KININOGEN AND FERRITIN IN TUMORIGENESIS

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

TABLES **Page**

CHAPTER II

CHAPTER III

FIGURES

CHAPTER I

CHAPTER III

LIST OF ABBREVIATIONS

APC	Allophycocyanin
bFGF	Basic fibroblast growth factor
BK	Bradykinin
BPAECs	Bovine pulmonary artery endothelial cells
BSA	Bovine serum albumin
CK1	Cytokeratin 1
CXCR4	CXC chemokine receptor 4
D5	Domain 5
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
Ft	Ferritin
gC1qR	Globular C1q receptor
GPI	Glycosylphosphatidylinositol
HK	High molecular weight kininogen
HKa	Cleaved high molecular weight kininogen
HMW uPA	High molecular weight uPA
HRP	Horseradish Peroxidase
HUVEC	Human umbilical vein endothelial cell
kD	Kilodalton

Kd	Equilibrium dissociation constant
LK	Low molecular weight kininogen
MAP	Mitogen-activated protein kinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
PMS	phenazine methosulfate
SFKs	Src family kinases
TIM-2	T cell immunoglobulin-domain and mucin-domain 2
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
Vn	Vitronectin

ABSTRACT

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KININOGEN AND FERRITIN IN TUMORIGENESIS

Thesis under the direction of Suzy V. Torti, Ph.D., Professor of Biochemistry and Frank M. Torti, MD, Professor of Cancer Biology

High molecular weight kininogen (HK) is a plasma glycoprotein which serves as a cofactor in the intrinsic coagulation cascade, and it is also known as an inhibitor of cysteine proteases. HKa can interact with a variety of cells including endothelial cells, neutrophils, monocytes and platelets. When bound to endothelial cells, HK can be cleaved by plasma kallikrein to release bradykinin and the two chain high molecular weight kininogen, HKa. HKa has been shown to inhibit angiogenesis both in vitro and in vivo. Ferritin is a 24-subunit iron-storage protein which is mostly located intracellularly. Small amounts of ferritin also exist in serum and are often elevated during inflammation and some malignancies. Previous studies have demonstrated that ferritin binds to HKa and antagonizes its antiangiogenic effects, enhancing the proliferation, migration and survival of HKa-treated endothelial cells. Thus far, several binding sites for HKa on the endothelial cell surface have been identified including urokinase plasminogen activator receptor (uPAR, CD87), cytokeratin 1(CK1), globular “head” of C1q (gC1q-R) and tropomyosin, of which uPAR is the most studied and most relevant to tumorigenesis. Tumor cells frequently overexpress uPAR. The work presented here focuses on the

effects of HKa and ferritin on cancer cells and the signaling pathways initiated by HKa and ferritin in endothelial cells.

HKa was shown to inhibit the proliferation, migration, adhesion and clonogenic growth of cancer cells. Ferritin did not block HKa's effects on these cells. To assess if uPAR plays a role in determining sensitivity to HKa, uPAR levels were detected in cancer cells using by western blot. Most of the cancer cells tested expressed uPAR. However, correlation analysis showed no linear correlation between the sensitivity of cells to HKa in terms of its effect on cell proliferation and uPAR level.

We also assessed HKa's effect on signaling pathways in endothelial cells. Both HKa and ferritin inhibited uPA-induced phosphorylation and activation of ERK1/2. HKa did not inhibit phosphorylation of FAK and paxillin. Neither HKa nor ferritin affected the phosphorylation and activation of Akt. Flow cytometry analysis showed that both HKa and ferritin bound to endothelial cells and that ferritin only slightly decreased HKa's binding to endothelial cells.

Overall, these findings defined the novel roles for HKa and ferritin in cancer cells and preliminarily explored the signaling pathways initiated by HKa and ferritin in endothelial cells.

CHAPTER I

INTRODUCTION

Angiogenesis

Angiogenesis, the process by which new blood vessels form from pre-existing blood vessels, plays an important role in physiological processes such as embryonic development and wound healing and pathological processes such as inflammation and cancer [1]. This process involves detachment of endothelial cells and migration into the perivascular space where they settle down, proliferate and form tube-like structures which eventually join and form new capillaries. Angiogenesis is orchestrated by a variety of activators and inhibitors. Activators of endothelial cell proliferation and migration are mainly receptor tyrosine kinase ligands, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Many angiogenic inhibitors, such as angiostatin (a fragment of plasminogen), HKa and endostatin (from collagen XVIII) are proteolytic fragments of plasma or extracellular matrix proteins that have no effect on angiogenesis [2]. In general, the balance between angiogenesis activators and inhibitors dictate whether an endothelial cell will be in a quiescent or an angiogenic state.

In addition to angiogenic activators and inhibitors, the ECM also plays an important role in angiogenesis [3]. It not only provides a mechanical support for endothelial cells, but also initiates various cellular signaling pathways through its contact with cell surface proteins such as integrins [4]. The ECM is also a reservoir for

angiogenic activators or inhibitors. For instance, bFGF can be deposited in the ECM, and HK has also been detected in atherosclerosis lesions [5].

Inhibition of angiogenesis has been used as a strategy for cancer therapy in the clinic. Developing antiangiogenic drugs is promising, but has encountered many difficulties mostly because the lack of the knowledge about the molecular mechanism for their anti-angiogenic effects. Understanding the mechanisms of angiogenesis will help to improve the antiangiogenic strategies and prevent reoccurrence.

HK and HKa

HK is a ~120 kD glycoprotein circulating in plasma at a concentration of ~90ug/ml (670nM). It was initially identified as a precursor of bradykinin (BK), which is a potent vasodilator implicated in many inflammatory diseases including asthma and cancer [6]. HK is also known as coactivator of intrinsic coagulation cascade. Cleavage of HK generates HKa, an angiogenesis inhibitor [7].

Structure and biochemical properties of HK and HKa

High molecular weight kininogen (HK) and low molecular weight kininogen (LK) are the products of a single gene due to alternative splicing [8]. The gene (27kb) is located on chromosome 3q26, and consists of 11 exons. The mRNA for HK and LK are 3.5 and 1.7 kb respectively. Structurally, HK contains six domains (designated D1-D6, respectively, Figure 1 [9]). D1-D3 forms a heavy chain and D5-D6 forms a light chain, and D4 links these two chains. HK binds to endothelial cells through D3 and D5 via a

Zn2+-dependent manner [10]. HK bound to endothelial cells serves as a substrate for plasma kallikrein, which cleaves HK into BK (from D4) and HKa. (Figure 1) HKa contains a heavy chain and a light chain which is linked by a single disulfide bond. Electron microscopy demonstrated that after being cleaved from HK, HKa underwent conformational changes and acquired new properties such as the ability to bind to anionic surface [11] and heparin [12]. Particularly, HKa showed a higher antiadhesive ability than HK probably due to the exposure of D5 to the molecule surface [13].

LK is a 68 kD beta-globulin with a plasma concentration of 220ug/ml [14]. It has identical D1-D4 , a different D5 with HKa and no D6. Relative to HK, LK does not have antiangiogenic activity which suggests that D5 is responsible for the antiangiogenic activity of HKa [7].

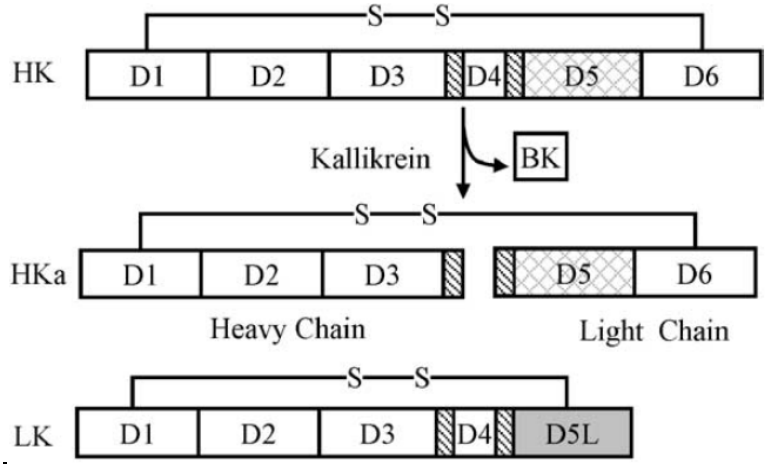

Figure 1. Domain structures of HK and LK. HK contains 6 domains, designated D1-D6. BK is D4 cleaved from HK by Kallikrein. The remaining portion of HK is designated as HKa, which contains a heavy chain and a light chain, linked by a disulfide bond. LK is the product of variant splicing from the same gene as HK. It contains the same D1-D4, a different D5 and has no D6.

Receptors on endothelial cells for HK and HKa

HK interacts with various cell types to exert discrete functions. HK binds to GPIb/IZ/V platelets through D3 to exert its antithrombotic effect [15]. Another receptor for HK on platelets is gC1qR which is also a receptor for HK on endothelial cells. HK binds to Mac-1 (αmβ2 integrin) on neutrophils and monocytes through D3 and D5 [16]. Three major receptors for HK/HKa on endothelial cells have been identified so far, including uPAR [17], gC1qR [18], and CK-1 [19]. Of these, uPAR (55-60 kD) has been shown to be the most important for angiogenesis and tumorigenesis [20]. uPAR contains three domains (designated D1-D3). As a GPI-anchored cell surface protein, it is mostly expressed in monocytes, endothelial cells and many tumor cells. One of uPAR's ligands, uPA cleaves plasminogen to generate plasmin which degrades ECM proteins such as laminin and fibronectin in the basement membrane[3]. Plasmin also activates matrix metalloproteinases that degrade the ECM protein collagen. Degradation of the basal membrane and ECM proteins is essential for tumor metastasis [21]. In addition to its role in uPA-uPAR-plasmin system, uPAR also induces signal transduction at the cell surface. Because uPAR has no transmembrane or cytosolic domains, it induces signalings through interacting with coreceptors such as integrins on the cell surface and vitronectin in the ECM [22]. Therefore it can act as an adhesion receptor for vitronectin and is also capable of transmitting signals through integrins. Previous studies have shown that HKa binds to endothelial cells through D5 of HKa and D2 and D3 of uPAR because an antibody against D2 and D3 of uPAR and soluble recombinant uPAR (suPAR) completely blocked HKa's binding to endothelial cells [17]. In contrast, HK preferentially binds to CK1 and

gC1qR. uPAR, CK1 and gC1qR form a complex at the endothelial cell surface. Therefore, the uPAR-CK1-gC1qR complex might mediate HK/HKa binding to endothelial cells.

