescence

Cells were grown on Lab-Tek II chamber slides (Nunc) until they were at least 80% confluent. The cells were fixed in 2% paraformaldehyde in 1X PBS for 30 minutes at room temperature, then washed 2 times in 1X PBS. Cells were next treated with 0.2 M glycine and incubated for 20 minutes at room temperature and then washed an additional 2 times in 1X PBS. Cells were blocked in 10% goat serum in 1X PBS, 1% BSA for 30 minutes at room temperature. This was followed by incubation with either 50 µg/mL anti-EPCR (JNK 1494) or 40 µg/mL PAR-1 (Zymed) in 1X PBS, 1% BSA for 1 hour at room temperature. Negative controls were treated with buffer containing no primary antibody. Cells were then washed 5 times with 1X PBS, 1% BSA and treated with 1:20 secondary anti-mouse IgG $F(ab')_2$ fragment-R-phycoerythrin sheep antibody (Sigma) in 1X PBS, 1% BSA for 1 hour at room temperature. Cells were finally washed 5 times with 1X PBS and stored covered at 4^oC in 1X PBS until photos were taken. Photographs were taken with an Olympus DP70 Microscope Digital Camera and DP70-BSW Software using an Olympus BX51WI fluorescent microscope with a TRITC filter. Photographs were taken at a 200X magnification with the same exposure time and sensitivity levels for each cell line and each antibody.

Statistical Analysis

For each transwell experiment, conditions were done in duplicate and averaged. Experiments were repeated as indicated in the figure legends. Averages of each condition were compared to No Treatment, APC, or α-IIa. All experiments were averaged and the percentages of No Treatment were reported. Averages of the comparisons of various APC or α-IIa treatments to APC or α-IIa alone treatments were also done (not reported). Statistical analysis was performed using a one sample T-test with a normal distribution, a theoretical mean of 100, and significance of $p < 0.05$ comparing back to No Treatment, APC, or α-IIa treatment.

Results

APC Increases Breast and Endothelial Cell Invasion and Chemotaxis

Incubation of the MDA-MB-231 cells with increasing concentrations of APC (1-50 μg/mL) increased both invasion and chemotaxis 150-300% compared to no treatment (Figure 3-1A) in the transwell assays. Unexpectedly, the same effect was seen with the MDA-MB-435 cell line when treated with increasing concentrations of APC (Figure 3-1B). APC increased the MDA-MB-435 cell invasion and chemotaxis 125-375% compared to no treatment. It was previously reported that APC increased ovarian cancer and choriocarcinoma cell invasion through Matrigel only when PAI-1 was present in the culture media [67]. This suggested that APC complexed to PAI-1, allowing uPA to increase activation of extracellular proteases. APC alone was believed to have no effect in the absence of PAI-1 [67]. From this study, we hypothesized APC would only increase invasion and chemotaxis in the MDA-MB-231 cells because they express PAI-1 (inset in Figure 3-1A), and have no effect on the MDA-MB-435 cells since they do not express PAI-1 (inset in Figure 3-1A).

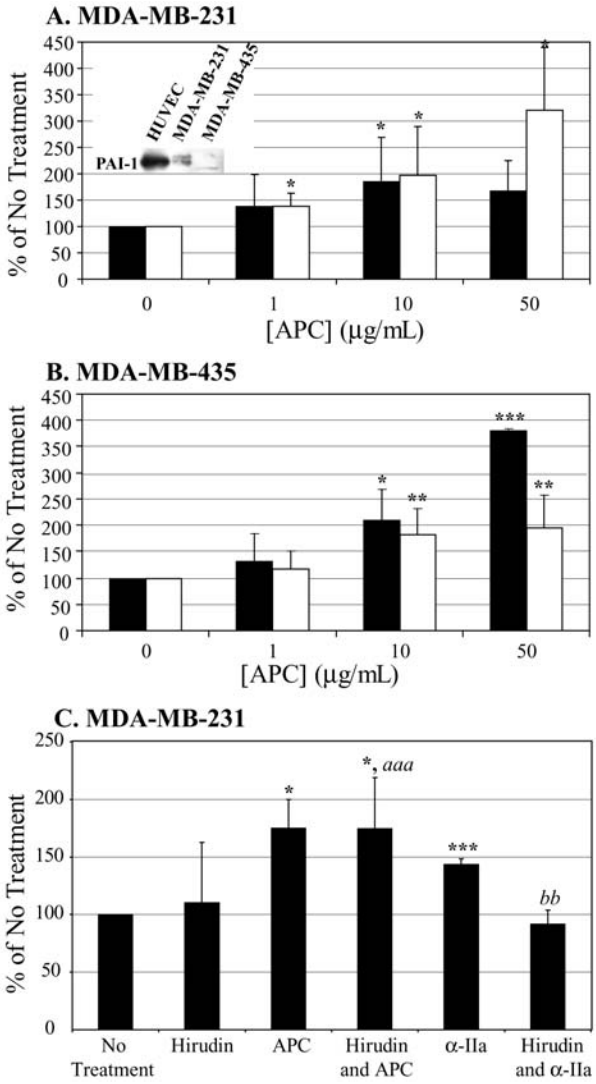

Figure 3-1. ***APC Increases Invasion and Chemotaxis of Breast Cancer Cells.*** Increasing concentrations of APC (0-50 μg/mL) are incubated with MDA-MB-231 cells (A) for 12 h transwell chemotaxis assay (white bars) and 24 h transwell invasion assay (black bars). Inset is a representative western blot showing the presence of PAI-1 in the conditioned media of HUVEC and MDA-MB-231 cells but PAI-1 is absent in the MDA-MB-435 cells. Increasing concentrations of APC were also incubated with MDA-MB-435 cells (B) for 24 h transwell chemotaxis assay (white bars) and 48 h transwell invasion assay (black bars). Cells migrate towards media containing 10% FBS as the chemotactic agent. The graphs represent the average of 8 separate experiments; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to No Treatment. (C) Hirudin (50 nM) is added with or without APC (10 μg/mL) to the MDA-MB-231 cells in a 12 h transwell chemotaxis assay. As control, 5 nM α-IIa is added with or without hirudin (50 nM) in a 12 h transwell chemotaxis assay to verify effectiveness of hirudin. Cells migrated towards media containing 10% FBS as the chemotactic agent. The graphs represent the average of 5 experiments; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to No Treatment, *aaa* $p<0.001$ compared to Hirudin Treatment, *bb* $p<0.01$ compared to α-IIa Treatment.

Because APC treatment of MB-435 cells since they do not express PAI-1 (inset in Figure 3-1A). Because APC treatment of both cell lines yielded the same result, it was concluded that APC was activating other mechanisms to increase cell invasion and chemotaxis in the transwell assay that are not dependent on the presence of endogenous PAI-1. Similar results were generated using HUVEC in the transwell assays, but with lower concentrations of APC, 0.1-10 μg/mL (data not included). These results indicate that the effects of APC on migration in the transwell assay are not isolated to cancer cells and, further, there is a common pathway among these three cell lines that is activated by APC. The remainder of the studies presented here will focus on the effects of APC on the MDA-MB-231 cells as a model system for the effects of APC on cellular invasion and chemotaxis.

We also verified that thrombin, which could potentially be present in the APC preparation, was not responsible for promoting the increase in migration seen with APC treatment. Cells were treated with hirudin, a specific thrombin inhibitor, and APC in the 12 h transwell chemotaxis assay. As seen in Figure 3-1C, hirudin alone has no effect on cell migration when plated with the cells. APC significantly increases chemotaxis of the MDA-MB-231 cells by 175% in either the presence or absence of hirudin. As a control, cells were also treated with α-IIa in the presence or absence of hirudin. α-IIa alone increases chemotaxis of the MDA-MB-231 cells by 144%. This effect is lost with α-IIa and hirudin. Therefore, the effect of APC on cellular migration is due to APC alone and not the presence of trace amounts of α-IIa.

Active Protease is Necessary to Increase Invasion and Chemotaxis of the MDA-MB-231 Cells.

It is important to determine if active protease is necessary to increase cell migration in the transwell assays. The MDA-MB-231 cells were treated with APC, inactive forms of APC, or PC in a 12 h transwell chemotaxis assay and a 24 h transwell invasion assay. Active APC (10 μg/mL) was the only protease that significantly increased cell invasion by 190% (Figure 3-2A). The addition of inactive forms of APC- DEGR-APC, active site mutant APC (S195A) and zymogen PC - all at the same concentration had no effect on cell invasion. The same results were seen in the transwell chemotaxis assay with the MDA-MB-231 cells (Figure 3-2B). APC activity was verified by measuring the rate of cleavage of an APC specific chromogenic substrate. Conditioned media was sampled at the beginning and end of the experiment to verify the activity of the active and the inactive forms of APC, as seen in Figure 3-2C and 3-2D pre- and post-experiment. These results indicate the active form of APC is necessary to increase invasion and chemotaxis in the MDA-MB-231 cells using the transwell system.

APC Increases Chemotaxis of the MDA-MB-231 Cells, but It Is Not a Chemotactic Agent.

α-IIa, another serine protease, has been shown to be both a chemotactic agent for the MDA-MB-231 cells [8] and an enhancer of the cell's response to chemotactic agents [8]. Using a modified "checkerboard" analysis, we next determined if APC could affect cell migration in the same manner as α-IIa. APC (10 μg/mL) was plated in the insert with the cells either in 10% FBS containing media or in SFM with 0.1% BSA. These conditions were varied with the conditions in the well below, where APC (10 μg/mL) was added with either 10% FBS containing media or SFM with 0.1% BSA. All assays were run for 24 h in order to give APC sufficient time as a putative chemotactic agent. In the transwell chemotaxis assay,

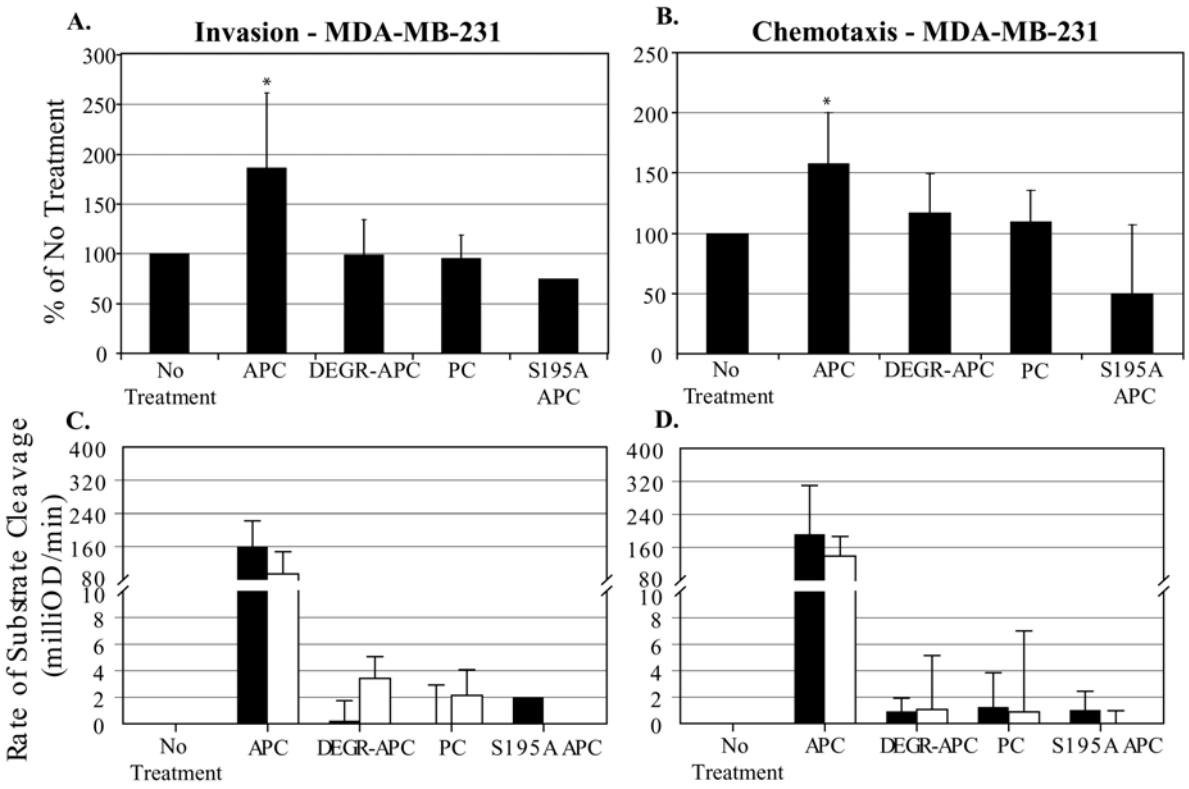

Figure 3-2. ***Active Protease is Necessary to Increase Invasion and Chemotaxis in the MDA-MB-231 Cells.*** 10 μg/mL APC, DEGR-APC, zymogen PC, and S195A APC were used in a 24 h transwell invasion assay (A) and 12 h transwell chemotaxis assay (B). Cells migrated towards media containing 10% FBS as the chemotactic agent. APC activity assays were done to verify the presence or absence of activity of each protease at the beginning (black bars) and at the end (white bars) of the transwell invasion (C) and chemotaxis (D) assays. The graphs represent the average of 4 separate experiments with the exception of S195A APC, which was done only 1-2 times due to the limited amount of protein available; *$p<0.05$ compared to No Treatment.

APC is not a chemotactic agent since there was no difference in the number of cells that migrated towards SFM, 0.1% BSA or 10% FBS containing media with or without APC (Table 3-1). When the cells were plated in 10% FBS containing media with APC, there was no difference in the number of cells that migrated either to SFM, 0.1% BSA with or without APC or 10% FBS containing media with or without APC. When cells were plated in SFM, 0.1% BSA with APC and migrated towards SFM, 0.1% BSA with or without APC, there was, again, no difference in the number of cells that migrated between the two conditions. In fact, there were very few cells that did migrate. The only difference seen with the modified checkerboard transwell chemotaxis assay was the increase in the number of cells that migrated when 10% FBS containing media was the chemotactic agent and APC was plated with the cells in SFM, 0.1% BSA. Similar results were seen in a modified checkerboard transwell invasion assay (data not shown). These results suggest that APC increases cell migration when plated with the MDA-MB-231 cells, enhancing the response of the cells to the chemotactic agent.

APC Binding to EPCR is Necessary to Increase Chemotaxis of the MDA-MB-231 Cells.

To study the role of EPCR in the pro-migratory effects of APC, we showed the presence of EPCR using both western blots of cell lysates (Figure 3-3A) and immunofluorescence staining of cell monolayers (Figure 3-3B) of HUVEC, as the positive control, and both breast cancer cell lines. A blocking antibody (JNK 1494) to the PC/APC binding site on EPCR [309-311] [156]was used in a 12 h transwell chemotaxis assay with the MDA-MB-231 cells. Initially, it was necessary to determine the optimal concentration of EPCR blocking antibody to use with the MDA-MB-231 cells. HUVEC, as a control, and

Table 3-1: ***Modified Checkerboard Analysis of APC on Chemotaxis of the MDA-MB-231 Cells***[a]

APC Concentration (μg/mL) Below Membrane	APC Concentration (mg/mL) Above Membrane			
	0 + SFM	10 + SFM	0 + 10% FBS	10 + 10% FBS
0 + SFM	5.0 ± 6.5	2.4 ± 2.3	113.4 ± 28.4	64.3 ± 33.9
10 + SFM	1.6 ± 1.7	1.9 ± 3.1	82.0 ± 29.1	104.2 ± 27.0
0 + 10% FBS	498.8 ± 112.9	590.7 ± 148.5	156.1 ± 46.2	188.1 ± 68.7
10 + 10% FBS	471.3 ± 120.9	341.7 ± 84.2	148.1 ± 42.6	144.1 ± 44.1

[a]Two different concentrations of APC (0 and 10 mg/mL) were added above or below the uncoated insert membrane in wither SFM-0.1% BSA or media containing 10% FBS. After a 24 h incubation, the number of cells that underwent chemotaxis were determined as described in the "Material and Methods". Data represents the mean number of cells that migrated ± standard deviation of 4 separate experiments, each done in duplicate.

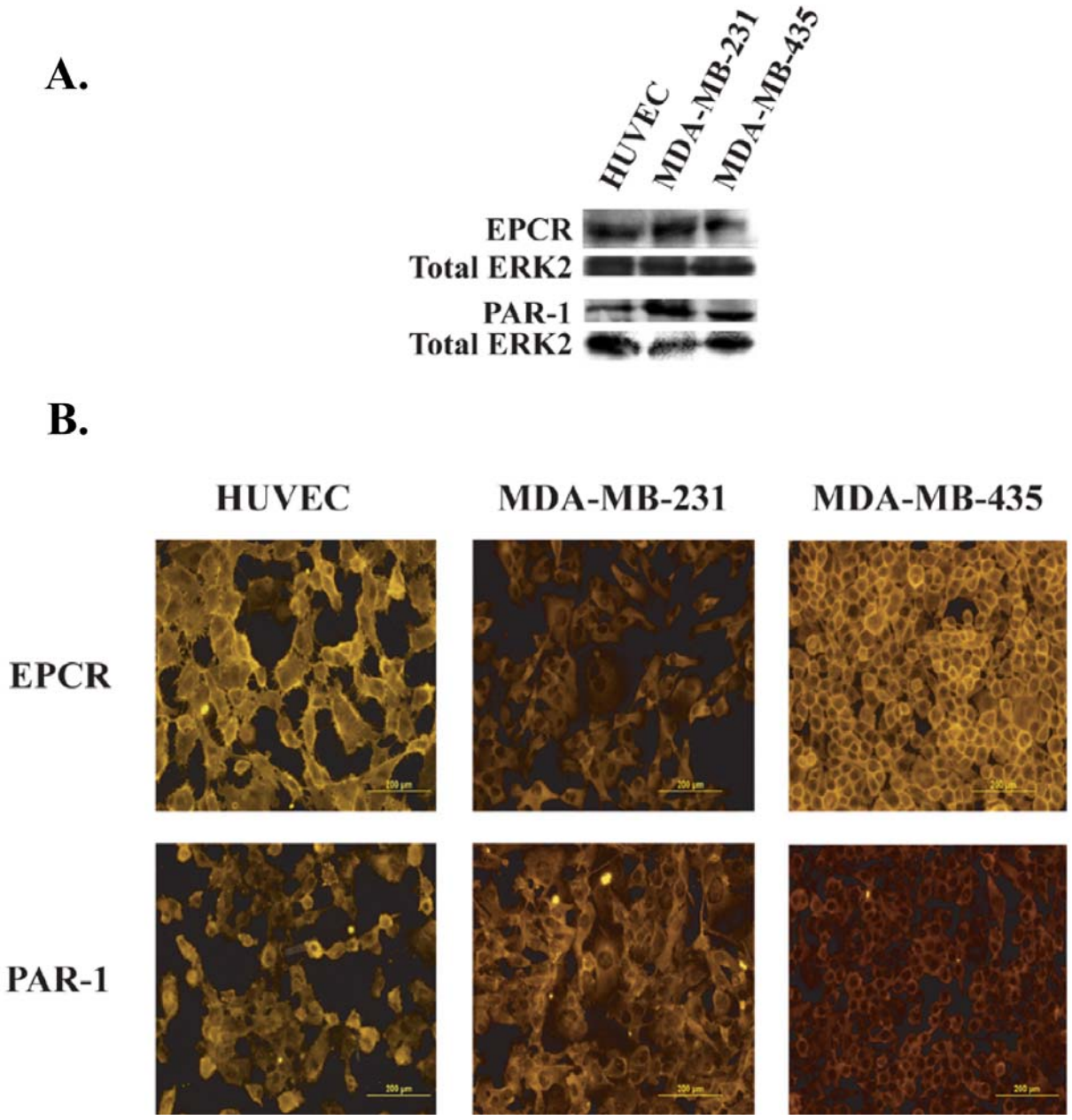

Figure 3-3. ***PAI-1, EPCR, and PAR-1 Expression in HUVEC, MDA-MB-231, and MDA-MB-435 Cell Lines.*** Representative western blots (A) showing the presence of EPCR and PAR-1 in cell lysates of HUVEC, MDA-MB-231, and MDA-MB-435 cells. Total Erk2 is used as a loading control. Immunofluorescence staining (B), as described in the Material and Methods, was done on the HUVEC, MDA-MB-231, and MDA-MB-435 cell lines to show the expression pattern of EPCR and PAR-1 on the cell surface.

MDA-MB-231 cells were treated with increasing concentrations of the EPCR blocking antibody to block the generation of APC on the cell surface [156, 309-311]. Figure 3-4A and 4B shows the generation of APC on both HUVEC and MDA-MB-231 cell surfaces increases over time in the absence and presence of control mouse IgG. The generation of APC is reduced in the presence of the anti-EPCR antibody, even at a concentration of 2 μg/mL. Reduction of APC generation was seen out to 24 h at concentrations as low as 4 μg/mL.

In the transwell chemotaxis assay, MDA-MB-231 cells were pre-incubated with 4 μg/mL control mouse IgG or anti-EPCR antibody for 15 minutes at room temperature prior to APC treatment. In Figure 3-5, there was a 150 and 130% increase in chemotaxis when the cells were incubated with 10 μg/mL APC or APC and control IgG, respectively, compared to no treatment. Neither control IgG alone nor anti-EPCR antibody alone had an effect on chemotaxis. However, anti-EPCR antibody (4 μg/mL) attenuated the effects of APC, reducing chemotaxis back to baseline. These results indicate that an interaction between APC and EPCR is necessary to increase chemotaxis in the MDA-MB-231 cells.

APC Interaction with PAR-1 is also Necessary to Increase Chemotaxis of the MDA-MB-231 Cells.

PAR-1 has been shown to be involved in mediating the non-hemostatic effects of APC [54, 63, 64, 79]. With western blots of cell lysates (Figure 3-3A) and immunofluorescence staining of cell monolayers (Figure 3-3B) of HUVEC, as a positive control, and both cancer cell lines, we verified the presence and expression of PAR-1 on the cell surface. To study the role of PAR-1 in the pro-migratory effects of APC with the MDA-

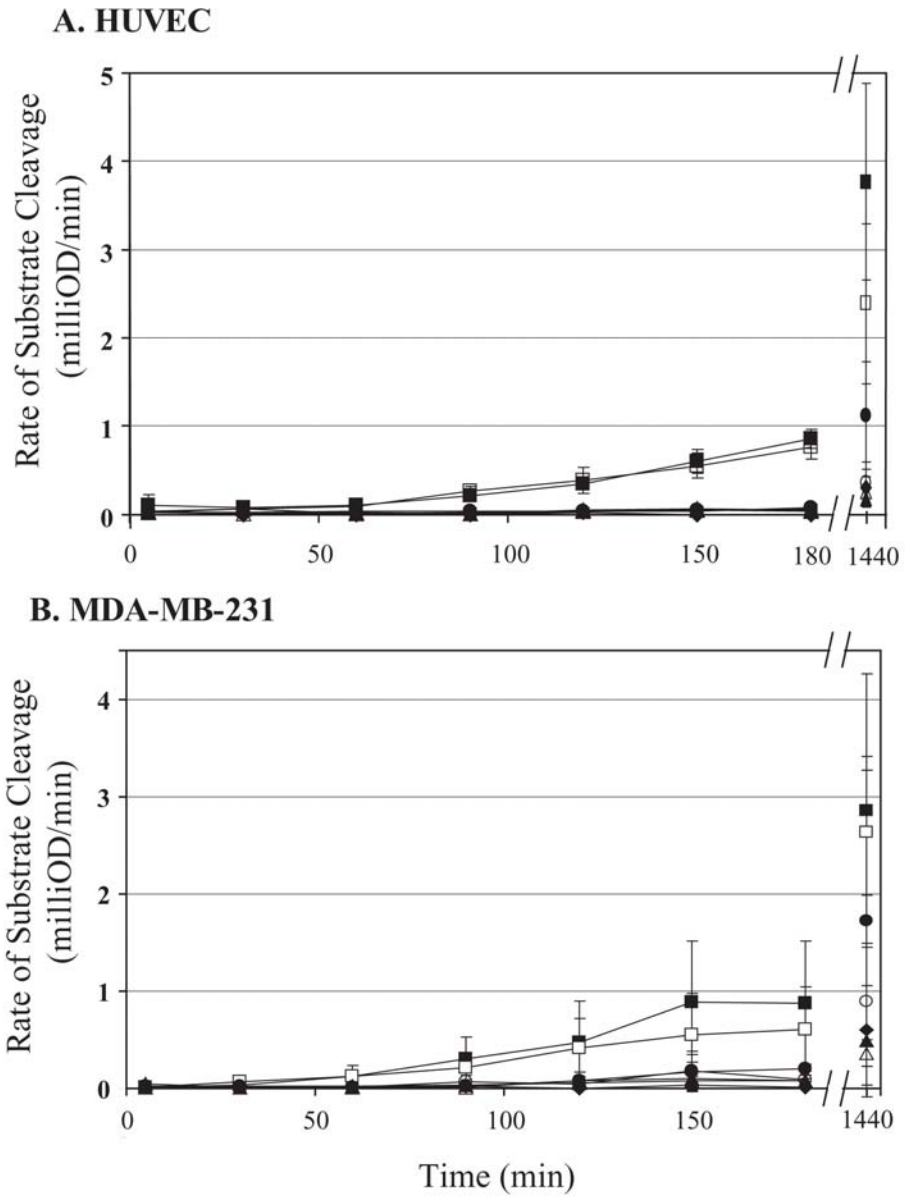

Figure 3-4. ***Generation of APC on Either HUVEC (A) or MDA-MB-231 (B) Cell Monolayer is Blocked With Anti-EPCR Antibody.*** In a 96-well plate, serum starved confluent monolayers were treated with either buffer, 20 μg/mL mouse serum IgG, or (0-20 μg/mL) anti-EPCR (JNK 1494) antibody for 15 minutes. Cells were then treated with zymogen PC (100 nM) for an additional 15 minutes. α-IIa (2 nM) was added and samples were taken from each well at various timepoints from 5 minutes – 24 h. Samples were added to hirudin (5 nM) and APC substrate (0.15 mM). The change in absorbance was read at 405 nm. The graphs represent the average of 3 separate experiments for HUVEC (A) and 4 separate experiments for MDA-MB-231 (B) done in triplicate. —◆— α-IIa only; —■— α-IIa and PC; —□— 20 μg/mL mouse serum IgG, α-IIa, PC; —▲— 2 μg/mL anti-EPCR IgG, α-IIa, PC; —△— 4 μg/mL anti-EPCR IgG, α-IIa, PC; —●— 10 μg/mL anti-EPCR IgG, α-IIa, PC; —○— 20 μg/mL anti-EPCR IgG, α-IIa, PC.

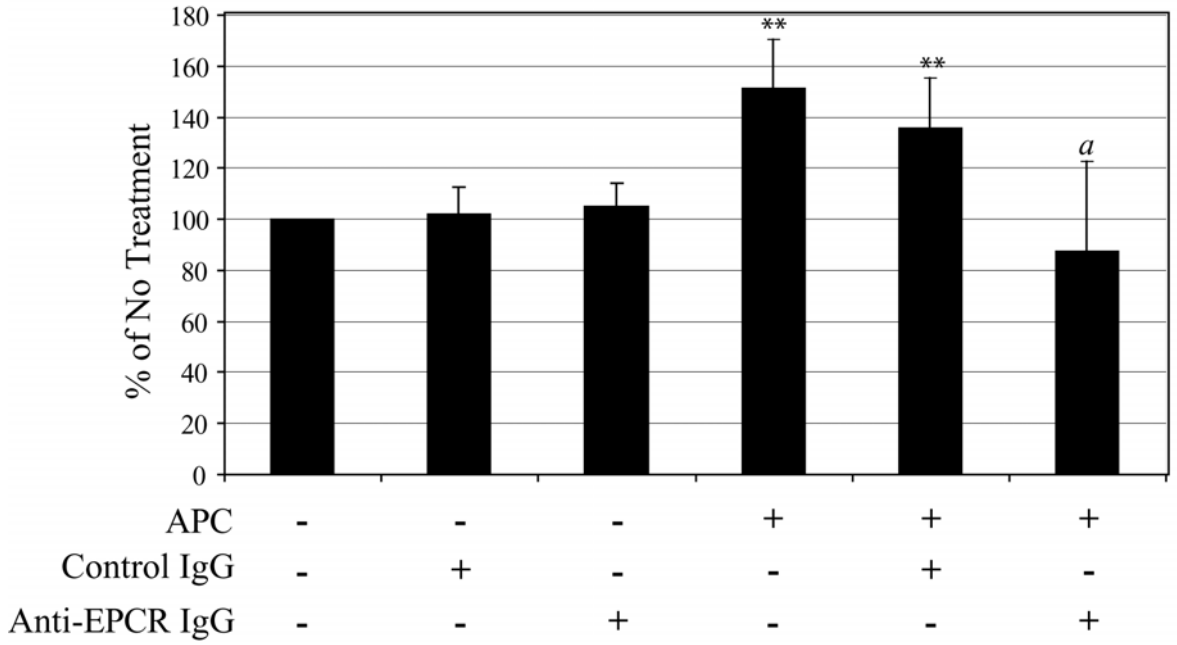

Figure 3-5. ***APC Binds to EPCR to Increase Chemotaxis in the MDA-MB-231 Cells.*** Cells were pretreated with either anti-EPCR IgG (JNK1494; 4 μg/mL) or mouse serum IgG (4 μg/mL) for 15 minutes prior to the addition of APC (10 μg/mL) in a transwell chemotaxis assay incubated for 12h. Cells migrated towards media containing 10% FBS as the chemotactic agent. The graphs represent an average of 6 separate experiments; **$p<0.01$ compared to No Treatment; *a* $p<0.01$ compared to APC treatment or APC and mouse serum IgG treatment.

MB-231 cells, two PAR-1 blocking antibodies were used in a 12 h transwell chemotaxis assay. The cells were pre-incubated with either PAR-1 blocking antibodies (wede15 and atap2) or control mouse IgG for 15 minutes at room temperature prior to APC treatment. In Figure 3-6, 10 μg/mL APC (± control IgG) increased chemotaxis approximately 150%. In the presence of PAR-1 blocking antibodies, the effects of APC was attenuated. To verify that the PAR-1 blocking antibodies were properly blocking the binding of the protease to the receptor, cells were also treated with 5 nM α-IIa, a known ligand of the PAR-1 receptor that will affect migration of the MDA-MB-231 through its activation [8, 312, 313]. α-IIa alone or α-IIa with control IgG increased chemotaxis approximately 150%, and this increase was attenuated in the presence of the PAR-1 blocking antibodies. These results suggest that binding of APC to EPCR and PAR-1 are necessary to increase chemotaxis in the MDA-MB-231 cells.

APC Does Not Increases Chemotaxis of the MDA-MB-231 Cells By Increasing Cell Number.

Na Butyrate was used as an inhibitor of proliferation to determine if APC increases the number of cells that migrate in the transwell assays by increasing proliferation. Treatment of the MDA-MB-231 cells with 10 mM Na Butyrate in 12 h reduces cell proliferation but does not induce apoptosis (data not shown). In a 12 h chemotaxis assay (Figure 3-7), Na Butyrate reduces chemotaxis compared to no treatment as expected due to differentiation and inhibition of undefined pathways in breast cancer cells. When APC is added with Na Butyrate, APC increases cell chemotaxis over Na Butyrate treatment alone (approximately 150% increase over Na Butyrate treatment alone), which is the approximately the same ratio as APC treatment alone compared to no treatment (approximately 130% increase over No

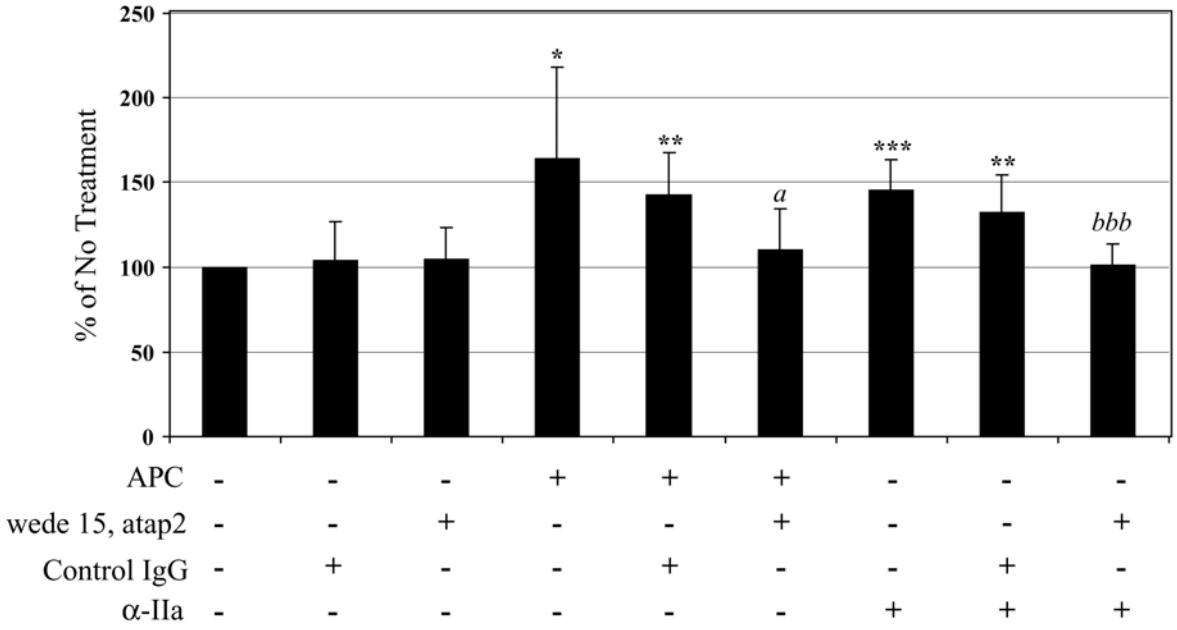

Figure 3-6. ***Interaction with PAR-1 is Necessary for APC to Increase Chemotaxis in the MDA-MB-231 Cells.*** Cells were pretreated with either PAR-1 blocking IgG (10 μg/mL atap2 and 20 μg/mL wede15) or 30 μg/mL mouse serum IgG for 15 minutes prior to addition of APC (10 μg/mL) or α-IIa (5 nM) in a transwell chemotaxis assay incubated for 12 h. Cells migrated towards media containing 10% FBS as the chemotactic agent. The graphs represent the average of 8 separate experiments for studies done with APC and 7 separate experiments done with α-IIa; *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to No Treatment; a $p<0.05$ compared to APC treatment or APC and mouse serum IgG treatment; *bbb* $p<0.05$ compared to α-IIa treatment or α-IIa and mouse serum IgG treatment.

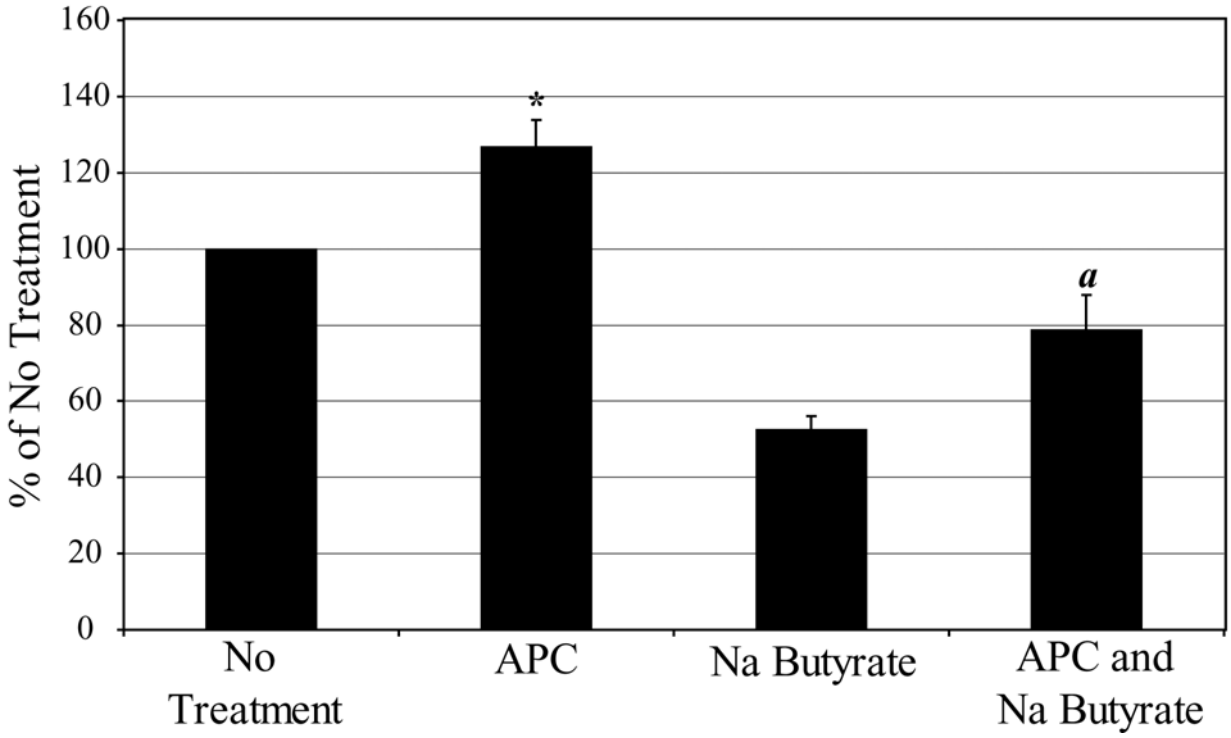

Figure 3-7. ***APC Does Not Increase Migration in the MDA-MB-231 Cells by Increasing Cell Number.*** Cells were pretreated for 1 h with 10 mM Na Butyrate prior to addition of APC in the transwell chemotaxis assay incubated for 12 h. Cells migrated towards media containing 10% FBS as the chemotactic agent. The graph represents an average of 3 separate experiments; *$p<0.05$ compared to No Treatment; *a* $p<0.05$ compared to Na Butyrate treatment.