HKa and Angiogenesis

HKa is an angiogenesis inhibitor. The first evidence came from a study in 2000 which showed that a recombinant D5 (GST-D5) inhibited endothelial cell proliferation and migration, two important processes of angiogenesis, and GST-D5 also showed antiangiogenic activity in an *in vivo* chicken chorioallantoic membrane assay (CAM) [7]. The antiangiogenic effect of HKa was further confirmed in two other *in vivo* models: the matrigel plug assay and the corneal micropocket angiogenesis assay. Further investigation revealed that HKa and D5 inhibited the de novo synthesis of DNA in proliferating endothelial cells stimulated by bFGF and this was associated with decreased cyclin D1, an important regulator for the G1/S phase transition during cell cycle [23]. HKa was also shown to induce endothelial cell apoptosis demonstrated by condensed and fragmented nuclei and multiple DNA fragments [24] .Therefore, HKa or D5 inhibited angiogenesis both *in vitro* and *in vivo*.

Despite strong evidence that HKa or D5 is an angiogenesis inhibitor, the underlying mechanism remains unclear. The first approach to defining this mechanism has been to identify the cell surface binding sites for HKa. uPAR, CK-1 and gC1qR have been reported as binding sites for HKa on a confluent monolayer of endothelial cells, while another study suggested that the antiangiogenic activity of HKa was mediated through tropomyosin on proliferating endothelial cells [25]. Tropomyosin is a regulator

of actin filaments in cells [26]. In this study, they showed that TM311, an antibody against tropomyosin, prevented HKa-inhibited bFGF-induced endothelial cell proliferation. In addition, TM311 blocked HKa-induced endothelial cell apoptosis [25]. These results suggested an additional binding site for HKa on endothelial cells might exist. Other mechanisms involve HKa-binding proteins and HKa-initiated signaling pathways which will be further interpreted in the following paragraphs.

Ferritin

Ferritin is a ubiquitous and highly conserved iron-binding protein. It is well known for its central role in the storage of iron in a nontoxic but available form. In vertebrates, ferritin contains 2 kinds of subunits, H and L. Twenty four H and L subunits assemble to form the apoferritin shell. Each ferritin molecule can sequester up to 4500 iron atoms [27]. The ratio of H subunit to L subunit in ferritin might vary depending on the tissue type and physiologic status of the cell. The L subunit is predominantly expressed in liver and spleen whereas the H subunit is mainly expressed in heart and kidney [28]. Ferritin H and L subunits are encoded by different genes [29]. Ferritin is mostly located intracellularly, but serum ferritin also occurs in small amount and is often elevated during inflammation [30] and malignancies [31-34]. Although serum ferritin is widely used as an indicator of total body iron level [35] , little is known about the source and function of this ferritin. A previous study from Dr. Torti demonstrated that HKa was a ferritin-binding protein [36]. Recently, Coffman *et al* found that both HK and HKa cound bind to ferritin. However, HKa bound to ferritin more tightly than HK (with a kD of 13nM vs. 140nM). Her study also demonstrated that ferritin antagonized HKa-inhibited

angiogenesis, by enhancing the proliferation and migration of endothelial cells and preventing their apoptosis. Furthermore, ferritin antagonized the antiangiogenic effect of HKa in an *in vivo* PC3 cell-injected mouse model [37]. These findings revealed a new role for serum ferritin in regulating angiogenesis. What remains to be determined is what effects HKa and ferritin might have on cancer cells, the signaling pathways through which they affect angiogenesis, and the mechanism by which HKa and ferritin interact on endothelial cells.

Few studies have been performed on ferritin receptors. It was reported that T cell immunoglobulin-domain and mucin-domain 2 (TIM-2) was expressed on B cells in rat liver and kidney and might be a receptor for H-ferritin endocytosis [38]. A chemokine receptor CXCR4 was suggested to be another receptor for H-ferritin in human embryonic kidney cells and Hela cells [39]. Receptors for ferritin on human endothelial cells have not been identified thus far. Therefore, how HKa and ferritin interact with each other on endothelial cells needs further investigation.

HKa and signaling pathways

ERK Signaling Pathway

VEGF and bFGF play important roles in angiogenesis through several signaling pathways including the mitogen activated protein (MAP) kinase pathway (Ras-Raf-MEK-ERK) [40]. Three types of MAP kinases have been identified so far [41-42]. ERKs are usually strongly activated by growth factors and are therefore the most important for cell proliferation and tumor growth. It has two isoforms– p44 (ERK1) and p42 (ERK2),

both of which can be activated by kinases. Activation of ERK requires phosphorylation of a threonine plus a tyrosine residue at its active sites. Growth factors, such as PDGF, EGF, FGF and uPA, activate ERK through the Ras-Raf-MEK-ERK signaling module [43]. More studies need to be done in order to determine the real role of the ERK signaling pathway in HKa/ferritin-mediated angiogenesis and tumorigenesis. Figure 3 is a schematic representation of the ERK signaling pathway.

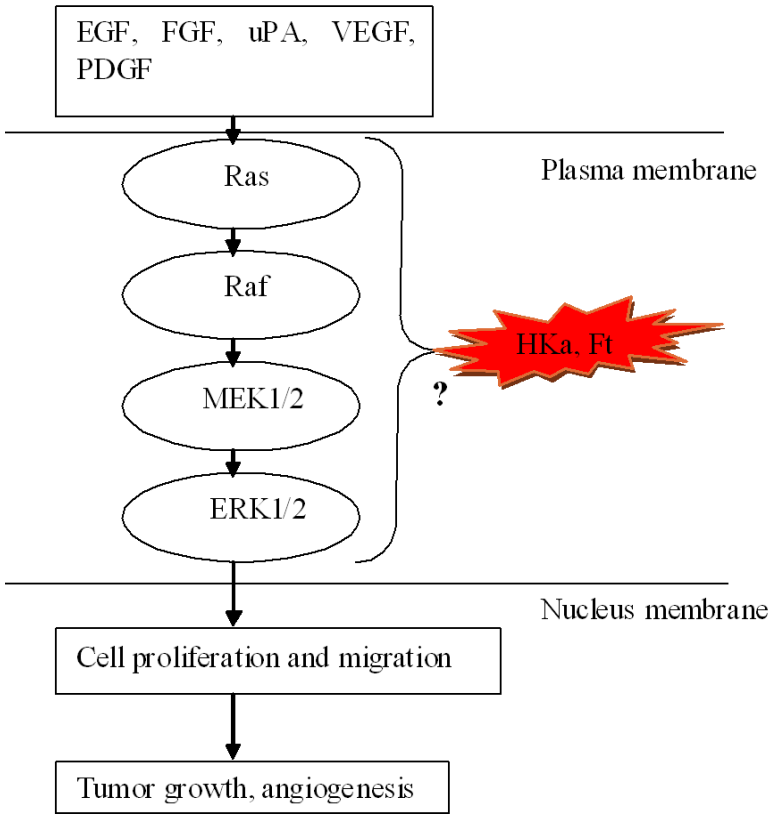

Figure 3. ERK signaling pathway. Growth factors such as EGF, FGF and VEGF etc bind to cell surface receptors and trigger the MAPK or ERK cascade, resulting in the activation of a Ras GTPase. Ras then activates Raf serine/threonine kinase, which in turn phosphorylates and activates MAPK kinases, exemplified by MEK1 and MEK2. MEK1 and MEK2 phosphorylate ERK1 and ERK2 on both threonine and tyrosine residues. Upon activation, ERK1 and ERK2 phosphorylate cytoplasmic targets and translocate into the nucleus, where they stimulate the activity of transcription factors, thereby altering the expression of genes involved in the regulation of cell proliferation, cell migration and cell differentiation. HKa and ferritin might be involved in the regulation of this pathway, thereby affecting the biological action of endothelial cells.

PI3K and Akt Signaling Pathway

HKa inhibited endothelial cell migration, and ferritin reversed the anti-migratory effects of HKa [37]. Endothelial cell migration is one essential step for angiogenesis. In order to block angiogenesis and thereby to limit tumor growth, it is critical to understand how HKa and ferritin regulate endothelial cell migration. Figure 4 is a schematic representation of PI3K/Akt signaling pathway [44].

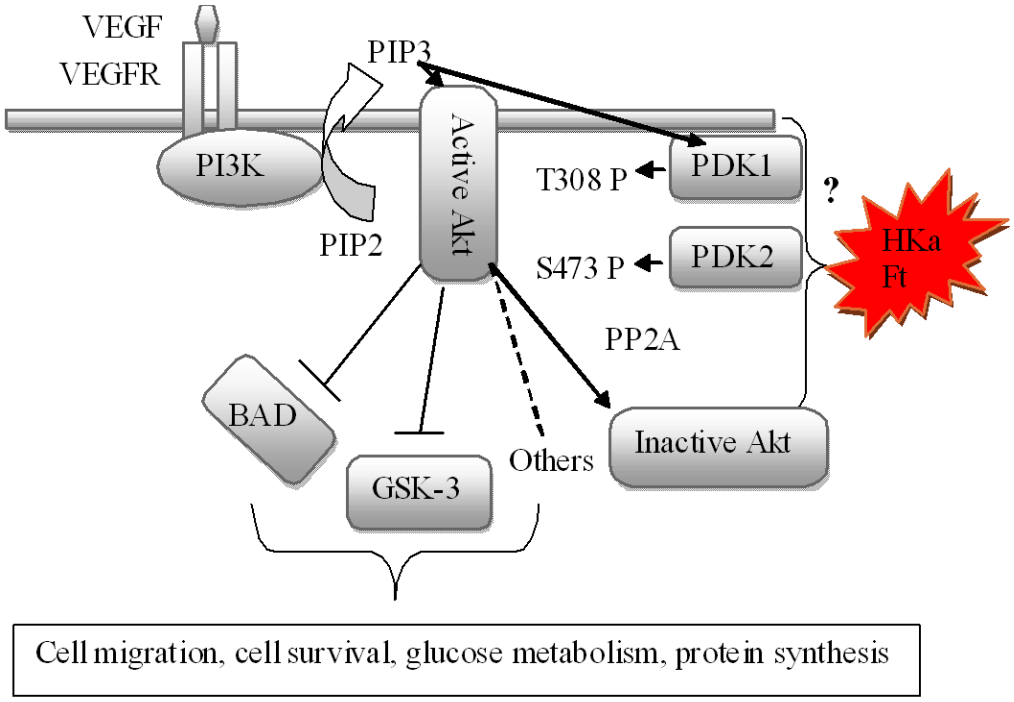

Figure 4. PI3K-Akt signaling pathway. VEGF binding to VEGFR2 activates its receptor tyrosine kinase activity which activates phosphatidylinositol-3 kinase (PI3K). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 activates PDK1, which activates and phosphorylates T308 of membrane-bound Akt. S473 of Akt is phosphorylated and activated by PDK2. Activated Akt affects many downstream targets. It inhibits pro-apoptotic protein BAD and glycogen synthase kinase 3 (GSK-3), thereby inhibiting apoptosis and glycogen synthesis. Akt can be dephosphorylated and inactivated by protein phosphatase 2A (PP2A). HKa and ferritin might mediate angiogenesis through the PI3K/Akt pathway. The dash line stands for other downstream signals regulated by Akt.

Focal adhesion kinase (FAK) and Paxillin

Focal adhesions are large macromolecular assemblies through which mechanical force and regulatory signals can be transmitted. They serve as the linkages for intracellular molecules to ECM. FAK and paxillin are focal adhesion-associated proteins. FAK is a nonreceptor tyrosine kinase, and paxillin is one of the substrates for FAK. Clustering of integrins promotes the recruitment of FAK to focal contacts and facilitates FAK activation by phosphorylating Tyr397. The activated FAK then becomes a substrate for SFKs (Src family kinases) and leads to the recruitment and activation of Src. FAK-Src complexes play a central role in downstream signaling contacts by paxillin and talin. Both FAK and paxillin in focal contacts play essential roles in cell migration, adhesion, proliferation and survival by transmitting signals downstream of integrins [45]. Figure 5 is a schematic representation of a focal adhesion.

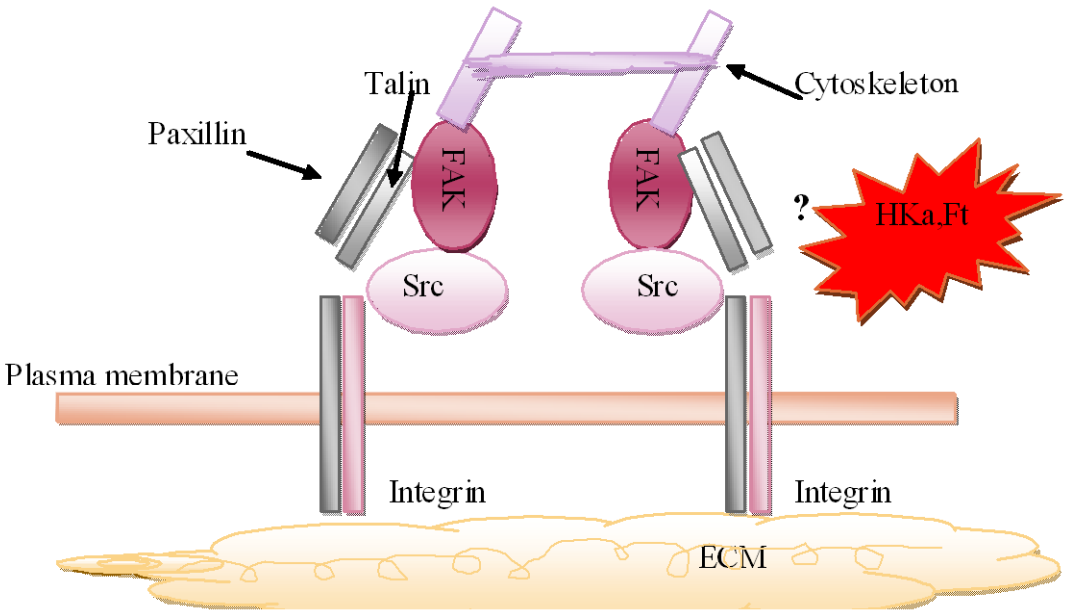

Figure 5. The ECM, integrins and the cytoskeleton interact at focal contacts. Integrin at focal adhesion recruits FAK which then recruits Src. FAK also can be recruited to focal adhesion by paxillin and talin, two adapter proteins. Focal adhesion proteins, including FAK and paxillin, transmit signal from ECM to cytoskeleton.

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CHAPTER II

MATERIAL AND METHODS

Reagents. HKa, bFGF, EGF and vitronectin were purchased from BD Biosciences. Human spleen ferritin was from the Scripps Research Institute. CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay was purchased from Promega. The 24-well cell invasion assay kit was from Calbiochem. VEGF was purchased from R & D Systems. HMW uPA and Anti-uPAR antibody were purchased from American Diagnostica Inc., (Stanford, CT, USA). Antibodies directed against total and phosphorylation-specific (T308) Akt, total and phosphorylation-specific (T202/Y204) ERK, were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Mouse Anti-phospho-FAK (Y397) monoclonal antibody was purchased from Chemicon International, INC. Total FAK antibody was purchased from Santa Cruz Biotechnology, INC. Anti-Paxillin [pY118] phosphospecific antibody was purchased from Invitrogen. Anti-paxillin antibody was from Millipore. GAPDH antibody was purchased from Fitzgerald. BCA protein assay kit, Poly-HRP conjugated secondary antibody, Restore western blot stripping buffer, SuperSignal West Pico Chemiluminescent Substrate and No-Weigh Sulfo-NHS-LC-Biotin and Biotin Quantitation Kit were purchased from Thermo Fisher Scientific. Phosphatase Inhibitor Cocktail 2 was purchased from Sigma. Protran Nitrocellulose transfer membrane was purchased from Perkin Elmer Life Sciences. Streptavidin conjugated APC was purchased from SouthernBiotech.

Cell culture. Pooled human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in endothelial cell growth medium-2 (EGM-2, Lonza) containing basal medium (EBM-2) and supplemental nutrition and growth factors. HUVECs from passage 3 to 7 were used for experiments. BG-1 and SKOV-3 ovarian cancer cell lines and THP-1 cell line were kind gifts from Dr. Greg Kucera, Dr. Larry Daniel and Dr. Darren Seals from Wake Forest University School of Medicine (WFUSM). A2780 was from Core culture room from WFUSM. HEC1A and AGS cell lines were from American Type Culture Collection (ATCC). All the other cell lines used in our experiments were from the Torti lab. Cells used in our experiments are listed in Table 1 with cell type and culture medium specified. All cells were grown in a humidified incubator at 37^{o}C with 5% CO2 and passed 2-3 times a week.

Table 1. Cells and media

Cell name	Cell type	Base medium	Antibiotics	Serum, growth factors and others
HUVEC	Human umbilical vein endothelial	EBM-2		EGM-2 SingleQuot Kit Suppl. & Growth Factors
A2780	Human ovarian cancer	RPMI 1640	1% P/S	10%FBS
BG-1	Human ovarian cancer	RPMI 1640	1% P/S	10%FBS
SKOV-3	Human ovarian cancer	McCoy's 5A or 1640	1% P/S	10%FBS
MDA-MB-231	Human breast cancer	DMEM	1% P/S	10% FBS
MCF-7	Human breast cancer	DMEM/F12	1% P/S	5% FBS
DU-145	Human prostate cancer	RPMI 1640	1% P/S	10% FBS
PC-3	Human	RPMI 1640	1% P/S	10% FBS

	prostate cancer			
Hela	Human cervical cancer	DMEM	1% P/S	10% FBS
HEC-1A	Human endometrial cancer	McCoy's 5A	1% P/S	10% FBS
H1299	Human non-small cell lung cancer	RPMI 1640	1% P/S	10% FBS
HCT116	Human colon cancer	McCoy's 5A	1% P/S	10% FBS
AGS	Human gastric cancer	RPMI 1640	1% P/S	10% FBS
CRL-1932	Human kidney cancer	McCoy's 5A	1% P/S	10% FBS
Caki 2	Human kidney clear cancer	McCoy's 5A	1% P/S	10% FBS
HME	Human mammary epithelial	Mammary Epithelial Growth Media (MEGM)		MEGM® SingleQuots®
THP-1	Human acute monocytic leukemia	RPMI 1640		10% FBS and 50uM 2-mercaptoethanol

Cell proliferation assay. 96-well plates were coated with vitronectin (2ug/ml in PBS) for 1 hour at RT. HUVECs and other cells were seeded onto the coated plates at a density of 3000-5000 cells/well in their respective basal medium containing 20ng/ml bFGF (HUVEC) and EGF (other cells) and 10uM ZnCl2. Cells were treated with various concentration of HKa with or without equal or higer molar ratios of human spleen ferritin, ferritin alone or saline control. Cells were incubated at 37^{o}C, 5% CO2 for 48 hours. Viability was assessed with CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay from Promega. Briefly, 2mg/ml MTS solution was mixed with 0.92mg/ml PMS at a ratio of 20:1 (MTS: PMS) immediately before addition to cells at a ratio of 5:1 (cell culture medium: MTS+PMS). Cells were incubated at 37^{o}C, 5% CO2 for 1-4 hours.