Treatment). Therefore, these results show APC increases chemotaxis by affecting other pathways involved in migration and not by increasing proliferation of the cells.

Discussion

Historically, studies on the protein C system have focused on the role of APC as an anticoagulant. However, there have been recent studies, such as the PROWESS trial [48], on the anti-inflammatory and anti-apoptotic role of APC. Many recent reports have looked into these non-hemostatic roles of APC *in vivo* and *in vitro*. In this study, we showed that APC affects breast cancer cell migration in the transwell invasion and chemotaxis assay. APC, in a concentration dependent manner, increases invasion and chemotaxis in both the MDA-MB-231 and MDA-MB-435 breast cancer cell lines. Previously, it had been published that APC increased cancer cell invasion through Matrigel coated membranes only in the presence of PAI-1 [67]. It was hypothesized that APC complexed with PAI-1 [67]. This stable complex removed active PAI-1 from the cell environment and allowed uPA to activate plasminogen to plasmin. Plasmin can go on to activate MMP-9 [71] and MMP-2 [72]. uPA can also activate MMP-2 and regulate MMP-9 expression [68-70]. Since the MDA-MB-435 cell line does not express PAI-1 but still was affected by APC treatment in the transwell assay, we concluded that another mechanism is activated by APC to increase cell migration.

As previously reported, IIa is approximately 10^4 fold more potent that APC at activating PAR-1 [314]. Cells were treated with hirudin in the absence and presence of APC to assess if trace amounts of IIa were influencing the APC-induced transwell migration assays. We showed that APC alone was able to increase chemotaxis of the MDA-MB-231 cells and this was not due to IIa. The amount of APC needed to promote cellular migration is

greater than physiological protein C blood plasma levels (~ 4μg/mL). In the PROWESS trial, sepsis patients that were treated with APC have a steady state level of 45 ng/mL [314]. However, our overall goal was to characterize the mechanism of APC to promote cell migration and we used similar APC concentrations as in previous *in vitro* studies (0.5-50 μg/mL) [54, 67, 79]. Interestingly, levels of APC used in HUVEC experiments are closer to physiological concentrations of APC (0.1-10 μg/mL) (data not shown).

As part of the anti-inflammatory action of APC, it had been previously reported that either APC or zymogen PC, through EPCR and EGFR, inhibits lymphocyte migration [58, 59]. Using zymogen PC, chemically inactivated DEGR-APC, and an active site mutant of APC (S195A) with the MDA-MB-231 cancer cells, one finding of our study showed that the active protease was needed to increase cell migration. Therefore, unlike the inhibitory role of APC with lymphocytes, the pro-migratory role of APC in the MDA-MB-231 cells requires the active site of the protease, most likely to bind and activate receptors, such as PAR-1 [54, 57, 63, 64, 79], and to activate extracellular matrix proteases, such as MMP-2 and MMP-9 [74, 77]. It is possible that when bound to EPCR, APC may undergo modifications to its macromolecular substrate recognition. Thus, APC bound to EPCR could promote migration and invasion through both activation of signaling pathways and activation of extracellular proteases.

Another finding in our study was that APC itself is not a chemotactic factor for the MDA-MB-231 cells, but it promoted chemotaxis and invasion through direct interactions with the cells. By contrast, an analogous serine protease, IIa, can either promote migration when treated with the cell or when used as a chemotactic factor. Using the transwell invasion and chemotaxis assay with the MDA-MB-231 cells and altering the treatment of APC from

the insert to the well, we found that APC must interact with the cells directly to allow for the increase in migration. Our results parallel what is known about uPA as a mediator of cell migration. This serine protease is not considered a chemotactic factor [315, 316]. When bound to uPAR, uPA is able to promote both cell migration and invasion. Therefore, APC does not act as a chemotactic agent, but functions similarly to uPA and not IIa.

Past studies have indicated that APC is either activating proteases that degrade the extracellular matrix [52, 77] and/or activating receptor(s) that initiate signaling pathways to increase invasion and chemotaxis. Other studies have shown that the ability of APC to interact with EPCR and PAR-1 is a critical aspect of the mechanism responsible for altering inflammation, proliferation, and apoptosis [54, 59, 64-66, 77, 79, 306]. We found that the relationship of APC, EPCR, and PAR-1 is similar to that shown in anti-apoptotic [54, 63] and anti-inflammatory [54] studies, where binding and activating of both receptors by APC is important to initiate the effects of APC on cell migration. Using blocking antibodies to both of these receptors, we showed that APC must bind to EPCR and PAR-1 in order to increase chemotaxis in the MDA-MB-231 cells. Blocking one or the other receptor completely attenuated the effects of APC on the MDA-MB-231 cells. We hypothesize that APC binds to EPCR, which localizes the protease and aids in the interaction of APC with PAR-1. Upon activation of PAR-1, the heterodimer of EPCR and PAR-1 initiates signaling pathways that increase MDA-MB-231 chemotaxis. It is important to note that this heterodimer pair is what allows for the unique effect of APC on the cell and distinguishes it from the effects of IIa on the cell. IIa signaling through PAR-1 is the opposite of APC in many aspects, such as IIa promotes both apoptosis and inflammation.

There are other studies that describe slight variances for the requirements of APC, EPCR, and PAR-1 to modify cell responses, suggesting either some diversity in the response or some cellular adaptation to APC as a modulator of cell function. In one setting it was shown that APC binding to EPCR is necessary for increases in HUVEC proliferation while PAR-1 activation by APC is only important for initial signaling events and not in maintaining the effect [79]. In a different setting, APC binding to EPCR and EGFR was critical for lymphocyte migration, not any PARs [58, 59]. It is still possible that other receptors, including EGFR [59], have a role in the effects of APC on MDA-MB-231 cell migration. Additional studies on the interactions of APC and the cell surface are needed to further our understanding of how APC affects various cellular processes, including migration.

Na Butyrate has been used as an inhibitor of proliferation in the MDA-MB-231 cells [317, 318]. Na Butyrate inhibits histone deacetylase, increases p21 expression, and decreases cyclin D gene expression [317] resulting in an arrest of the cell in G2 [318]. Na Butyrate also induces cell differentiation [317], as shown with an increase in lipid accumulation, and a reduction in migration across multiple extracellular matrices [319]. We found that unlike the effects of APC on HUVEC proliferation, in breast cancer cells, binding to EPCR and activation of PAR-1 by APC increases migration but not proliferation. APC is able to increase MDA-MB-231 cell migration over Na Butyrate treatment alone by the same ratio as APC treatment compared to control. Further, the pathways activated by Na Butyrate [319, 320] are not the same pathways utilized by APC to increase migration in these cells. Other pathways, such as MAPK and PI3K pathways [79], that have been implicated in the mechanism by which APC increase HUVEC proliferation may have a role in increasing migration in the MDA-MB-231 cells. Within the context of the MDA-MB-231 cells as a

model system of cell migration, our results imply that in the presence of APC a pro-migratory process is initiated that is not dependent on cell proliferation.

Chapter 4

ACTIVATED PROTEIN C INDUCES CELL MOTILITY THROUGH ENDOTHELIAL CELL PROTEIN C RECEPTOR (EPCR), PAR-1, EGFR, MMP-2, AND MMP-9 INTERACTIONS.

with Lea M. Beaulieu , Mark W. Gramling, and Frank C. Church

Submitted for Publication

Summary

Activated protein C is an anticoagulant serine protease that regulates hemostasis. Activated protein C also has several non-hemostatic functions related to inflammation, proliferation, apoptosis, and migration. Using the MDA-MB-231 breast cancer cell line as a model system, we investigated the mechanism by which activated protein C promotes chemotaxis and invasion. An active site inhibitor of urokinase and blocking antibodies to both urokinase and plasminogen activator inhibitor-1 did not alter the ability of activated protein C to increase MDA-MB-231 cell invasion. However, inhibitors to matrix metalloproteases-2 and -9 significantly reduced the ability of activated protein C to promote MDA-MB-231 invasion. Activated protein C-promoted cellular invasion is also attenuated in the presence of blocking antibodies to endothelial cell protein C receptor, protease activated receptor-1, and epidermal growth factor receptor. Cells treated with pharmacological inhibitors showed that activated protein C increased migration through the mitogen-activated protein kinase and the phosphatidylinositol 3 kinase pathways, but not the c-Jun NH2-

terminal kinase pathway. Our results suggest that activated protein C promotes cell motility (i) by binding to endothelial cell protein C receptor, (ii) by activating both protease activated receptor-1 and epidermal growth factor receptor to interdependently activate the phosphatidylinosital 3 kinase and mitogen-activated kinase pathways intracellularly, and (iii) by interacting with matrix metalloproteases-2 and -9 to increase matrix degradation extracellularly.

Introduction

Activated protein C (APC) is a liver-derived serine protease [298], which circulates in the blood as a zymogen [299, 300], with known functions *in vivo* as an anticoagulant. Zymogen protein C (PC) is localized to the endothelium by endothelial cell protein C receptor (EPCR) [154]. Upon activation by thrombin (IIa) bound to thrombomodulin (TM) [156, 302] on the endothelial cell surface, APC proteolytically inactivates factors Va and VIIIa [301, 303] in the presence of protein S (PS) [304, 305]. The importance of this pathway is seen in both mouse models and human disease. $PC^{-/-}$ mice do not survive 24 h after delivery due to formation of thrombi in the brain and liver, resulting in a consumption of coagulation factors and hemorrhage [321]. In humans, severe thrombophilia occurs due to deficiencies in PC or PS or due to a mutation in factor Va that prevents its inactivation by APC, known as Factor V Leiden [322].

In the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis Study (PROWESS), patients diagnosed with sepsis and acute organ dysfunction were treated with recombinant human APC, resulting in a mortality reduction of 19.4% [48]. This study and others show APC has anti-inflammatory activity [47, 48, 50, 58, 59]. APC inhibits

apoptosis through upregulation of anti-apoptotic Bcl-2 [54, 60, 64], and down-regulation of p53, Bax [54, 64], and caspases 3[54, 64, 65], and 8 [64, 65] all dependent on interactions with EPCR and protease activated receptors (PARs). In a murine focal ischemic stroke model, human and mouse APC treatment restores blood flow, reduces infarct volume and edema, eliminates neutrophil infiltration, and reduces fibrin deposition [54, 64-66, 306]. These *in vivo* neuroprotective effects of APC are shown to be mediated through EPCR [54], PAR-1 [54, 64], and PAR-3 [64].

APC is shown to have a role in regulating migration of various cell types. Studies done by Kobayashi, *et al*. (1994) originally report APC increases invasion of ovarian cancer and choriocarcinoma cells only in the presence of the serine protease inhibitor (serpin), plasminogen activator inhibitor-1 (PAI-1). They suggested that APC forms a stable complex with PAI-1, thereby removing a potent inhibitor of uPA from the system [67]. Then free uPA can subsequently activate plasminogen to plasmin, an extracellular matrix (ECM) serine protease to promote invasion. uPA is also known to activate matrix metalloprotease (MMP)-2 and to regulate MMP-9 expression [68] [69, 70], which are zinc-dependent proteases that degrade the ECM. Plasmin can also activate both MMP-9 [71] and MMP-2 [72]. Previously, APC has been shown to activate MMP-2 from an intermediate form to a fully active protease [73, 74] [75, 76]. *In vitro* cell culture assays with keratinocytes show that APC promotes cell migration by increasing both the expression and activation of MMP-2 [52, 77]. In HUVEC, APC increases proliferation in a concentration dependent manner through activation of the mitogen activated protein kinase (MAPK), phosphatidylinositol 3 kinase (PI3K), and endothelial nitric oxide synthase (eNOS) pathways primarily via binding to EPCR rather than to PAR-1 [79]. With MDA-MB-231 breast cancer cells, the active form of APC increases

migration in a concentration dependent manner by binding to EPCR and activating PAR-1, priming the cell to respond to a chemotactic factor [323]. As part of its anti-inflammatory actions, APC/PC reduces the migration of immune cells towards multiple chemoattractants through its binding to both EPCR [58] and to epidermal growth factor receptor (EGFR) [59]. *In vivo,* APC promotes angiogenesis and wound healing in a rat skin healing model through a reduction in inflammation, an increase in VEGF expression, and MMP-2 activation [52]. In an *in vivo* mouse cornea angiogenesis assay, APC promotes angiogenesis via the eNOS pathway [79].

Previous studies suggest that APC regulates cell motility through various mechanisms. The first potential mechanism involves the extracellular manipulation of the plasminogen activation system by APC, which includes uPA and PAI-1. The second possible mechanism implicates the activation of MMPs by APC to increase motility. The third potential mechanism relies on the activation of intracellular signaling pathways, such as the MAPK and PI3K pathways, through EPCR, PAR-1, and/or EGFR. The results described here provide evidence that APC promotes cell motility by binding to EPCR, activating PAR-1 and EGFR to promote intracellular signaling through MAPK and PI3K pathways, and by extracellular interactions with MMP-2 and -9 to promote ECM degradation.

Experimental Procedures

Cell Culture

Assays were performed using the MDA-MB-231 breast cancer cell line obtained from the University of North Carolina – Chapel Hill Tissue Culture Facility. These cells were maintained in Minimum Essential Media (MEM; Gibco) with 10% fetal bovine serum (FBS;

Sigma), 1% sodium pyruvate (Gibco), and 1% antibiotic/antimycotic (Gibco). Cells were kept in an incubator at 37 ^{o}C, 5% CO_2.

Transwell Invasion and Chemotaxis Assays

Migration was evaluated as previously described using the transwell invasion and chemotaxis assays [323]. Human recombinant APC (also known as Xigris®; Eli Lilly and Co.) was plated with the cells. Blocking antibodies used to study the potential receptors bound by APC were also plated with the cells and allowed to incubate at room temperature for 15 min prior to the addition of APC. These antibodies include mouse EPCR antibody (provided by Dr. Charles T. Esmon, OMRF, Oklahoma, OK), mouse PAR-1 antibodies (atap2 and wede15; Zymed and Immunotech, respectively), mouse EGFR antibody (Upstate), and mouse serum IgG (Sigma). Blocking antibodies used to study the role of the plasminogen activation system, including goat PAI-1 antibody (American Diagnostica), mouse uPA antibody (American Diagnostica), and serum mouse or goat IgG (Sigma), were plated with the cells and allowed to incubate at room temperature for 1 h prior to the addition of APC. Amiloride, an inhibitor of uPA, was also plated with the cells and preincubated for 1h prior to the addition of APC. Pharmacological inhibitors that block specific signaling pathways were plated with the cells and also allowed to preincubate at room temperature for 1 h prior to treatment with APC. These compounds include PD 98059 (Biomol), LY 294002 (Biomol), SP 600125 (Biomol), and DMSO alone. Finally, inhibitors for MMPs, GM 6001 (Biomol), SB-3CT (Biomol), and DMSO, were plated with the cells and preincubated for 15 min prior to the addition of APC. To determine if selective inhibitors of MMPs were affecting APC activity levels, conditioned media from the transwell assays were tested for

APC activity using an APC-specific chromogenic substrate (final concentration of 0.15 mM; Centerchem) as previously described [323].

In each experiment, conditions were done in duplicate and averaged. Experiments were averaged together and graphed as a comparison to No Treatment, set at 100%.

Western Blots

25 μg total protein from cell lysates created in complete RIPA buffer were run on 12% SDS-PAGE gels and transferred onto PVDF (Millipore). Cell lysates were probed for P-Erk1/2 (Santa Cruz Biotechnology), total ERK2 (Santa Cruz Biotechnology), P-Akt (Cell Signaling), total Akt (Cell Signaling), P-c JUN (Cell Signaling), total c JUN (Cell Signaling), tubulin (Sigma), tubulin (Sigma), and actin (Santa Cruz Biotechnology).

Immunofluorescence

Cells were grown to form a confluent monolayer in 2-chambered slides (Lab Tek II by Nunc). Cell monolayers were then washed in 1X DPBS and fixed for 30 minutes in 2% paraformaldehyde in 1X DPBS. After the paraformaldehyde was aspirated off, glycine (0.2 M) was added to the cell monolayers for 20 minutes to block any potential interactions with free aldehyde groups from the paraformalhehyde. Cells were blocked in 10% goat serum in 1X DPBS, 1% BSA for 30 minutes and treated for 1 hour with mouse EGFR antibody or serum mouse IgG (5 μg/mL) in 1X DPBS, 1% BSA. After multiple washes in 1X DPBS, 1% BSA, cells were treated with sheep anti-mouse IgG $F(ab')_2$ fragment-R-phycoerythrin antibody (1:20) in 1X DPBS, 1% BSA for 1 hour. Cells were then washed multiple times in 1X DPBS and stored in 1X DPBS at 4^oC. Photographs were taken with an Olympus DP70

Microscope Digital Camera with DP70-BSW Software using an Olympus BX51WI fluorescent microscope with a TRITC filter. Photographs were taken at a 200X magnification with the same exposure time.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Assays Using Epidermal Growth Factor

Cell viability was measured using a MTT assay. 10,000 cells/well were plated in a 96-well plate, incubated overnight at 37^{o}C, 5% CO_2. Cells were treated with SFM containing 1% sodium pyruvate, 1% antibiotic/antimycotic with or without mouse EGFR antibody or mouse serum IgG for 15 min followed by the addition of recombinant human epidermal growth factor (EGF; 1nM final concentration; Invitrogen). After a 48 h incubation, cells were treated with SFM containing 1% sodium pyruvate, 1% antibiotic/antimycotic and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (0.5 mg/mL; Sigma) for 2 h. DMSO was added to each well to dissolve formazan crystals formed by living cells for 15 min at 37^{o}C, 5% CO_2. The absorbance of each well was read at 560 nm. In each experiment, conditions were done in triplicate and compared to No Treatment.

Statistical Analysis

For each transwell experiment, conditions were done in duplicate and averaged. Averages of each condition were compared to No Treatment, APC alone, or blocking antibody/inhibitor alone. Experiments were repeated as indicated in the figure legends and averaged together. Results show the percentages compared to No Treatment. Statistical analysis was performed on each comparison – to No Treatment, APC, or blocking

antibody/inhibitor – using a one sample T-test with a normal distribution, a theoretical mean of 100, and significance of $p < 0.05$.

Results

APC increased invasion of the MDA-MB-231 cells, independent of the balance between uPA and PAI-1.

To determine if APC is altering the endogenous expression ratio of PAI-1 to uPA to increase invasion in the breast cancer cell line, MDA-MB-231, reverse transcription-pcr was performed on samples treated with or without APC. Results showed that APC did not alter mRNA levels of either uPA or PAI-1 (data not shown). Transwell invasion assays were performed with amiloride and blocking antibodies to uPA and PAI-1 along with APC. In figure 1A, APC or APC and DMSO increased invasion of the MDA-MB-231 cells greater than 150%. The amiloride treatment alone reduced invasion to approximately 70% of No Treatment. This result was expected since uPA was inhibited, reducing the activation of plasminogen and MMPs. If uPA was a key component to the mechanism by which APC promotes invasion of the MDA-MB-231 cells, then the treatment of APC and amiloride should have the same result as amiloride treatment alone. However, APC still increased invasion of the MDA-MB-231 cells even when uPA was inhibited, indicating that APC promotes cell invasion through another mechanism other than through uPA. APC activity assays verified that APC was not inhibited by amiloride (data not shown). To further confirm that uPA was not involved in the mechanism, a blocking antibody to the active site of uPA was then used in the transwell invasion assay (Figure 4-1B). As was found with amiloride,

even in the presence of the uPA blocking antibody, APC was able to increase cell invasion approximately 150% compared to No Treatment.

To determine if PAI-1 had a role in the increase in invasion by APC, a blocking antibody to PAI-1 was used along with APC in the transwell invasion assays (Figure 1C). In contrast to control goat serum IgG, the blocking antibody to PAI-1 increased invasion approximately the same amount compared to APC alone. With no PAI-1 to inhibit uPA, the serine protease can more freely activate proteases that degrade the ECM, such as plasmin and MMPs. Interestingly, the addition of APC with the blocking antibody to PAI-1 had an additive effect, increasing invasion further (approximately 200%) over PAI-1 blocking antibody alone (Figure 4-1C). These results suggest that APC promotes motility of the MDA-MB-231cells by a mechanism distinct from that involved in altering the ratio between PAI-1 and uPA.

APC increased invasion of the MDA-MB-231 cells via MMP-2 and MMP-9.

A broad-spectrum hydroxamate inhibitor of MMPs, GM 6001 was used to determine if MMPs have a role in APC promoted cell invasion. As shown in Figure 4-2A, GM 6001 treatment reduced invasion compared to No Treatment in a concentration dependent manner as expected due to a reduction in active proteases to degrade the ECM. GM 6001 and APC together yielded similar results. As the concentration of GM 6001 increased, the effects of APC were reduced further and further below No Treatment (Figure 4-2A). These results imply that the increase in invasion caused by APC involves MMPs. Because the GM 6001 MMP inhibitor is a broad-spectrum inhibitor, we also used a specific MMP-2 and -9

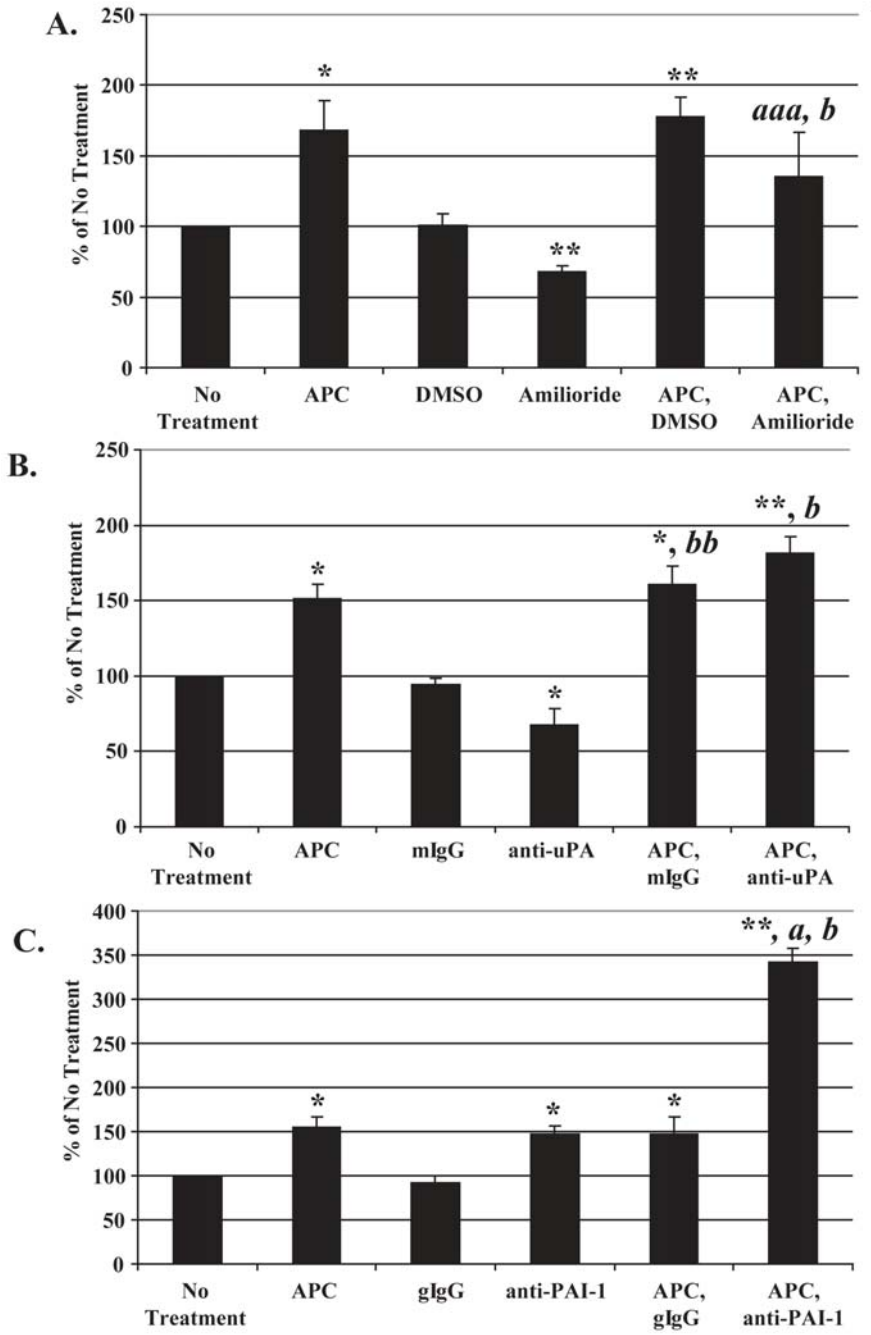

Figure 4-1. ***APC Does Not Increase Invasion of the MDA-MB-231 Cells Through the Plasminogen Activator System.*** Cells were pretreated with 0.2 mM amiloride (A), 20 μg/mL anti-uPA (B), 20 μg/mL anti-PAI-1 (C), and corresponding control antibodies for 1 h prior to the addition of 10 μg/mL APC. Cells invaded and migrated towards 10% FBS containing media for 24 h. Graphs represent the average of 3 separate experiments; * $p<0.05$, ** $p<0.01$ compared to No Treatment; *a* $p<0.05$, *aaa* $p<0.001$ compared to APC treatment; *b* $p<0.05$, *bb* $p<0.01$ compared to amiloride/anti-uPA/anti-PAI-1 treatment.

inhibitor, SB-3CT, to determine if these MMPs were involved in the increase in cell invasion caused by APC. Invasion was reduced in a concentration dependent manner upon SB-3CT treatment alone as expected (Figure 4-2B). With SB-3CT and APC treatment, we see continued concentration dependent reduction in invasion and abrogation of the increase seen with APC treatment alone (Figure 4-2B). APC activity assays verified that neither GM 6001 nor SB-3CT had an effect on APC activity (data not shown). These results imply that APC promotes invasion of the MDA-MB-231 cells is dependent on MMP-2 and MMP-9 degradation of the ECM.

APC Increased Invasion of the MDA-MB-231 Cells by Binding to EPCR and PAR-1.

Previously, we showed that APC binds to both EPCR and PAR-1 to increase chemotaxis of the MDA-MB-231 cells [323]. Using blocking antibodies to both receptors, we next wanted to determine if both EPCR and PAR-1 are also required for APC promoted cell invasion. Blocking antibody to EPCR abrogated the effects of APC on invasion (Figure 3A). The PAR-1 blocking antibodies also abrogated the effects of APC, reducing the level of invasion back to No Treatment (Figure 4-3B). Solution-phase APC could interact with latent and intermediately active MMPs, specifically MMP-2 and -9. However, although there may be an increase in active MMP-2 and -9, it is not enough to promote invasion. These results suggest that APC must both bind to the cell surface through EPCR and PAR-1 and increase MMP-2, -9 activities to promote invasion of the MDA-MB-231 cells.

APC Increased Invasion and Chemotaxis of the MDA-MB-231 Cells Through EGFR.

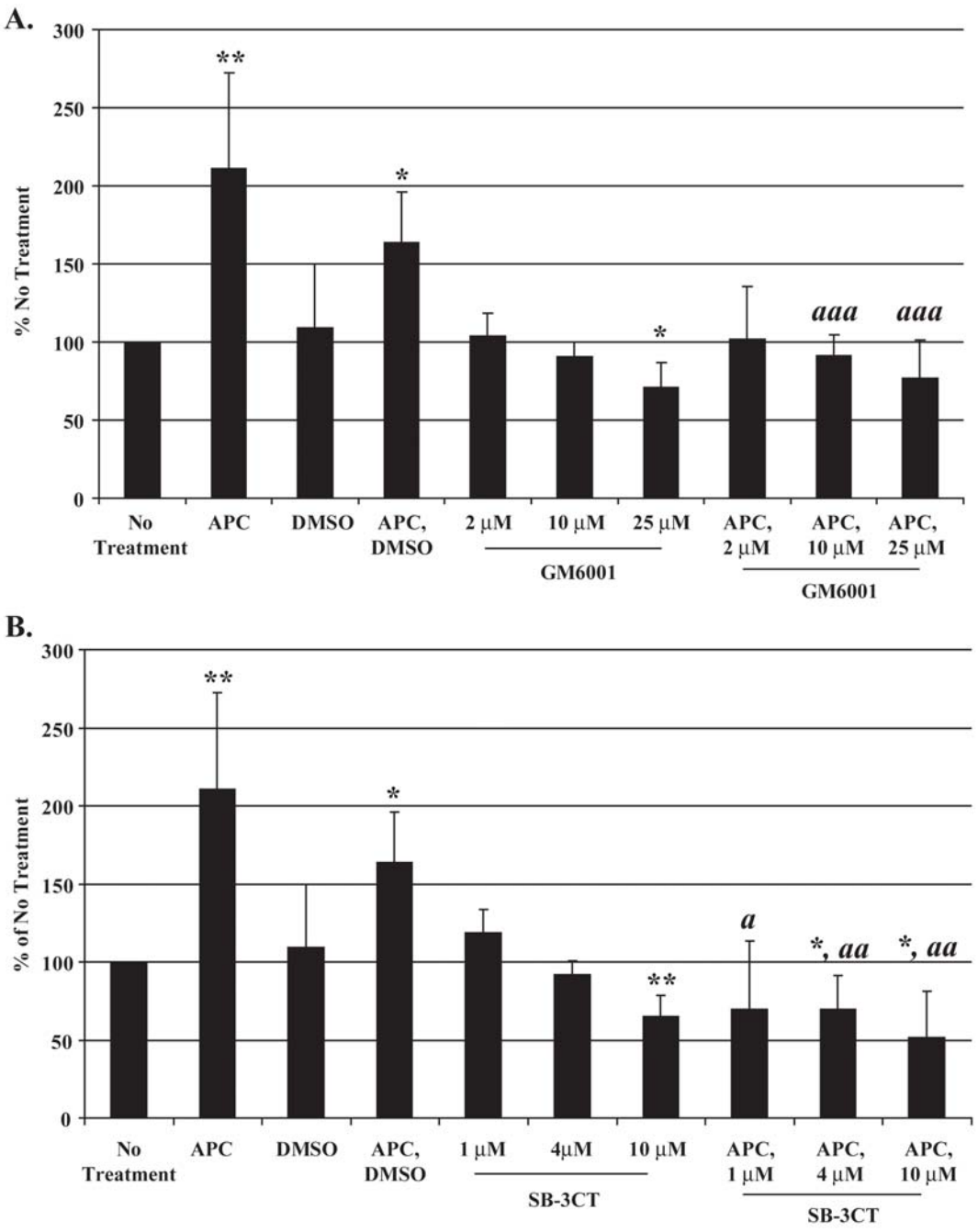

Figure 4-2. ***APC Increases Invasion of the MDA-MB-231 Cells Through the MMP-2 and MMP-9 Degradation of the Extracellular Matrix.*** Cells were pretreated with increasing concentrations of GM 6001 (0-25 μM; A) or SB-3CT (0-10 μM; B) for 15 minutes prior to the addition of 10 μM APC. Cells invaded and migrated towards 10% FBS containing media for 24 h. Graphs represent the averages of 8 separate experiments; * $p<0.05$, ** $p<0.01$ compared to No Treatment; *a* $p<0.05$, *aa* $p<0.01$, *aaa* $p<0.001$ compared to APC treatment.

It had been previously shown that APC downregulated migration of lymphocytes by binding to EPCR and EGFR. Thus, we became interested in exploring the role of EGFR during APC-promoted MDA-MB-231 cell motility. First, we verified that our MDA-MB-231 cells expressed EGFR (Figure 4-4A). Second, EGF induced cell proliferation was reduced in the presence of an EGFR blocking antibody (20 μg/mL) (Figure 4-4B). Finally, to see if EGFR had a role in the increase of cell migration by APC, MDA-MB-231 cells were pretreated with the EGFR blocking antibody (20 μg/mL) prior to the addition of APC (10 μg/mL) in the transwell assays. Treatment with EGFR blocking antibody alone had no effect on cell migration (Figure 4-4C). By contrast, the EGFR blocking antibody substantially reduced APC-promoted invasion and chemotaxis of the MDA-MB-231 cells (Figure 4-4C). These results indicate that EGFR is activated by APC on the MDA-MB-231 cells, along with EPCR binding and PAR-1 activation, to increase invasion and chemotaxis.

APC Increased Chemotaxis of the MDA-MB-231 Cells by Activating PI3K and MAPK Pathways.

To determine if the PI3K and MAPK pathways are activated by APC in order to increase MDA-MB-231 cell motility, western blots were run on lysates of cells collected at multiple timepoints up to 12 h. As seen in Figure 4-5A, there was an increase in phosphorylation of ERK, which was used as an indicator of the activation of the MAPK pathway. At 2 h, there was no difference in P-ERK levels between 0 and 10 μg/mL APC. By the 6 h timepoint, APC treatment consistently increased the level of P-ERK over no APC treatment. Blots were also probed for P-Akt as an indication of the activation of the PI3K pathway, and they showed a similar consistent increase in the levels of P-Akt upon treatment

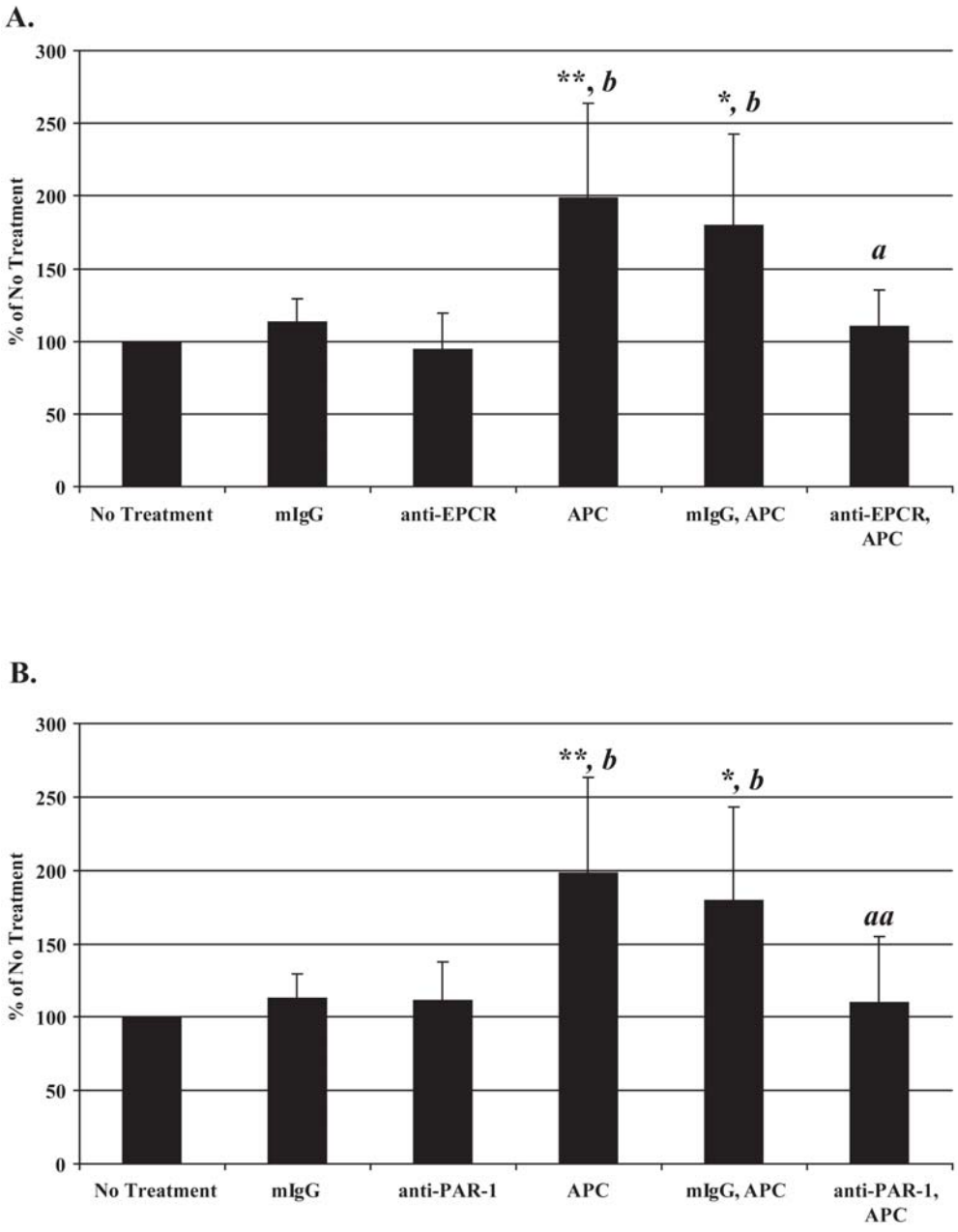

Figure 4-3. ***APC Increases Invasion of the MDA-MB-231 Cells by Binding to EPCR and PAR-1.*** (A) Cells were pretreated with 4 μg/mL EPCR antibody (JNK 1494) or control mouse serum IgG for 15 minutes prior to the treatment of 10 μg/mL APC. (B) In a separate experiment, cells were pretreated with 10 μg/mL atap 2 and 20 μg/mL wede15 (PAR-1 blocking antibodies) for 15 minutes prior to the addition of 10 μg/mL APC. Cells invaded and migrated toward 10% FBS containing media for 24 h. The graphs represent the average of 8 separate experiments for both (A) and (B); * $p<0.05$, ** $p<0.01$ compared to No Treatment; *a* $p< 0.05$, *aa* $p<0.01$ compared to APC treatment; *b* $p<0.05$ compared to mIgG treatment.

with APC at 6 h (Figure 4-5A). Based on these results, western blots were run on cell lysates from MDA-MB-231 cells that were treated for 6 h with 10 μg/mL APC, PC, or chemically inactived APC-DEGR. As seen in Figure 4-5B, active protease, APC, was able to consistently increase the level of both P-ERK and P-Akt at 6 h compared to no treatment, while PC and APC-DEGR have similar if not lower levels of P-ERK and P-Akt as no treatment. This result suggests that APC interactions at the cell surface require active protease to activate of the PI3K and MAPK pathways. Western blots were also performed on cell lysates from MDA-MB-231 cells treated with APC (10 μg/mL) and either control mouse serum IgG (mIgG; 4 μg/mL) or EPCR blocking antibody (4 μg/mL) for 6 h (Figure 4-5B). The EPCR blocking antibody prevented any increase in P-Akt levels in the presence of APC compared to the EPCR blocking antibody treatment alone. The same is true for P-ERK (Figure 4-5B). This result suggests that APC binds to EPCR and activates both the PI3K and MAPK pathways in order to increase motility of the MDA-MB-231 breast cancer cells.