Absorbance was then measured at 490nm filter in a spectrophotometer. Control cells were normalized to 100% viability and compared with HKa, ferritin, or HKa plus ferritin treatments.

Clonogenic assay. Cells were trypsinized to single cell suspension and diluted to low concentration. 200-250 cells were seeded into each well of 6-well plate in duplicate or triplicate. Cells were allowed to attach overnight at 37^{o}C, 5% CO2 humidified atmosphere then treated without (control) or with different concentration of HKa, ferritin, and HKa plus ferritin with the addition of 10uM ZnCl2. Treatment medium was changed to regular cell culture medium after 24 hours and then changed to fresh cell culture medium every 2-3 days. When most cell formed colonies of over 50-100 cells (10-14 days), cells were fixed with in acetic acid, methanol, H_2O at a 1:1:8 ratio for 10 minutes and stained with 0.4% crystal violet for 10 minutes. After drying up in air, cell colonies were scanned with Epson Perfection 2450 Photo scanner and then counted with an ImageJ software.

Cell scratch assay. Cells were allowed to form monolayer in a 24-well plate, and then scratched with a 200ul pippet tip. Cells were untreated or treated with different concentrations of HKa or ferritin or HKa plus ferritin with the addition of 10uM ZnCl2. Marks were made in the scratch and imaged with an Olympus microscope. Cells were kept in 37^{o}C, 5% CO2 humidified incubator for another 10-12 hours. After the incubation, images of the scratch matching the marking points were acquired again. Migrated distance between one side of scratch and the other was measured using Image-Pro Plus

software. Migrated distance in the control cells untreated with HKa or ferritin was compared to all the other treatments.

Cell invasion assay. Inserts in the 24-well invasion assay kit were rehydrated with warm base culture medium for 2 hours at 37^{o}C before using. 500ul AGS and DU145 cell suspensions (2.5X10^{4} cells) untreated or treated with 100nM HKa (plus 10uM ZnCl2) were added to the top chambers and 10% FBS contained medium was added to the bottom chamber for 24 hours. Cell staining solution was made by mixing calcein-AM with cell detachment buffer at a ratio of 1:300 and added to new wells of the plate. The inserts with invaded cells were moved to these wells and kept at 37^{o}C for 30 minutes. After incubation, 200ul of the solution containing the dislodged cells were transferred to a 96-well plate in duplicate for fluorescence readings at 485/530nm.

Cell adhesion assay. 2ug/ml vitronectin was used to coat a 96-well plate for 1 hour at RT. DU145 cells untreated (control) or treated with different concentrations of HKa or ferritin or HKa plus ferritin with the addition of 10uM ZnCl2 were seeded at a density of 1X10^{4} cells/well for 1 hour. Unattached cells were removed, and an MTS assay was performed on the attached cells as an indication of adherent cells. Each plate was read for absorbance at 490nm in a spectrophotometer.

Western blot. *Cell treatment for HKa and ferritin's effect on uPA-induced P-ERK.* Five 100mm dishes of HUVECs were starved with serum-free M199 medium for 5 hours and then treated with serum-free M199 medium without (control) or with 100nM HKa or

100nM HKa plus 100nM ferritin (plus 10uM ZnCl2) for 1 hour at 37^{o}C, 5% CO2. All cells, except control cells, were stimulated with 15nM HMW uPA for 30 minutes.

Cell treatement for HKa and ferritin's effect on VEGF-induced P-Akt. HUVECs were trypsinized and seeded into 2ug/ml vitronectin-coated dishes for 2 hours. Cells were then left untreated (control) or treated with 100nM HKa, or 100nM HKa plus 100nM ferritin, 100nM ferritin (plus 10uM ZnCl2) for 30 minutes followed by a stimulation of VEGF (20ng/ml) for 5 minutes.

Cell treatment for HKa's effect on P-FAK and P-paxillin. 150mm dishes were coated with 2ug/ml vitronectin for 1 hour at RT. HUVECs were left untreated (control) or treated with 100nM HKa with the addition of 10uM ZnCl2 and 20ng/ml bFGF for 30 minutes, 60 minutes and 3 hours.

Cells with the above treatments and other subconfluent cells (the latter is for detection of uPAR expression) were collected by scraping with a cell lifter and centrifuged at 2800RPM for 15 minutes. 1XPBS was used to wash the pellet before another round of centrifuging at the same speed. Cell lysis buffer containing 1% SDS, 10mM Tris-Cl, 150mM NaCl 0.5mM EDTA plus protease and phosphatase inhibitor cocktail (from Sigma) was added to cell pellets and kept on ice for solubilization for 30 minutes with intermittent vortexing. Cell extracts were sonicated for 10 seconds, centrifuged for 15 minutes at 14,000 g, and the supernatant (cell lysate) was then recovered. Protein concentration was determined by use of a BCA kit (Pierce, Rockford, IL). Equal amounts

of protein for each sample (40μg per lane) were run on 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The transferred protein was reversibly stained with Ponceau S to ensure equivalent loading of protein in each lane. Nitrocellulose membrane was blocked with 5% nonfat dry milk and 0.5% BSA in Tris-buffered saline containing 0.05% Tween 20 and then probed with antibodies to phospho-ERK (1:500) and ERK (1:1000), phospho-Akt (1:500), Akt (1:1000), phospho-FAK (1:500), FAK (1:1000), phospho-paxillin (1:500), paxillin (1:1000), uPAR (1:300) and GAPDH (1:10,000). Bound antibodies were detected with SuperSignal West Pico Chemiluminescent Substrate and analyzed with densitometry using Adobe Photoshop cs2 software.

Biotinylation of HKa and ferritin. 100ul HKa (1.2mg/ml) and 100ul ferritin (2mg/ml) were dialyzed respectively into 1L 1XPBS containing no potassium using a Slide-A-Lyzer Mini Dialysis Unit for 2 hours at 4^{o}C. 180ul ultrapure water was added and mixed into No weight Sulfo-NHS-LC-Biotin to make 10mM concentration. 50-fold molar excess of biotin was added to dialyzed HKa and ferritin. The reaction was incubated on ice for 2 hours on ice and then dialyzed to remove non-reacted biotin. Biotin Quantitation Kit was performed to determine the level of biotin incorporation before use in flow cytometry.

Flow cytometry. HUVECs were trypsinized and centrifuged at 700 PRM for 5 minutes. Cell pellets were dissolved in 250ul 2X EBM-2 basal medium containing 50uM ZnCl2. Cell suspensions were left untreated (control) or treated with 1uM bio-HKa, 1uM bio-

ferritin, 1uM bio-HKa plus 1uM unlabeled ferritin for 1 hour at 37^{o}C, 5% CO2 incubator. Then Streptavidin-conjugated APC (Strep-APC) was added to cells for 30 minutes on ice in darkness. Treated cells were finally dissolved in 500ul 1% BSA/PBS and run through a flow cytometer.

Statistical analysis. Results were reported as the mean value and 95% confidence interval. F test and student T-test were used to compare different treatment conditions. P values were also reported ($\alpha<0.05$ or <0.001).

CHAPTER III

RESULTS

HKa inhibited angiogenesis both in *vitro* and *in vivo*. Ferritin antagonized HKa's effects on angiogenesis. We used *in vitro* assays such as cell proliferation, migration, invasion, adhesion and clonogenic assays to assess the effects of HKa and ferritin on cancer cells.

HKa inhibited the proliferation of cancer cells. We first assessed the effect of HKa on the viability of various cancer cells including A2780, BG-1, SKOV-3, MDA-MB-231, MCF-7, PC3, DU145, Hela, HEC1A, H1299, HCT116, AGS, CRL-1932, and Caki2 cells, and three other cell lines including HME, THP-1 cells and HUVECs. The type of cells and their culture medium were summarized in Table I (METHODS). We seeded cells on a 96-well plate at a concentration of 3000-5000 cells/well and then treated them without or with HKa of different concentrations from 10nM to 200nM. 10uM ZnCl2 was included in the treatment medium because it is required for HKa binding to the endothelial cell surface. After 48hrs, cell viability was assessed. As seen in Figure 1A, to 90.0±2.9% (p=0.0007), SKOV-3 cells to 96.8±4.7% (p=0.0196), MDA-MB-231 cells to 83.0±26.0% (p=0.108), MCF-7 cells to 40.4±21.3%, (p=0.0003), PC3 cells to 87.8±12.9 (p=0.086), DU145 cells to 47.8±9.4% ($p=9.4X10^{-6}$), Hela cells to 86.2±6.5% (p=0.001), and HEC1A cells to 78.0±13.2% (p=0.0005) of control cells. In Figure 1B, 100nM HKa inhibited viability of H1299 cells to 73.2±14.8% (p=0.017), HCT116 cells to 48.6±3.6% (p=0.039), AGS cells to 75.3±15.5% (P=0.002), CRL-1932 cells to 45.9±16.8%

(p=0.00018), and Caki2 cells to 82.1±18.4% (p=0.099) of control cells. In Figure 1C, 100nM HKa inhibited viability of HME cells to 88.0±14.8% (p=0.029), THP1 cells to 84.3±8.9% (p=0.017) of control cells, and as a positive control, the viability of HUVECs was inhibited by 10nM HKa to 49.7±18.9% ($p=4.1X10^{-6}$) of control HUVECs, which was consistent with the reported IC50 of HKa on endothelial cells. The effect of HKa on the viability of cells in figure 1 was summarized in Table 1.