Three signaling pathways (PI3K, MAPK, and JNK) were then studied in the transwell assays to determine if they had an impact on APC promoted cell migration. LY 294002 was used to inhibit PI3K activity and reduce phosphorylation of Akt. LY 294002 reduced P-Akt levels (Figure 4-6A) with an accompanying loss of migration (Figure 4-6B), as expected since this is an important signaling pathway for cell migration (under these treatment conditions cell survival was not reduced). If this pathway was not activated by APC, we would expect that combined APC and LY 294002 treatment would be increased 150% over LY 294002 alone. However, APC and LY 294002 treatment was the same as LY 294002 treatment alone (Figure 4-6B). These results show that APC increases cell migration through a PI3K dependent pathway.

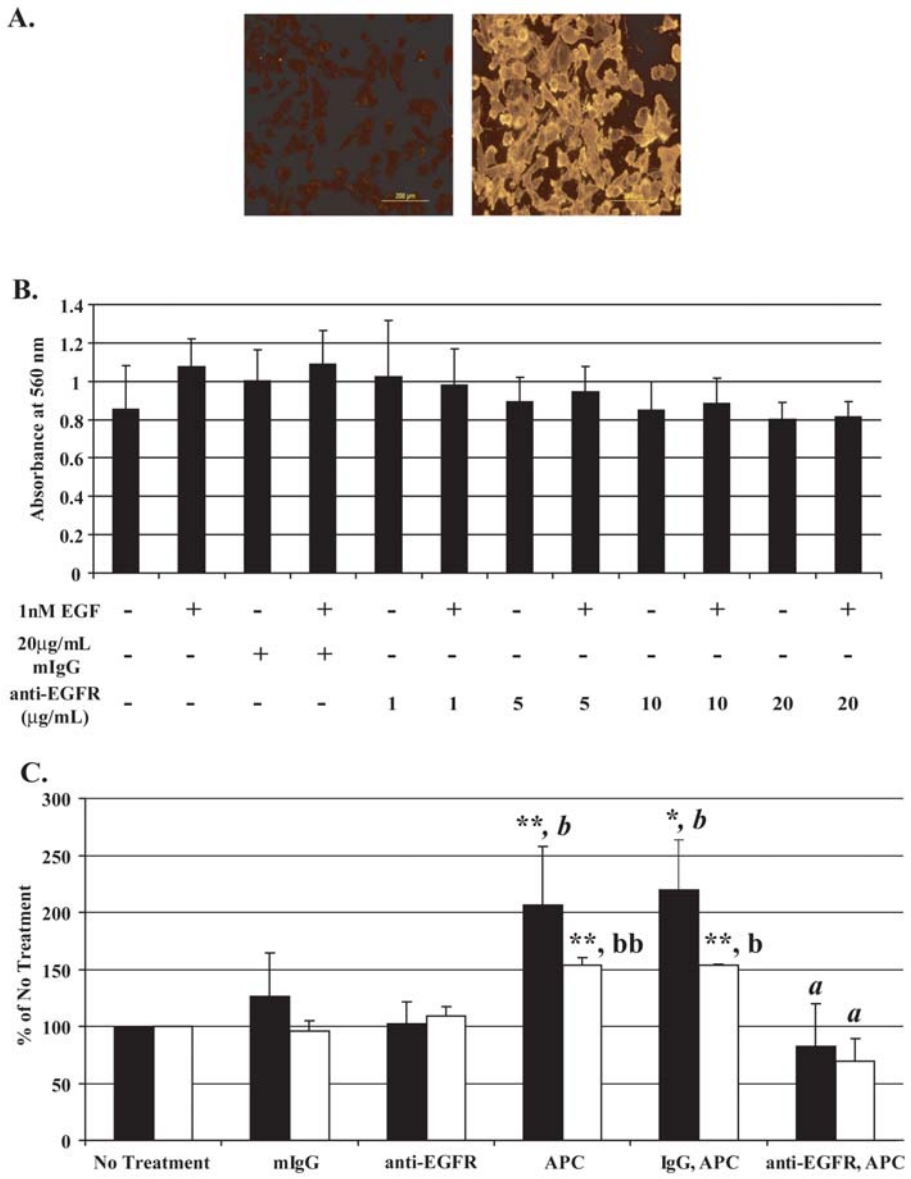

Figure 4-4. ***APC Increases Invasion and Chemotaxis of the MDA-MB-231 Cells by Activating EGFR.*** (A) Immunofluorescence done on the MDA-MB-231 cell monolayer for EGFR. (B) Cells were pretreated with increasing concentrations of EGFR antibody or control mouse serum IgG (0-20 μg/mL) prior to the addition of 1 nM EGF in SFM. Cells were allowed to grow for 48 h and then treated with MTT in SFM and analyzed as described in "Experimental Procedures" to determine cell viability. Graphs represent the average absorbance for each condition for 4 separate experiments. (C) Cells were pretreated with 20 μg/mL anti-EGFR or control mouse serum IgG for 15 minutes prior to the addition of APC (10 μg/mL). Cells invaded for 24 h and chemotaxed for 12 h towards 10% FBS containing media. Graphs represent the average of 4 separate experiments for invasion and 3 separate experiments for chemotaxis assays; * $p<0.05$, ** $p<0.01$ compared to No Treatment; *a* $p<0.05$ compared to APC treatment; *b* $p<0.05$, *bb* $p<0.01$ compared to mIgG treatment.

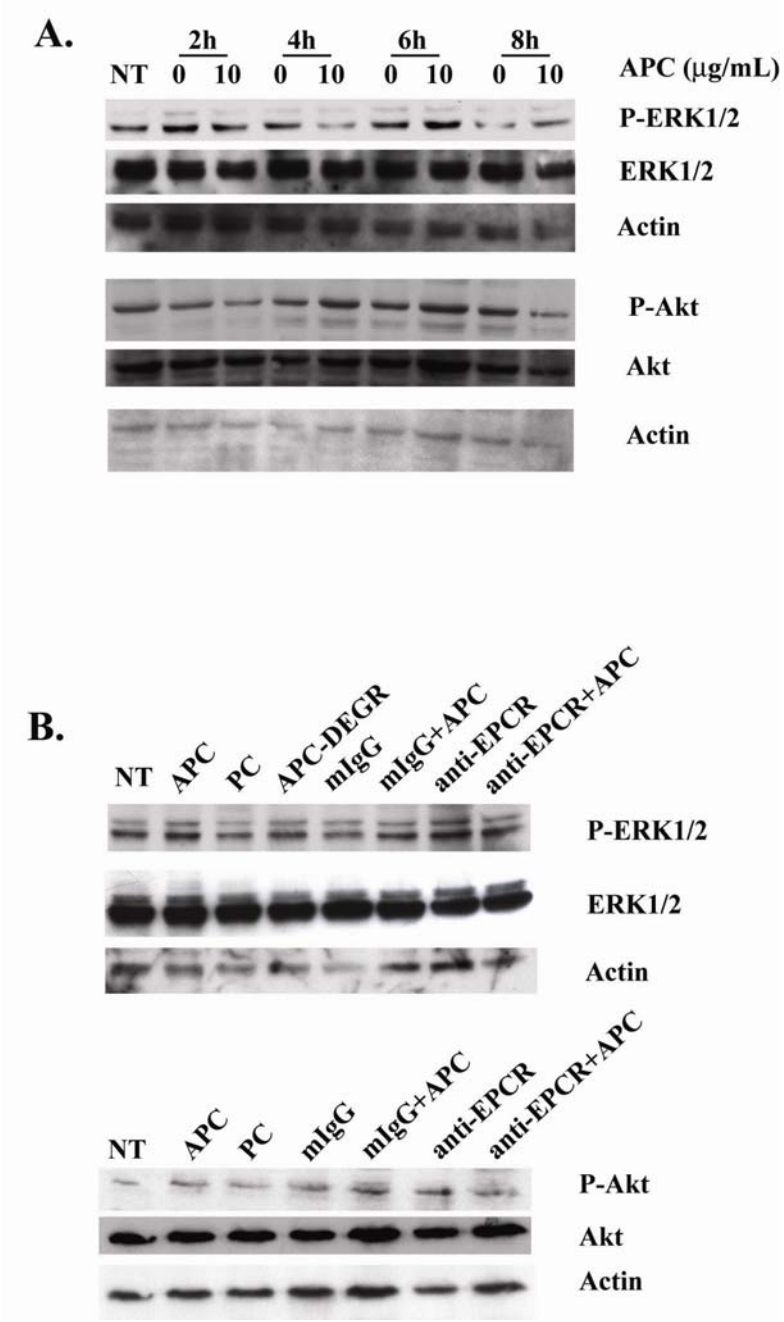

Figure 4-5. ***APC Treatment of MDA-MB-231 Cells Results in the Increased Phosphorylation of ERK1/2 and Akt at 6 h.*** (A) Representative western blots showing timpoints from 2 h to 8 h of cells treated in SFM with or without APC (10 μg/mL). 25 μg total protein from cell lysates were probed for pERK1/2, total ERK2, p-Akt, total Akt, and actin. Westerns were run on 5 separate experiments. (B) Representative western blots of cells treated with 10 μg/mL of APC, PC, APC-DEGR, 4 μg/mL mouse serum IgG, and 4 μg/mL anti-EPCR blocking antibody for 6 h. 25 μg of total protein from cell lysates were probed for pERK1/2, total ERK2, pAkt, total Akt, and actin. Westerns were run on 5 separate experiments.

The PD 98059 compound was used to inhibit MEK1/2 phosphorylation of ERK1/2 (Figure 4-6C). Because the MAPK pathway is an important signaling pathway for cell migration, treatment with the PD 98059 compound alone reduced chemotaxis (Figure 4-6D). APC and PD 98059 treatment was also reduced to the same level as PD 98059 treatment alone, suggesting that the MAPK pathway is activated by APC.

The JNK pathway is involved in cell survival and cell migration [23-25], and is activated by EGFR [26] and G protein-coupled receptors [27].The SP 600125 compound inhibited JNK phosphorylation of c JUN (Figure 4-6E). SP 600125 treatment alone had no effect on cell migration (Figure 4-6F). However, APC and SP 600125 treatment was increased 150% compared to No Treatment, suggesting that the JNK pathway is not required for APC promoted cell migration.

DISCUSSION

APC has been previously shown to increase chemotaxis and invasion of breast cancer cells, particularly the MDA-MB-231 and MDA-MB-435 cell lines [323] toward a chemoattractant. In this study, we look into the mechanism by which APC increases cellular invasion and chemotaxis. Extracellularly, APC does not interact with the plasminogen activation system to promote invasion as previously reported [67]. Instead, APC promoted cellular invasion is dependent on MMP-2 and MMP-9 degradation of the ECM. However, extracellular degradation by MMP-2 and -9 is not sufficient for APC promoted cellular invasion. Using blocking antibodies in the transwell invasion assay, we show that APC interacts with EPCR and PAR-1, as was previously shown to be important in chemotaxis [323]. Not only is APC binding to EPCR and activating PAR-1, APC treatment also leads to

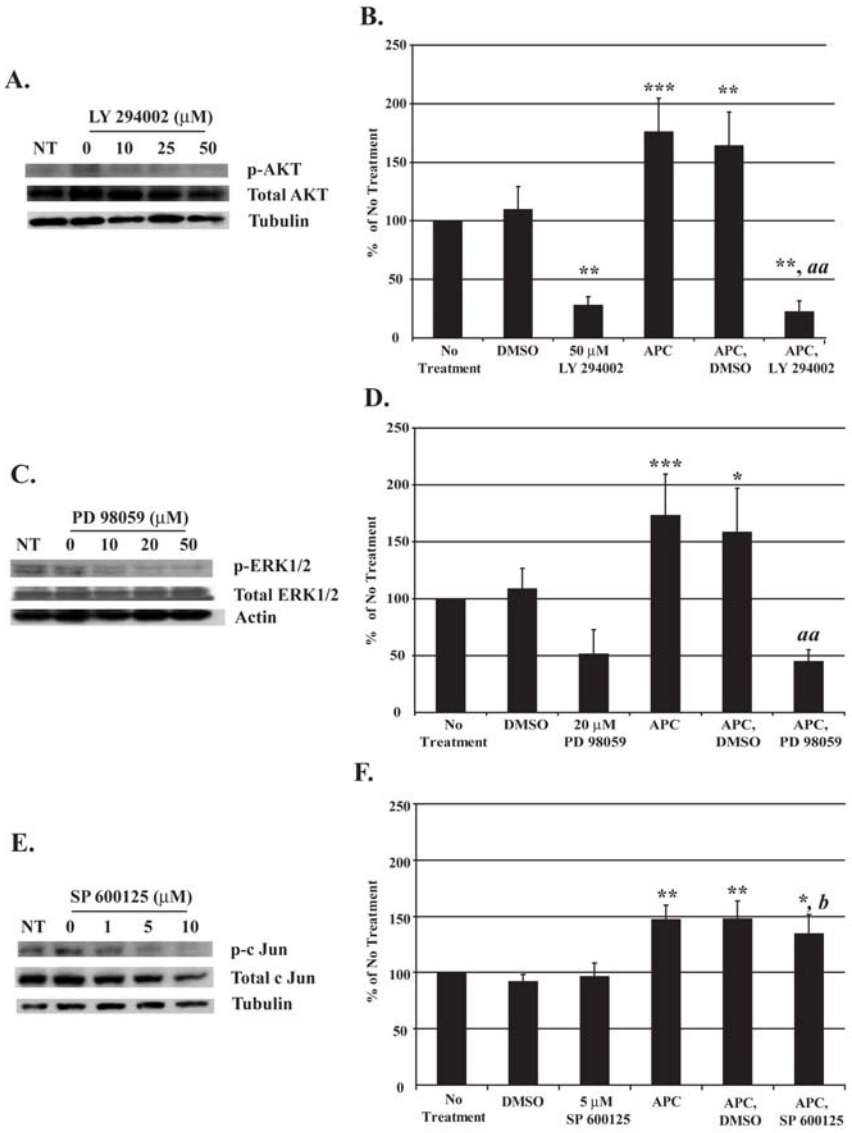

Figure 4-6. ***APC Increases Chemotaxis of the MDA-MB-231 Cells by Activating both the PI3K and MAPK Pathways, but not the JNK Pathway.*** Cells were treated with increasing concentrations of LY 294002 (0-50 μM; A), PD 98059 (0-50 μM; C), or SP 600125 (0-10 μM; E) for 12 h to determine the optimal concentration of each inhibitor to be used in a 12 h transwell chemotaxis assay. Cell lysates were probed for phosphorylated and total AKT (A), ERK (C), and c Jun (E), tubulin, and actin. Western blots shown are representative of 3 separate experiments. In 12 h transwell chemotaxis assay, cells were pretreated with 50 μM LY 294002 (B), 20 μM PD 98059 (D), or 5 μM SP 600125 (F) for 45 minutes prior to the addition of 10 μg/mL APC. Cells migrated towards 10% FBS containing media for 12 h. Graphs represent the averages of 9 separate experiments for (B), 8 separate experiments for (D), and 4 separate experiments for (F); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to No Treatment; *aa* $p<0.01$ compared to APC treatment; *b* $p<0.05$ compared to SP 600125 treatment.

the transactivation of EGFR through PAR-1 to increase both invasion and chemotaxis. The interaction of these 3 receptors leads to the increased phosphorylation of ERK and Akt, but not c JUN, implicating that APC promotes cell migration via PI3K and MAPK, and not JNK, dependent pathways (Figure 4-7).

Kobayashi, et al. (1994) found that APC increased invasion of cancer cells *in vitro* by forming a stable complex with the serpin, PAI-1 [67]. Removing the major inhibitor in the system allowed for increased protease activity and degradation of the ECM. uPA, a serine protease inhibited by PAI-1, would be free to activate plasminogen to plasmin, which would not only degrade the ECM but also activate MMPs that could degrade the ECM. These experiments added both PAI-1 and APC exogenously [67]. In experiments presented here, APC is added exogenously and we study how endogenous uPA and PAI-1 expressed by the MDA-MB-231 breast cancer cells interacts with APC. Some APC could be inhibited, forming a complex with PAI-1 to allow for uPA to activate both MMPs and plasminogen to degrade the ECM. However, this is not the major mechanism utilized by APC to increase invasion. Blocking uPA with either the chemical inhibitor amiloride or a blocking antibody to its active site did not alter the ability of APC to increase invasion of the breast cancer cells. Additionally, in the presence of a PAI-1 blocking antibody and APC, the effect was essentially additive. When either the general MMP inhibitor GM 6001 or specific MMP-2 and -9 inhibitor SB-3CT compounds are used in conjuncture with APC, the increase in invasion of the breast cancer cells is lost. APC, therefore, increases invasion through MMP degradation of the ECM, not through uPA. There is prior evidence that APC directly activates MMPs, specifically MMP-2. Through zymography, APC was shown to activate MMP-2 from the intermediate to fully active form [73-76], independent of MT1-MMP

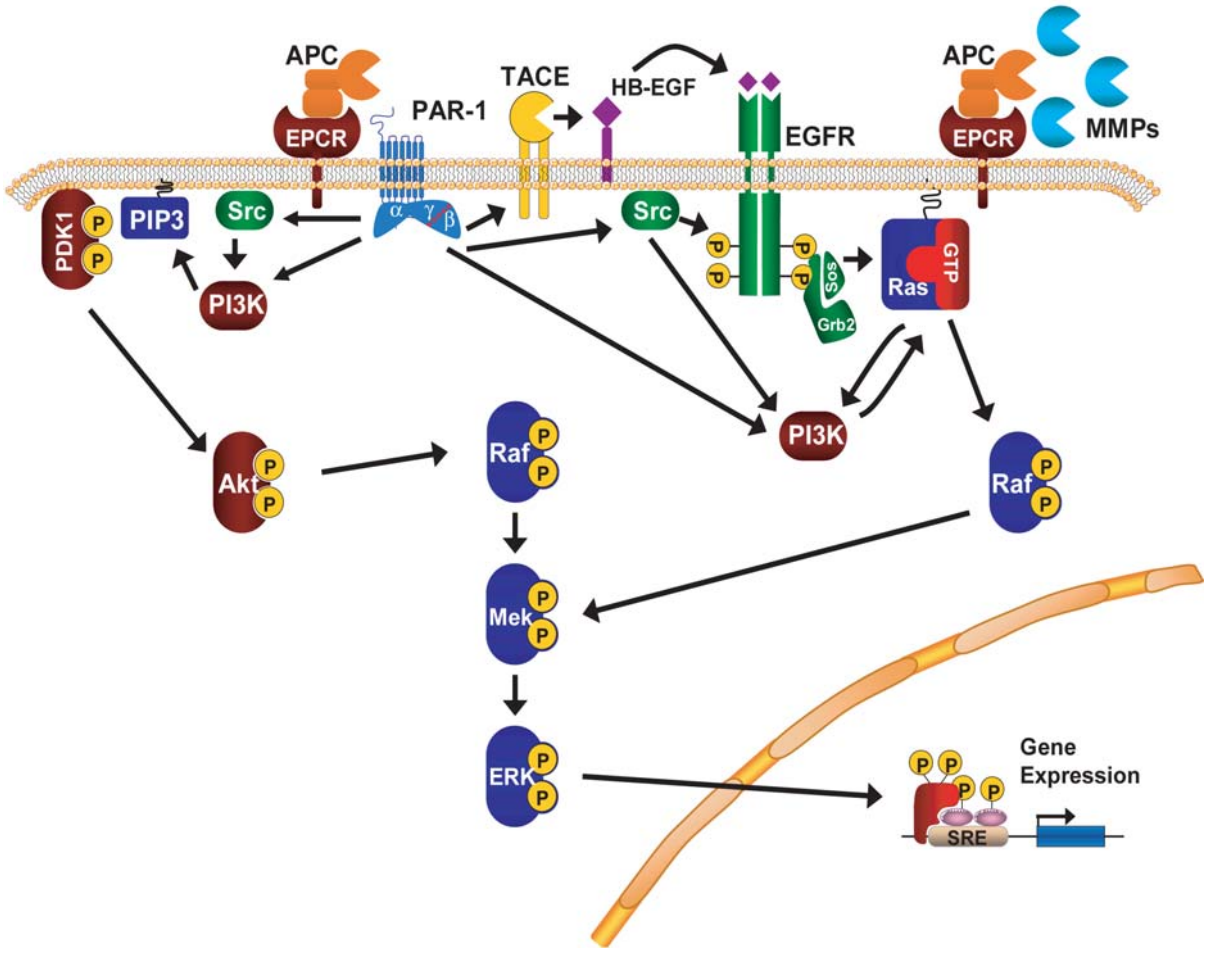

Figure 4-7. ***Schematic of the Proposed Intracellular and Extracellular Mechanism Utilized by APC to Increase Cellular Migration in the MDA-MB-231 Cells.***

[74, 75]. Additionally, it has also been shown that solution phase APC [74-76] and APC generated on the cell surface in the presence of IIa bound to TM can activate MMP-2 [73].Therefore, APC increases invasion through extracellular interactions either directly or indirectly to activate MMP-2 and -9 and this does not involve altering the balance between uPA and PAI-1.

As previously shown in the transwell chemotaxis assays, APC binds EPCR on the cell surface and is localized to a nearby PAR-1 receptor, which it cleaves to activate and promote cell motility [323]. The same is true in the transwell invasion assay. Using blocking antibodies to either EPCR or PAR-1, the effects of APC on invasion are inhibited. These results suggest that the degradation of the ECM by MMPs is not the only mechanism utilized by APC to increase invasion. Activation of PAR-1 by EPCR-bound APC, in addition to the MMP-mediated degradation of the ECM, is required to substantially promote invasion.

In addition to APC binding to EPCR and activating PAR-1, it is shown using a blocking antibody in both the transwell invasion and chemotaxis assays that APC also activates EGFR to increase cellular motility. Previously, APC was shown to inhibit lymphocyte migration through both EPCR and EGFR [59], characteristic of its anti-inflammatory role. In our model, EGFR promotes cell migration. These results suggest that the effects of APC are cell type specific. Immune cell migration is inhibited by APC, while cancer cell migration is promoted. There are three likely mechanisms by which APC could lead to the activation of EGFR. One, APC could bind directly to EGFR, like recently shown for another serine protease, tissue plasminogen activator [324]. Second, APC could bind to EPCR and activate PAR-1, which then would promote the transphosphorylation of EGFR. In this pathway, PAR-1 activation would lead to Gα or β/γ subunits activating c-Src, which

would phosphorylate Tyr 845 on the cytoplasmic tail of EGFR [36]. Via EPCR and PAR-1, APC has been shown to transactivate another receptor, sphingosine-1-phosphate$_1$ receptor, which affects vascular permeability of endothelial cells [159]. And third, APC could also bind to the cell through EPCR, and activate PAR-1. PAR-1 would then activate the TNF-alpha converting enzyme (TACE; ADAM-17), a membrane-bound disintegrin metalloprotease that processes membrane-associated cytokines such as heparin bound-EGF (HB-EGF) or other EGFR-family member ligands to transactivate EGFR [38] [39]. HB-EGF would bind to the ligand binding domain of EGFR, leading to the phosphorylation of the receptor [40-42]. Our results do not favor the direct activation of EGFR by APC, but suggest that APC-EPCR interactions through PAR-1 promotes either the transphosphorylation of EGFR (since the EGFR blocking antibody reduced motility in the presence of APC) or by ADAM-17-directed ligand transactivation (since the MMP inhibitor GM 6001 is known to inhibit ADAM-17).

Since PAR-1 and EGFR are both activated by APC to increase invasion and chemotaxis, there are many signaling pathways that could be involved, including the MAPK, PI3K, JNK, NFκB, or eNOS pathways. Previously, the MAPK, PI3K, and eNOS pathways have been implicated in APC induced proliferation of HUVEC and, additionally, the eNOS pathway was implicated in APC induced angiogenesis [79]. Based on these previous studies, we focused on the role of the MAPK, PI3K, and JNK pathways in the APC induced cellular motility. Using pharmacological inhibitors and western blots, we show that APC activates the PI3K and MAPK pathways, to increase motility of the MDA-MB-231 cells. The JNK pathway, which has been shown to be activated by EGFR [325], is not involved in the APC promoted cellular motility of the breast cancer cells. Therefore, we propose a model in which

APC is focused at the MDA-MB-231 cell surface through its binding to EPCR. PAR-1 is subsequently cleaved by APC-bound to EPCR [54]. Either through intracellular or extracellular transactivation, EGFR is activated. EGFR will activate Ras and the MAPK pathway [16, 17]. Ras can also activate PI3K, which will lead to the activation of Akt and the MAPK pathway [16, 20, 32]. Additionally, PAR-1 can also activate PI3K through c-Src , which would also result in the activation of Akt and the MAPK pathways [17, 32]. Ultimately, the resultant signal amplification culminates in the promotion of cellular migration (schematically shown in Figure 4-7).

In summary, our results show an interesting interdependent nature of the pathways that are activated by APC to increase motility of the MDA-MB-231 breast cancer cells. Blocking of any one of the three involved receptors (EPCR, PAR-1 or EGFR) or by inhibiting MMP-2 and MMP-9 results in a loss of the increase in motility promoted by APC. This leads us to speculate that the extracellular and intracellular events induced by APC are dependent on one another to facilitate increased motility (Figure 4-7). Our results also imply that APC binding to EPCR not only localizes the protease to aid its interaction with PAR-1 but also in its interactions with MMPs. Overall, the effect of APC is coordinated with both intracellular activation of signaling pathways, which promote motility, and with extracellular activation of MMPs, which primes the MDA-MB-231 cells to respond to chemoattractants at an increased rate.

Chapter 5

APC Increases Endothelial Cell Motility, Tube Formation, and Angiogenesis

Summary

Activated protein C (APC) is an anticoagulant serine protease involved in the regulation of hemostasis, inflammation, apoptosis, and cell migration. The mechanism used by APC to alter endothelial cell functions was studied using the transwell chemotaxis and invasion assay, a tube formation assay, and an *ex vivo* aortic ring assay. In the transwell invasion and chemotaxis assay, APC increased both types of cell migration of human umbilical vein endothelial cells (HUVEC) that peaked at 0.1 and 0.5 μg/mL, respectively, which are near physiologic concentrations. APC also augmented the formation of tube-like structures with HUVEC, which did require active protease. Finally, APC promoted the formation of sprouts from aortic rings of wild-type C57BL/6 mice. This sprout formation required active APC and was not a result of any trace contamination by thrombin. Furthermore, the increase in sprout formation from murine aortic rings involved the activation of both epidermal growth factor receptor (EGFR) and phosphatidylinositol 3 kinase (PI3K) signaling pathways, along with the requirement for the increased degradation of the extracellular matrix by the action of matrix metalloproteases (MMPs). Collectively, these results suggest that APC promotes endothelial cell motility and angiogenesis through a similar mechanism shown previously for breast cancer cell migration and invasion, which

points to essentially the same interdependent extracellular and intracellular signaling process for both cell motility and angiogenesis.

Introduction

Activated protein C (APC) is a serine protease [298] involved in the regulation of thrombin (IIa) generation. Zymogen protein C (PC), produced in the liver, is found in circulation [299, 300]. At the site of injury in the blood vessel, PC will be sequestered to the endothelium surface through the binding to endothelial cell protein C receptor (EPCR) [154]. PC bound to EPCR is localized to IIa bound to thrombomodulin (TM). IIa will cleave PC to activate it [156, 302]. APC, with the aid of protein S [304, 305], will inactivate factors Va and VIIIa [301, 303] on the periphery of the clot to control excessive IIa formation and prevent the occlusion of the blood vessel. It is also here on the endothelial cell surface, bound to EPCR, that APC can interact and modulate the cellular processes, including inflammation, apoptosis, proliferation, and migration.

Much research is currently focused on characterizing the non-hemostatic role of APC and the other components of the protein C pathway, including EPCR, PC, and IIa bound to TM. APC reduces the inflammatory response by reducing chemoattractant expression [47, 48, 51], migration [58, 59] and adhesion of immune cells [47, 48] all shown to be mediated through EPCR [53, 54, 58, 59], protease activated receptor-1 (PAR-1) [54], and epithelial growth factor receptor (EGFR) [59]. APC has also been shown to promote survival by up-regulating anti-apoptotic genes, such as Bcl-2 [54, 60, 64], and down-regulating pro-apoptotic genes, such as p53 [64] and BAX [54, 64]. APC also prevents the activation of caspases 3 [63-65] and 8 [64, 65]. Again, these anti-apoptotic effects of APC are shown to

occur through EPCR [54, 63], PAR-1 [54, 63, 64] in neurons and endothelial cells, and PAR-3 in mouse neurons [64].

APC has also been shown to modulate cellular migration and proliferation. In HUVEC, APC increases proliferation through EPCR and PAR-1 activation of the MAPK, PI3K, and eNOS pathways [79]. APC increases keratinocyte proliferation by interacting with EPCR and PAR-1 [78] and increasing the expression of mitogenic factors, interleukin-6 and interleukin-8 [77]. APC increases cellular migration of various cell types. In cancer cells, APC increases chemotaxis and invasion. Extracellularly, it has been shown that this increase occurs because APC is inhibited by plasminogen activator inhibitor-1 (PAI-1) [67], allowing another serine protease, urokinase plasminogen activator (uPA), to activate other extracellular matrix (ECM) proteases [68-71]. Using zymography, APC is shown to directly activate MMP-2 from the intermediate to fully active form [73-76], independent of MT1-MMP [74, 75]. It has also been shown that APC [74-76] and APC generated on the cell surface in the presence of IIa bound to TM can activate MMP-2 [73]. Additionally, we have shown that APC increases cancer cell chemotaxis and invasion through EPCR [323, 326], PAR-1 [323, 326], and EGFR [326] activation of the MAPK and PI3K pathways [326], along with the increase activation of MMP-2 and -9 [326]. In keratinocytes, APC increases migration, again, through MMP-2 [77, 78] activity and inhibition of NFκB, a promoter of senescence in keratinocytes [77]. Through EPCR and PAR-1, APC is able to increase the expression of MMP-2 and the activation of ERK and p38 in keratinocytes [78]. In fibroblasts and endothelial cells, APC was shown to increase MMP-2, VEGF, and MCP-1, all of which are pro-angiogenic factors [52]. From these studies, we can focus on specific extracellular and intracellular events that APC could be utilizing to affect endothelial cell migration.

In vivo, APC has been shown to increase angiogenesis in wound healing assays, concluded to be through MMP-2 [52]. APC has also been shown to increase the formation of blood vessels in a murine cornea angiogenesis assay through the eNOS pathway [79]. Finally, in the murine focal ischemic stroke model, APC has been shown to better the recovery through the reduction of inflammation [54, 66], apoptosis of endothelial cells and neurons[64, 65], and decreased fibrin deposition [54], while increasing cerebral blood flow post-onset of stroke and the recovery of neuromuscular function [54, 65, 66]. Additionally, APC reduced the expression of ICAM-1 on vessel walls in the area affected, thereby reducing the adhesion of neutrophils to the endothelium [66]. The benefits of APC in stroke were shown to be modulated through EPCR, PAR-1 [54, 64] and PAR-3 in mice [64].

The work presented in this chapter aims to characterize the function of APC to promote endothelial cell migration. I used multiple *in vitro and ex vivo* endothelial cell migration assays: transwell invasion and chemotaxis assay; tube formation assay; and an *ex vivo* aortic ring assay. I also characterized the role of other receptors, extracellular proteases, and signaling pathways involved in the modulation of APC promoted endothelial cell migration. The results show that APC increases HUVEC chemotaxis and invasion towards VEGF at closer to physiological levels of APC than was found for MDA-MB-231 cell motility. APC also promotes the formation of tube-like structures in HUVEC, dependent on active protease (inactive forms of APC were unable to promote tube formation). Finally, APC increased sprouting of murine aortic rings, independent of any trace amounts of IIa potentially present in the assay. Active APC is also required to increase the formation of sprouts. APC, through activation of EGFR and PI3K and degradation of the ECM by MMPs, is able to increase sprout formation in this *ex vivo* assay. These extracellular factors and

intracellular signaling network function interdependently to cause the increase in sprout formation by APC since elimination of any single factor by a pharmacological inhibitor prevents the APC-promoted increase in sprouting.

Material and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVEC), obtained from Cambrex, were grown according to manufacturer's specifications. Cells were grown in endothelial cell basal media with 2% fetal bovine serum, bovine brain extract (BBE) with heparin, GA-1000, hEGF, and hydrocortisone (Cambrex). Cells were kept in an incubator at 37^{o}C, 5% CO_2.

Transwell Invasion and Chemotaxis Assay

Based on previous work [307, 308, 323], matrigel (1.67 mg/mL; BD Biosciences) coated and uncoated inserts (BD Bioscience) with 8 μm diameter pores were hydrated and coated with serum-free minimal essential media (Gibco) containing 0.1% bovine serum albumin (Sigma), 1% sodium pyruvate (Gibco), and 1% antibiotic/antimycotic (Gibco) for 2h at 37^{o}C. The chemotactic agent, 1 ng/mL VEGF (R&D Systems) in endothelial cell basal media with 2% FBS, BBE with heparin, GA-1000, hEGF, and hydrocortisone, was added to the well. 50,000 cells/membrane with increasing concentrations of APC (0-10 μg/mL; Xigris ®; Eli Lilly and Co.) in endothelial basal media with 2% FBS, BBE with heparin, GA-1000, hEGF, and hydrocortisone was added to the insert, which was placed in the well. Each condition was done in duplicate and allowed to incubate for 24 h at 37^{o}C, 5% CO_2 . Cells that migrate through were fixed in 100% methanol then stained with a fluorescent nuclear dye,

Hoechst 3342 (1:20000 in 1X PBS; Molecular Probes). Membranes were cut from the insert and mounted in 50% glycerol. Using a Nikon Eclipse E600 with a UV1A filter, 4-400X fields were counted and totaled to represent the number of cells that migrated per membrane. Duplicates were averaged together and compared to No Treatment, set at 100%.

Tube Formation Assay

In a 24-well plate, wells were coated with Growth Factor-Reduced Matrigel (BD Biosciences) and incubated for 1 h at 37^{o}C. 80,000 cells per well were plated with increasing concentrations of APC (0-10 μg/mL), PC (10 μg/mL), APC-DEGR (10 μg/mL), or VEGF (100 ng/mL) as the positive control and incubated at 37^{o}C for 18 h. Photographs with Adobe Photoshop and a Kodak DC290 Digital Camera mounted on an inverted microscope were taken at 6, 12, and 18 h. The number of tube-like structures (defined at a structure no more than 3 cells wide with junction points on each end) were counted in 4-100X views. Each condition was done in triplicate, averaged, and compared to No Treatment set at 100%.

Murine Ex Vivo Aortic Ring Assay

Wildtype C57BL/6 mice were maintained by the Veterinary Staff of the University of North Carolina at Chapel Hill Department of Laboratory Animal Medicine and all mouse protocols were reviewed and approved by the University's Institutional Animal Care and Use Committee (IACUC). All mouse protocols were reviewed and approved by IACUC.

Based on previous work [327], male and female mice, at varying ages, were anesthetized with 1.25% tribromethanol (0.2 mL/10g) and monitored by toe reflex response and respiratory rate. Once properly anesthetized, an incision was made midline and the

organs were removed to expose the aorta from the heart to the renal artery branch point. The aorta was dissected from the heart to the point above the split to the renal arteries. The dissected aorta were placed in a 100 mm dish containing Dulbecco's Modified Eagle Media (DMEM; Gibco) containing 1% antibiotic/antimycotic (Gibco) and cleaned of connective tissue and fat. Cleaned aortas were then sectioned into 1-2 mm pieces and placed into a new 100 mm dish containing DMEM with 1% antibiotic/antimycotic and set in an incubator for approximately 1 h at 37^{o}C, 5% CO_2 until implantation.

In a 48-well plate, 200 μL of Growth Factor-Reduced Matrigel was plated in each well and put in an incubator at 37^{o}C, 5% CO_2 for 10 minutes. One aortic section per well was placed on top of the gelatinized Matrigel, covered with an additional 200 μL of Growth Factor-Reduced Matrigel, and incubated for 10 minutes. 200 μL of endothelial basal media with 2% FBS, BBE with heparin, GA-1000, hEGF, and hydrocortisone was added to each well with either no additional treatment (negative control), 100 ng/mL VEGF (positive control), or increasing concentrations of APC (0-10 μg/mL). To eliminate any effects of IIa on sprout formation, 50 nM of hirudin was added to the aortic sections with or without APC (10 μg/mL). To determine if the active form of APC was needed to increase sprout formation, aortic sections were also treated with PC (10 μg/mL) with or without hirudin (50 nM) or APC-DEGR (10 μg/mL). In studies for EGFR, PI3K, and MMPs in APC-promoted sprout formation, pharmacological inhibitors, AG 1478 (10 μM) [328], LY 294002 (10 μg/mL) [329], and GM 6001 (10 μg/mL) [330], were added to the aortic sections 30 minutes prior to the addition of APC (10 μg/mL). Every 24 h for 5 days, media from each well was removed and replaced with fresh conditioned media. On days 3-5, the number of sprouts extending form the periphery of the aortic sections was counted and digital photographs were

taken as described above. Each condition was done in either duplicate or triplicate, averaged, and compared to No Treatment set at 100%.

Immunofluorescence

After 5 days in culture, media covering the implanted aortic ring was aspirated off and each ring was washed with 1X PBS. Aortic sections were fixed in 4% paraformaldehyde in 1X PBS for 30 minutes on ice. After 2 washes in 1X PBS, the sections were permeabilized with 0.5% Triton X-100 in 1X PBS for 15 minutes on ice and washed as before. To block any reactive aldehyde groups, the sections were treated with 0.2 M glycine for 20 minutes on ice and washed as before. Each section was blocked overnight in 10% goat serum, 1X PBS, at 4^{o}C. The following day each section was washed in 1X PBS and then treated with a rabbit polyclonal anti-von Willebrand factor (vWF) antibody (1:50 in 1X PBS, 1% BSA; Dako) or normal rabbit serum, overnight at 4^{0}C. The next day, each aortic section was washed 5 times with 1X PBS, 1% BSA, then allowed to sit in this wash solution overnight at 4^{o}C. Each section was treated with goat F(ab´)$_2$ anti-rabbit IgG conjugated to Lissamine Rhodamine-B (1:50 in 1X PBS, 1% BSA; BioSource International), covered, and stored overnight at 4^{o}C. The following day, each aortic section was washed 5 times with 1X PBS and then stored in 1X PBS and covered at 4^{o}C, until photographs could be taken with the Olympus IMT2 Inverted Flourescence Microscopy using a TRITC filter.

Statistical Analysis

For each experiment, conditions were performed in duplicate or triplicate and averaged. Averages of each condition were compared to No Treatment, which was set to

100%. Experiments were repeated as indicated in the figure legends. Statistical analysis was then performed using a one-sample T-test with a normal distribution, a theoretical mean of 100, and a significance of $p<0.05$.

Results

APC Increased HUVEC Invasion and Chemotaxis in the Transwell Assays

HUVEC were treated with increasing concentrations of APC in the transwell chemotaxis and invasion assay with VEGF as the chemotactic agent. As seen in Figure 5-1, APC increased invasion of HUVEC at 0.1 μg/mL and increased chemotaxis at 0.5 μg/mL. APC increased HUVEC migration compared to No Treatment at optimal concentrations that were below the physiological levels of PC found in plasma (~4 μg/mL). The highest concentration of APC (10 μg/mL) used to treat the HUVEC in this assay reduced migration compared No Treatment. These results suggest that in these experimental conditions, endothelial cells migration can be increased at physiologically relevant concentrations and inhibited at much higher levels.

APC Increased Tube Formation of HUVEC Requires Active Protease

HUVEC plated onto growth factor-reduced Matrigel will form a network of tube-like structures (consisting of 1-3 cells wide) that intersect at junction points. Upon the addition of a stimulus, such as VEGF, HUVEC will form a more extensive network of tube-like structures that last longer in culture. As seen in the transwell assays, upon treatment with increasing concentrations of APC (0-10 μg/mL), the increase in the formation of tube-like

structures occurs at an optimal concentration (1 μg/mL) and at an optimal timepoint (12 h) similar to the effects of VEGF (data not shown).

To understand if the effects of APC are due to its proteolytic activity, HUVEC in the tube formation assay were also treated with 2 inactive forms of APC, the zymogen PC (1 μg/mL) and the chemically inactive protease, APC-DEGR (1 μg/mL). At the 12 h timepoint, only the active protease is able to significantly increase the formation of tube-like structures compared to No Treatment (Figure 5-2A and B). These results suggest that APC must proteolytically cleave another protease or receptor to increase the formation of tube-like structures to a level similar to that of VEGF. The concentration of APC (1 μg/mL) needed to promote this formation is, again, closer to physiologically relevant levels of PC.

APC Increased Sprout Formation of Murine Aortic Rings Requires Active Protease

In the *ex vivo* aortic ring assay, murine aortic sections will form sprouts, composing of endothelial cells that will lengthen over time. Upon treatment with a stimulus, such as VEGF, the formation of endothelial cell sprouts will increase in number, grow longer, and last in culture. As seen in Figure 5-3A, increasing concentrations of APC (0-10 μg/mL) increased the formation of sprouts compared to No Treatment, but only the highest concentration of APC (10 μg/mL) was able to significantly increase this formation. The highest concentration was also able to increase the number of sprouts that form significantly earlier that even VEGF treatment. Photographs depicted in Figure 5-3B are from day 4. They show the increase in the number of sprouts that form around the periphery of the aortic section compared to No Treatment. These sprouts were also longer than those for No Treatment. The ability to form sprouts at 10 μg/mL APC even exceeded the effect of the

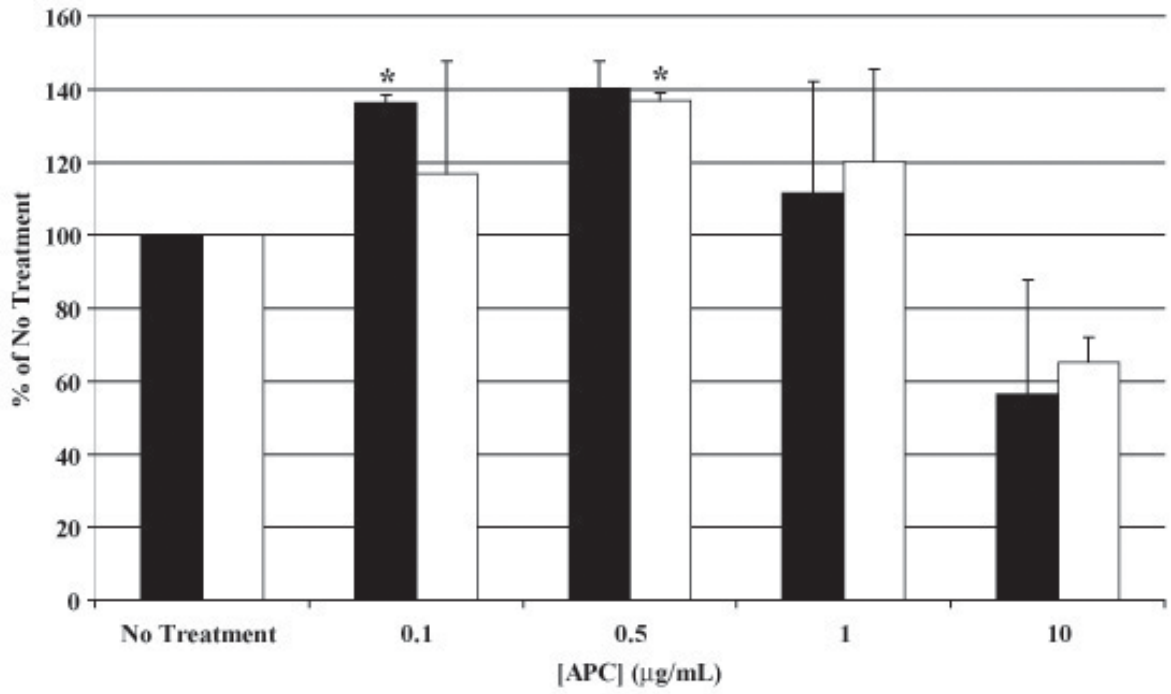

Figure 5-1. ***APC Increases HUVEC Invasion and Chemotaxis.*** HUVEC were treated with increasing concentrations of APC (0-10 μg/mL) for 24 h, using 1 ng/mL VEGF as the chemotactic agent. Black bars depict the results for invasion and white bars depict the results for chemotaxis. Graphs represent the average of 3 separate experiments. * $p<0.05$ compared to No Treatment.

control proangiogenic factor VEGF. Immunofluorscence staining for vWF, an endothelial cell-specific marker, was done to verify that the cells responding to APC treatment were in fact endothelial cells. As seen in Figure 5-3C, both VEGF and APC were able to increase sprout formation of endothelial cells, and they both stained positive for vWF. Therefore, APC increased the formation of endothelial cell sprouts on murine aortic rings similar to the effects of VEGF.

To determine if the effects shown with APC are dependent on APC and not any IIa present in the sample, aortic sections were also treated with hirudin (50 nM), a potent and specific inhibitor of IIa that has been previously shown to inhibit IIa-promoted cell migration [323]. As seen in Figure 5-4, at day 4, APC increases sprout formation, in the presence or absence of hirudin. The effects of hirudin alone are not significant. Therefore, APC is increasing endothelial sprout formation, independent of any IIa present.

To further verify that in the *ex vivo* aortic ring assay, the active form of APC was required to increase the sprout formation, PC (10 μg/mL) and APC-DEGR (10 μg/mL) were used to treat the aortic sections. Because these aortic sections are in culture for 5 days, it is possible any IIa present in the media can bind to the endothelial cell surface and activate soluble PC added at high concentrations. Therefore, to inhibit the effects of IIa, hirudin (50 nM) was also added with PC. As seen in Figure 5-4, at day 4, there was an increase in sprout formation with just the addition of PC to the aortic sections that was not statistically significant. When hirudin was added with PC, there was no effect on sprout formation. The same was true with the addition of APC-DEGR. Therefore, these results suggest that APC promotes endothelial cell sprout formation through its proteolytic activity, as seen with the transwell and tube formation assays.

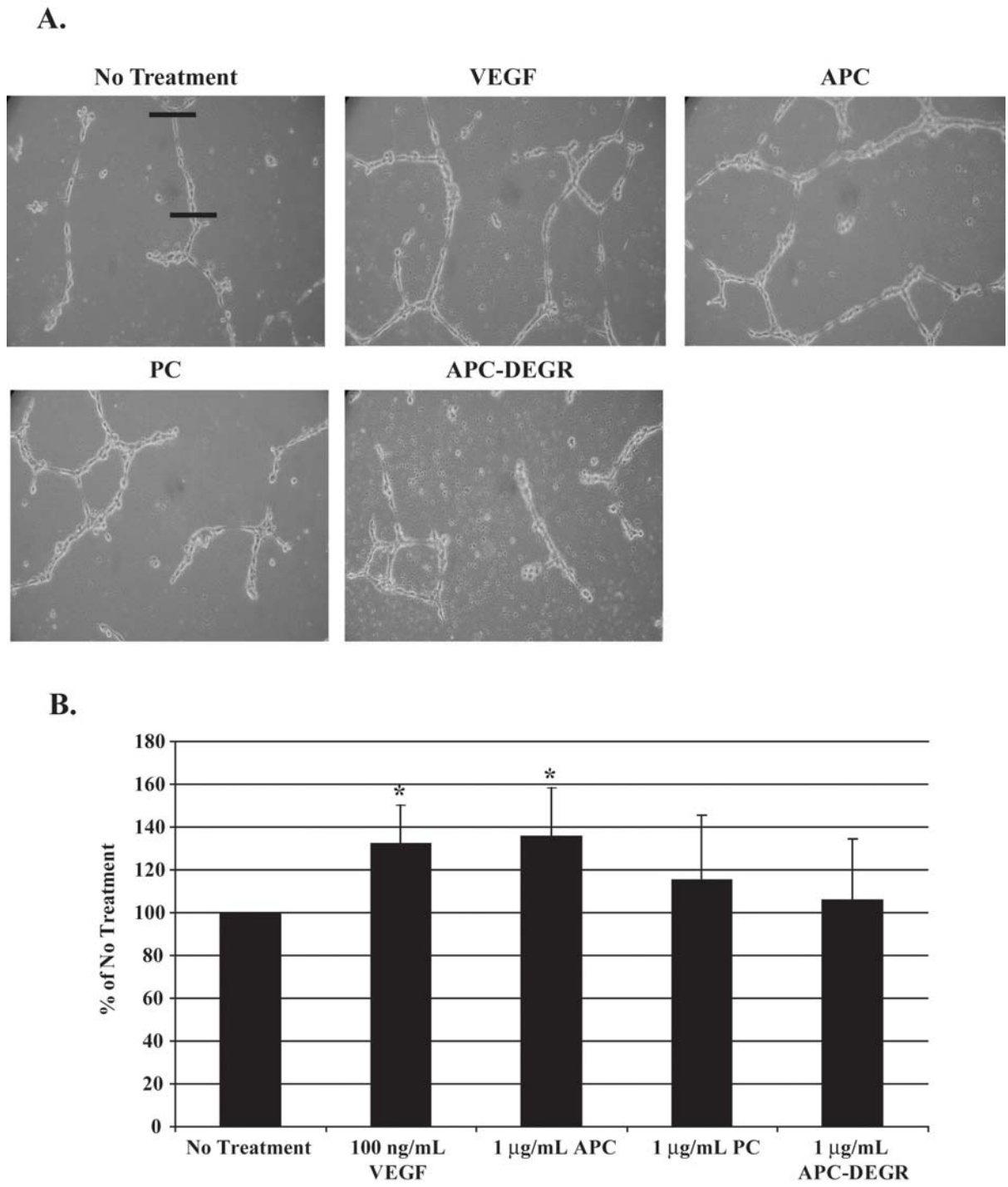

Figure 5-2. ***APC Increases Tube Formation of HUVEC.*** HUVEC were plated onto Growth Factor-Reduced Matrigel and treated with APC (1 µg/mL), PC (1 µg/mL), APC-DEGR (1 µg/mL), or VEGF (100 ng/mL; positive control) for 12 h. (A) Representative photographs were taken at 12 h. Black bars in No Treatment photograph depicts what was considered a vessel-like structure. (B) Graphs represent the average of 3 separate experiments. * $p<0.05$ compared to No Treatment.

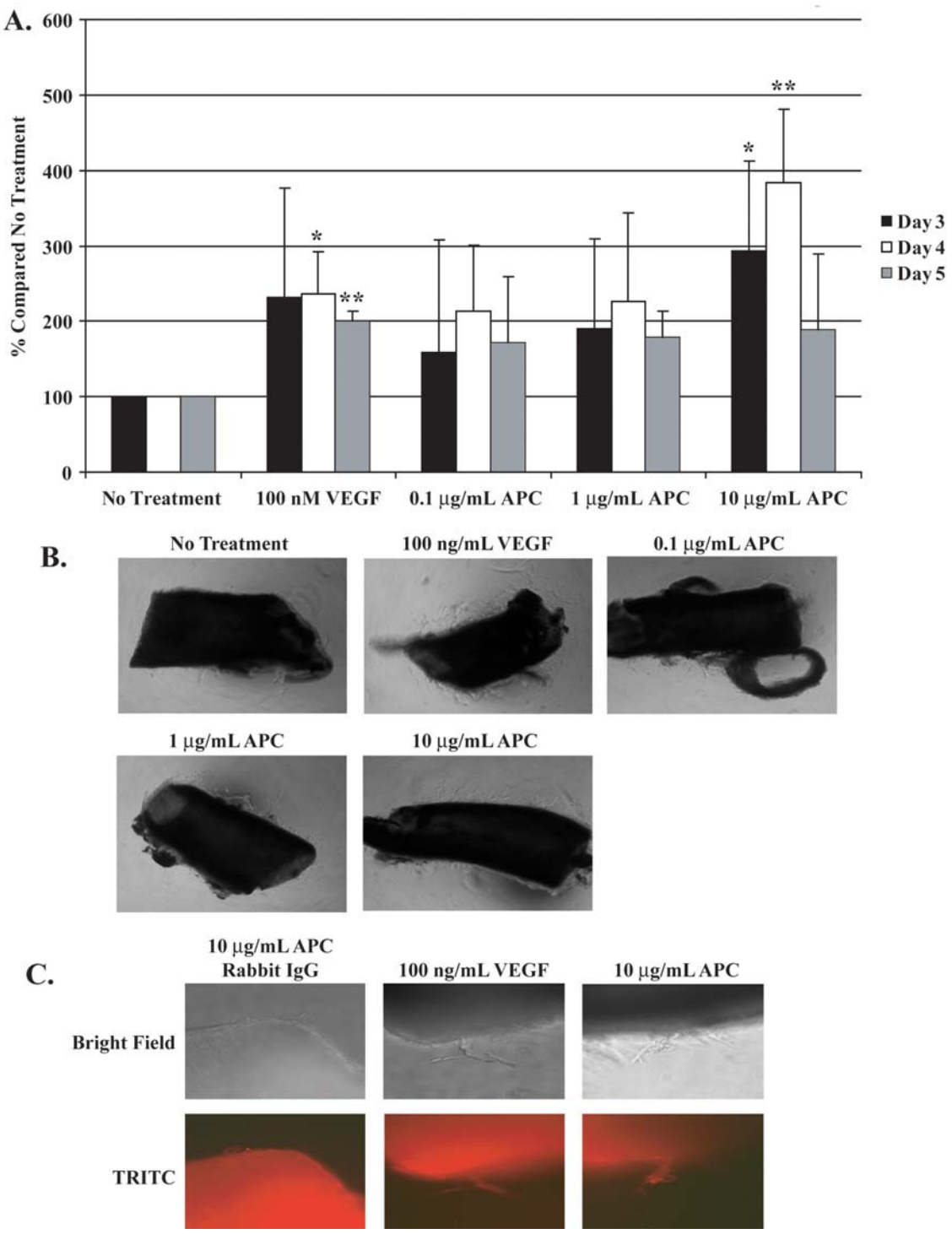

Figure 5-3. ***APC Increases Sprout Formation of Murine Aortic Rings.*** (A) Murine aortic rings were treated with increasing concentrations of APC (0-10 μg/mL) or VEGF (100 ng/mL) for 5 days. On days 3-5, the number of sprouts that projected from the periphery of the aorta was counted. Graphs represent the average of 4 separate experiments. * $p<0.05$, ** $p<0.01$ compared to No Treatment. (B) Representative photographs of aorta taken on day 4. (C) Representative immunofluorescent staining of aortic rings stained for vWF.

APC Increased Endothelial Sprout Formation through EGFR, PI3K, and MMPs

To further characterize the effects of APC on endothelial cell sprout formation, pharmacological inhibitors were used to block the binding or function of specific factors that have been shown to be important in APC-mediated events in other experimental settings. First, EGFR has shown to be important to mediate the effects of APC on inflammation, by reducing the migration of lymphocytes [59]. It has also been shown through EGFR, APC increases breast cancer cell chemotaxis and invasion [326]. Aortic rings were pretreated with a chemical inhibitor, AG 1478, that blocks ligand binding to EGFR. As seen in Figure 5-5A, at day 4, AG 1478 alone reduces the formation of sprouts because of the importance of this receptor in angiogenesis. If this receptor is not important to the mechanism utilized by APC to increase endothelial cell sprout formation, then APC treatment with the inhibitor will be increased over No Treatment. In the presence of the EGFR inhibitor, AG 1478, APC is unable to increase endothelial cell sprout formation. As shown in Figure 5-5B, AG 1478 treatment reduces the number and the length of the endothelial cell sprouts that form.

Second, the PI3K pathway has been implicated in mediating the effects of APC on HUVEC proliferation [79] and breast cancer cell invasion [326] and chemotaxis [323]. Using the pharmacological inhibitor, LY 294002, that blocks PI3K phosphorylation of Akt, there was a complete loss of endothelial cell sprout formation, as seen in Figure 5-5A. If the PI3K pathway is not important to the mechanism utilized by APC to increase endothelial cell sprout formation, then there should be an increase in sprouts, both numerically and visually (Figure 5-5B). However, as seen in Figure 5A, LY 294002 was able to abrogate the effects of

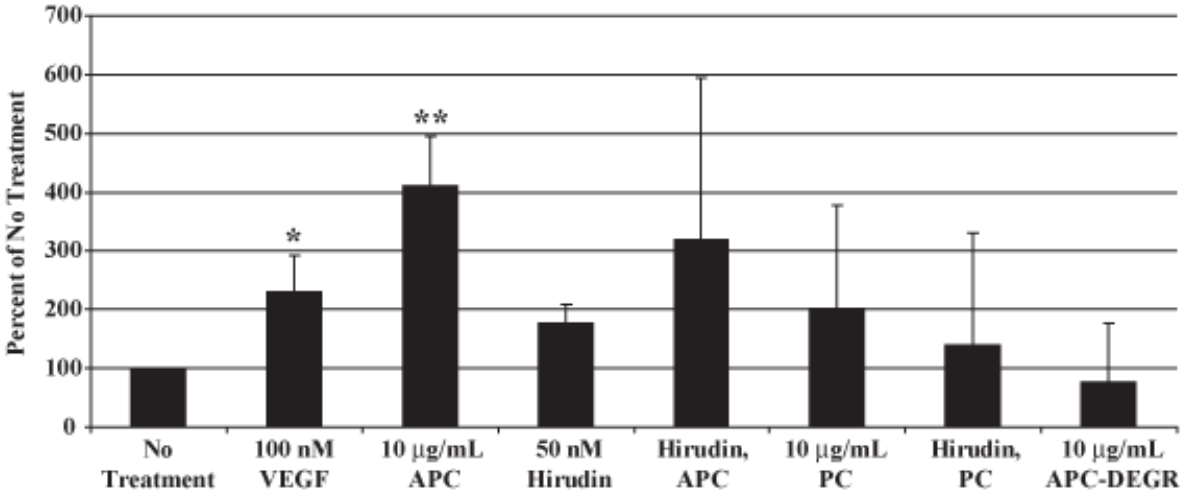

Figure 5-4. ***Active Protease is Required to Increase Sprout Formation of Murine Aortic Rings.*** Murine aortic rings were treated with 10 μg/mL APC with or without Hirudin (50 nM), 10 μg/mL PC with or without Hirudin (50 nM), and 10 μg/mL APC-DEGR for 5 days. Graphs represent the average of 2-3 separate experiments from data collected on day 4. * $p<0.05$, ** $p<0.01$ compared to No Treatment.

APC on sprout formation. Figure 5-5B shows an almost complete lack of endothelial sprouts around the periphery at day 4. If any sprouts are formed, they are only approximately one cell in length, unlike the sprouts formed with APC alone.

Finally, MMPs have been implicated in modulating the effects of APC on migration. MMP-2, specifically, has been shown to have a role in APC-promoted wound healing [77, 78]. MMP-2 and -9 also have been implicated in APC-promoted invasion of breast cancer cells [326]. Using a general MMP inhibitor, GM 6001, there was little effect on sprout formation compared to No Treatment (Figure 5-5A) at day 4. Photographs also taken on day 4 (Figure 5-5B) show few sprouts with some length around the periphery of the aortic section. However, with APC present, GM 6001 blocks the effect of APC on endothelial cell sprout formation (Figure 5-5A). Photographs (Figure 5-5B) show that there is no difference between treatment with GM 6001 and GM 6001 with APC. There are sprouts that form around the periphery of the aortic section, however, these sprouts are neither as numerous, nor as long as the ones that form with APC treatment alone. Taken together, these results imply that APC through EFGR activation of PI3K and degradation of the ECM by MMPs, increases endothelial cell sprout formation in the *ex vivo* aortic ring assay.

Discussion

APC had been previously shown to increase proliferation of HUVEC through binding to EPCR and activating PAR-1 to activate the MAPK, PI3K, and eNOS pathways [79]. APC also increases tube formation of HUVEC, dependent on active protease [79] at a concentration of 300 nM [79]. Using the transwell assays, we present data that shows APC is able to increase HUVEC invasion and chemotaxis at an optimal concentration of 0.1 μg/mL

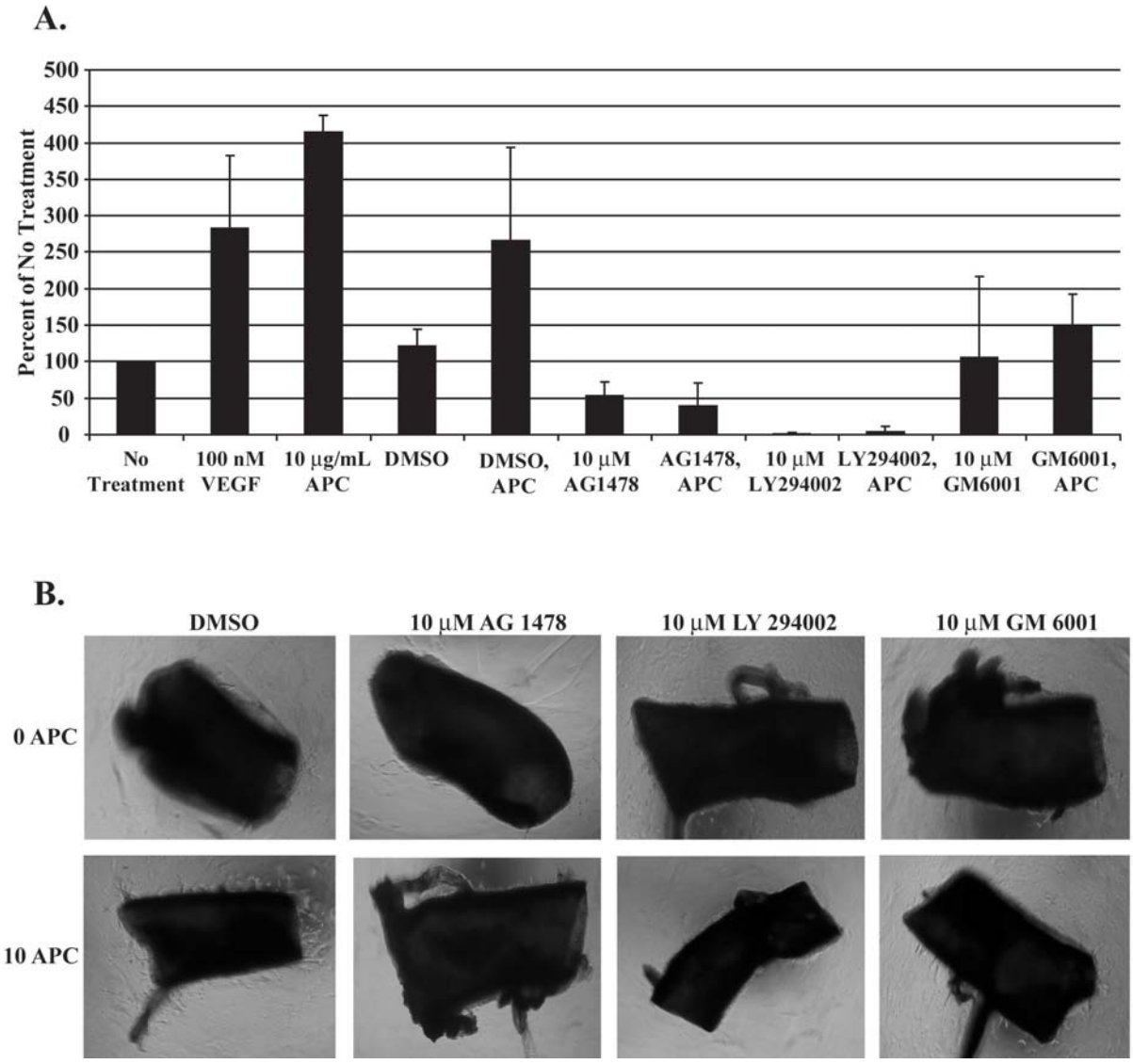

Figure 5-5. ***APC Increases Sprout Formation of Murine Aortic Rings Through EGFR, PI3K, and MMPs.*** (A) Murine aortic rings were pretreated on day 1 with 10 μM of AG 1478, LY 294002, or GM 6001 for 30 minutes prior to the addition of APC (10 μg/mL). Treatments continued out for 5 days. Graphs represent the average of 2 separate experiments from data collected on day 4. (B) Representative photographs of aorta taken on day 4.

and 0.5 μg/mL, respectively. These concentrations, equal to 1.77 nM and 8.83 nM, respectively, are much less then what has been previously used and closer to the physiological level of PC in plasma (~4 μg/mL or ~70.8 nM). The amount of APC administered to patients in the PROWESS study resulted in a steady state level of 45 ng/mL (~0.8 nM) [314]. Also, APC increases the formation of tube-like structures in HUVEC, verifing what had been previously shown [79]. APC increases tube formation of HUVEC through its proteolytic activity, at a lower concentration (17.7 nM) than used in previous studies (300 nM) [79]. Therefore, the effects of APC described here are more relevant to physiologically concentrations of protein C/APC.

HUVEC responded to an optimal concentration of APC in these assays. Endothelial cells respond to an optimal concentration of a pro-migratory factor, such as VEGF, during angiogenesis. As blood vessels form, oxygen and nutrients are brought closer to the tissue releasing VEGF, which promotes a decrease in any further release of this pro-angiogenic factor. With less VEGF present, endothelial cell migration is halted and differentiation into a mature blood vessel will occur. Higher concentrations of VEGF will result in vessels that are leaky and have large lumens [331]. Unlike what has been shown in breast cancer cells, where APC is able to continuously increase migration as the concentration of APC increases [323], endothelial cells appear to respond to an optimal concentration of APC in both the transwell assays and the tube formation assay. The highest concentration of APC (10 μg/mL) actually reduces endothelial cell migration in the transwell assays. Thus, depending on the experimental setting, there is an optimal concentration of APC that will increase endothelial cell invasion, chemotaxis, and tube formation, with higher or lower concentrations having either no effect or a negative effect on HUVEC migration.

Using the *ex vivo* aortic ring assay, APC is shown to increase the formation of vWF-positive endothelial cell sprouts at a higher concentration than was shown with human cells in culture. I used only human APC in my work, as was also initially used in the murine ischemic stroke models [66]. The concentration of human APC that effectively reduced the volume of cerebral infarct and increased cerebral blood flow post-onset of stroke was 2 mg/kg [66]. Similar studies done with murine APC showed that the same beneficial effects can be seen using 10 times less, 0.2 mg/kg [63, 306]. Therefore, the level of APC used to increase endothelial cell sprout formation in the assays presented here (10 μg/mL) would theoretically be comparable to 1 μg/mL of murine APC.

The proteolytic active form of APC is required to increase endothelial cell sprout formation, since both inactive forms of APC, zymogen PC (with hirudin) and APC-DEGR, were unable to promote sprouting. It should be noted that hirudin was added to eliminate any effects of IIa on the system, such as activation of PAR-1 or activation of PC to APC. These results both showed that it was APC and not IIa that was promoting sprout formation, and this increase required active protease. It has been previously shown in endothelial cells and other model systems that APC must cleave PAR-1 to initiate signaling events in the cell to promote proliferation [79], inhibit apoptosis [54, 63-66], decrease the inflammatory response [54, 65, 66], and increase recovery after the onset of a stroke [54, 65, 66]. It has also been shown that APC cleaves pro-MMP-2 to its fully active form [73-76]. Therefore, it is concluded that the proteolytic effects of APC are also required for the formation of endothelial sprouts, potentially activating PAR-1 and initiating signaling pathways to promote chemotaxis and invasion, and/or activate MMPs to promote endothelial cell invasion.

APC increases endothelial cell sprout formation through EGFR. AG 1478 has been used on murine tissue or cells to block binding to EGFR [328, 332]. Endothelial cells express members of the ErbB receptor family [333-335] that are responsive to AG 1478 [334, 335]. EGFR has previously been shown to modulate the anti-inflammatory effects of APC by inhibiting lymphocyte migration [59]. EGFR has also been shown by our laboratory to be involved in APC-promoted invasion and chemotaxis of breast cancer cells [326]. APC may be directly activating the receptor, similar to the effects of tissue-type plasminogen activator on EGFR [324]. APC may also be transphosphorylating EGFR [38, 39] through EPCR and PAR-1. APC has been previously shown to transactivate another receptor, sphingosine 1-phosphate receptor-1, through EPCR and PAR-1 [159]. The results presented here show that APC is activating EGFR, and that EGFR activation is important not only for the increase in the number of sprouts that form on the periphery of the aorta, but also the lengthening of each sprout, as seen in the representative photographs taken on day 4.

APC also increases endothelial cell sprout formation through the activation of the PI3K pathway. The LY 294002 compound, which inhibits PI3K from phosphorylating its downstream effecter Akt, almost completely inhibits the formation of any sprouts on the periphery of each aortic section. The PI3K pathway has been shown to be important for angiogenesis [16]. Therefore, blocking this key pathway blocks APC-promoted sprout formation. Furthermore, APC has been shown to activate PI3K to increase HUVEC proliferation [54] and to increase breast cancer cell migration [326]. APC must also utilize this pathway to increase endothelial cell migration, as shown in the *ex vivo* aortic ring model.

Finally, APC also increases endothelial cell sprout formation through MMP degradation of the ECM. GM 6001 is a broad-spectrum inhibitor of MMPs, including MMP-

2 and -9 that have been shown to be important for angiogenesis [336]. Using the ex vivo aortic ring sprouting assay, unlike that seen for AG 1478 and LY 294002 above, my results show that GM 6001 alone did not suppress sprout formation compared to No Treatment. However, when GM 6001 was added with APC, there was no increase in sprout formation, particularly no increase in the number of sprouts and no increase in length of the sprouts as seen with APC treatment alone. Therefore, as seen in previous cell motility studies, MMP degradation of the ECM is important for the APC-promoted increase in sprout formation. Previously it was shown that APC increased expression of MMP-2 [52, 78]. Through MMP-2, APC enhances wound healing [52, 77]. APC has also been shown to directly activate MMP-2 [73-76]. Previously, our laboratory has shown that MMP-2 and -9 degradation was important for APC-increased breast cancer cell invasion [326]. It is speculated that APC is either increasing the expression of MMP-2 or directly activating MMP-2, particularly since the active form of APC is required to increase sprout formation.

In conclusion, the work presented here gives further insight into how APC is affecting endothelial cell motility and angiogenesis. APC, and not the zymogen PC, activates EGFR or a member of the ErbB family of receptors on the endothelial cell surface. The activation of this receptor leads to the activation of the PI3K pathway to increase endothelial cell migration. The effect of APC on endothelial cell migration also involves the degradation of MMPs to allow for the increased number and lengthening of the sprouts. All of these components are required to increase sprout formation, since blocking just one completely inhibits the effects of APC.

Chapter 6

Breast Cancer and Metabolic Syndrome Linked Through the Plasminogen Activator Inhibitor-1 Cycle

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Summary

Plasminogen activator inhibitor-1 (PAI-1) is a physiological inhibitor of urokinase (uPA), a serine protease known to promote cell migration and invasion. Intuitively, increased levels of PAI-1 should be beneficial in down-regulating uPA activity, particularly in cancer. By contrast, *in vivo*, increased levels of PAI-1 are associated with a poor prognosis in breast cancer. This phenomenon is termed the "PAI-1 paradox." Many factors are responsible for the up-regulation of PAI-1 in the tumor microenvironment. We hypothesize that there is a breast cancer predisposition to a more aggressive stage when PAI-1 is up-regulated as a consequence of Metabolic Syndrome (MetS). MetS exerts a detrimental effect on the breast tumor microenvironment that supports cancer invasion. People with MetS have an increased risk of coronary heart disease, stroke, peripheral vascular disease, and hyperinsulinemia. Recently, MetS has also been identified as a risk factor for breast cancer. We hypothesize the existence of the "PAI-1 cycle". Sustained by

MetS, adipocytokines alter PAI-1 expression to promote angiogenesis, tumor-cell migration, and procoagulant microparticle formation from endothelial cells, which generates thrombin and further propagates PAI-1 synthesis. All of these factors culminate in a chemotherapy-resistant breast tumor microenvironment. The PAI-1 cycle may partly explain the PAI-1 paradox. In this hypothesis paper, we will discuss further how MetS up-regulates PAI-1 and how an increased level of PAI-1 can be linked to a poor prognosis.