As seen in Figure 1 and Table 1, HKa has a comprehensive inhibitory effect on the proliferation of cells including cancer cells, endothelial cells and HME cells. Specifically, 100nM HKa significantly inhibited the proliferation of human umbilical vein endothelial cells (HUVECs) and most of the cancer cells we have tested. Though it did not significantly affect the proliferation of several other cell lines, such as MDA-MB-231, PC3 and Caki2 cells, an inhibition trend by HKa was observed when we increased the concentration of HKa as shown in Figure 1.

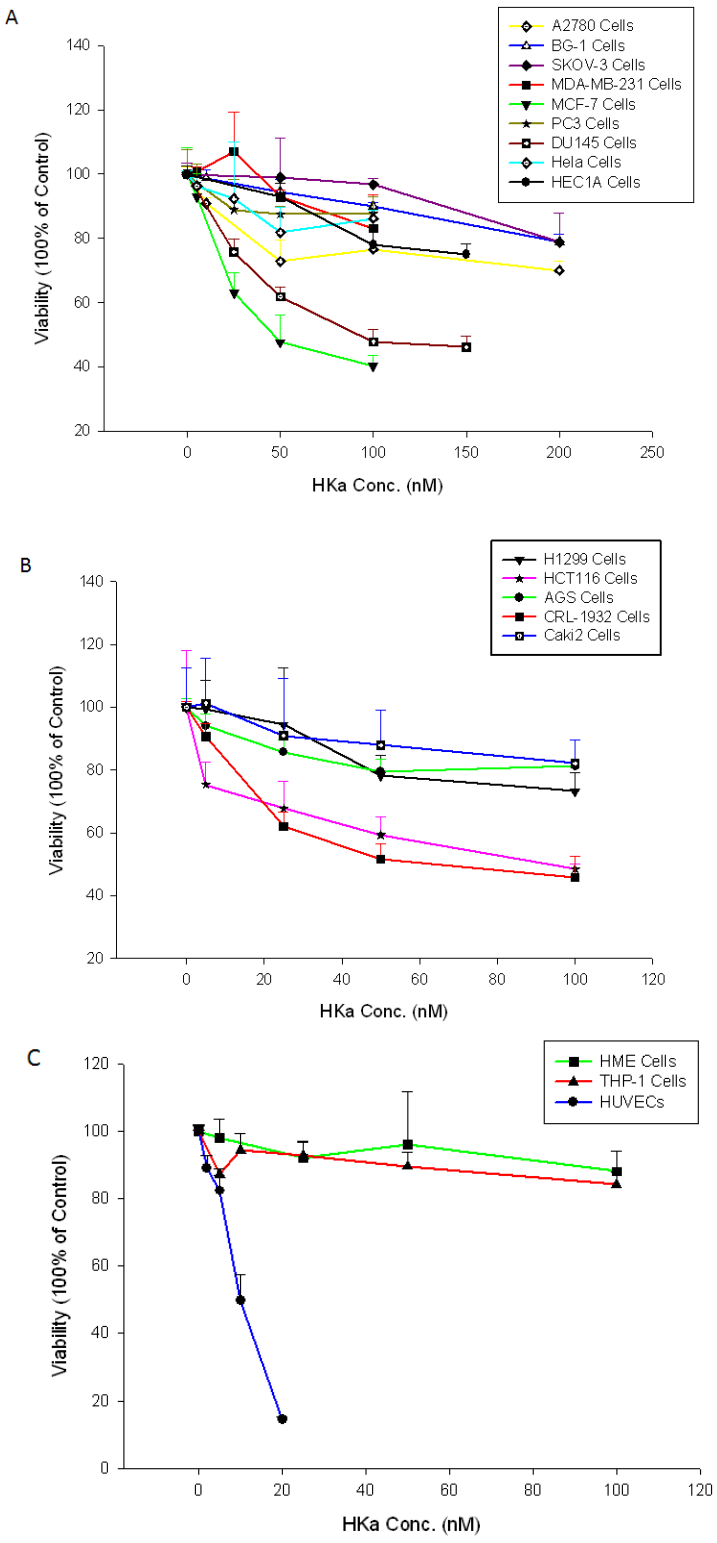


Figure 1. Effect of HKa on cancer cell proliferation. A. A2780, BG-1, SKOV-3, MDA-MB-231, MCF-7, PC3, DU145, Hela, HEC1A cells stimulated with 20ng/ml EGF were treated with different concentration of HKa. After 48 hours, levels of viability were assessed with a MTS assay. Viability was presented as a percentage of the control cells for each treatment. Values are the means and standard deviation of 3 independent experiments. B and C. H1299, HCT116, AGS, CRL-1932, Caki2 cells and HME, THP-1, HUVECs were treated the same manner as in A. HUVECs were used as positive control which were stimulated with 10ng/ml bFGF and treated with a lower concentration gradient up to 20nM due to their strong sensitivity to HKa. Cell viability was compared between 100nM HKa treatment and control except for HUVECs in which comparison was between 10nM HKa and control.

Table 1. Inhibition of Cell proliferation by HKa (100nM)

Cell name	Cell type	100% of control viability
A2780	ovarian cancer	70.1±6.8%***
BG-1	ovarian cancer	90.0±2.9%***
SKOV-3	ovarian cancer	96.8±4.7% *
MDA-MB-231	breast cancer	83.0±26.0%
MCF-7	breast cancer	40.4±21.3%*
PC3	prostate cancer	87.8±12.9%
DU145	prostate cancer	47.8±9.4%* **
Hela	cervical cancer	86.2±6.5%*
HEC-1A	endometrium cancer	78.0±13.2%*
H1299	lung cancer	73.2±14.8%*
HCT116	colon cancer	48.6±3.6%*
AGS	gastric cancer	75.3±15.5%*
CRL-1932	kidney cancer	45.9±16.8%* **
Caki2	kidney cancer	82.1±18.4%
HME	mammary epithelial	88.0±14.8%*
THP-1	acute monocytic leukemia	84.3±8.9%*
HUVEC	human umbilical vein endothelial	49.7±18.9%*

Table 1: The viability of cells treated with 100nM HKa after 48 hours presented as the percentage of untreated control cells. Means and 95% confidence interval are shown; * $p<0.05$, *** $p<0.001$.

Effect of Ferritin on HKa-inhibited cell proliferation.

We assessed the effect of ferritin plus HKa on the viability of DU145, CRL-1932, MCF-7 and endothelial cells. We seeded cells onto a 96-well plate at 3000-5000 cells/well and then treated without (control) or with HKa alone, ferritin alone or both proteins. In Figure 2, we see similar result as in Figure 1, 100nM HKa significantly decreased the viability of DU145 cells; 50nM HKa significantly decreased the viability of CRL-1932 cells; 40nM HKa significantly decreased the viability of MCF-7 cells; and 10nM HKa significantly decreased the viability of HUVECs. Ferritin alone did not affect the viability of all these three cancer cell lines, but increased the viability of HUVECs.

Co-treatment of HUVECs with HKa and ferritin at a 1:1 or 1:2 molar ratio (HKa: Ft) significantly reversed the viability of HUVECs compared to HKa alone, which confirmed the result from Coffman's study [37]. Contrary with endothelial cells, co-treatment of cancer cells with both HKa and ferritin at a 1:1 molar ratio or higher (Ft: HKa) did not increase the viability of DU145, CRL-1932 and MCF-7 cells.

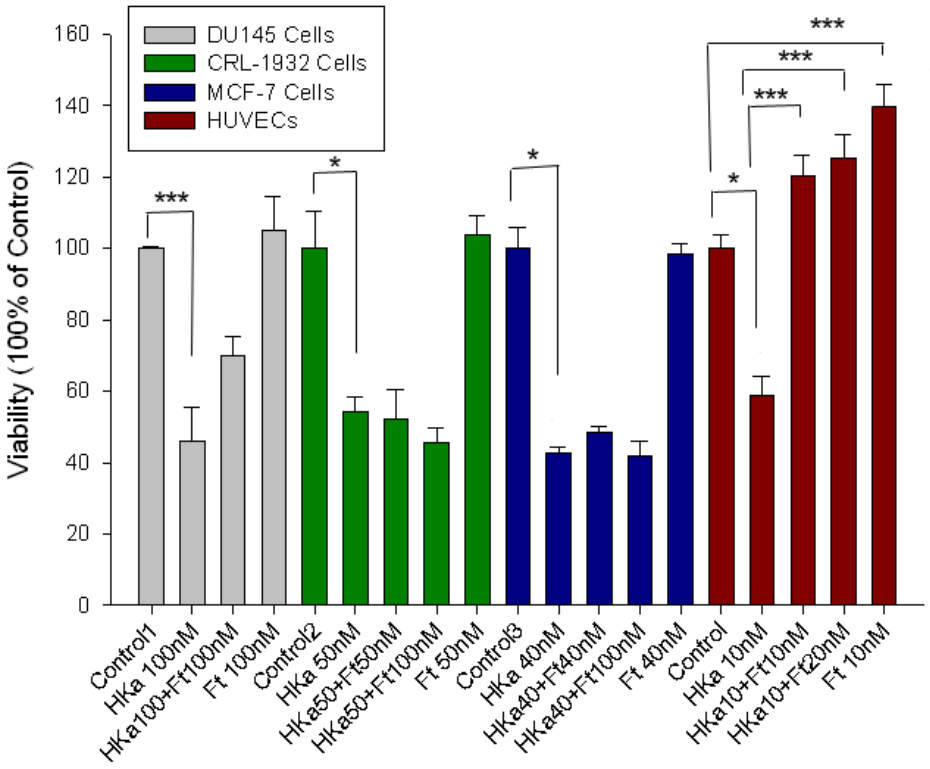


Figure 2. Effect of HKa and ferritin on cancer cell proliferation. DU145, CRL-1932, MCF-7 and HUVECs, whose proliferation was significantly inhibited by HKa, were treated with HKa alone, ferritin alone, HKa plus ferritin (at 1:1 ratio or high ration). HKa or Ft alone treatment was compared to a non-treated control. HKa plus Ft treatment was compared to HKa alone treatment. After 48 hours, cell viability was assessed with a MTS assay. Viability was presented as a percentage of the control cells for each treatment. Values are the means and standard deviation of 3 independent experiments. * $p<0.05$, *** $p<0.001$, HUVECs were used as positive control.