Introduction: Western Diet, Metabolic Syndrome (MetS) and Breast Cancer

"When diet is wrong medicine is of no use. When diet is correct medicine is of no need."
Ancient Ayurvedic Proverb

Breast cancer is the second leading cause of cancer deaths among women in the United States. It is predicted that there will be more than 178,000 new cases of invasive breast cancer and more than 40,000 estimated deaths from breast cancer in 2007 [349]. The lifetime probability of a woman developing breast cancer is 1 in 7 [350]. The duration of exposure to estrogen is a major risk factor for breast cancer [350]. Modifiable risk factors for breast cancer include obesity, excessive alcohol use, and physical inactivity [350]. Invasion and metastasis are significant hindrances to the effective treatment of women with breast cancer [351, 352]. Despite advances in detection and therapy, many women will return with distant metastases and eventually succumb to their disease. Understanding the mechanisms that promote or permit invasion is crucial to the development of effective therapies.

Obesity and the resulting associated metabolic pathologies, now termed Metabolic Syndrome (MetS), affect more than 50% of adults in the United States. MetS is a group of

metabolic risk factors, including central obesity, dyslipidemia, hyperinsulinemia, hypertension, and prothrombotic/proinflammatory states [353, 354]. MetS increases the risk of coronary heart disease, stroke, peripheral vascular disease, and hyperinsulinemia [355, 356]. There is growing epidemiological data to suggest a correlation between high-fat consumption from a Western diet and breast cancer in humans [357-359]. Recently, it was shown that obesity and weight gain are associated with increased breast cancer recurrence and cancer mortality [360-362].

In the breast, adipocytes are responsible for the synthesis of adipocytokines, potent cellular modulators up-regulated during MetS [355, 356, 363-367]. Besides adipocytes, there are vascular endothelial cells and stromal fibroblasts that can be regulated by adipocytokines [368]. In addition, a prominent feature of MetS is the up-regulation of the serine protease inhibitor (serpin), plasminogen activator inhibitor-1 (PAI-1; systematic name, *SERPINE1*) [282, 368-370]. PAI-1 is a physiological inhibitor of urokinase-type plasminogen activator (uPA), a serine protease involved in the promotion of cellular de-adhesion, migration/invasion, and activation of plasmin from plasminogen [168, 371-373]. uPA is up-regulated in breast cancer as it progresses to a metastatic disease. Increased levels of the protease are associated with a poor prognosis [374].

Instinctively, one would predict that increased expression of PAI-1 might be beneficial in breast cancer, since it potently inhibits uPA proteolytic activity. *In vitro*, PAI-1 has been shown to inhibit the effects of uPA in cancer [168, 371-373]. However, several clinical studies have found that increased PAI-1 expression in breast cancer is associated with a grim prognosis [351]. In the following sections, we present a hypothesis to possibly explain the relationship of MetS to breast cancer, via a link with PAI-1. We hypothesize that

MetS and adipocytokines alter the microenvironment surrounding the tumor cell by changing the expression of various factors, including PAI-1, that promote breast cancer cell migration and invasion (Figures 6-1 and 6-2).

Plasminogen Activator System and Breast Cancer

The plasminogen activator (PA) system plays an important role in promoting tumor cell invasion [168, 371-373]. uPA is a serine protease involved in many cellular processes including motility, activation of plasminogen to plasmin, and activating pro-matrix metalloproteases (MMP's). While bound to the urokinase receptor (uPAR), uPA activates the zymogen plasminogen to the serine protease plasmin at the cell surface. Plasmin activates MMP's and degrades the surrounding extracellular matrix (ECM) [168, 371-373]. Serpins control proteolytic activity during normal and pathological processes [178, 179]. There are four serpins that regulate the serine proteases of the PA system, PAI-1, plasminogen activator inhibitor-2, plasminogen activator inhibitor-3 (also known as protein C inhibitor), and α_2-plasmin inhibitor. An imbalance in the ratio of protease to the inhibitor allows for increased degradation of the ECM and increased invasion of tumor cells, which explains why many components of the PA system are associated with poor prognosis in several types of cancer [168, 371-373]. There is an inherent balance among uPA, uPAR, and PAI-1 to regulate focal ECM proteolysis and invasion [375]. However, the known biochemical function of PAI-1 contradicts its pathological function in cancer. An increased level of PAI-1 would be expected to inhibit uPA generation of plasmin, protecting the ECM from proteolysis, and thus inhibiting invasion. By contrast, high PAI-1 levels correlate with

a poor prognosis and reduced survival in many cancers including breast cancer [351]. Analyses of pooled data from 18 separate studies showed unequivocally that high levels of PAI-1 and uPA in the tumor are associated with an increase in relapse and metastasis, and a decrease in survival, indicative of a poor prognosis in primary breast cancer [351]. Furthermore, a prospective randomized investigation found that the levels of PAI-1 and uPA in primary breast cancer are predictive of disease recurrence [374].

PAI-1 is expressed in many cell types including endothelial cells, fibroblasts, adipocytes, smooth muscle cells, numerous tissue epithelial cells, keratinocytes, granulosa cells, and platelets. PAI-1 is the primary inhibitor of the PA system targeting both uPA and tissue-type PA (tPA). PAI-1, in the presence of either VN or heparin, also inhibits the serine protease thrombin, although the importance of this inhibition reaction in cancer biology is not well understood. PAI-1 is found in trace levels in peripheral blood because it is a potent uPA and tPA inhibitor and its latent and protease-complexed forms are rapidly cleared. PAI-1 is conformationally unstable, rapidly decaying to an inactive form within 1 to 2 hours [376]. Binding to VN will stabilize PAI-1 for 4 to 6 hours [377, 378]. PAI-1 will rapidly inhibit uPA, which is bound to urokinase receptor (uPAR) on the cell surface. The PAI-1:uPA:uPAR ternary complex interacts with the low density lipoprotein receptor-related protein (LRP), which internalizes the quaternary complex [379]. uPAR and LRP will recycle to the cell surface while PAI-1 and uPA are degraded [380].

The “PAI-1 paradox” exists because increased levels of PAI-1, which presumably would down-regulate uPA activity, should be considered good for cancer. However, in vivo, PAI-1 is associated with a poor prognosis in breast cancer. An increase in PAI-1 levels in the

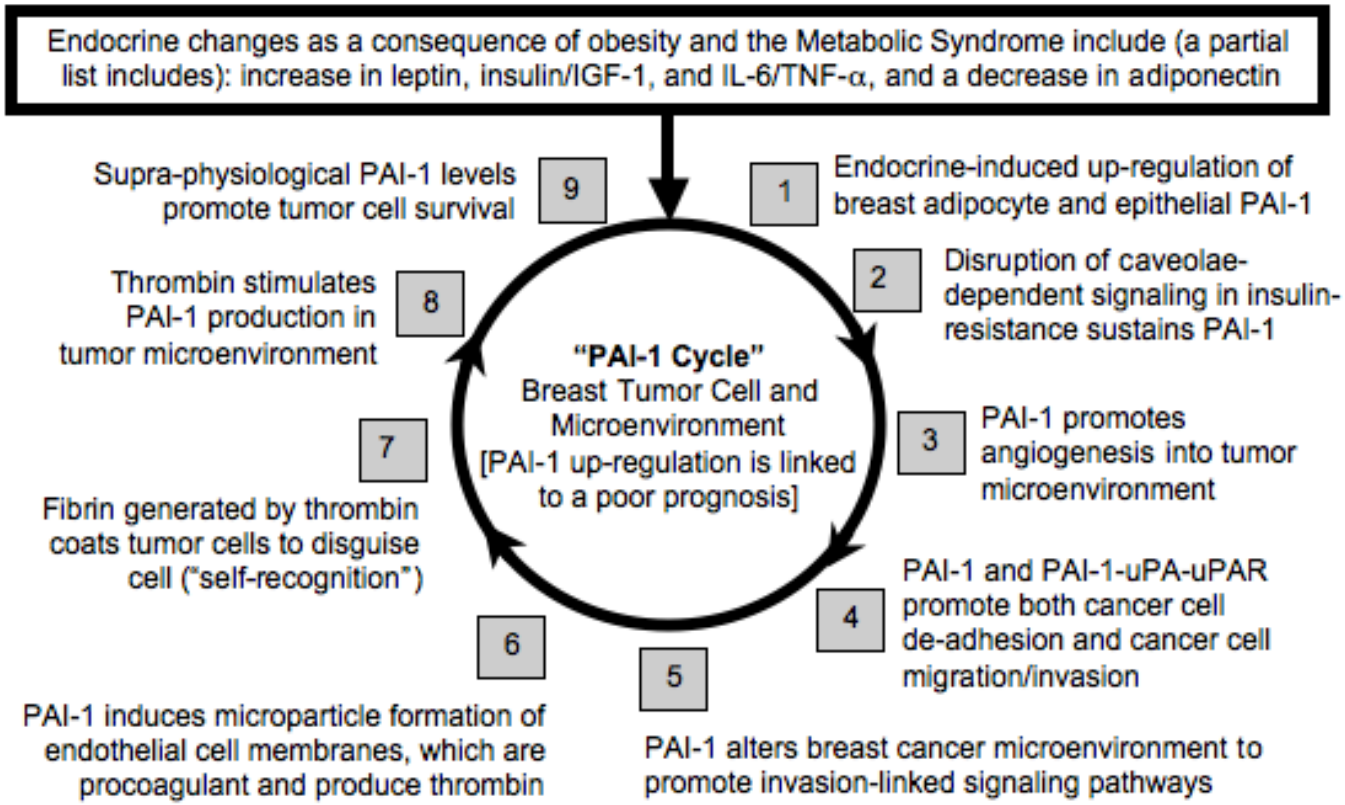

Figure 6-1. ***Breast Cancer, MetS and the PAI-1 cycle.***

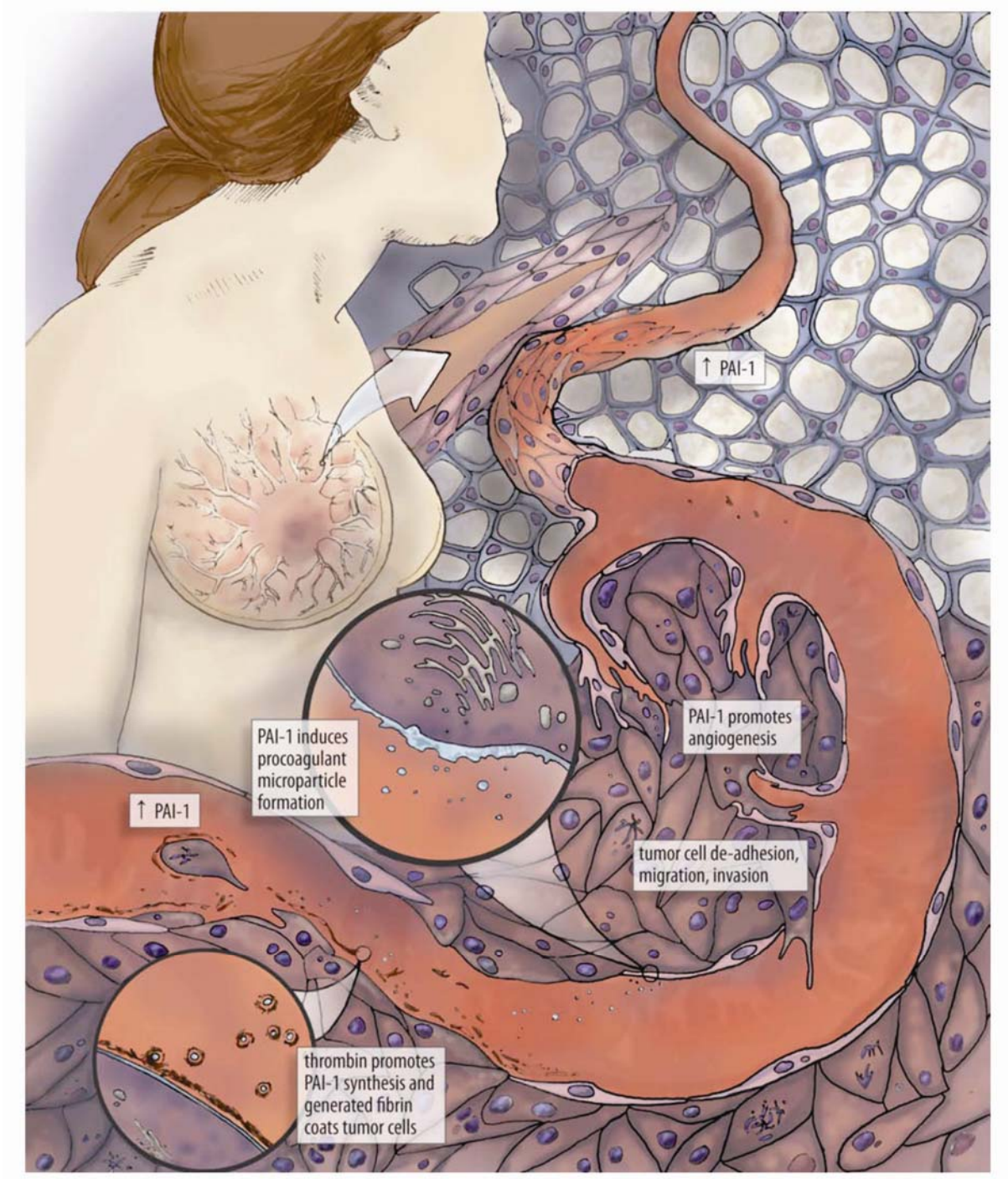

Figure 6-2. ***Metastatic Breast Cancer and the PAI-1 Cycle.*** Adipocytokines as a consequence of MetS will increase PAI-1 expression in breast adipocytes and surrounding tumor microenvironment. The increased levels of PAI-1 will support tumor angiogenesis, promote tumor cell adhesion/de-adhesion, and activate signaling pathways to promote tumor cell invasion. PAI-1 will promote the generation of pro-coagulant microparticles from Fibrin will coat the tumor cells to help the tumor cells avoid immune surveillance. PAI-1 will also prevent apoptosis, and provide a chemotherapy-resistant breast tumor microenvironment. Ultimately, the PAI-1 cycle will lead to sustained PAI-1 synthesis.

breast tumor environment will affect cell adhesion, migration and invasion, apoptosis and proliferation, cell signaling, and tumor angiogenesis via a mechanism that is partly independent of its inhibitory site [168, 371-373, 381]. We suggest the concept that increased levels of PAI-1 involve both "traditional" and "non-traditional" roles for PAI-1 in cancer biology.

The Hypothesis: PAI-1 Cycle

PAI-1 cycle links MetS with breast cancer. We propose that PAI-1 is up-regulated as a consequence of MetS. This increase in PAI-1 exerts a detrimental effect on breast tumor cells, adipocytes, stromal fibroblasts, and vascular endothelial cells to promote tumor cell invasion. The "PAI-1 cycle" is sustained by MetS, resulting in the increased expression of PAI-1 in the tumor microenvironment (Figure 6-1). This PAI-1 will advance numerous pathological processes of breast cancer (Figure 6-2). However, we do not presume that all people with MetS will be predisposed to breast cancer. Adipocytokines, whose production is promoted by MetS, will alter PAI-1 expression [355, 356, 366, 382-384]. The induction of insulin-resistance in adipocytes by MetS will also sustain PAI-1 expression [385, 386]. Increased levels of PAI-1 will support tumor angiogenesis [387-389], promote tumor cell adhesion/de-adhesion, and activate signaling pathways to promote tumor cell invasion [168, 371-373, 390, 391]. PAI-1 will also promote the generation of pro-coagulant microparticles from endothelial cells. These microparticles can support the activation of prothrombin to thrombin [392-394]. Thrombin facilitates tumor cell migration by activating protease-activated receptors to further promote PAI-1 synthesis [395, 396]. Additionally, fibrin produced near the tumor by thrombin will coat the tumor cells, disguising the tumor from the immune system [397, 398]. PAI-1 will also counteract apoptosis [399, 400], which could increase breast

cancer cell survival when treated with a chemotherapeutic drug. Ultimately, the PAI-1 cycle would provide a chemotherapy-resistant breast tumor microenvironment primed for invasion/metastasis [399-403] (Figures 6-1 and 6-2). We will further discuss the various pathophysiological aspects of the PAI-1 cycle and its role in cancer.

Tumor Angiogenesis

PAI-1 has been shown to be both pro-angiogenic and anti-angiogenic. PAI-$1^{-/-}$ mice, described by Devy, et al. [404] and McMahon, et al [405], have a reduction in angiogenesis, up to 60% [405], compared to wild-type mice. Addition of wild-type (wt)-PAI-1 at physiological concentrations, up to ~1 nM, restores/increases angiogenesis up to 3-fold, as seen in two different angiogenic assays [404, 405], while wt-PAI-1 above 1 to 2 nM reduces angiogenesis in these same assays [404, 405].

How PAI-1 affects angiogenesis is not fully understood. Adenoviral gene transfer of wt and mutant forms of PAI-1 that either bind ineffectively to vitronectin (VN) or are unable to inhibit the PA system show angiogenesis is promoted through PAI-1 inhibition of uPA and tPA [404, 406]. uPA and tPA activate plasminogen to plasmin. Excessive plasmin activity could lead to vessel destabilization [406]. Others have shown that the interaction of PAI-1 with the surrounding ECM is a key parameter affecting the progression of angiogenesis. In neuroblastoma tumors, PAI-1 co-expresses with integrins, specifically $\alpha_v\beta_3$, on endothelial cells [407]. PAI-1 binds to VN and prevents VN-integrin cell interactions, resulting in endothelial cell migration towards fibronectin [407].

Cell Adhesion and Proliferation

The PA system is involved in both promoting and preventing cell adhesion. Cells can bind VN through integrins, such as $\alpha_v\beta_3$ and uPAR [408, 409]. uPAR on the cell surface binds directly to VN [409, 410] and this interaction will up-regulate integrin binding to the ECM. uPA bound to uPAR also promotes the interaction of uPAR to VN and strengthens cell adhesion to the ECM [127, 411]. Occupied uPAR will also associate with integrins to further strengthen the cell's adhesion to VN [reviewed in [412, 413]].

PAI-1 inhibits PA system mediated cell adhesion by disrupting the interactions of uPAR and integrins with the ECM [408, 409, 414]. PAI-1 competes with uPAR for VN binding [409]. PAI-1 will lose the ability to bind to VN when it complexes with uPAR-bound uPA [409, 415]. PAI-1 inhibition of uPA prevents the serpin from interacting with the cell and ECM, promoting cell adhesion to VN, fibronectin, and laminin [316, 416]. By removing PAI-1 from VN, cell adhesion is promoted through integrin interaction with the ECM. PAI-1 inhibition of uPA can also promote cell de-adhesion. Internalization of the integrin:uPAR: uPA:PAI-1 complex by LRP removes key components needed to adhere the cell to the ECM [124].

We have found that over-expressing wild-type PAI-1 (wt-PAI-1) in the MDA-MB-435 cells, a breast cancer cell line, decreased the rate of proliferation when compared to either parental cells or the inactive mutant P14 PAI-1-expressing MDA-MB-435 cells (P14 is T333R PAI-1) [416-418]. Anchorage-dependent colony forming efficiency was used as a measure of cell proliferation. The wt-PAI-1-expressing MDA-MB-435 cells had a decreased ability to form colonies compared to the parental, Neomycin vector control, and P14-PAI-1-expressing MDA-MB-435 cells (Table 1). Anchorage-independent proliferation, a hallmark of metastatic potential, had similar results. Wild-type-PAI-1-expressing MDA-MB-435 cells

had a reduced ability to form colonies in soft agar when compared with the other MDA-MB-435 cells (Table 6-1). Although PAI-1 has been associated with a negative prognosis in breast cancer, these results show it to be a negative regulator of proliferation of breast cancer cells *in vitro*.

A possible explanation for the decrease in proliferation by PAI-1 may be through an established function of the cell signaling protein, Akt. Akt is involved in cell survival and proliferation [reviewed in [419]]. PAI-$1^{-/-}$ endothelial cells have an increased level of phosphorylated Akt and an increased rate of proliferation [420]. Wild type-PAI-1-expressing MDA-MB-435 cells have less phosphorylated Akt compared to inactive P14-PAI-1-expressing MDA-MB-435 cells, parental MDA-MB-435 cells, and Neomycin vector control MDA-MB-435 cells (data not included). Our laboratory and others have recently shown that PAI-1 levels are increased in cells that have had the phosphatidylinositol 3-kinase/Akt-Akt signaling axis inhibited [386, 421]. Why a negative indicator of breast cancer survival slows down cell proliferation is not fully understood. It is possible that PAI-1 may serve in a protective role; thus, slowing tumor cell growth could permit more genetic alterations to occur advancing breast cancer to a metastatic stage.

Cell Migration and Invasion

All components of the PA system are involved in regulating cell migration and invasion. These interactions of the PA system can be complex, cell type-specific, and often context-specific. uPA stimulates migration of breast cancer cells, dependent on uPAR ligation and subsequent activation of cell signaling pathways by integrins [422, 423]. There is a reduction in tumor cell migration and metastasis when uPA is inhibited by PAI-1. When

Table 6-1. Stable Expression of wt-PAI-1 Decreases MDA-MB-435 Cell Proliferation*

MDA-MB-435 Cell type	Anchorage-dependent proliferation (% CFE)	Anchorage-independent proliferation (% CFE)
Parental	100 ± 5	100 ± 6
Neomycin vector control	112 ± 19	90 ± 8
wt-PAI-1-expressing	53 ± 23**	58 ± 14*
P14-PAI-1-expressing	121 ± 9	113 ± 10

*PAI-1-expressing [wildtype (wt) and inactive T333R (P14)] and control MDA-MB-435 cells were generated as described previously [416-418]. For anchorage-dependent proliferation, cells were plated at 50 cells per 60 mm^2 dish in 3 ml growth media and grown for 14 days. Colonies were fixed in 3:1 methanol:acetic acid and stained with Giemsa. Colonies of >50 cells were counted and expressed as a % of the number of colonies in the parental MDA-MB-435 population to yield colony-forming efficiency (CFE). For anchorage-independent proliferation, soft agar assays were performed by plating 20,000 cells per 60 mm^2 dish. Cells were suspended in a thin layer of 0.33% noble agar, which was overlaid on a 0.5% agar layer containing 10% FBS in 2x MEM. CFE's were assessed after 3 weeks by counting colonies with >50 cells. The data represent the average of 4 experiments performed in triplicate. * $p<0.05$, ** $p<0.01$, compared with the parental MDA-MB-435 cells.

uPA is over-expressed, altering the ratio of protease to inhibitor, cell migration *in vitro* is promoted [424].

PAI-1 has been shown to promote cell migration. Chazaud et al. [425] showed PAI-1 attaches to cells through uPA:uPAR, which alters cell morphology and promotes breast cancer cell migration. This increase in migration can only occur when all of the components of the PA system are present [425]. Wild-type PAI-1-expressing MDA-MB-435 cells are shown to have significantly increased chemotaxis to VN and fibronectin compared to control cells [316, 416]. Alternatively, PAI-1 can support cell migration by preventing cell adhesion to VN [127, 426]. In smooth muscle cells, PAI-1 increases migration by binding to LRP, which results in morphology changes, cytoskeleton reorganization, and alterations in signaling pathways [427].

PAI-1 also inhibits tumor cell migration [132, 415, 417, 428, 429]. HT1080 cells expressing wt-PAI-1 have reduced cell migration *in vitro* and do not form many lung metastases in vivo [424]. The same is true in glioma cells expressing PAI-1 with a reduction in invasion in vitro compared to wildtype [426]. Over-expressing PAI-1 in breast and ovarian cancer cells reduces both cell migration and invasion, dependent on the active form of PAI-1 [417]. PAI-1 will complex with uPA and through the formation of a ternary complex with uPAR, will also bind LRP. This quaternary complex is internalized resulting in a loss of components of the PA system and reduction in cell migration [429]. Treatment with either receptor-associated protein (RAP), a LRP binding protein that competitively inhibits ligand binding, or LRP blocking antibodies reverses these effects on migration. However, PAI-1-mediated inhibition of migration can also be independent of its uPA-inhibitory role. A mutant form of PAI-1 that is unable to inhibit uPA also prevented cell migration by

disrupting the interactions of VN and integrins [317, 415]. Smooth muscle cell migration can also be inhibited in the presence of PAI-1 through the disruption of VN binding to the cell [428].

Apoptosis and the Gene Expression Profile of PAI-1-expressing Cells

Components of the PA system may regulate apoptosis. PAI-1 has been shown to inhibit apoptosis when added exogenously to both normal and tumor cells, again, due to its inhibition of uPA [399]. This fact alone might explain the PAI-1 paradox. Excess PAI-1 protects cancer cells from apoptosis. As a result, PAI-1 could promote the formation of a more aggressive tumor. A closer examination of PAI-1's inhibition of apoptosis showed that PAI-1 inhibits caspase-3-mediated apoptosis in vascular smooth muscle cells [400].

Slower proliferating cells tend to have a decreased sensitivity to chemotherapeutic agents [430]. We believe that endogenously produced PAI-1 confers a "survival advantage" upon cancer cells. It has been shown that the disruption of the cytoskeleton with chemotherapeutic drugs will alter the balance of protease-to-protease inhibitor in favor of PAI-1 [431]. PAI-1 expression is affected by the perturbation of the actin cytoskeleton by cytochalasin D [401, 402]. Although multiple chemotherapeutic drugs are used to treat breast cancer, paclitaxel (Taxol®) has shown efficacy in patients with drug resistance cancers, including breast cancer [432]. In our studies with the PAI-1-expressing MDA-MB-435 cells, paclitaxel initially induces cell death in both wt-PAI-1-expressing and P14-PAI-1-expressing MDA-MB-435 cells (data not shown). However, when the cells were allowed to recover for 48 hours after treatment in complete medium, wt-PAI-1 expressing MDA-MB-435 cells had an increase in survival compared to P14-PAI-1 expressing MDA-MB-435 cells, although when plated onto either vitronectin or fibronectin surfaces the increase in survival

between cell types was less different (data not included). These results imply that PAI-1 increases cell survival that depends on the integrity of the inhibitory site.

The up-regulation of PAI-1 in a cancer cell is clearly conducive to promoting tumor cell progression to a malignant state. A cDNA microarray analysis was done on RNA extracted from the PAI-1-expressing MDA-MB-435 cells compared to the parental and vector control MDA-MB-435 cells. Of those genes, a list was generated of genes that were up-regulated ≥ 2.5 or down-regulated ≤ 0.5 compared to the MDA-MB-435 Neo cells (Table 6-2). Of the 23 genes up-regulated in the wt PAI-1-expressing MDA-MB-435 cells, many are associated with adhesion, motility, and angiogenesis, thereby bringing nutrients to the tumor and providing a means for metastasis to occur. PAI-1 expression reduces cell proliferation by both up-regulating genes associated with senescence (G protein-coupled receptor 1) and down-regulating genes associated with promoting proliferation. These results agree with what has been published about the effects of PAI-1 on cancer.

PAI-1 expression in the MDA-MB-435 cells increases the expression of genes that are typically up-regulated in various cancers, including breast cancer (Table 6-2). ELAV results in unstable mRNA in breast cancer [433] and eta polypeptide, a 14-3-3 protein involved in various signal transduction pathways, binds to gremlin 1, which is up-regulated in numerous cancers, including breast cancer [434].

Two genes that were up-regulated in the wt-PAI-1-expressing MDA-MB-435 cells would make the cells more responsive to stimuli produced by the MetS (Table 6-2). Protein phosphatase 2C, magnesium-dependent, catalytic subunit is a mitochondrial enzyme regulated by insulin [435]. Niemann-Pick disease, type C1 is a lysosomal membrane protein

involved in cholesterol trafficking [436], and most importantly in drug resistance by its drug efflux and sequestration [437].

The genes down-regulated in the wt-PAI-1-expressing MDA-MB-435 cells were less numerous (Table 6-2). These genes are associated with proliferation, differentiation, and adhesion. Apolipoprotein D regulates both proliferation [438] and cell migration [439]. Follistatin is typically down-regulated in cancer, and is an inhibitor of anti-proliferative pathways [440]. G protein-coupled receptor 126, which is associated with adhesion [441], is also down-regulated by PAI-1 expression.

Concluding Remarks

"What is food to one man may be fierce poison to others." Lucretius (99 B.C.-55 B.C.)

Obesity is a worldwide problem that contributes to the risk and prognosis of many cancers, including breast cancer. With a high-caloric and high-fat Western diet, the resulting MetS will enhance the synthesis of PAI-1 and other components of the PA system in the tumor microenvironment. Adipocytokines (insulin, insulin-like growth factor 1, tumor necrosis factor-alpha, interleukin-6, and leptin/adiponectin) and other by-products of MetS (such as glucose and cholesterol) will alter PAI-1 expression not only in breast tissue adipocytes and in endothelial cells but also in breast cancer cells to potentially favor invasion and metastasis. The complex role and interaction between obesity and MetS linked to PAI-1 expression will clearly be studied for many years to come, as many study the pathological consequences of increased levels of PAI-1.

We hypothesize that increased expression of PAI-1 and other components of the plasminogen activator system (uPA and uPAR) confers a survival advantage upon breast

Table 6-2. The Genetic Profile of wt-PAI-1-expressing MDA-MB-435 Cells Genes Upregulated[a]					
Wt-PAI-1-expressing MDA-MB-435:MDA-MB-435 Neo	**MDA-MB-435:MDA-MB-435 Neo**	**UG Cluster**	**Gene Symbol**	**Gene**	**Function**
18.63	1.22	Hs.414795	SERPINE1	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (PAI-1)	Serine protease inhibitor; PAI-1
5.67	0.96	Hs.12379	ELAVL1	ELAV	RNA transporter/stabilizer; upregulation results in unstable mRNA in breast cancer
4.45	0.71	Hs.149609	ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	Adhesion/cell surface mediated signaling; interacts with uPAR; IGF-1 decreases its adhesion strength in breast cancer
4.08	1.23	Hs.414332	OAS2	2'-5'-oligoadenylate synthase 2, 69/71 kDa	Antiviral activity of interferons
3.77	0.86	Hs.194710	GCNT3	glucosaminyl (N-acetyl) transferase 3, mucin type	Inflammation/immune system
3.64	0.83	Hs.201641	BASP1	brain abundant, membrane attached signal protein	Regulate actin cytoskeleton/neuronal outgrowth in mammory gland
3.62	1.38	Hs.111779	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	Extracellular matrix, adhesion; increased expression in breast cancer; Also associated with increased cell motility
3.52	1.16	Hs.519385	FOXD1	forkhead box D1	Involved in the increase in PAI-1 gene expression
3.5	1.19	Hs.173894	CSF1	Colony stimulating factor 1 (macrophages)	Regulate differentiation, activity, survival of osteoclasts and monocytes/macrophages; Role in monocyte/macrophage mediated tumor angiogenesis
3.15	0.97	Hs.22265	PPM2C	Protein phosphatase 2C, magnesium - dependent, catalytic subunit	Mitochondrial enzyme regulated by insulin
2.96	0.87	Hs.262960	TRPC4	transient receptor potential cation channel, subfamily C, member 4	Component of Ca2+ signaling pathway; Activated by EGFR stimulation
2.96	0.86	Hs.528634	OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	Antiviral activity of interferons
2.94	0.96	Hs.525392	SYNE2	spectrin repeat containing, nuclear envelope 2	Nuclear envelope scaffolding
2.91	0.95	Hs.517601	RAC2	Ras-related C3 botulimun toxin substrate 2 (rho family, small GTP binding protein Rac2)	Ras superfamily GTPase involved in cell growth, cytoskeletal reorganization, and protein kinase

					activation
2.83	1.27	Hs.78068	CPZ	carboxypeptidase Z	Member of metallocarboxypeptidase gene family; Associated with extracellular matrix and Wnt binding
2.73	0.83	Hs.184907	GPR1	G protein-coupled receptor 1	Senescence associated gene
2.72	0.86	Hs.464779	NPC1	Niemann-Pick disease, type C1	Lysosomal membrane protein involved in cholesterol trafficking Associated with drug clearance in the cell
2.66	0.97	Hs.468410	EPAS1	endothelial PAS domain protein 1	Hypoxia induced protein involved in angiogenesis; Regulates PAI-1 and VEGF gene expression
2.65	0.87	Hs.249125	TLX3	T-cell Leukemia, homeobox 3	Involved in autonomic nervous system development; Gene rearrangement (t(5;14)) upregulated in acute lymphoblastic leukemia
2.63	0.95	Hs.144795	KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	Component of a conductance, voltage, and Ca^{2+} sensitive channel
2.61	1.36	Hs.13155	ITGB5	Integrin, beta 5	Adhesion to extracellular matrix; Cell migration
2.58	0.79	Hs.474751	MYH9	Myosin, heavy polypeptide 9, non-muscle	Cytoskeleton and cell migration
2.57	1.24	Hs.226755	YWHAH	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	14-3-3 protein involved in signal transduction; Found to be bound to gremlin 1, which is upregulated in numerous carcinomas including breast
Genes Down-Regulated[a]					
Wt-PAI-1-expressing MDA-MB-435:MDA-MB-435 Neo	**MDA-MB-435:MDA-MB-435 Neo**	**UG Cluster**	**Gene Symbol**	**Gene**	**Function**
0.29	0.85	Hs.522555	APOD	apolipoprotein D	Regulate both proliferation and cell migration in response to growth factors
0.41	1.04	Hs.318894	GPR126	G protein-coupled receptor 126	G-protein coupled receptor involved in cell adhesion
0.42	1.14	HS.9914	FST	Follistatin	Downregulated in cancer; Inhibits activin, a member of the TGF-beta superfamily involved in regulating proliferation

0.44	1.39	Hs.25597	ELOVL1	elongation of very long chain fatty acids (FEN/Elo2, SUR4/Elo3, yeast)-like 2	Unknown
0.47	0.78	Hs.368226	SOX6	SRY (sex determining region Y)-box 6	Transcription factor involved in various developmental processes; Upregulated in gliomas and not other cancers

[a]The MDA-MB-435 cell line was transfected using pcDNA3.1 vectors (Invitrogen) containing wt PAI-1 using Effectene (Qiagen) according to manufacturer's instructions) [416-418]. Clones were selected for resistance to neomycin analogue, G418 (Invitrogen). In collaboration with the Microarray Core Facility at NHGRI, a cDNA microarray was used to compare the gene expression profiles among the parental MDA-MB-435, Neomycin vector control MDA-MB-435 (MDA-MB-435 Neo), and wt-PAI-1-expressing MDA-MB-435 cells. A standard human chip was used that contains over 29,000 elements: 28,200 human sequence verified clones and 800 controls. These genes represent over 24,400 unique Unigene clusters. One sample of RNA was labeled with Cy3 and the other with Cy5. The mixture of the two-labeled RNAs was competitively hybridized to a glass slide containing the cDNAs. For analysis, only genes in which the ratio between MDA-MB-435 and MDA-MB-435 Neo was between 0.7-1.4 were considered. From this list of genes the above tables were generated that list the genes upregulated $\geq$ 2.5 fold and those genes that are reduced by $\leq$0.5 compared to the MDA-MB-435 Neo cells.

cancer cells by decreasing sensitivity to chemotherapeutic agents, regulating adhesion, increasing tumor angiogenesis, and increasing cell migration (Figures 6-1 and 6-2). As many researchers and clinicians have been and are still trying to fully understand the role of PAI-1 in cancer, the PAI-1 cycle hypothesis may further explain the paradox as to why a protease inhibitor of ECM degradation is detrimental for women with breast cancer (Figure 6-2).

Chapter 7

Discussion and Future Direction

APC is not only an anticoagulant serine protease that regulates IIa generation, but it is also involved in modulating inflammation, apoptosis, proliferation, and cell migration. The goal of this Dissertation was to investigate the role of APC in breast cancer cell invasion and endothelial cell angiogenesis. I tested a hypothesis that APC would increase both breast cancer and endothelial cell migration through similar pathways. I used various migration assays along with blocking antibodies and pharmacological inhibitors to characterize the effects of APC and the mechanism by which APC modulates migration.

Like many other serine protease, serpins, and receptors involved in the coagulation pathway, APC has other non-hemostatic functions. Other serine proteases, such as IIa, tPA, and uPA also modulate other cellular processes in numerous cell types. IIa is involved in inflammation, proliferation, and cell migration of endothelial and breast cancer cells. uPA is used as a diagnostic marker in breast cancer. tPA is used clinically to reduce fibrin deposition in the brain related to strokes. Similarly, serpins have been found to have their own functions on various cell processes. PAI-1 has been shown to have a negative role in various pathological processes including cardiovascular disease, cancer, and diabetes. PAI-1 is also proposed to link Metabolic Syndrome to breast cancer. Factors, such as hormone and inflammatory cytokines that are upregulated as a result of the Metabolic Syndrome, also increase the expression of PAI-1. PAI-1 increases tumor angiogenesis, tumor cell migration,

and survival. Another serpin, PCI, is involved in retinoic acid transport and regulating proteases in the testes to ensure proper spermatogenesis. Finally, receptors involved in the coagulation pathway also have non-hemostatic functions associated to activating various signaling pathways. The expression pattern of these receptors occurs not just on the endothelium, but these receptors can be found on cancer, immune, and epithelial cells. More than just the coagulation pathway, but pathways including inflammation also modulate the expression of these proteases, serpins and receptors. Therefore, it is possible that APC can modulate much more that just coagulation and have influence in cell migration and angiogenesis.

Based on previous work, I focused my studies of APC on cell migration and look at specific receptors, ECM proteases, and signaling pathways that had been implication in other functions of APC. First, I studied the effects of APC on breast cancer cells. APC increased breast cancer cell migration in a concentration dependent manner. This increase in both invasion and chemotaxis was dependent on active protease and on interactions with EPCR, PAR-1, and EGFR. I hypothesized that APC was localized to the cell surface by EPCR. APC bound to EPCR then cleaved PAR-1 at the same site as another serine protease, IIa. The activation of PAR-1 would then lead to the transphosphorylation of EGFR either through extracellular release of an EGFR ligand by TACE or through intracellular signaling by c-Src. Through this trimer of receptors (EPCR, PAR-1, and EGFR), APC is able to activate the PI3K pathway, which will lead to the activation of the Raf-MEK1/2-ERK1/2 pathway. These pathways will lead to actin polymerization, formation of leading edge protrusions, and modulations in FAK and integrin interactions with the ECM and other cells. Through Raf-MEK1/2-ERK1/2 pathway, there is an increase in MMP-2 expression. Interestingly, I found

that APC does not increase breast cancer cell invasion through uPA and PAI-1, as previously described. APC increases invasion through MMP-2 and -9 degradation of the ECM. I hypothesized that APC upregulates the expression of MMP-2 and/or -9. Bound on the cell at EPCR on MDA-MB-231 cells, APC activated the zymogen to the fully active form of these ECM proteases. Importantly, all of these components work interdependently. Blockage of any single pathway, receptor or cell component results in the complete abrogation of the effects of APC. Thus, when APC is in solution and able to interact with MMPs, it must still bind to the cell surface and activate signaling pathways to increase cell migration.

In another aspect of this research, I showed that APC increases endothelial cell migration, resulting in an increase in angiogenesis. Unlike the breast cancer cells, the endothelial cells only responded to a specific concentration of APC. Knowing that the endothelial cell migration and invasion could be enhanced by APC, I then used a tube formation assay and found that APC enhanced the network of tube-like structures and junction points, representative of a network of blood vessels. Again, APC increased the formation of these tube-like structures dependent on active protease. Finally, using an *ex vivo* aortic ring assay, I explored whether or not APC could promote endothelial cells to de-differentiate, migrate, and proliferate at an increased rate, representative of the beginning stages of angiogenesis. APC increased the number of vWF-positive endothelial cell sprouts to form on the periphery of the aortic sections and also increased their length comparable to the effects of VEGF. As was seen above, this increase was also dependent on active APC, activation of both EGFR and PI3K-dependent signaling pathways, and degradation of the ECM by MMPs. Thus, I hypothesized that APC binds to the endothelium through EPCR, where it can cleave and activate PAR-1. The activation of PAR-1 leads to the activation of

EGFR. This network of receptors including EPCR, PAR-1, and EGFR, will then activate the PI3K pathway, resulting in the increase in cell migration. APC will also increase the expression and activation of MMPs, specifically MMP-2 and -9, to increase degradation of the ECM. All of these components were again shown to be interdependent on each other. If even just one were inhibited, the effects of APC were abrogated.

There remain several questions that have evolved from the work presented in this Dissertation. First, three receptors, EPCR, PAR-1, and EGFR, have been identified as having a role in mechanism utilized by APC to promote cell migration. There is only speculation of how APC binds to and activates these receptors. Further work using fluorescently tagged proteins and cell models that quantitatively identify the activation of specific signaling pathways would help us understand how APC is interacting with the cell surface. Second, using siRNA, pharmacological inhibitors, and quantitatively measuring the phosphorylation of specific proteins in the signaling pathways examined would allow us to more fully map out the entire signaling pathway(s) activated by APC. Third, using real time rt-PCR we could determine if APC is increasing the expression of MMPs, specifically MMP-2 and -9. Also, using activity assays specific for these ECM proteases and by using zymography, we could directly determine how APC is involved in activating MMP-2 and -9 to increase invasion. Fourth, I found that endogenous levels of PAI-1 and uPA of breast cancer cells were not involved in the mechanism utilized by APC to increase cell migration. However, the question still remains whether or not APC bound to the cell surface can be inhibited by serpins, specifically PCI and PAI-1. Using wild-type serpins and serpins mutated at residues important for protease interaction, we could determine if these serpins inhibit the non-hemostatic functions of APC and the specific points of interactions. Finally, as explored in

more detail in Chapter 4, additional work that could be linked to Chapter 5 includes the involvement of murine EPCR and PAR-1, activation of MAPK, MMPs, role of murine tPA/uPA/PAI-1 and further verification that endothelial cells are migrating in response to APC. This would allow for us to more completely determine how similar the pathways to increase breast cancer cell and endothelial cell migration using murine endothelial cells and the aortic ring model of sprouting.

In conclusion, APC is able to increase cell migration, shown using breast cancer cells and endothelial cells, through both extracellular and intracellular processes. APC binds to the cell surface through EPCR, activates PAR-1 and EGFR, which lead to the activation of the PI3K and MAPK pathways. APC also increases invasion with the help of MMP-2 and -9 degradation of the ECM. APC could be both beneficial by promoting angiogenesis. However, in another setting, APC could be detrimental by promoting the spread of cancer cells through the same pathways utilized to promote angiogenesis.

Appendix

1. Beaulieu, L.M. and F.C. Church (2006) Is protein C inhibitor (PCI) antithrombotic and protective in pulmonary hypertension? ***J. Thromb. Haemost.*** **4:** 2327-2330.

2. Whitley, B.R., L.M. Beaulieu, J.C. Carter, and F.C. Church (2007) Phosphatidylinositol 3-kinase/Akt regulates the balance between plasminogen activator inhibitor-1 and urokinase to promote migration of SKOV-3 ovarian cancer cells. ***Gynecol. Oncol.*** 104(2): 470-479.

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COMMENTARY

Is protein C inhibitor antithrombotic and protective in pulmonary hypertension?

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See also Nishii Y, Cesar Gabazza E, Fujimoto H, Nakahara H, Takagi T, Bruno N, D'Alessandro-Gabazza CN, Maruyama J, Maruyama K, Hayashi T, Adachi Y, Suzuki K, Taguchi O. Protective role of protein C inhibitor in monocrotaline-induced pulmonary hypertension. This issue, pp 2331–9.

In this issue, Nishii *et al.* [1] from Mie University in Japan provide interesting evidence that mice over-expressing protein C inhibitor (PCI) are protected from monocrotaline-induced pulmonary hypertension, due in part to the ability of PCI to inhibit thrombin and down-regulate coagulation. The transgenic mouse over-expressing PCI has enhanced lung secretion of PCI, and the clinical, biochemical and pathological parameters show a reduction in pulmonary hypertension in this experimental model.

The pathophysiological role of the human plasma serine protease inhibitor (serpin), protein C inhibitor (PCI, also named plasminogen activator inhibitor-3, systematic name of *SERPINA5*) has remained elusive since its description in the 1980s [2,3]. From its name, PCI is an inhibitor of activated protein C (APC) [4–14]. However, PCI also inhibits thrombin (IIa) [8–10,12], factor Xa and factor XIa [8,11–14], kallikrein [8,10,12,15–17], urokinase-plasminogen activator and tissue-plasminogen activator (t-PA) [8,18–23], acrosin [24–26], prostate specific antigen [27,28], and remarkably, thrombin-thrombomodulin [29–31], which is responsible for generating APC (Fig. 1). The broad protease inhibitory profile of PCI has led many to postulate both specific and generic roles for this serpin. To further complicate matters, the tissue distribution of PCI in humans compared with mice is quite different. Humans show a broad tissue expression pattern for PCI, including the liver, kidney, pancreas, prostate, testes and ovaries [32–37]. Thus, this explains why human PCI (hPCI) is found not only in circulating blood, but also in urine, saliva, amniotic fluid, milk, tears and other body fluids [32]. In contrast, the mouse and rat express PCI only in the reproductive organs and it is not found in the circulating blood [34,38–42]. Through the creation of a PCI knockout mouse by homologous recombination, one non-hemostatic function of PCI was determined [26]. Male $PCI^{-/-}$ mice were infertile due to abnormal spermatogenesis caused by loss of the Sertoli cell barrier. Unopposed proteolytic activity in these mice brought about the degradation of the cell barrier [26]. Two transgenic mouse models expressing hPCI have been developed. The first was described by Wagenaar *et al.* [43], in which hPCI was expressed in the liver and found in the circulation. The second hPCI transgenic mouse was described by Hayashi *et al.* [44] and it expressed hPCI not only in the liver, but also in the kidney, heart, brain, lung and reproductive organs.

Concerning human health, the presence of PCI in various lung diseases has been described [45]. Fujimoto *et al.* [45] reported that bronchoalveolar lavage fluid contained increased amounts of both PCI and thrombin-activatable fibrinolysis inhibitor (TAFI) in patients with interstitial lung disease (ILD), particularly in patients with cryptogenic-organizing pneumonia, collagen vascular disease-associated ILD, and sarcoidosis. One explanation of their findings was that the levels of intra-alveolar PCI inhibited both APC activity and activation, which contributed to the pathogenesis of ILD. Therefore, a key question asked in the current study by Nishii *et al.* [1] was concerning the contribution of PCI to the pathogenesis of pulmonary hypertension. This study uses the hPCI over-expressing transgenic mouse described by Hayashi *et al.* [44] to begin to address this question regarding pulmonary hypertension, and also provides data on the physiological function of PCI (Fig. 1).

Nishii *et al.* [1] treat mice with monocrotaline to induce pulmonary hypertension. This murine model is representative of pulmonary hypertension caused by a known etiology and not a secondary consequence of cardiovascular disease. Overall, hPCI reduces the disease state in the mouse lung compared with the wild-type mouse. The increase in pressure associated with pulmonary hypertension is not seen in the hPCI over-expressing transgenic mice. Pulmonary hypertension also results in endothelium dysfunction. The vessels in the lungs

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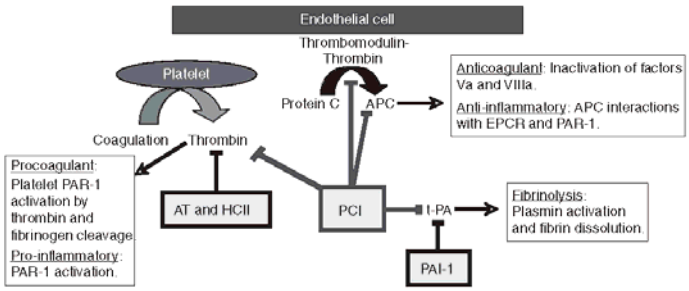

Fig. 1. Role of protein C inhibitor and other serpins (antithrombin, heparin cofactor II, and plasminogen activator inhibitor-1) in the regulation of serine proteases thrombin, activated protein C (APC), APC generation by thrombin-thrombomodulin, and tissue plasminogen activator. All are key proteases in blood coagulation, fibrinolysis, and inflammatory processes.

are hypercoagulant as a result of a decrease in prostaglandin and nitric oxide production. Platelets become activated and will adhere to the vessel wall. Hypercoagulability can be assessed by measuring the formation of thrombin:AT (TAT) complex. Although there is an increase of TAT complex in the hPCI over-expressing transgenic mice when treated with monocrotaline, this increase is significantly less than that in wild-type animals. These results suggest that either there is a decrease in thrombin production that would reduce TAT levels, or the increased presence of PCI is competing with AT to inhibit thrombin, which would also reduce the TAT levels. Either way, the hPCI over-expressing transgenic mouse does not exhibit an increase in activation of coagulation upon treatment with monocrotaline. Although PCI can be procoagulant through its inhibition of the protein C system of proteases, when hPCI is over-expressed in the mouse, its anticoagulant function is more prominent. Fibrinolysis is increased in mice upon treatment with monocrotaline, as indicated by an increase in t-PA activity. As PAI-1 levels are similar between the wild-type and hPCI over-expressing transgenic mice when treated with monocrotaline, the only explanation for a decrease in free t-PA and lowered fibrinolysis in the transgenic mice is the increase in hPCI.

Pulmonary vascular endothelium dysfunction also results in the release of various cytokines, such as tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1), and growth factors, such as platelet-derived growth factor (PDGF) and interleukin-13 (IL-13), that will promote inflammation and vascular wall thickening. The monocrotaline-treated wild-type mice show an increase in all of these protein levels. The hPCI over-expressing transgenic mice treated with monocrotaline exhibit little to no change in these same proteins. Therefore, hPCI reduces the endothelial dysfunction and inflammation associated with pulmonary hypertension. Furthermore, measurements of the pulmonary arteries in the hPCI over-expressing transgenic mouse show a smaller change in vessel wall and lumen area upon monocrotaline treatment. Overall, their results suggest that hPCI inhibits thrombin, a pro-inflammatory and pro-migratory factor, thus, reducing the effects of monocrotaline-induced pulmonary hypertension.

The data presented in this paper raise more questions than answers about the *in vivo* protease specificity of PCI. Their results suggest that PCI exerts both anti-inflammatory and anticoagulant action by inhibiting thrombin, a known participant in coagulation, inflammation and tissue remodeling. This function of PCI is more prominent than its role as an inhibitor of APC. Inhibition of APC alone would result in an increase in coagulation and inflammation, and a reduction of tissue remodeling, which was not seen in the mouse model described. PCI is also antifibrinolytic through its inhibition of t-PA. Whether or not PCI can be used therapeutically for treating pulmonary hypertension remains to be studied. With the aid of new ELISAs reported for PCI-protease complexes [46–49], these tools can provide answers regarding the role of PCI in coagulation and inflammation.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Phosphatidylinositol 3-kinase/Akt regulates the balance between plasminogen activator inhibitor-1 and urokinase to promote migration of SKOV-3 ovarian cancer cells

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Abstract

Objectives. Increased levels of urokinase-type plasminogen activator (uPA) are associated with shortened overall survival in ovarian cancer patients. Additionally, elevated levels of the serine protease inhibitor (serpin), plasminogen activator inhibitor-1 (PAI-1), a uPA inhibitor, have also been correlated with an unfavorable prognosis in ovarian cancer. Therefore, it is critical to understand the signaling pathways that regulate PAI-1 and uPA expression in cancer cell migration-invasion.

Methods. We studied the PI3K/Akt, Rho kinase/ROCK, p38 MAPK and MEK pathways and their modulation of PAI-1 and uPA expression and wound-induced cell migration in SKOV-3 ovarian cancer cells. The PI3K/Akt pathway was further examined using pharmacological inhibitors (LY294002 and wortmannin), Akt siRNA, constitutively active Akt adenovirus and treatment with IGF-1/insulin in the SKOV-3 cells.

Results. The PI3K/Akt pathway negatively regulates PAI-1 expression and positively correlates with migratory abilities and uPA expression in SKOV-3 cells. A reduction in active Akt results in an increase in PAI-1 expression coupled with a decrease in uPA expression to ultimately result in reduced cell migration and invasion. By contrast, an increase in Akt activity reduces PAI-1 expression and results in an increase in SKOV-3 wound-induced cell migration. Furthermore, IGF-1 and insulin stimulated SKOV-3 migration by altering the balance between uPA and PAI-1 to favor uPA, and the enhanced migration was attenuated by treatment with LY294002 indicating PI3K/Akt in this pathway.

Conclusions. These results suggest an overall ovarian tumor-protective role for PAI-1, and that the PI3K/Akt signaling pathway regulates the ratio of PAI-1:uPA to either increase or decrease cell migration.

Keywords: Plasminogen activator system; PAI-1; Urokinase; Migration and invasion; SKOV-3 cells; PI3K/Akt signaling system

Introduction

Ovarian cancer accounts for 4% of all cancers among women but it is the leading cause of gynecological cancer deaths [1]. Advanced ovarian cancer is characterized by a high frequency of metastasis to lymph nodes and invasive growth into multiple organs due to peritoneal dissemination [2,3]. Invasive ovarian cancers demonstrate increased levels of the serine protease, urokinase-type plasminogen activator (uPA), and its serine protease inhibitor (serpin), plasminogen activator inhibitor-1 (PAI-1), compared with benign ovarian cancer or normal ovary. These increased amounts of uPA and PAI-1 are typically correlated with a more aggressive phenotype of ovarian cancer and are linked to a poor prognosis [4–8]. Upon binding its cell surface urokinase receptor (uPAR), uPA activates plasminogen into plasmin to facilitate extracellular matrix degradation and tumor cell invasion. In addition to proteolytic activity and regulation, uPA, PAI-1 and uPAR are implicated in cell signaling pathways controlling cell proliferation, migration and invasion.

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The PI3K/Akt cell signaling pathway is implicated in cell migration and invasion [9–11]. The PI3K/Akt pathway regulates uPA expression; selective inhibition of the PI3K/Akt pathway in numerous cell types decreases uPA expression and/or activity with a subsequent decrease in cell invasion [12–14]. Urokinase itself has been reported to stimulate PI3K activity and activates the downstream effectors Akt [15,16] and Rac1 [17]. Inversely, antisense uPA in glioblastoma cells causes a decrease both in wound migration and in PI3K/Akt activity [18]. Conversely, looking at PAI-1 levels, both hypoxia-induced PAI-1 expression [19] and nerve growth factor-induced PAI-1 expression [20] can be inhibited by PI3K inhibitors. A link between PAI-1 and phosphorylated Akt was recently demonstrated in aortic endothelial cells from the PAI-1 knockout mouse, which showed increased phosphorylated Akt levels compared to wild-type aortic endothelial cells [21]. Furthermore, both insulin-like growth factor-1 (IGF-1) and insulin modulate expression of uPA and PAI-1 through PI3K/Akt in breast cancer cells and in adipocytes [22,23]. IGF-1 and insulin are involved in cell survival, proliferation and cell migration; thus, their interaction with PI3K/Akt and the changes in expression of uPA and PAI-1 are being studied in many different disease settings.

The PI3K pathway is important in ovarian carcinogenesis [24]. Akt has been shown to be amplified or over-expressed in ovarian cancer, implying that it also has a role in ovarian carcinogenesis [25–28]. PI3K is constitutively activated in the SKOV-3 ovarian cancer cell line [27]. The PI3K/Akt pathway is an important signaling pathway to examine in the context of ovarian cancer and in relation to both PAI-1 and uPA expression. Using the SKOV-3 ovarian cancer cell line as a model for ovarian cancer in in vitro migration assays, we sought to better understand the relationship of the PI3K/Akt pathway to PAI-1 and uPA. This study describes the effects of a selection of signaling pathway inhibitors on both basal unstimulated SKOV-3 and on insulin- and IGF-1-treated SKOV-3 cell migration.

Materials and methods

Cell Culture

SKOV-3 cells were obtained from the University of North Carolina Tissue Culture Facility in the Lineberger Comprehensive Cancer Center and maintained as monolayer culture in minimal Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic in a humidified chamber with 5% CO_2 at 37°C.

Wound-induced migration assay

Confluent SKOV-3 monolayers were scratched with a sterile yellow pipette tip, washed and then treated with serum-free media containing the given concentrations of signal pathway inhibitors (Y27632 and SB203580 were obtained from Calbiochem, San Diego, CA; LY294002, wortmannin and PD98059 were from Biomol, Plymouth Meeting, PA) and/or blocking antibodies (PAI-1 or uPA antibody, both from American Diagnostica, Stamford, CT). Wound-induced migration assays with Akt siRNA or Akt adenovirus-treated SKOV-3 cells were performed in 1% FBS-containing media. Additional wound-induced migration assays with IGF-1 and insulin were performed on serum-starved SKOV-3 monolayers, as described previously [29]. Cells were pre-treated for 1 h with sterile DMSO (Sigma, St. Louis, MO) or 50 μM LY294002 in serum-free media, scratched, washed and then treated with 100 nM insulin (Invitrogen, Carlsbad, CA) or 50 ng/mL IGF-1 (R&D Systems, Minneapolis, MN) in the absence or presence of 50 μM LY294002 in serum-free media for 24 h.

Migration of cells into the wound was monitored, beginning at 0 h (immediately following wounding) using a Kodak MDS290 camera and software. Wound closure was quantified by measuring the distance in pixels between wound edges (10 lines per wound) at each time point, using the measuring tool in Adobe Photoshop® with a grid superimposed on the image to guide the measurements. The wound width was normalized to 100% at 0 h for each treatment condition and presented as a percent of wound remaining at the given time point.

Immunofluorescent staining

SKOV-3 cells were plated on glass-bottom dishes overnight, scratched with a sterile yellow pipette tip and treated with indicated concentrations of cell signaling inhibitors (Y27632, SB203580, LY294002, wortmannin and PD98059) for 6 h. Non-permeabilized cells were fixed in 1% paraformaldehyde, then incubated with antibodies to PAI-1 (polyclonal, #528216, Calbiochem) and uPA (monoclonal, #394, American Diagnostica). AlexaFluor-conjugated secondary antibodies (Molecular Probes, Carlsbad, CA: PAI-1, AlexaFluor 647; uPA, AlexaFluor 594) were used to visualize immunofluorescence staining with an Olympus FV500 confocal laser-scanning microscope (Microscopy Services Laboratory, Department of Pathology and Laboratory Medicine, Chapel Hill, NC).

Cell-secreted indirect uPA assay

An indirect uPA activity assay was performed using a synthetic plasmin substrate measuring plasminogen activation by uPA as described previously [30]. Briefly, unconcentrated conditioned media from treated cells was added to buffer containing plasminogen and plasmin substrate (S-2251, Chromogenix, West Chester, OH). Triplicate samples were incubated for 60 min at 37°C and the reaction was monitored spectrophotometrically. The data presented are the average of 2 separate experiments and represents the amount of uPA activity remaining compared to the untreated SKOV-3 cells (normalized to 100%).

Western blot

Cell lysates were harvested and protein was separated on a 10% polyacrylamide gel, transferred and blotted for active Akt (phospho-Ser 473, Cell Signaling Technology, Danvers, MA), total Akt (Cell Signaling Technology), total ERK2 (Santa Cruz, Santa Cruz, CA) or tubulin (Sigma). Conditioned media from the cells were concentrated using centrifugal filtering devices (Millipore, Billerica, MA) with a molecular weight cutoff of 30 kDa and treated the same as the lysates but run on either 10% or 12% gels, and blotted for PAI-1 (Molecular Innovations) and uPA (American Diagnostica). Total protein concentration was determined using a dye-binding assay (Biorad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Densitometry of the Western blots was performed with ImageJ software from NIH and normalized to either Akt, ERK2, tubulin, total protein or no treatment, as indicated.

Cell migration and invasion assays

Migration experiments were conducted using BD BioCoat™ culture inserts (BD Bioscience, Bedford, MA), while invasion experiments utilized BD BioCoat™ Matrigel™ Invasion Chambers (BD Bioscience), both with an 8-μm diameter pore size membrane in a 24-well companion plate, as described previously [30]. Briefly, membranes were re-hydrated with 0.1% BSA and 1% antibiotic/antimycotic in serum-free medium prior to the experiment. The chemoattractant (complete medium containing 10% FBS) was added to the lower well of the plate. Cells were seeded onto the culture insert in serum-free medium with the given concentrations of the various cell signaling inhibitors and incubated for either 6 or 24 h, as specified, at 37°C. After the incubation period, the media were removed from the insert, cells on the upper surface of the membrane were removed with a cotton-tipped applicator and cells that migrated or invaded to the lower surface of the membranes were fixed with 100% methanol. Inserts were washed with PBS, stained with Hoechst (1:20,000 in 1×

PBS, Molecular Probes), and the membranes were excised from the insert, inverted and mounted on glass microscope slides. The total number of nuclei were counted in four fields at 40× magnification using UV fluorescence microscopy. The data presented are normalized to untreated SKOV-3 cells.

siRNA transfection

SKOV-3 cells were transiently transfected using SignalSilence™ Akt siRNA (Cell Signaling Technology) and the GeneEraser™ siRNA transfection reagent (Stratagene, LaJolla, CA), following manufacturer's guidelines. Briefly, a mixture of Opti-MEM (Gibco, Carlsbad, CA) and GeneEraser was incubated 15 min at room temperature. Then Akt siRNA (final concentration of 125 or 250 nM) was added and incubated for 15 min at room temperature. This mixture was added to SKOV-3 cells at approximately 80% confluency for 8 h, and all siRNA experiments were performed under these same conditions. Fresh media were then added to prevent cell toxicity by the transfection reagent. Protein expression and the wound-induced migration assay were performed 48 h post-transfection.

Akt adenovirus

The constitutively active (myristolyated) Akt adenovirus (Myr Akt) was a generous gift from Dr. Kenneth Walsh (Boston University Medical Center, Boston, MA). SKOV-3 cells were infected with a CMV control adenovirus or Myr Akt adenovirus at an MOI 50 for 24 h. Protein expression and wound-induced migration were measured 24 h post-infection.

Statistical analysis

Statistical analyses were performed using Instat (GraphPad Software, Inc, San Diego, CA). Assuming normal distribution, a one-way analysis of variance test (ANOVA) ($p<0.05$) was used followed by a Dunnett multiple comparison test. If the p value was less than 0.05 after the post-test, it was concluded that the differences observed were not due to a type I error and with a 95% confidence interval, the difference between the means was true. In Fig. 8, a Student's t-test was done comparing individual treatments to SKOV-3 cells treated with vehicle (DMSO). Assuming normal distribution, $p<0.05$ was concluded to be significant and have a 95% confidence interval; that is, the differences between the means were true.

Results

Effect of a selection of pharmacological inhibitors on PAI-1 and uPA expression and wound-induced migration of SKOV-3 ovarian cancer cells

We used pharmacological inhibitors of Rho-kinase/ROCK, p38 MAPK, MEK and PI3K to better understand the signaling pathway(s) involved in regulating both PAI-1 and uPA expression and cell migration, using a wound-induced migration

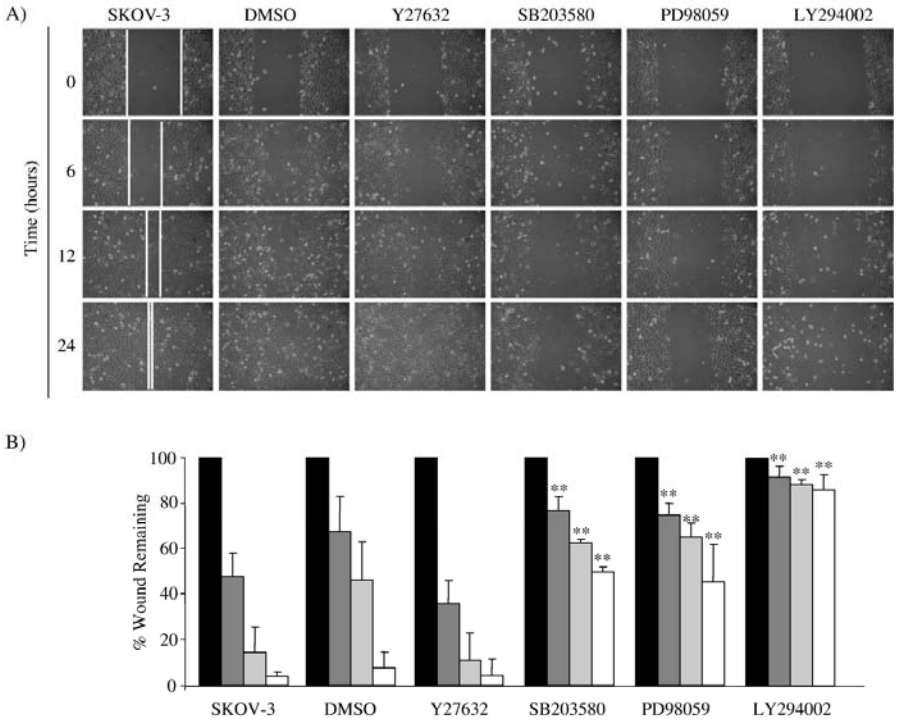

Fig. 1. Rho kinase/ROCK, p38 MAPK, MEK and PI3K inhibitors differentially alter wound-induced SKOV-3 cell migration. (A) A wound-induced migration assay was performed on SKOV-3 cells in the absence and presence of DMSO vehicle control, 10 μM Y27632, 10 μM SB203580, 25 μM PD98059 or 25 μM LY294002. Photomicrographs of the treated cells are shown for the initial wounding (0 h) and at 6, 12 and 24 h post-wounding. (B) The distance the cells migrated into the wounded area was quantified as described in Materials and methods and presented as the percent wound remaining at the given times. $^{**}p<0.01$, compared with untreated SKOV-3 cells at the given time point, 0 h (black bar), 6 h (dark grey bar), 12 h (light grey bar) and 24 h (white bar).

assay in the highly invasive SKOV-3 ovarian cancer cell line. The Rho kinase/ROCK inhibitor (Y27632) did not alter SKOV-3 wound-induced migration (Fig. 1A and B). However, the p38 MAPK inhibitor (SB203580) and the MEK inhibitor (PD98059) reduced SKOV-3 wound-induced migration by approximately 50% (Fig. 1A and B). The PI3K inhibitor (LY294002) reduced SKOV-3 migration by approximately 90% (Fig. 1A and B).

By immunofluorescence staining, there was an apparent increase in PAI-1 in SKOV-3 cells treated with LY294002 and PD98059, but there was no change noted in cell surface PAI-1 expression in SKOV-3 cells treated either with Y27632 or with SB203580 (Fig. 2A). Unlike that seen for PAI-1, a decrease in uPA expression was found in SKOV-3 cells treated with all of the pharmacological inhibitors (Fig. 2A).

A functional uPA activity assay was then used with conditioned media of SKOV-3 cells. This assay confirmed that all four pharmacological inhibitors altered the balance between uPA and PAI-1, reflected by the changes in functional uPA measured. Listed is the relative order of potency of the inhibitors on reducing uPA activity (at the highest dose tested): Y27632<PD98059 ~ SB203580<LY294002 (Fig. 2B).

Collectively, these results reveal that the various signaling pathways reduce wound-induced migration of SKOV-3 cells to varying extents, which is manifested by different changes with regards to both PAI-1 and uPA expression.

Inhibition of PI3K increases PAI-1 expression and decreases uPA expression in SKOV-3 cells

The PI3K pathway was examined in more detail due to the contrasting change in PAI-1 and uPA levels in SKOV-3 cells. Western blot analysis of LY294002-treated SKOV-3 cells shows a decrease in phosphorylated Akt, from 40% to 80% with increasing doses, as a measure of PI3K activity (Fig. 3). We found a substantial increase in PAI-1 secreted (2- to >6-fold) by SKOV-3 cells in the conditioned media upon LY294002 treatment (Fig. 3). As previously shown by others [12–14], we also found an accompanying decrease in the amount of uPA secreted (55–70%) when SKOV-3 cells were treated with LY294002 (Fig. 3). These results imply that changes in both PAI-1 and uPA expression are a direct result of PI3K inhibition since both LY294002 and wortmannin (data not included) had similar effects.

PI3K inhibitors decrease both SKOV-3 wound-induced migration and transwell invasion and migration

The dose response of both LY294002 and wortmannin on wound-induced SKOV-3 cell migration was performed. At 12 h, untreated SKOV-3 cells migrated into the denuded area to essentially close the wound (Fig. 4A). Increasing doses of LY294002 (5–50 μM) significantly reduced SKOV-3 wound-

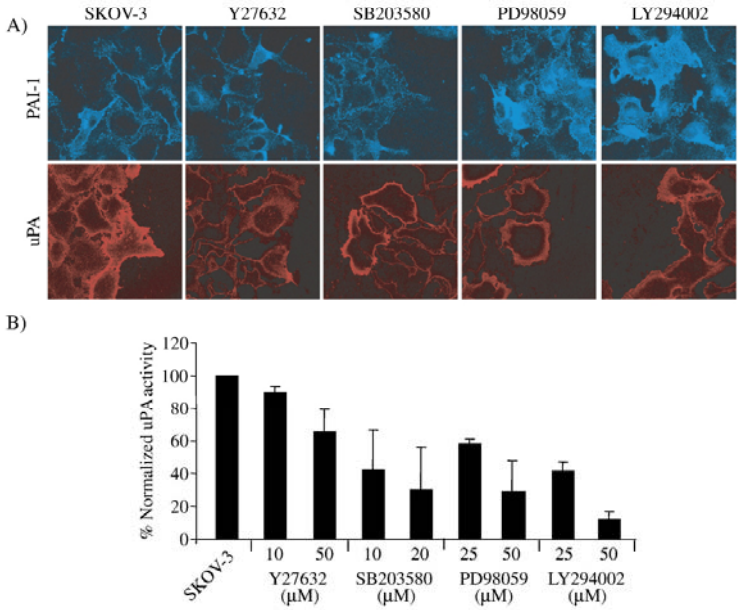

Fig. 2. Rho kinase/ROCK, p38 MAPK, MEK and PI3K inhibitors differentially alter the expression of PAI-1 compared to uPA in SKOV-3 cells. (A) SKOV-3 cells were wounded and treated with 10 μM Y27632, 20 μM SB203580, 25 μM PD98059 or 25 μM LY294002 for 6 h, then the cell surface expression of PAI-1 and uPA was detected with immunofluorescence staining of non-permeabilized cells. (B) SKOV-3 cells were treated with the given concentrations of the same panel of cell signaling inhibitors for 24 h before uPA activity in the conditioned media was measured. uPA activity was normalized to the untreated SKOV-3 cells (results are given as mean values of triplicate assays done in two separate experiments).

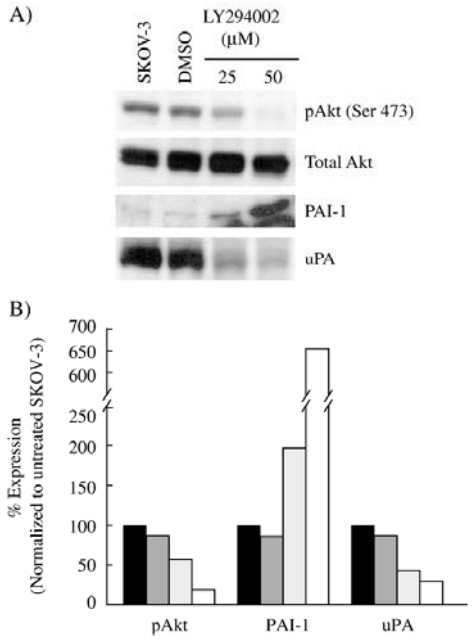

Fig. 3. Inhibition of PI3K increases PAI-1 expression and decreases uPA expression in SKOV-3 cells. SKOV-3 cells were treated for 24 h in serum-free media with two doses of the PI3K inhibitor, LY294002. (A) Lysates were harvested and equal protein was separated on a 10% polyacrylamide gel, transferred and blotted for active Akt (phospho-Ser473) and total Akt. Conditioned media from the cells were concentrated and treated the same as the lysates, except blotted for PAI-1 and uPA, loaded by total protein. (B) The bands detected by Western blot were quantitated by densitometry as described in Materials and methods as SKOV-3 cells untreated (black bar), DMSO-treated control SKOV-3 cells (dark grey bar), 25 μM LY294002 (light grey bar) and 50 μM LY294002 (white bar).

induced migration from 20% to 80%, and wortmannin (0.25–1 μM) similarly affected SKOV-3 migration (Fig. 4A).

As expected, treatment with a PAI-1 blocking antibody increased the migration of LY294002-treated SKOV-3 cells compared to SKOV-3 cells treated only with LY294002 or with LY294002 and a non-specific IgG control antibody (Fig. 4B). Likewise, the uPA-blocking antibody decreased SKOV-3 cell migration even further following treatment with LY294002 (Fig. 4B). These results suggest that some of the LY294002-induced migration changes are mediated by alteration in the levels, and thus the balance, of PAI-1 and uPA in SKOV-3 cells.

It is possible that the signal pathways required in cell migration over a solid surface, as in a wound-induced migration assay, may differ from those required in transwell assays. Addition of LY294002 (Fig. 5A and B) or wortmannin (Fig. 5C and D) to the SKOV-3 cells during transwell invasion and migration assays resulted in a dose-dependent decrease in both invasion and migration after 6 h, with a maximum reduction of 80%. The experiments were carried out for 6 h, to ensure that any changes measured were not the result of loss in cell viability induced by the compounds. This would also allow for direct comparison with uPA and PAI-1 expression after 6 h of treatment (Fig. 2A). These results suggest that the effect of PI3K inhibitors was similar to the wound-induced migration assay with SKOV-3 cells; thus, inhibition of PI3K/Akt reduces cell invasion and migration by altering the existing levels of PAI-1 and uPA to change the PAI-1:uPA ratio.