HKa inhibited the clonogenic growth of cancerous and endothelial cells and ferritin did not affect HKa-inhibited clonogenic growth of cancer cells.

Many cancer cells can undergo “unlimited” division and form colonies from a single cell. The clonogenic assay is an *in vitro* method to determine cell reproductibility after treatment with ionizing radiation or cytotoxic agents. To determine the effect of HKa on the clonogenic growth of cancer cells and endothelial cells, we first seeded 200-250 HUVECs, AGS, HEC1A, HCT116, DU145, A2780, BG-1 and SKOV-3 cells onto a 6-well plate in duplicate or triplicate. The cells were allowed to attach overnight, and were then treated without (control) or with different concentrations of HKa. After ten 10 days, cells were fixed, stained and counted for colony numbers. We treated HEC1A, AGS and HCT116 cells without (control) or with 100nM and 300nM HKA, and treated the other cells with various concentrations of HKa ranging from 0nM to 100nM. Our results in Figure 3A show that the clonogenic growth of cells were all inhibited significantly by the highest concentration of HKa used for each cell line. 300nM HKa significantly inhibited the clonogenic growth of HCT116, AGS and HEC1A cells. 50nM HKa significantly inhibited the clonogenic growth of A2780, BG-1, SKOV-3 and endothelial cells. 100nM HKa significantly inhibited the clonogenic growth of DU145 cells. We further assessed the effect of cotreatment of HKa and ferritin on the clonogenic growth of cancer cells and endothelial cells. Cells were seeded the same way as above. Then we treated cells with HKa alone and HKa plus ferritin. For DU145 and HUVEC cells, we also treated cells with ferritin alone. As shown in figure 3B, 20nM HKa significantly inhibited the clonogenic growth of BG-1 cells and DU145 cells, and 5nM HKa significantly inhibited the clonogenic growth of endothelial cells. 25nM HKa and 20nM HKa significantly inhibited the clonogenic growth of SKOV-3 and A2780 cells. 20nM HKa plus 20nM ferritin did not block HKa-inhibited clonogenic growth of BG-1

cells. Similarly, 25nM HKa plus 25nM ferritin did not reverse 25nM HKa-inhibited clonogenic growth of SKOV-3 cells; 20nM HKa plus 20nM ferritin, 5nM HKa plus 5nM ferritin did not block the inhibition of HKa on the clonogenic growth of A2780 and endothelial cells. Both 20nM HKa plus 20nM ferritin and 20nM HKa plus 40nM ferritin did not reverse the inhibition of HKa on the clonogenic growth of DU145 cells.

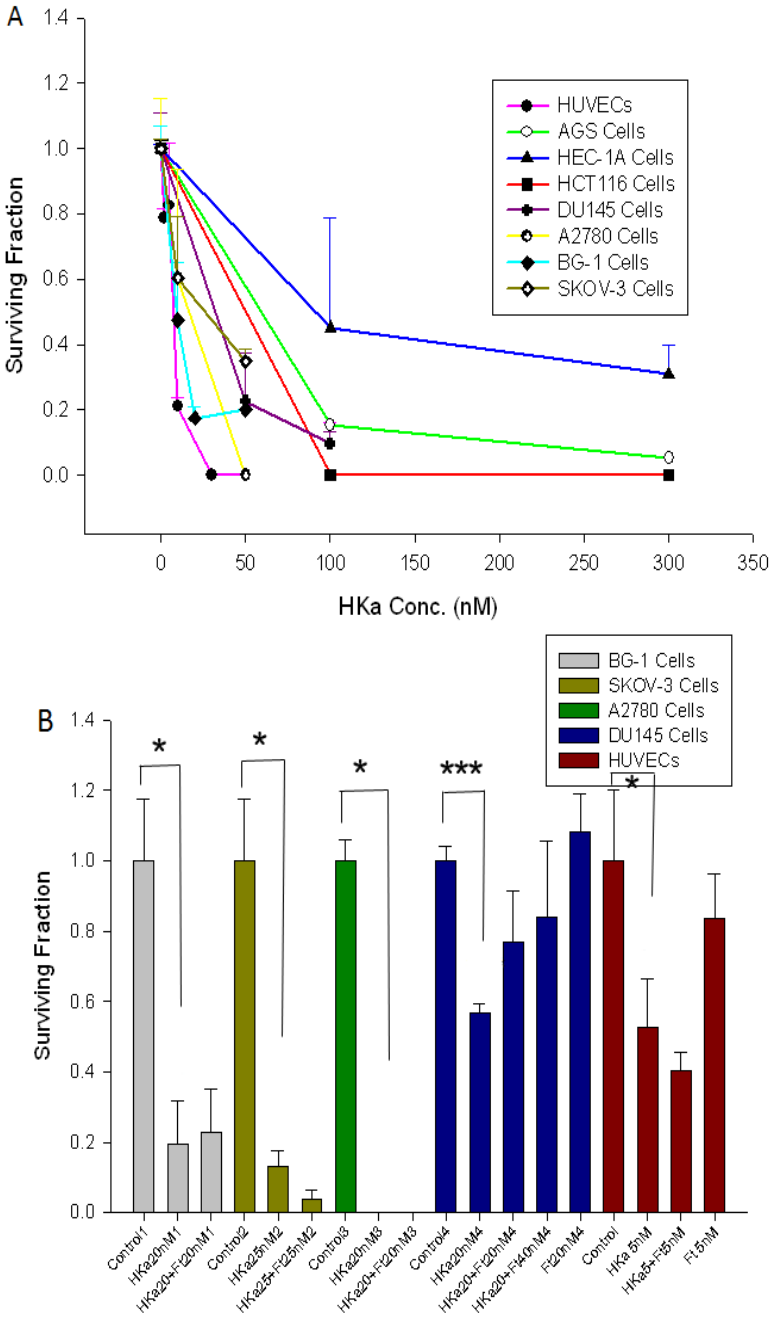


Figure 3. Effect of HKa and ferritin on clonogenic growth of cancer and endothelial cells.

A. *Effect of HKa on clonogenic growth of cells*. 200-250 HUVEC, AGS, HEC1A, HCT116, DU145, A2780, BG-1 and SKOV-3 cells were seeded onto a 6-well plate in duplicate or triplicate and allowed to attach overnight. Cells were treated with different concentrations of HKa. Then cells were cultured for 8-10 more days. Cells were fixed with acetic acid, methanol and H_2O at a ratio of 1:1:8 and stained with 0.4% crystal violet. Colony number was counted using ImageJ software after cells were air dried overnight and scanned. A cell cluster containing over 50 or 100 cells was defined as one colony. In each treatment, colonies were normalized to untreated control cells.

B. *Effect of HKa and ferritin on the clonogenic growth of cancer and endothelial cells.* BG-1, SKOV-3, A2780, DU145 and HUVEC cells were seeded as above and treated with HKa alone, HKa plus ferritin at an equal ratio (DU145 cells were also treated with Ft at a higher ratio; HUVECs were also treated with Ft alone). HKa or Ft alone was compared to untreated control. HKa plus Ft treatment was compared to HKa alone treatment. * $p<0.05$, *** $p<0.001$, difference from control.

Effect of HKa and ferritin on the migration of cancer cells

Cell migration is a central step for metastasis and angiogenesis. The *in vitro* scratch assay is used to measure cell migration. We first made a scratch in a monolayer of CRL-1932, AGS and DU145 cells, treated them without (control) or with HKa at different concentrations ranging from 0 to 300nM, and captured the images of the scratches at the beginning of experiment and after 10-12 hours. We then measured the migrated distance with ImagePro plus software and compared the migrated distance among different treatments. Figure 4A is the representative images of AGS cells scratched at 0hr and 10hr. Migrated distance was measured from the left margin of the scratch to the right. We can see that the migrated distance in AGS cells treated with HKa was less than control cells in 10 hours. Figure 4B showed that HKa did not inhibit the migration of CRL-1932 cells but inhibited the migration of DU145 cells in a concentration-dependent manner, specifically, 300nM HKa significantly inhibited the migration of DU145 cells. To further determine whether ferritin has an effect on HKa-inhibited cancer cell migration, we treated DU145 cells without HKa, with 100nM HKa alone, with 100nM ferritin alone and with 100nM HKa plus 100nM ferritin. We found that (Figure 4C) 100nM HKa inhibited the migration of DU145 cells to 69.9±31.2% of control cells. Ferritin alone did not significantly affect the migration of these cells compared to control. 100nM HKa plus

100nM ferritin treatment showed a migrated distance of 62.1±49.3% of control, which was not significantly different with HKa alone treatment.