Modulation of Akt alters SKOV-3 wound migration, PAI-1 expression and uPA expression

We used siRNA to specifically down-regulate Akt and then re-assessed wound-induced migration and levels of Akt, PAI-1 and uPA expression in the SKOV-3 cells. Transient transfection of SKOV-3 cells with Akt siRNA reduces total Akt expression by 30% when compared to SKOV-3 cells transfected with GeneEraser™ transfection reagent alone. As a result, there was a dose-dependent up-regulation of PAI-1 (>200%) and a down-regulation of uPA expression (60–75%) (Fig. 6A). Despite the incomplete siRNA silencing of Akt expression, the change in uPA and PAI-1 levels was similar to that in SKOV-3 cells

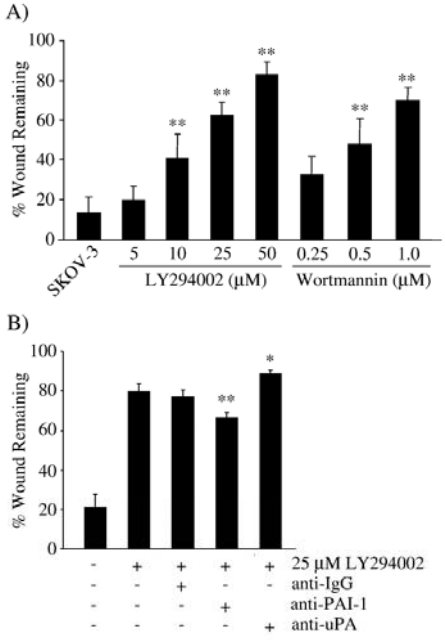

Fig. 4. Inhibition of PI3K, PAI-1 and uPA alter SKOV-3 wound-induced migration. (A) Wound-induced migration in the presence of increasing doses of LY294002 or wortmannin was measured for 12 h. *$p<0.05$, **$p<0.01$ compared with untreated SKOV-3 cells. (B) Wound-induced migration in the presence of 25 μM LY294002 and uPA- and PAI-1-blocking antibodies (each at 20 μg/ml) for 12 h. The graphs represent the average of 2–4 separate experiments performed in triplicate. *$p<0.05$, **$p<0.01$ compared with SKOV-3 cells treated with 25 μM LY294002.

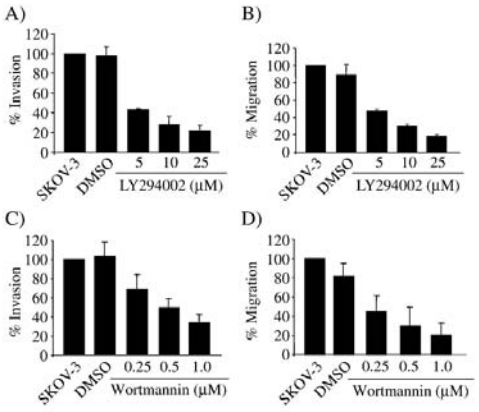

Fig. 5. Inhibition of PI3K decreases SKOV-3 invasion and migration. SKOV-3 transwell invasion and migration were measured in the presence of increasing doses of LY294002 (A and B) or wortmannin (C and D) for 6 h. Cell migration and invasion assays were performed as described in Materials and methods. Graphs presented are the average of 2 experiments performed in duplicate.

following LY294002 treatment (compare to 25 μM LY294002 in Fig. 3). Furthermore, transient transfection of the SKOV-3 cells with Akt siRNA has a dose-dependent reduction (50–70%) in wound closure compared to SKOV-3 cells in the presence of the transfection reagent alone (Fig. 6B). Again, the reduction in migration by Akt siRNA is similar to that seen when SKOV-3 cells are treated with LY294002 (compare to 10 μM LY294002 in Fig. 4). These results further support an association between PI3K/Akt and PAI-1 and uPA expression to influence cell migration in SKOV-3 cells.

We then utilized a constitutively active Akt construct (Myr Akt) to further define the relationship of active Akt, PAI-1 and uPA expression and wound-induced migration in SKOV-3 cells. Greater than two-fold increased levels of Akt in SKOV-3 cells infected with the Myr Akt adenovirus correlated with a greater than 50% decrease in PAI-1 expression (Fig. 7A). The change in uPA expression is minor compared with our results when Akt was down-regulated by siRNA; however, the balance between inhibitor and protease is still shifted, and in this case, in favor of uPA. In addition to changes in protein expression, Myr Akt significantly increased wound-induced migration of SKOV-3 cells, from 30% to 4% wound remaining (Fig. 7B). These results help to further establish the link between the plasminogen activator system as components in the PI3K/Akt signaling pathway regulating cell migration and invasion.

IGF-1 and insulin modulate SKOV-3 wound migration and uPA/PAI-1 expression

Given the established link between IGF-1 and insulin with the PI3K/Akt pathway in many cell systems [23,31], we next examined the influence of these growth factors on uPA and PAI-1 levels and their ability to modulate SKOV-3 cell migration. Urokinase expression in SKOV-3 cells was increased by insulin and IGF-1 with a concomitant decrease in PAI-1 (Fig. 8A). Under serum-free conditions, the addition of LY294002 alone revealed a similar pattern of increased PAI-1 levels described earlier (Fig. 8A). The addition of IGF-1 with LY294002, but not the combination of insulin with LY294002, also showed the trend to increase PAI-1 expression (Fig. 8A). The effects of IGF-1 and insulin on the activity of PI3K, with or without LY294002, were confirmed by Western blot analysis of phosphorylated Akt (data not shown). Insulin and IGF-1 significantly increased the wound-induced migration of SKOV-3 cells (from 70% to 20% and 40% wound remaining, respectively), while LY294002 eliminated this enhanced cell migration (Fig. 8B). These results imply that insulin and IGF-1

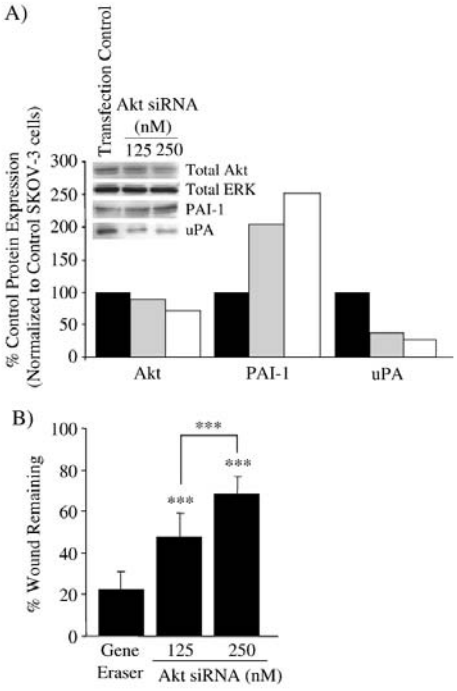

Fig. 6. Modulation of Akt in SKOV-3 cells affects PAI-1 and uPA expression and cell migration. SKOV-3 cells were transiently transfected with Akt siRNA. Total Akt, ERK2, PAI-1 and uPA protein expression were monitored in cells transfected with GeneEraser transfection reagent alone or with Akt siRNA (125 nM or 250 nM). (A) Total Akt, total ERK, PAI-1 and uPA expression were monitored and densitometry was used to quantify the change in protein expression detected by Western blot, normalized to ERK2 as a total protein loading control, and presented as the percent expression compared to GeneEraser transfection control SKOV-3 cells. SKOV-3 cells with GeneEraser transfection reagent alone (black bar); SKOV-3 cells with GeneEraser plus 125 nM Akt siRNA (grey bar); SKOV-3 cells with GeneEraser plus 250 nM Akt siRNA (white bar). (B) The ability of Akt siRNA-transfected SKOV-3 cells to migrate was monitored in a wound-induced migration assay in 1% FBS conditions, and the results are presented as the percent wound remaining at 12 h. ***$p<0.001$ compared with SKOV-3 cells with GeneEraser alone, or between Akt siRNA concentrations.

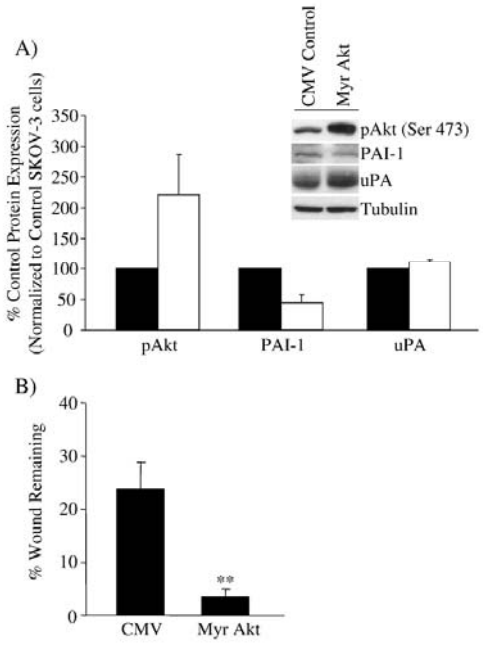

Fig. 7. Over-expression of Akt in SKOV-3 cells affects PAI-1 expression and cell migration. SKOV-3 cells were infected with control CMV or Myr Akt adenovirus at MOI 50. (A) Active Akt (pAkt), PAI-1 and uPA expression were monitored, and densitometry used to quantify the change in protein expression detected by Western blot, normalized to tubulin as a protein loading control; the results are presented as the percent expression compared to control CMV adenovirus-treated SKOV-3 cells. Results are presented as the percent expression of Myr Akt-infected SKOV-3 cells (white bar) compared to CMV control adenovirus-infected SKOV-3 cells (black bar). (B) The effect of Myr Akt expression on SKOV-3 cell migration was measured in a wound-induced migration assay in 1% FBS conditions, and the results are presented as the percent wound remaining at 12 h. $^{**}p<0.01$ compared with SKOV-3 cells with control CMV alone.

alter the balance between uPA and PAI-1 in favor of uPA, thus enhancing cell migration. LY294002 attenuates this pro-migratory activity, which further supports an association between PI3K/Akt and PAI-1:uPA levels as an influence on SKOV-3 cell migration.

Discussion

There is a need to develop new approaches in chemoprevention, early detection and innovative treatments for ovarian cancer, the leading cause of gynecological cancer deaths. Defining the genetic aberrations and their underlying molecular changes can help in the development of new detection methods and treatments for ovarian cancers. Increased expression of PAI-1 and uPA in ovarian cancers suggests that they are markers linked to a poor prognosis [4–8]. Therefore, it is imperative to understand the regulation of PAI-1 and uPA expression through signal pathways involved in migration and invasion of cancer cells that contribute to the progression and mortality of ovarian cancer.

The PI3K pathway is involved in many cellular processes, including proliferation, survival, apoptosis, migration, invasion and cytoskeletal rearrangements. The results presented in this paper dissect the importance of this pathway, using pharmacological inhibitors, targeted deletion or deliberate over-expression of active Akt in SKOV-3 ovarian cancer cell migration and invasion with respect to regulation of PAI-1 and uPA expression. The balance between PAI-1 and uPA expression is delicate, but extremely important in regulating cell behavior. A shift in the balance towards PAI-1, whether due to an increase in PAI-1, a decrease in uPA or a combination of both, tends to prevent in vitro migration and invasion of cancer cells, as we and others have shown previously [30,32–35]. Likewise, down-regulation of PAI-1, up-regulation of uPA or both would shift the balance in favor of uPA and presumably increase in vitro migration and invasion. This concept helps to explain our results using a survey of pharmacological inhibitors to signaling pathways known to affect cell migration. No matter the change

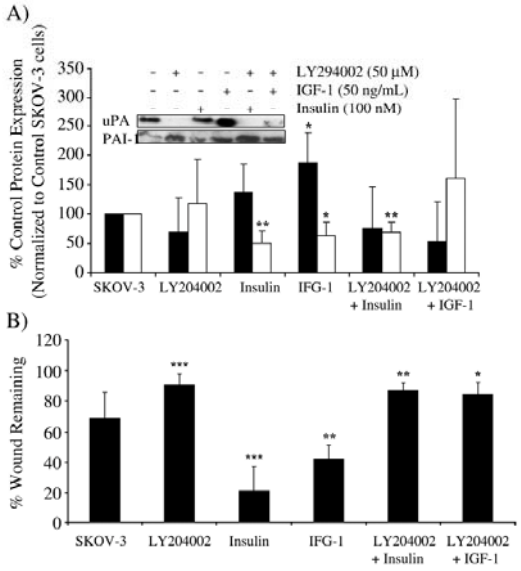

Fig. 8. IFG-1 and insulin enhance cell migration and alter the balance between uPA and PAI-1 in SKOV-3 cells. SKOV-3 cells were treated with LY294002 and insulin (100 nM) or IGF-1 (50 ng/ml) as described in Materials and methods. (A) PAI-1 and uPA expression (secreted in the conditioned media) were monitored, and densitometry used to quantify the change in protein expression detected by Western blot (uPA, black bar; PAI-1, white bar); data were presented as the percent expression compared to control-treated SKOV-3 cells. $^{*}p<0.05$ or $^{**}p<0.01$ for uPA or PAI-1 compared with untreated SKOV-3 cells for each respective protein. (B) The effect of insulin and IGF-1 in combination with LY294002 was measured in a wound-induced migration assay under serum-free conditions, and results are presented as the percent wound remaining at 12 h. $^{*}p<0.05$, $^{**}p<0.01$ or $^{***}p<0.001$ compared with untreated SKOV-3 cells.

in PAI-1 expression, the inhibitors of Rho kinase/ROCK, p38 MAPK, MEK and PI3K all decrease uPA expression in SKOV-3 ovarian cancer cells, effectively shifting the PAI-1:uPA balance in favor of PAI-1. Only the p38 MAPK, MEK and PI3K inhibitors decrease wound-induced SKOV-3 cell migration. The lack of effect of the Rho kinase/ROCK inhibitor may be due to only a small decrease in uPA expression. Collectively, our results support the finding that various signaling pathways positively and negatively alter both PAI-1 and uPA expression to profoundly regulate SKOV-3 cell wound-induced migration.

Through our experiments, a new link emerges between PAI-1 expression and levels of phosphorylated Akt, which alters both cell migration and cell invasion. SKOV-3 cells treated with LY294002 showed a dose-dependent decrease in phosphorylated Akt, a dose-dependent increase in PAI-1 and a dose-dependent decrease in uPA. Inhibition of PI3K activity also resulted in a dose-dependent decrease in cell migration and invasion in a transwell assay, and a dose-dependent decrease in migration measured in a wound-induced migration assay. Likewise, specific down-regulation of Akt by siRNA resulted in an increase in PAI-1 expression, a decrease in uPA expression and a decrease in wound-induced migration. By contrast, expression of constitutively active Akt caused the opposite effects on SKOV-3 cells: an increase in phosphorylated Akt levels correlated with a decrease in PAI-1 expression and an increase in wound-induced migration. The changes in SKOV-3 cell migration that accompanied the increase (constitutively active Akt adenovirus) or decrease (LY294002 and wortmannin treatment, Akt siRNA) in active Akt levels were similar to previously published studies [11–15,36–41]. It will be important to further these observations using different ovarian cancer cell lines, especially those that are not dependent on PI3K/Akt for migration and invasion. However, in further support of our results, a recent study showed a correlation between decreased phosphorylated Akt levels and decreased invasion in SKOV-3 cells [42]. Likewise, the regulation of uPA expression and activity by the PI3K/Akt pathway that we showed confirmed previously published results [12–15,39,40]. Finally, Venugopal et al. [23] showed in an in vivo study that plasma PAI-1 was up-regulated in Akt-deficient mice (protein kinase B or PKBα $^{-/-}$), which would attenuate the PI3K/Akt signaling pathway.

Potential initiators of the PI3K/Akt pathway that could alter the plasminogen activator system are insulin and IGF-1. Elevated levels of IGF-1 have been associated with an increased risk in development of ovarian cancer [43,44]. The relationship of insulin is of importance since obesity and metabolic syndrome have been linked to various cancers [45]. Recently, it was shown that insulin-induced PAI-1 levels in 3T3L1 adipocytes were increased by treatment with the PI3K inhibitor LY294002 [23]. Using IGF-1 and insulin, which are both known to increase uPA levels, in a wound-induced migration assay, we found that these growth factors increased SKOV-3 cell migration and this increase was attenuated upon treatment with LY294002. Our results are supported by work showing that IGF-1 affects invasion and proliferation in ovarian and cervical cancer cells through activation of Akt and ERK1/2 [29], resulting in an increase in uPA activity in ovarian cancer [46]. Overall, the novel finding here is that PI3K/Akt activity alters cell migration due to changes in both PAI-1 and uPA expression in SKOV-3 cells, indicating that the PI3K/Akt signaling pathway negatively regulates PAI-1 expression while it up-regulates uPA expression, and this action is further modulated by IGF-1 and insulin.

The "traditional" function of PAI-1 is to inhibit uPA and thus prevent plasmin generation and matrix degradation. However, the "non-traditional" functions for PAI-1, including cell adhesion, proliferation, angiogenesis, apoptosis and cell signaling, are likely contributing to the detrimental role played by PAI-1 and why this inhibitor is associated with a grim prognosis in many cancers (reviewed in [47,48] and references cited therein). Based on the experimental end-points that we measured, the decrease in SKOV-3 migration and invasion implies a more favorable scenario to prevent further metastasis. However, since it is well established that increased levels of PAI-1 are associated with a poor prognosis in ovarian cancer [4–8], this apparent contradiction seen here might be better understood by evoking a combination of both traditional and the non-traditional functions of PAI-1. Increased levels of PAI-1, in conjunction with inhibition of the PI3K pathway, would guard the primary tumor mass from host fibrinolytic proteases, effectively decrease localized cell migration–invasion by inhibition of tumor plasminogen activator capability, promote neovascularization and help to maintain an anti-apoptotic environment in order to allow for genetic changes toward a metastatic phenotype. As the tumor begins to over-express PI3K/Akt, the PAI-1:uPA balance changes to now favor uPA and influence tumor invasion and metastasis. Thus, our results expand the relationship between PAI-1 and uPA that is regulated by PI3K/Akt in the highly invasive SKOV-3 ovarian cancer cell line.

Acknowledgments

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Caffeine and human DNA metabolism: the magic and the mystery

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Abstract

The ability of caffeine to reverse cell cycle checkpoint function and enhance genotoxicity after DNA damage was examined in telomerase-expressing human fibroblasts. Caffeine reversed the ATM-dependent S and G2 checkpoint responses to DNA damage induced by ionizing radiation (IR), as well as the ATR- and Chk1-dependent S checkpoint response to ultraviolet radiation (UVC). Remarkably, under conditions in which IR-induced G2 delay was reversed by caffeine, IR-induced G1 arrest was not. Incubation in caffeine did not increase the percentage of cells entering the S phase 6–8 h after irradiation; ATM-dependent phosphorylation of p53 and transactivation of $p21^{Cip1/Waf1}$ post-IR were resistant to caffeine. Caffeine alone induced a concentration- and time-dependent inhibition of DNA synthesis. It inhibited the entry of human fibroblasts into S phase by 70–80% regardless of the presence or absence of wildtype ATM or p53. Caffeine also enhanced the inhibition of cell proliferation induced by UVC in XP variant fibroblasts. This effect was reversed by expression of DNA polymerase η, indicating that translesion synthesis of UVC-induced pyrimidine dimers by DNA pol η protects human fibroblasts against UVC genotoxic effects even when other DNA repair functions are compromised by caffeine.

Keywords: Caffeine; Checkpoints; DNA repair; ATM; ATR; Ionizing radiation; Ultraviolet radiation

1. Introduction

Caffeine occupies an important niche in the cell cycle checkpoint field. Not only does it help bleary-eyed scientists concentrate on their experiments, it directly inhibits the checkpoint kinases, ATM and ATR [1,2]. Indeed, caffeine's ability to reverse delays in cell proliferation [3] was known before ATM and ATR were identified [4–6] and even before the concept of checkpoints was originated [7]. Caffeine is not a specific inhibitor of ATM and ATR, however, and many of its physiologic activities derive from its action on other enzymes, such as cyclic AMP phosphodiesterase [8]. Studies examining the effects of caffeine on cellular responses to DNA damage are motivated by reproducible demonstrations that it enhances the toxicity of radiations [9,10] and chemical carcinogens [11,12].

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Caffeine inhibits ATM and ATR in a dose-dependent fashion. The concentration that inhibits ATM by 50% in vitro is 1 mM and the 50% inhibitory concentration for ATR is about 3 mM [1]. These concentrations define the range at which caffeine effectively reverses cell cycle checkpoints and enhances cytotoxicity in carcinogen-damaged cells [3,9,13] without significant toxicity of its own. Lower concentrations are less effective at reversing checkpoint function and higher concentrations cause a reduction in DNA synthesis associated with cytotoxicity [13].

The enhancing effects of caffeine on cytotoxicity in carcinogen-damaged cells may derive from its reversal of checkpoints that act in S or G2. For ionizing radiations (IR), caffeine produces maximal enhancement of cytotoxicity on late S/G2 cells [9], whereas for UVC maximal enhancement occurs in S [14]. These results are rationalized by the knowledge that DNA repair protects cells against the lethal effects of carcinogen-induced damage [15] and the expectation that reducing the time available for repair in proliferating cells reduces repair efficiency. DNA double strand breaks are believed to be the lethal lesion induced by ionizing radiation, and this complex lesion may require an hour or more for repair [16]. Provision of an extra hour or two for repair before entry into mitosis, when chromosome condensation likely blocks further repair and permanently fixes the double strand break, would appear to be beneficial. Several studies have shown that reversal of checkpoint function using caffeine [10,17] and UCN-01 [18] doubled the toxicity of IR in human cancer cell lines.

Caffeine enhances the cytotoxicity of ultraviolet radiation (UVC) by its effect on damaged S phase cells. Here the issue is complicated by the nature of UVC-induced cellular damage and its mechanism of toxicity. UVC-induced cyclobutane pyrimidine dimers and 6–4 pyrimidine–pyrimidone adducts in DNA are repaired by nucleotide excision repair. It is during replication of UVC-damaged DNA that mutations and chromosomal aberrations are induced [19–21], and repair of photoproducts before DNA replication can provide significant protection against such genotoxic outcomes. However, inactivation of colony formation in human skin fibroblasts is rather invariant across the cell cycle [19], suggesting that the blockage of transcription of vital genes, and not DNA replication errors, accounts for inactivation of colony formation.

Nucleotide excision repair and especially transcription-coupled repair provide significant protection against UVC-induced genotoxicity, reducing the lethal effects of UVC by about 10-fold [22]. A system of post-replication repair that includes efficient translesion synthesis of cyclobutane pyrimidine dimers by DNA pol η also provides a measure of protection. Accurate translesion synthesis by DNA pol η reduces mutagenesis by a factor of 4 [20], and decreases clastogenesis and cytotoxicity by a factor of two-fold or less [20,23]. Caffeine is also known to inhibit the repair of gaps in daughter-strand DNA in UVC-damaged cells [24]. The effects of caffeine are especially notable in XP variant cells [24], which lack DNA pol η and must rely upon other more error-prone mechanisms of replication through UVC-induced photoproducts. Thus, while caffeine is now known to reverse an ATR-dependent S checkpoint that slows the rate of replicon initiation in UVC-treated cells [25], it may have an additional effect on pol η-independent bypass of cyclobutane pyrimidine dimers, which could involve translesion synthesis by other DNA polymerases or recombinational repair of daughter-strand gaps.

The studies reported here were undertaken with a motivation to define more fully the biological effects of caffeine in normal human fibroblasts (NHF). We applied sensitive and quantitative measures of cell cycle checkpoint function to monitor DNA damage responses in G1, S and G2 cells. While caffeine was found to reverse the S and G2 checkpoint responses to IR, it had very little effect on G1 checkpoint function, implying that ATM is less sensitive to caffeine in G1. Moreover, caffeine itself was found to induce an ATM- and p53-independent G1 delay, which was saturated at 2 mM concentration of the drug. The mechanism of this biological response remains a mystery. While caffeine also reversed the ATR-dependent S checkpoint response to UVC, it produced very little enhancement of UVC-induced inhibition of cell proliferation in normal human fibroblasts (NHF). In contrast, caffeine enhanced the UVC-induced inhibition of XP variant cell proliferation by eight-fold, and this hypersensitivity was corrected by transduction of DNA pol η. This result is consistent with an emerging model that ATR (and downstream effectors) help to stabilize blocked growing points to prevent replication fork collapse and lethal cytogenetic damage [26,27].

2. Materials and methods

2.1. Cell lines and culture conditions

Normal human fibroblasts (NHF1) were derived from neonatal foreskin [28]. Ataxia telangiectasia (AT) and xeroderma pigmentosum variant (XP-V) correspond to dermal fibroblasts from affected individuals. The original mutant fibroblast strains were obtained from the NIGMS Human Genetic Cell Repository (GM02052A, AT) and the American Type Culture Collection (CRL1162, XP-V, strain XP4BE). Immortalized cell lines from these strains of human fibroblasts were obtained by ectopic expression of human telomerase (hTERT), as previously described [25]. The derivative cell lines described below are also immortalized. For simplicity, we refer to the immortalized normal human fibroblasts as F1-hTERT. XP-V fibroblasts (CRL1162-hTERT) were complemented for expression of wildtype human DNA pol η (CRL1162 + XPV) by infection with a replication-defective retrovirus carrying the XPV cDNA (a generous gift from Dr. Fumio Hanaoka, Osaka University) and the neomycin-resistance gene under control of the same viral promoter (pLXIN). An isogenic cell line carrying the empty vector (CRL1162 + LXIN) was derived in parallel. The same strategy was used to derive NHF1 fibroblasts expressing human papilloma virus (HPV) type 16 E6 oncoprotein (F1-hTERT + E6) or the vector control (F1-hTERT + LXIN). Detailed characterization of these derivative cell lines will be published elsewhere.

Fibroblasts were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 2 mM glutamine (Invitrogen) and, in NHF1 cultures, 10% fetal bovine serum (FBS, Sigma), in AT cultures, 15% FBS, and, in XP-V cultures, 10% FBS and 200 μg/ml Geneticin (Invitrogen). All cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Cell treatment and irradiation

Caffeine (concentrations indicated in figure legends) was added to the culture medium 30 min prior to irradiation and remained for the duration of the experiment. For UVC irradiation, culture medium was removed and reserved. Cultures were washed once with warm Hank's balanced salt solution (HBSS, Invitrogen), then placed uncovered under a UV lamp emitting primarily 254 nm radiation at a fluence rate of 0.5 J/m^2/s. Reserved medium was added back and the cultures incubated for the indicated periods of time. Cells were exposed to ionizing radiation (gamma rays) in their culture medium, using a ^{137}Cs source at a dose rate of 0.84 Gy/min.

2.3. DNA synthesis assay

Logarithmically growing cells were plated at a density of 2.5×10^5 cells per 60 mm dish and grown at 37 °C for 36–48 h in medium containing 5 nCi/ml of [^{14}C] thymidine (ICN Radiochemicals) to uniformly label DNA. Radioactive medium was replaced with fresh medium to chase [^{14}C]-labeled precursors into DNA for at least 3 h. The effect of caffeine concentration on DNA synthesis was measured by adding to the culture medium 5% volume of solvent (PBS) containing caffeine at various concentrations and incubating the cultures at 37 °C for 30 min, and then pulse-labeling DNA with 10 μCi/ml [^{3}H] thymidine for 15 min. The time course of inhibition of DNA synthesis was measured by adding solvent only or 2 mM caffeine to the cultures, incubating them for 30 min to 8 h (see figure legend), and then pulse-labeling DNA as indicated above. Radioactive medium was removed and plates washed twice with cold PBS, before adding 3 ml of cold 4% trichloroacetic acid (TCA) and incubating at 4 °C for 30 min. After washing the plates with cold 4% TCA and air drying at 4 °C, the fixed cells were dissolved in 0.4 M NaOH and transferred to test tubes. Acid-insoluble material was collected on GF/C microfibre glass filters for measurement of radioactivity by liquid scintillation counting. Net ^{3}H radioactivity corrected for ^{14}C spillover was normalized for cell number (total ^{14}C radioactivity). Average ^{3}H/^{14}C ratios from triplicate cultures were taken as the specific activity of incorporation of [^{3}H] thymidine in DNA and as measurements of DNA synthesis rates in cells treated with caffeine.

2.4. Velocity sedimentation analysis of nascent DNA

The velocity sedimentation methodology used to determine the steady-state distribution of sizes of nascent DNA 30–45 min after irradiation of log phase

cultures with either 1 J/m^2 UVC or 1.5 Gy IR has been described previously [23,29].

2.5. Western-blot analyses

These were performed essentially as described [25]. Briefly, logarithmically growing cells were seeded at 10^6 per 100 mm dish and incubated for 40 h. Cultures were treated as specified in the figure legends, harvested by trypsinization, washed once in PBS, and resuspended in lysis buffer (10 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% NP40, supplemented with 10 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 10 mM β-glycerophosphate, 10 mM sodium orthovanadate, and 10 μg/ml of leupeptin and aprotinin). Protein concentrations were determined using the Bio-Rad D_C Protein Assay (Bio-Rad Laboratories). Samples containing equal amounts of protein were mixed with an equal volume of 2× Laemmli sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol) containing 5% β-mercaptoethanol, boiled, and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and probed with antibodies against p53 (DO1; Santa Cruz), $p21^{Cip1/Waf1}$ (Labvision), phospho-serine-15 p53 (Cell Signaling), actin (Santa Cruz), and DNA pol η (a generous gift from Dr. Fumio Hanaoka, Osaka University [30]).

2.6. G1 checkpoint analysis

Cells were irradiated and/or treated with caffeine as indicated in the figure legends. Six hours after irradiation or continuous incubation in caffeine, 10 μM 5′-bromo-2′-deoxyuridine (BrdU; Sigma) was added to the medium and the cultures incubated for another 2 h. Cells were harvested, washed with PBS, and fixed in PBS containing 67% ethanol. Cells were stained with propidium iodide and fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Becton Dickinson) as described [31]. Flow cytometric analyses were done on a FACScan machine using Summit software (Cytomation Inc.).

2.7. G2 checkpoint analysis

Mitotic cells were quantified via flow cytometric analysis by enumerating cells with 4N DNA that stained positive for the mitotic cell-specific epitope phospho-histone H3, as previously described [32,33]. Briefly, cells were harvested by tripsinization, washed with PBS, and fixed in 1% formaldehyde in PBS. Fixed cells were then rinsed once with PBS containing 67% ethanol and resuspended in PBS containing 0.5 μg of anti-phospho-histone H3 antibody (Upstate Biotechnology), 5% FBS, 0.1% sodium azide, and 150 mM NaCl (IFA solution). Cells were washed twice in IFA and stained with propidium iodide and FITC-conjugated anti-mouse antibody (Santa Cruz). Flow cytometric analyses were done on a FACScan machine using Summit software (Cytomation Inc.).

2.8. Cytotoxicity assay

UVC-induced inhibition of cell proliferation was measured as an index of cytotoxicity as previously described [34]. Briefly, logarithmically growing cells were plated at a density of 1×10^4 (F1-hTERT) or 1.5×10^4 (CRL1162-hTERT and derivative cell lines) cells per well in 6-well dishes. Twenty-four hours later, the cells were irradiated with the indicated fluences of UVC and fed with fresh medium ±1 mM caffeine. After 2 days, the old medium was replaced with fresh medium ±1 mM caffeine. The following day (72 h after UVC irradiation), cells were incubated with 1 μCi/ml [^{3}H] thymidine for 1 h. As described above, cells were washed and fixed with 4% TCA, and radioactivity in acid-insoluble material measured by liquid scintillation counting. Reduction in the incorporation rate of [^{3}H] thymidine (average of triplicate cultures) was taken as an indirect measurement of the degree of inhibition of proliferation of cells exposed to UVC. Preliminary experiments established that under the culture conditions described here, control cultures remained in log phase and did not reach confluence prior to the end of the experiment. This assay produced results in concordance with the UVC-induced inhibition of colony formation in human fibroblasts, which is a common measure of cytotoxicity in this cell type [35,36].

Colony formation was measured in logarithmically growing F1-hTERT cells that were plated at 500 or 750 cells per 100 mm diameter dish and incubated for 18 h or less before exposure to increasing fluences of UVC (5–10 dishes per fluence). Cells were incubated for 2 weeks, with one medium change on day

8, then cell colonies were fixed with methanol:acetic acid (3:1 (v/v)) and stained with Giemsa. Only colonies with >50 cells were counted. The relative colony-forming efficiency of UVC-treated cells was expressed as a fraction of the sham-treated controls. This index of cell proliferation (clonal expansion) was compared to that determined in the short-term assay based on [^{3}H] thymidine incorporation described above.

3. Results

3.1. Caffeine inhibits G2 checkpoint function

Incubation with caffeine following exposure to IR was shown previously to reverse radiation-induced G2 delay in HeLa cells [3]. It also enhanced IR-induced cytotoxicity in a cycle phase-dependent manner with synchronized G2 cells displaying the greatest increment of cell killing [9,37]. Flow cytometry was used to quantify G2 checkpoint function in diploid human fibroblasts that were immortalized by expression of telomerase [38]. Normal fibroblasts displayed 2% of cells with 4N DNA content and expression of a mitosis-specific phospho-histone H3 (Fig. 1A). Treatment with 1.5 Gy of IR 2 h before cell harvest reduced the fraction of these mitotic cells to 0.1%, corresponding to 5% of control and reflecting a radiation-induced G2 delay. AT fibroblasts displayed an attenuation of this response to IR with a mitotic fraction in irradiated cells that was 50% of that in the sham-treated control culture (Fig. 1B). These results confirm previous reports that the radiation-induced G2 delay is a checkpoint response and that it is defective in AT cells [39,40]. Incubation with 2 mM caffeine for 2.5 h had little effect on the mitotic fraction in cultures of sham-treated normal and AT fibroblasts, but reversed the radiation-induced inhibition of mitosis by 90–100% (Fig. 1).

AT cells displayed a 50% inhibition of mitosis post-irradiation, and this inhibition likely reflects the activity of the ATM-and rad3-related checkpoint kinase, ATR. We and others have shown that expression of a kinase-inactive ATR also attenuates the G2 checkpoint response to IR [5,38]. Our analyses suggest that ATM and ATR contribute equally to the IR-induced G2 delay. Inactivation of either enzyme attenuates the G2 checkpoint response by about 50%. The full ablation by caffeine of G2 checkpoint response has been attributed to its inhibition of ATM [41]. A recent publication disputes the interpretation that ATM and ATR are directly inhibited by caffeine in cultured cells [42]. The new findings show that ATM autophosphorylation and ATM/ATR-dependent phosphorylation of Chk1 are resistant to caffeine. These results imply that caffeine inhibits the signaling cascades in the G2 checkpoint response that are downstream of ATM, ATR and Chk1.

3.2. Caffeine inhibits S checkpoint function

Caffeine-enhancement of cytotoxicity by UVC also is cycle phase-dependent but S phase cells display the greatest sensitivity. Synergistic lethality by caffeine with nitrogen mustard was correlated with reversal of the mustard-induced inhibition of replicon initiation in S phase cells [12,13]. The IR-induced S checkpoint response of inhibition of replicon initiation is ATM-dependent, as shown in Fig. 2. While normal fibroblasts responded to IR with a selective inhibition of incorporation of radiolabeled precursor in low molecular weight nascent DNA intermediates, AT cells were resistant to this effect, displaying the phenotype of radioresistant DNA synthesis. Treatment with caffeine reversed the S checkpoint response to IR in normal fibroblasts. AT cells displayed a normal S checkpoint response to UVC, with 1 J/m^2 producing the same selective inhibition of replicon initiation in normal and AT cells. We have recently demonstrated that the UVC-induced inhibition of replicon initiation is an ATR- and Chk1-dependent S checkpoint response [25]. Incubation with caffeine post-UVC reversed the inhibition of replicon initiation in normal and AT fibroblasts (Fig. 2). Thus, caffeine also ablated the S checkpoint responses to radiation-induced cellular damage.

In view of the recent report that caffeine does not inhibit ATM and ATR in cells [42], we performed a western immunoblot analysis of Chk1 phosphorylation in HeLa cells after treatment with UVC and IR. While both 1 J/m^2 UVC and 5 Gy IR induced the phosphorylation of Chk1 at ser317 within 45 min after treatment, these effects were not blocked by caffeine (results not shown), confirming the observations by Cortez [42].

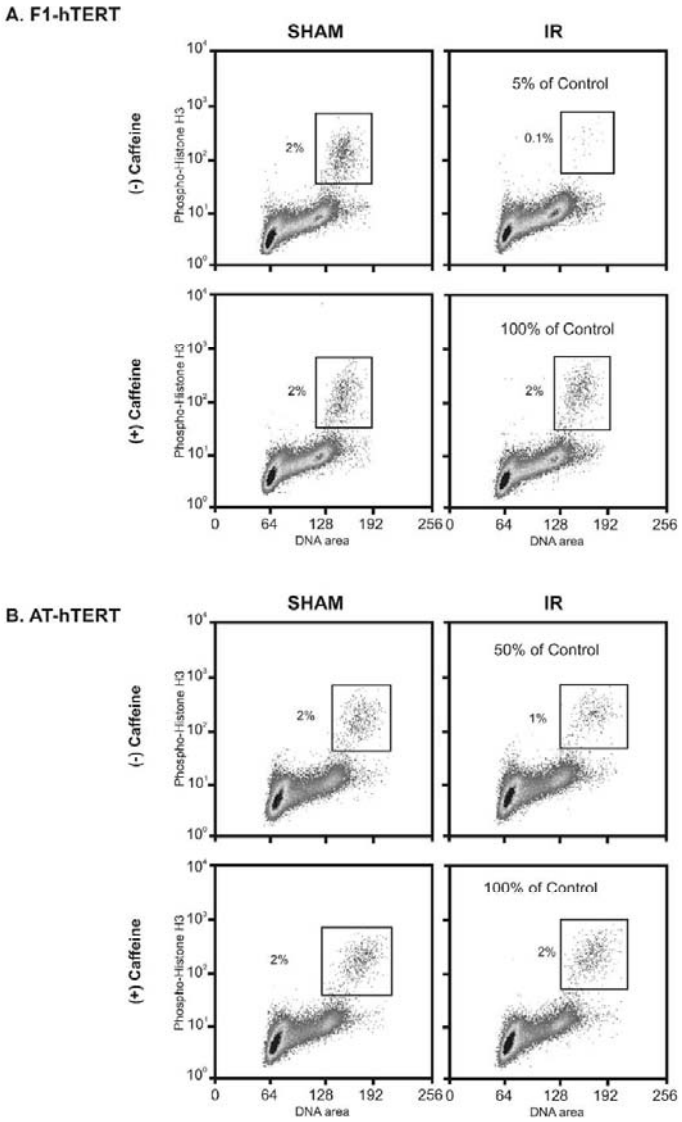


Fig. 1. Caffeine inhibits G2 checkpoint function. Logarithmically growing F1-hTERT (A) and AT-hTERT (B) fibroblasts were pretreated with 2 mM caffeine for 30 min prior to either being sham-treated or irradiated with 1.5 Gy. Two hours post-IR the cells were harvested and processed as described in Section 2 for determination of mitotic index. Propidium iodide was used to stain DNA (*X*-axis) and an antibody specific for phospho-histone H3 was used to stain mitotic cells (*Y*-axis).

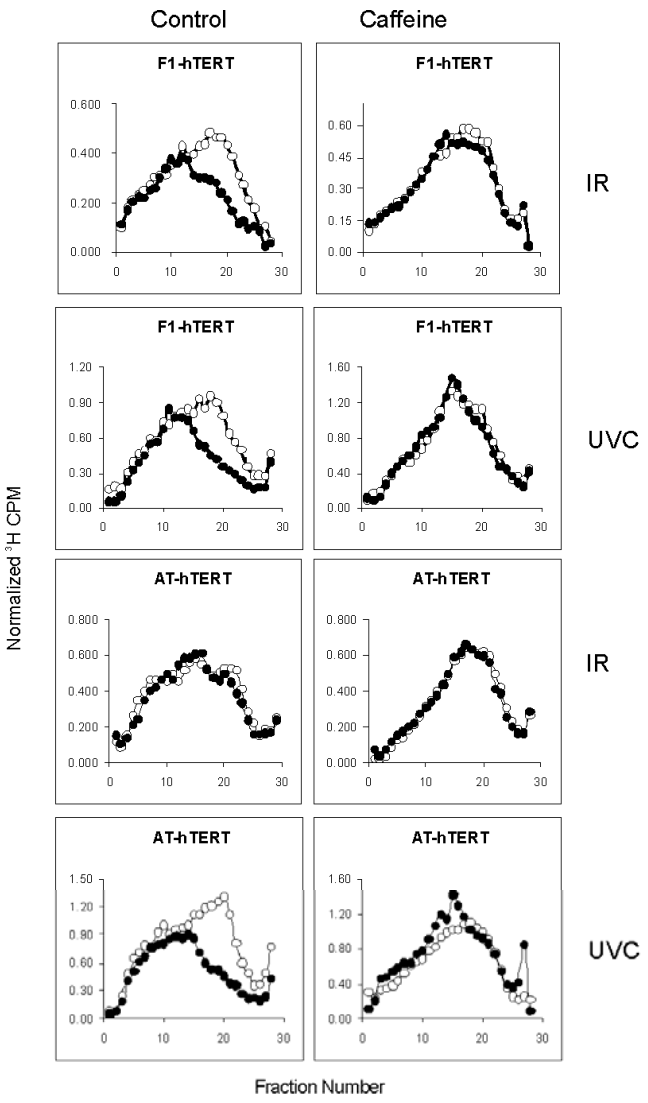


Fig. 2. Caffeine abrogates the ATM- and ATR-dependent S checkpoints. F1-hTERT and AT-hTERT fibroblasts were uniformly labeled with [^{14}C] thymidine as described and incubated for 30 min in medium alone or medium containing 2 mM caffeine. Cells were sham-treated or irradiated with 1 J/m^2 UVC or 1.5 Gy IR, incubated in reserved medium for 30 min and then pulse-labeled with [^{3}H] thymidine for 15 min. Cells were harvested and nascent DNA separated by velocity sedimentation (see Section 2). Net ^{3}H radioactivity corrected for ^{14}C spillover was normalized to cell number (total ^{14}C radioactivity). Open circles: sham-treated cells; closed circles: irradiated cells. The UVC profiles illustrated in this figure have been previously published [25].

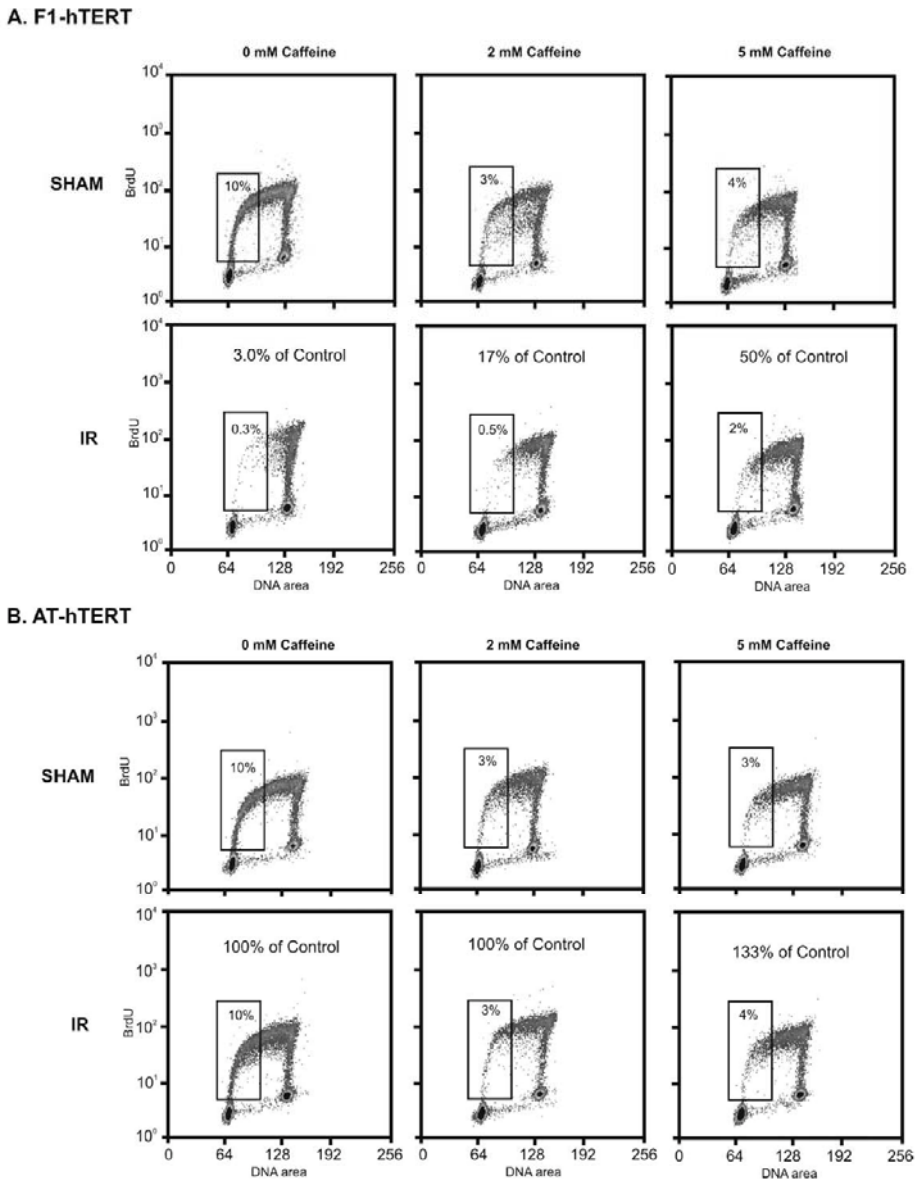
3.3. Caffeine does not inhibit G1 checkpoint function but induces G1 delay

To complete our survey of caffeine effects on cell cycle checkpoint function in diploid human fibroblasts, we quantified radiation-induced G1 checkpoint function in the presence or absence of caffeine. Treatment of normal human fibroblasts with 1.5 Gy IR produced a selective 97% reduction of cells in the first half of S phase, as seen by labeling cells with BrdU 6–8 h after irradiation (Fig. 3A). This selective emptying of the early S compartment is a checkpoint response to IR, as AT fibroblasts were resistant to this effect (Fig. 3B). No significant increment of S phase cells was observed in cultures of irradiated normal human fibroblasts upon post-irradiation incubation with caffeine (Figs. 3A and 4A). However, caffeine alone at 2 mM reduced the fraction of cells in early S phase by 70–80% in normal and AT fibroblast cultures (Figs. 3 and 5A). Quantification of G1 checkpoint function in three independent experiments using normal human fibroblasts is shown in Fig. 4. While IR reduced the mean fractions of fibroblasts in the first half of S phase from 12 to 0.7%, a similar fraction of 0.7% of cells were in early S phase following post-irradiation incubation with 2 mM caffeine. Caffeine alone reduced the fraction of cells in early S phase from 12 to 4%. Consequently, when expressed as a percentage of the sham-treated control (Fig. 3), caffeine-treated cultures appeared to have an attenuation of the G1 checkpoint response to IR. Such an attenuation became more evident when the caffeine concentration was increased to 5 mM (Fig. 3A), a concentration that significantly inhibits DNA synthesis in human fibroblasts, as discussed below (Fig. 6). In comparison to the 90–100% reversal of G2 (Fig. 1) and S (Fig. 2) checkpoint responses to IR produced by 2 mM caffeine, the G1 checkpoint response to IR (Fig. 3A) was substantially non-responsive to this concentration of caffeine (Figs. 3A and 4A). In the same experiments, quantification of cells in the G2/M compartment 8 h post-IR revealed the expected IR-induced accumulation associated with G2 delay (Fig. 4B). Post-irradiation incubation with caffeine reversed this effect, indicating that in the same population of cells in which G1 arrest was little affected by caffeine, the G2 checkpoint response was completely abrogated.

Flow scattergrams and quantitation of cells in the first half of S indicated that 2 mM caffeine alone induced a significant G1 delay in normal and AT fibroblasts (Figs. 3 and 5A). To determine whether the G1 delay that was induced by caffeine required p53 signaling, we tested whether inactivation of p53 with HPV16E6 oncoprotein would eliminate this G1 delay. Incubation of E6-expressing and vector-control fibroblasts with 2 or 5 mM caffeine for 8.5 h produced the same 70–80% reduction of cells in the first half of S phase (Fig. 5A). This finding indicated that p53 function, and any other function affected by HPV16E6, was not required for the caffeine-induced G1 delay.

ATM phosphorylates p53 on ser15, and ATM signaling is required for induction of p53 protein after IR. Activation of p53 induces synthesis of the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ as the effector of the ATM- and p53-dependent G1 checkpoint response. Western immunoblot analysis was done to test whether caffeine affected p53 signaling in response to IR. While treatment with IR induced p53 protein, p53-ser15 phosphorylation and $p21^{Cip1/Waf1}$ expression in normal fibroblasts, these effects were not reversed by incubation in caffeine (Fig. 5B). Incubation with caffeine induced neither p53 or p21 but, rather, reduced expression of $p21^{Cip1/Waf1}$ in both irradiated and unirradiated cells. As $p21^{Cip1/Waf1}$ is, in part, growth-regulated, this may reflect the inhibition of cell proliferation induced by caffeine as described above. The caffeine-induced G1 delay therefore appears to be independent of the ATM/p53/$p21^{Cip1/Waf1}$ signaling pathway.

Fig. 3. Caffeine does not inhibit G1 checkpoint function but induces a G1 delay. Logarithmically growing F1-hTERT (A) and AT-hTERT (B) fibroblasts were pretreated with 0, 2, or 5 mM caffeine for 30 min prior to being sham-treated or irradiated with 1.5 Gy. Cells were incubated for 6 h and then pulsed with 10 mM BrdU for 2 h. Cells were harvested and analyzed for G1 checkpoint function by flow cytometry as described in Section 2. Propidium iodide was used to stain for DNA content (*X*-axis) and anti-BrdU-FITC to stain BrdU-labeled cells (*Y*-axis). Numbers within the boxes represent the fraction of total cells in the first half of S phase. The indicated percent of control was calculated from the matched sham profile (non-irradiated profile from cells incubated in the same concentration of caffeine).


A. F1-hTERT
0 mM Caffeine
2 mM Caffeine
5 mM Caffeine
SHAM
IR
BrdU
10%
3%
4%
3.0% of Control
17% of Control
50% of Control
0.3%
0.5%
2%
DNA area
B. AT-hTERT
100% of Control
100% of Control
133% of Control

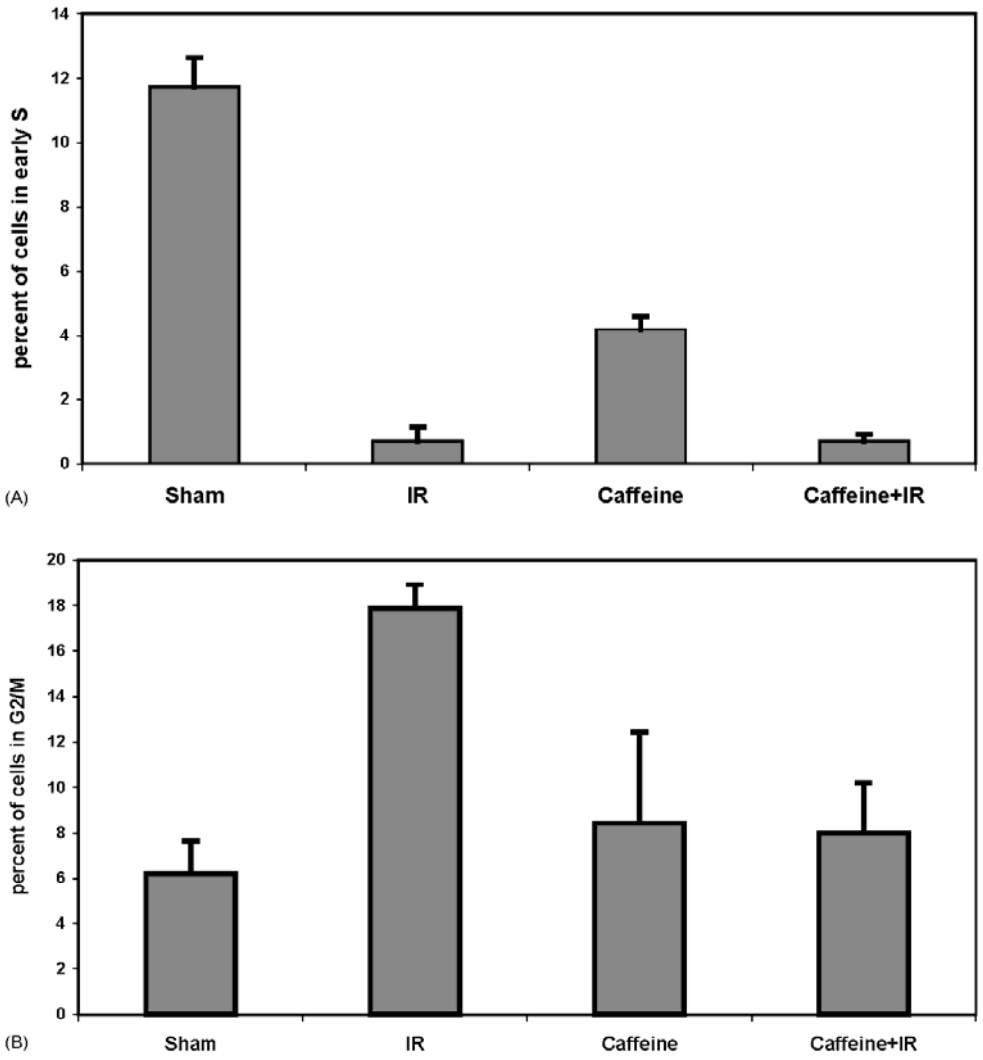


Fig. 4. Caffeine reverses IR-induced G2 delay but not G1 arrest. The effect of IR and caffeine (2 mM) on G1 and G2 checkpoint function in F1-hTERT fibroblasts was determined by flow cytometric studies as illustrated in Fig. 3. Results from several experiments were graphed as the average percent of cells in early S phase (A) or the percentage of cells in G2/M (B) (mean ± S.D., $n = 3$).

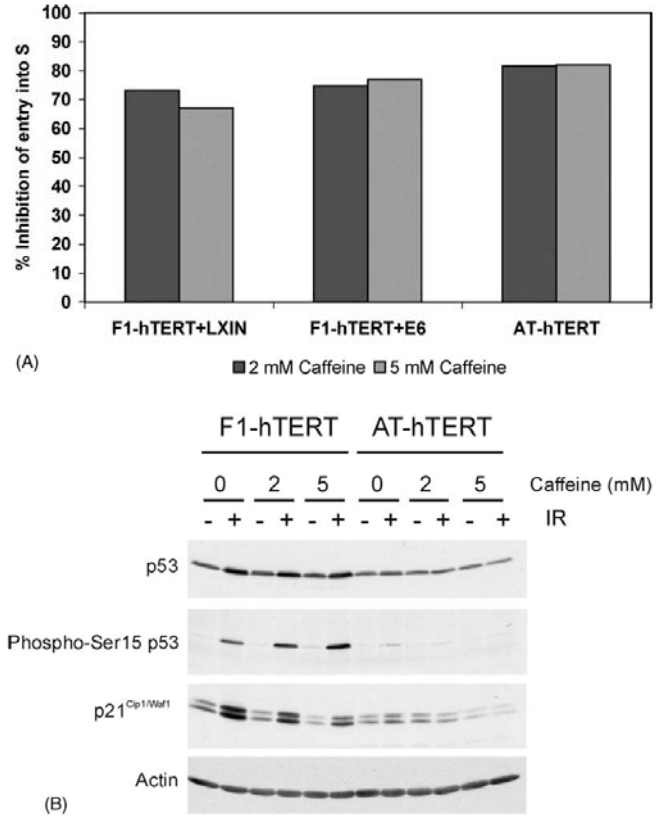


Fig. 5. Caffeine-induced G1 delay is independent of p53. (A) Logarithmically growing F1-hTERT-LXIN, F1-hTERT-E6, and AT-hTERT fibroblasts were treated with either 0, 2, or 5 mM caffeine for 6 h and then pulsed with BrdU for an additional 2 h. Cells were harvested and the effect of caffeine on S phase entry analyzed by flow cytometry as described in the legend to Fig. 3. In this graph, the *Y*-axis shows the percent inhibition of entry of cells into early S by caffeine. The results shown represent the average of two experiments. (B) F1-hTERT and AT-hTERT fibroblasts were incubated for 30 min in medium alone or medium containing 2 mM caffeine and then either sham-treated or irradiated with 1.5 Gy IR. Cells were incubated for 6 h and then harvested. Protein extracts were prepared and 100 μg of total protein analyzed by western immunoblot analysis.

3.4. *Caffeine inhibits DNA synthesis*

Previous studies of caffeine using transformed or cancer cell lines showed that concentrations higher than 3 mM inhibited DNA synthesis and inactivated clonal expansion [13]. To explore this dose-dependence on diploid human fibroblasts, we monitored incorporation of [^{3}H] thymidine after a brief incubation with various concentrations of caffeine, or after prolonged incubation with 2 mM caffeine (Fig. 6). Normal and AT fibroblasts were both sensitive to inhibition of incorporation of [^{3}H] thymidine by high concentrations of caffeine. While 1–2 mM inhibited DNA synthesis on average by about 30%, 30 min incubation with 5 mM reduced [^{3}H] thymidine incorporation by 60% and 10 mM reduced it by >80% (Fig. 6A). During the first 2 h of incubation with 2 mM caffeine, incorporation of [^{3}H] thymidine

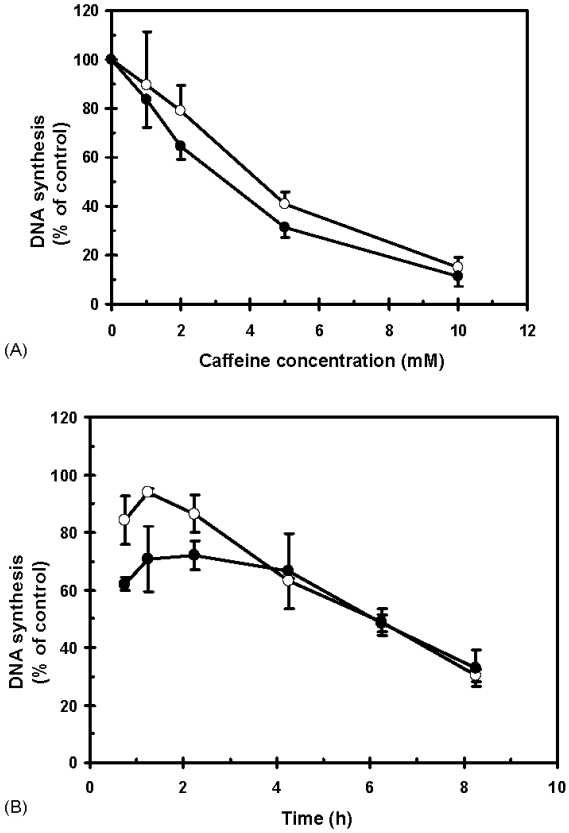


Fig. 6. Caffeine inhibits DNA synthesis. F1-hTERT and AT-hTERT fibroblasts were grown in the presence of [^{14}C] thymidine for ~40 h to label DNA uniformly, and then in non-radioactive medium for ≥3 h. (A) Cells were treated with 0, 1, 2, 5, or 10 mM caffeine, incubated at 37 °C for 30 min, and then labeled for 15 min in medium containing [^{3}H] thymidine. (B) In time course studies, cells were treated with 0 or 2 mM caffeine for 0.5, 1, 2, 4, 6, or 8 h and then labeled for 15 min in medium containing [^{3}H] thymidine. Net ^{3}H radioactivity corrected for ^{14}C spillover was normalized to cell number (total ^{14}C radioactivity). Normalized ^{3}H CPM was graphed as a percent of sham controls. Open circles: F1-hTERT; closed circles: AT-hTERT.

varied from 60 to 70% of control in AT cells and 85–95% of control in normal fibroblasts (Fig. 6B). Thereafter, incorporation declined more or less continuously, such that incorporation was reduced to 30% of control after 8 h incubation in caffeine. This degree of inhibition (70%) was similar to the reduction in S phase cells that were labeled with BrdU 6.5–8.5 h after addition of caffeine. The inhibition of incorporation of [^{3}H] thymidine 4–8 h after addition of 2 mM caffeine therefore reflects the caffeine-induced G1 delay.

3.5. *DNA pol η suppresses the enhancement of UVC cytotoxicity by caffeine*

Although a variety of data suggest that caffeine enhancement of cytotoxicity may be due to abrogation

of protective cell cycle checkpoint function, other effects of caffeine on DNA repair might also contribute. For example, pol η-dependent translesion synthesis of UVC-induced pyrimidine dimers provides a modest increment of protection against cytotoxicity over that afforded by nucleotide excision repair [43,44]. Caffeine has long been known to interfere with processes commonly known as post-replication repair [24,45,46], inhibiting in particular an ill-defined post-replication repair pathway that is operational in XP variant cells [35,47,48]. As shown in Fig. 2, caffeine does not appear to interfere with pol η-dependent translesion synthesis, as the inhibition of DNA chain elongation was not enhanced in normal human and AT fibroblasts by post-UVC incubation with caffeine. It is conceivable that caffeine may inhibit a pol η-independent post-replication repair process, as suggested by one notable study. Lehmann [46] showed that the average size of nascent DNA strands that were synthesized in pol η-defective XP variant cells in the presence of caffeine was equivalent to the average spacing between pyrimidine dimers. This observation suggested that the caffeine-sensitive post-replication repair pathway retained by XP variant cells involves pol eta-independent mechanism(s) of repair of daughter-strand gaps formed across UVC-induced DNA photoproducts. To disclose an effect of caffeine on post-replication repair, we employed an assay that quantified DNA synthesis in diploid normal and XP variant fibroblasts 72 h after treatment with UVC. As illustrated in Fig. 7A, this short-term assay for inhibition of cell proliferation [34] agreed very well with an assay of colony formation as surrogates of radiation-induced cytotoxicity. Normal human fibroblasts displayed a dose-dependent decrease in colony-formation efficiency or inhibition of DNA synthesis post-UVC with a 60% reduction at 8 J/m^2 and 90% reduction at 12 J/m^2. Addition of 1 mM caffeine for the 72 h post-UVC had only a small effect in normal human fibroblasts. XP variant fibroblasts (CRL1162-hTERT) displayed increased sensitivity to UVC with 70% inhibition of DNA synthesis after 4 J/m^2 and >90% inhibition after 8 J/m^2 (Fig. 7B). In contrast to the minor enhancement seen in normal fibroblasts, incubation of XP variant cells with caffeine post-UVC produced a significant increment of inhibition of DNA synthesis. While 4 J/m^2 reduced synthesis to 30% of the control in the absence

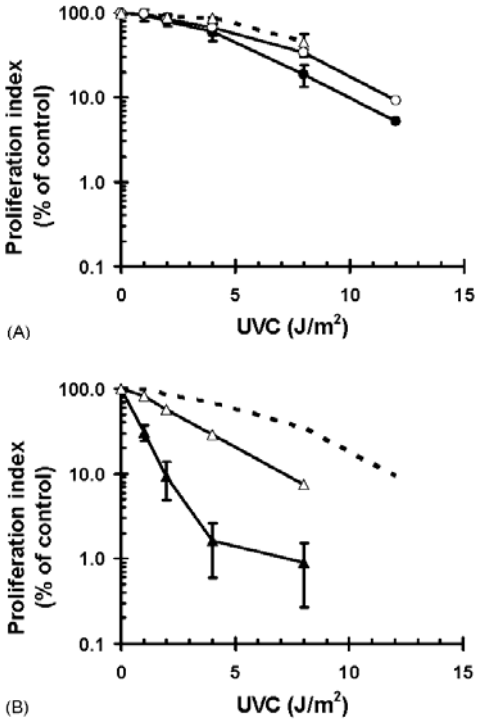


Fig. 7. Caffeine enhances UVC-induced cytotoxicity. Log cultures of F1-hTERT (A) and CRL1162-hTERT (B) were irradiated with the indicated fluences of UVC and then incubated for 72 h in medium containing either 0 or 1 mM caffeine. Cells were pulse labeled with 1 mCi/ml [^{3}H] thymidine for 1 h, and acid-insoluble DNA collected on microfibre filters. [^{3}H] thymidine incorporation was graphed as a percent of sham controls. (A) F1-hTERT grown in the absence (open circles) or the presence (closed circles) of 1 mM caffeine following exposure to UVC; dashed line with open triangles represents the colony-forming efficiency of F1-hTERT in the absence of caffeine. (B) CRL1162-hTERT grown in the absence (open triangles) or the presence (closed triangles) of 1 mM caffeine after irradiation; the dashed line represents the results with F1-hTERT grown in the absence of caffeine (open circles in A).

of caffeine, in its presence 4 J/m^2 reduced synthesis to 2% of control. Similarly, in the absence of caffeine 2 J/m^2 reduced synthesis to 70% of control but in the presence of caffeine 2 J/m^2 reduced synthesis to 10% of control. In summary, the short-term assays [34], in full agreement with measurements of colony formation [49], confirmed that XP variant cells display

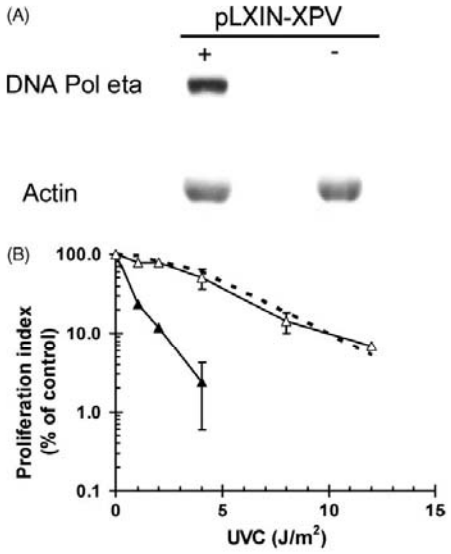


Fig. 8. DNA pol eta suppresses the enhancement of UVC cytotoxicity by caffeine. (A) Log cultures of CRL1162-pLXIN (−) and CRL1162-pLXIN-XPV (+) fibroblasts were harvested and whole cell extracts were prepared. Protein (50 μg) was separated on an 8% SDS-PAGE and then probed with specific antibodies against DNA pol η and actin. (B) Log cultures of CRL1162-pLXIN (closed triangles), CRL1162-hTERT-XPV (open triangles), and F1-hTERT (dashed line) fibroblasts were irradiated with indicated fluences of UVC and incubated for 72 h in medium containing 1 mM caffeine. The degree of cytotoxicity was determined as described in the legend to Fig. 7. The dashed line represents the results observed with F1-hTERT in the presence of caffeine (Fig. 7A, closed circles).

hypersensitivity to caffeine enhancement of cytotoxicity by UVC.

The CRL1162-hTERT line was transduced with DNA pol η or the empty vector by infection with a replication-defective retrovirus. Fig. 8A illustrates the expression of DNA pol η in the CRL1162+XPV cell line. XP variant cells that were infected with the empty vector alone displayed the expected hypersensitivity to UVC. XP variant cells that were transduced with DNA pol η displayed sensitivity to UVC equivalent to that seen in the F1-hTERT normal fibroblast line (Fig. 8B). Thus, caffeine-enhancement of cytotoxicity in XP variant cells was related to the defect in expression of DNA pol η.

4. Discussion

This survey of the effects of caffeine on DNA metabolism in diploid human fibroblast strains confirmed the inhibition of ATM- and ATR-dependent S and G2 checkpoint function and enhancement of UVC cytotoxicity in DNA pol η-defective XP variant cells. Unexpectedly, especially in view of a previous report [50], caffeine at 2 mM concentration did not inhibit ATM-dependent G1 checkpoint function. There was no decrement of IR-induced G1 arrest under conditions in which G2 delay was ablated. This result suggests that ATM signaling in G1 cells may be resistant to caffeine. The fact that caffeine also induced G1 delay in the absence of damage reveals a potential limitation in the use of checkpoint inhibitors to enhance cytotoxicity of chemo- and radiotherapies. Although the magical reversal of checkpoint function by caffeine is now well established, several of the biological effects of caffeine identified here remain a mystery.

Previous studies had indicated that caffeine inhibited ATM and ATR signaling in checkpoints acting in G1, S and G2 [2,41,51]. We were not surprised therefore to find that caffeine fully abrogated ATM- and ATR-dependent S and G2 checkpoint responses in IR- and UVC-treated human fibroblasts. We were surprised to find that caffeine did not abrogate ATM- and p53-dependent G1 checkpoint response to IR under conditions in which G2 delay was fully reversed. ATM-dependent phosphorylation of p53 at ser15 and p53-dependent induction of $p21^{Cip1/Waf1}$ were not inhibited by caffeine, indicating that ATM function in G1 cells was insensitive to caffeine. Recently, Cortez [42] showed that caffeine did not inhibit ATM autophosphorylation nor ATM- and ATR-dependent phosphorylation of Chk1 in cells with DNA damage, even though it inhibited the G2 checkpoint response to DNA damage. We confirmed that caffeine did not block ATM- and ATR-dependent phosphorylation of Chk1. These results suggest that caffeine must interfere with signaling downstream of ATM, ATR, and Chk1 to abrogate S and G2 checkpoint responses to DNA damage.

The S checkpoint response to IR-induced DNA damage has been shown to involve signaling through ATM and Chk2 to Cdc25A [52], and the G2 checkpoint response to IR includes signaling from ATM, ATR and Chk1 to Cdc25C [53]. The Cdc25 protein

phosphatases (Cdc25A, Cdc25B and Cdc25C) are dual specificity phosphatases that activate Cdk1 and Cdk2 by removing inhibitory phosphotyrosine and phosphothreonine residues from the ATP binding site [54]. One arm of DNA damage checkpoint responses includes ATM-dependent phosphorylation of Cdc25's by Chk1 and Chk2. Phosphorylation of Cdc25A leads to its proteolysis [52] while phosphorylation of Cdc25C creates a 14-3-3 binding site to sequester the protein in the cytoplasm and possibly inhibit activity [53,55]. The net effect of phosphorylation of Cdc25's is to sustain inhibition of Cdk1 and Cdk2, which are required for initiation of mitosis and DNA synthesis, respectively. As caffeine does not block intracellular ATM autophosphorylation and phosphorylation of Chk1 and Chk2 [42], the Cdc25 pathway does not appear to be the target of checkpoint abrogation by caffeine.

Inactivation of the Cdc25C regulatory pathway by expression of Cdk1AF, a non-phosphorylatable form of Cdk1, produced only a modest reversal of G2 checkpoint function [56]. Subsequent studies demonstrated that cyclin B1/Cdk1 complexes were also regulated by cellular compartmentalization, with the Crm1 nuclear exporter actively sequestering cyclin B1 in the cytoplasm [57,58]. Phosphorylation of cyclin B1 by the Polo-like kinase, Plk1, blocks Crm1-mediated nuclear export allowing nuclear accumulation of cyclin B1 [59]. Plk1 is inhibited by IR-induced DNA damage by an ATM-dependent mechanism [60,61]. The net effect of ATM/Plk1/cyclin B1 signaling is to block nuclear accumulation of cyclin B1/Cdk1 complexes, thereby preventing entry into mitosis. The ATR-dependent decatenation checkpoint also appears to rely upon inhibition of Plk1 to block the onset of mitosis [62]. Caffeine has been shown to block the IR-induced inhibition of Plk1 [61] suggesting at least one mechanism whereby caffeine abrogates G2 checkpoint function.

Quantification of [^{3}H] thymidine incorporation is a sensitive measure of UVC-induced genotoxicity and cytotoxicity. Caffeine enhanced genotoxicity in UVC-treated XP variant cells and this effect was fully reversed by expression of DNA pol η. We had originally interpreted this phenomenon of caffeine enhancement of genotoxicity to reflect a requirement for ATR to prevent the demise of replication forks blocked at cyclobutane pyrimidine dimers, in analogy to the requirement for Mec1 in alkylated yeast strains [63]. In view of the apparent insensitivity of intracellular ATR to caffeine this hypothesis in untenable. Treatment of XP variant cells with UVC leads to a condition in S phase cells with many blocked growing points and replicative gaps due to the absence of the bypass polymerase pol η. This condition may be similar to that produced by hydroxyurea in pol η-expressing cells, and the combination of hydroxyurea and caffeine is particularly lethal in mammalian cells [64]. These observations imply that there is a caffeine-sensitive pathway that stabilizes arrested replication forks or replicative gaps. The work of Lehmann [46] implies that this pol η-independent pathway may facilitate bypass of cyclobutane pyrimidine dimers. Work must now focus on the caffeine-sensitive steps of processing of nascent DNA at arrested replication forks and growing points. It will be particularly interesting to determine whether the caffeine-sensitive regulation of Plk1 intersects with signaling at arrested growing points.

The mechanism of caffeine-induced G1 delay remains to be determined. Caffeine has long been known to induce a G1 delay in mammalian cells [65–67], and we showed that caffeine-induced ~75% inhibition of G1/S progression in diploid human fibroblast strains. ATM and p53 were not required as AT cells and cells expressing HPV16E6 to inactivate p53 also arrested in G1 when incubated in caffeine. The caffeine-induced G1 delay was not associated with induction of $p21^{Cip1/Waf1}$. Previous studies of caffeine effects on the cell cycle have been devoted largely to cancer cell lines, which often display defects in G1 checkpoint function deriving from inactivation of p53 and pRB regulatory pathways. For example, HeLa cells express HPV18E6 and E7 to inactivate p53 and pRB. The fact that caffeine produced only a modest G1 delay in HeLa cells suggests that pRB may be required for the caffeine-induced G1 delay [3]. We had entertained the thought that the effect could be a DNA damage response. However, because p53 was not required and $p21^{Cip1/Waf1}$ was not induced by caffeine, this possibility appears unlikely. Caffeine might affect some component of the signaling pathway that inactivates pRB to promote S phase entry. Clearly caffeine does not inhibit cyclin B1/Cdk1 which is the cyclin-dependent kinase that promotes G2/M progression. As caffeine did not induce a dramatic inhibition of DNA synthesis at 1–2 mM, it seems unlikely that it inhibits the cyclin E/Cdk2 and cyclin A/Cdk2 complexes that are required for initiation of replication and

progression through S. Perhaps the cyclin D1/Cdk4,6 complexes that are required for inactivation of pRB are sensitive to caffeine.

In summary, caffeine has the magical property of reversing S and G2 checkpoint function and reducing DNA repair in irradiated cells. Mysteries remain concerning how intracellular ATM and ATR checkpoint kinases resist caffeine, the nature of the caffeine-sensitive steps in the S and G2 checkpoints, and how caffeine induces G1 delay.

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