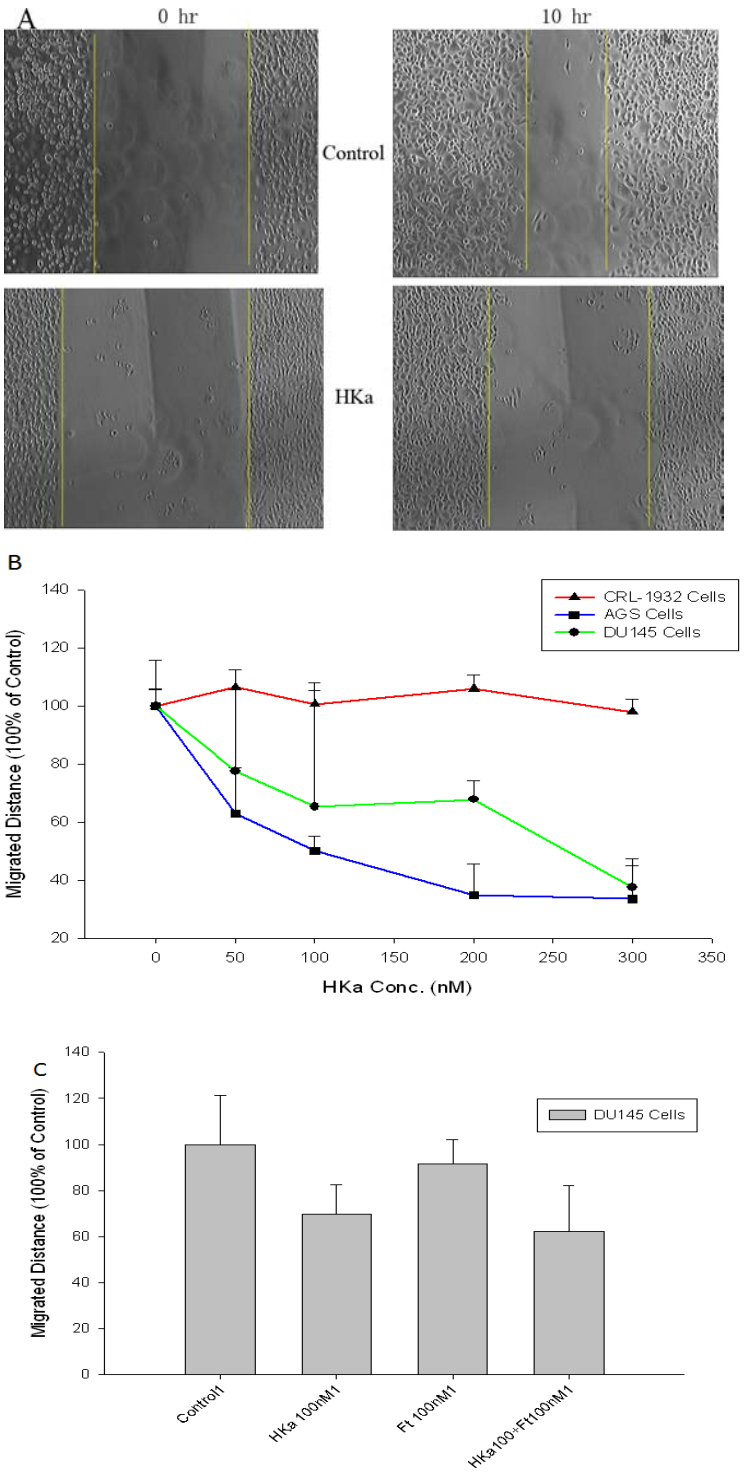
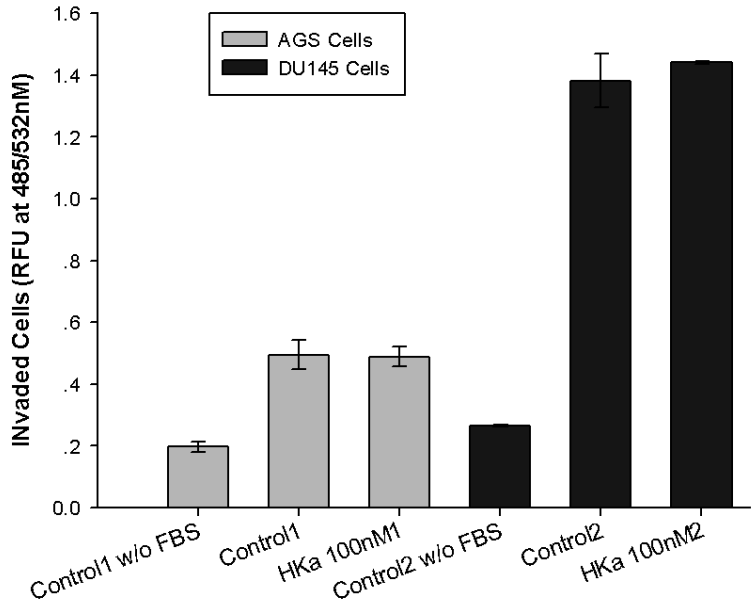


Figure 4. Effect of HKa and ferritin on cancer cell migration. A. *Representative image of AGS cell migration inhibited by HKa*. Top and bottom panels are control and HKa-treated AGS cells at 0 hr and 10hr. The straight line indicates the scratch margin. B. *Effect of HKa on cancer cell migration.* Monolayer of CRL-1932, AGS and DU145 cells were scratched with a 200ul pipette tip and treated with different concentration of HKa. Scratched cells were imaged at 0 hour and at 10-12 hours. ImagePro Plus was used to measure the migrated distance which was then compared between each treatment and nontreated control C. *Effect of HKa and ferritin on DU145 cell migration.* Similar cell scratch assay was performed on DU145 cells, wich were treated without or with 100nM HKa, 100nM Ft, or 100nM HKa plus 100nM Ft. Migrated distance was compared with nontreated control.

HKa did not inhibit the invasion of AGS and DU145 cells through Matrigel.

Cancer cell invasion into the basement membrane and ECM is an important step in the multistep process of angiogenesis. $2.5x10^4$ AGS or DU145 cells were seeded in the top chamber of Matrigel invasion kit; culture medium containing 10%FBS was put in the bottom chamber. After 24 hours, inserts with invaded cells were transferred to new wells containing a mixture of cell detachment buffer and calcein-AM for 30 minutes. Fluorescence was read at 485nm/530nm. Figure 5 showed that 100nM HKa did not inhibit the invasion of either AGS or DU145 cells through Matrigel.

A



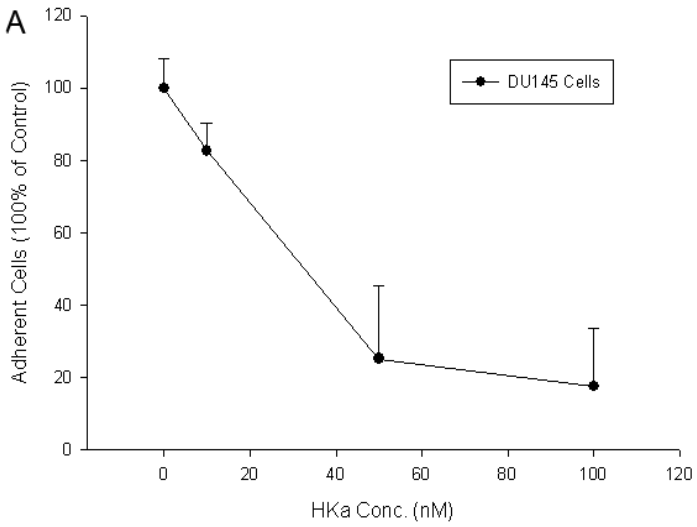
B

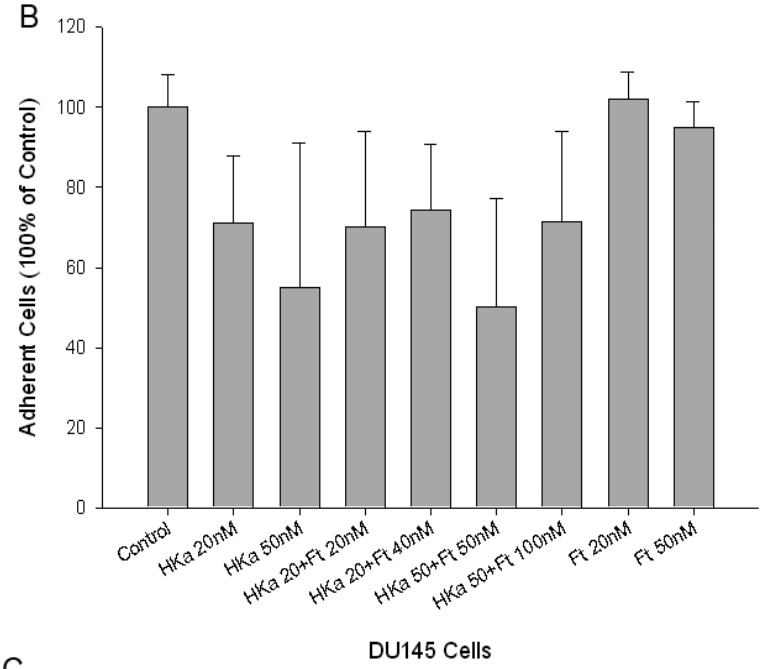
Cell line	AGS			DU145		
	Control1 w/o FBS	Control1	HKa100nM1	Control2 w/o FBS	Control2	HKa100nM2
Fluorescence1	0.209	0.462	0.466	0.263	1.320	1.437
Fluorescence2	0.186	0.528	0.512	0.266	1.443	1.443

Figure 5. Effect of HKa on cancer cell invasion. A. AGS and DU145 cancer cells treated with or without HKa were seeded into the top of Matrigel invasion chambers and allowed to invade toward 10% FBS in the bottom chamber for 24 hours. Inserts with invaded cells were detached and stained with a mixture of detachment buffer and calcein-AM at a ratio of 300:1 for 30 minutes. 200ul of solution containing dislodged cells were transferred to a 96-well plate and fluorescence was read at 485/530nm. B. Fluorescence readings of calcein from cells treated as in A. Fluorescence1 and fluorescence2 are duplicate wells.

HKa inhibited the adhesion of DU145 cells to vitronectin and ferritin did not affect HKa-inhibited DU145 cell adhesion.

Cell adhesion is required for angiogenesis and metastasis. We tested the effect of HKa on the adhesion of DU145 cells to vitronectin, a provisional ECM protein. First DU145 cells untreated (control) or treated with different concentration of HKa were allowed to attach for 1 hour, then a MTS assay was performed to measure the viability of adherent cells. As seen from Figure 6A, HKa inhibited the adhesion of DU145 cells to vitronectin in a concentration-dependent manner, specifically, 100nM HKa significantly inhibited DU145 cell adhesion to vitronectin ($p<0.05$). To assess ferritin's effect, these cells were left untreated (control) or treated with HKa or ferritin alone or combined at two different concentrations (20nM and 50nM). Although both 20nM HKa and 50nM HKa did not inhibit the adhesion of DU145 cells signficantly probably because of the big variance in each treatment, an inhibitory trend by HKa could be seen from these results (Figure 6B). Ferritin alone did not show much effect on the adhesion of these cells compared to control. All HKa plus ferritin treatments did not prevent the inhibition of HKa on DU145 cell adhesion to vitronectin. The actual obsorbance values from the MTS assays for the results in 6B was also listed in 6C.





C

DU145	Control	HKa20nM	Ft20nM	HKa20+Ft20	HK20+Ft40	HKa50nM	Ft50nM	HKa50+Ft50	HKa50+Ft100
OD1	1.3	1.02	1.314	1.069	1.057	0.99	1.222	0.852	1.075
OD2	1.159	0.729	1.195	0.652	0.774	0.359	1.111	0.38	0.683

Figure 6. Effect of HKa and ferritin on the adhesion of DU145 cells to vitronectin. A. *Effect of HKa on DU145 cell adhesion*. DU145 cells were left untreated (control) or treated with different concentrations of HKa (from 0-100nM) and seeded onto vitronectin-coated 96-well plates in triplicate for 1 hour, then a MTS assay was performed to measure the viability of adherent cells. B. *Effect of ferritin on HKa-inhibited DU145 cell adhesion*. DU145 cells was left untreated (control) or treated with HKa and/or Ft as indicated. Adhesion was measured as described. C. Absorbance readings of DU145 cells treated in duplicate as in B.

uPAR was expressed in various cell lines.

Urokinase plasminogen activator receptor (uPAR), also known as uPA receptor/CD87, is a glycosylphosphotidylinositol (GPI) anchored glycoprotein. uPAR was shown to be a main biding site for HKa on the endothelial cell surface. uPAR has been involved in various processes including cancer. It is often found overexpressed in cancer tissues or cells. The expression of uPAR is often used as a marker for bad prognosis for most cancer cells we tested in our cell proliferation assay, including ovarian, breast, prostate, cervical, endometrial, lung, colon, gastric, kidney, THP-1 cells. We blotted cell lysates from A2780, BG-1, SKOV-3, DU145, HepG2, HME, HUVEC, CRL-1932, HEC1A, AGS, HCT116, MCF-7, MDA-MB-231 and PC3 cells for uPAR. We found that uPAR was expressed more or less in all the cells we tested (Figure 7A, 7D). We then quantified uPAR expression in A2780, BG-1, SKOV-3, DU145, HepG2, HME and HUVEC cells by normalizing uPAR expression in HUVEC to 1 using Photoshop cs2 software. Our data showed that some cell lines, such as HME and HepG2 had lower uPAR expression, while other cell lines, such as DU145, had higher uPAR expression than endothelial cells (Figure 7B). A correlation analysis was performed to assess the possible correlation between uPAR expression and HKa's effect on cell proliferation. The R square is far from 1 (<0.1) which indicates that no linear correlation exists between uPAR expression and HKa's effect on cell proliferation (Figure 7C). uPAR expression in those that were not normalized and analyzed as in 7B and 7C was shown in 7D as western blots.

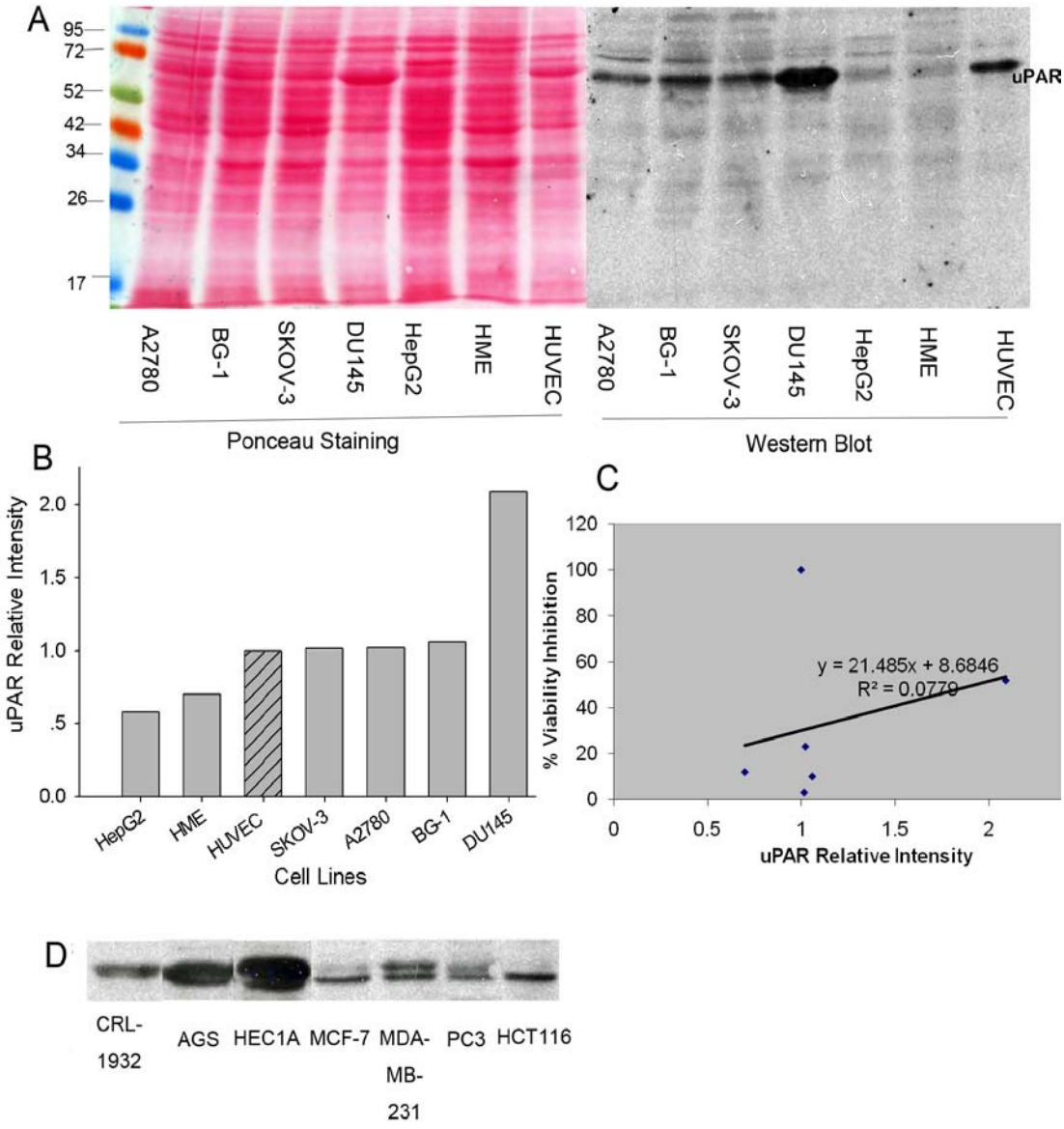


Figure 7. uPAR expression in cancer and endothelial cells. A. The indicated cell lines were cultured to 80% confluent and cell lysates were collected to detect uPAR expression using western blot. The left panel in A is ponceau staining and the fight is western blot. B. uPAR expression in the cells listed in A was presented as relative intensity compared to HUVECs which was normalized to 1 using Photoshop. C. The correlation between cells' sensitivity to HKa in cell proliferation assay and uPAR expression in each of these cell lines listed in A was analyzed using EXCEL. R2 is the correlation coefficient. D. uPAR expression in these cell lines was also tested by western blot yet not normalized. The blots were from the same experiment yet the order was rearranged.

Signaling pathways initiated by HKa and ferritin

Effect of HKa and ferritin on uPA-induced ERK phosphorylation (PERK) in endothelial cells. MAPK/ERK pathway is a signaling pathway that couples a lot of intracellular responses to growth factors binding to cell surface. ERK1/2 is required for the proliferation of many cells. The activation of this pathway was mediated through ERK1 and ERK2 (p44 and p42). Phosphorylation of Threonine 202 and Tyrosine 204 residues indicates the activation of these two molecules. HUVECs were cultured for several days until 90% confluency was reached. Cells were starved for 5 hours and treated without (control) or with 100nM HKa, or 100nM Ft, or 100nM HKa plus 100nM Ft for 1 hour and then stimulated with HMW 15nM uPA for 30 minutes. Cell lysates were collected for western blot. As seen from Figure 8, uPA strongly stimulated the expression of phosphorylated ERK1/2. HKa, even ferritin blocked HKa-inhibited uPA-induced P-ERK. Ferritin plus HKa treatment showed no difference with HKa alone treatment. Total ERK level was comparable between each treatment and control cells.

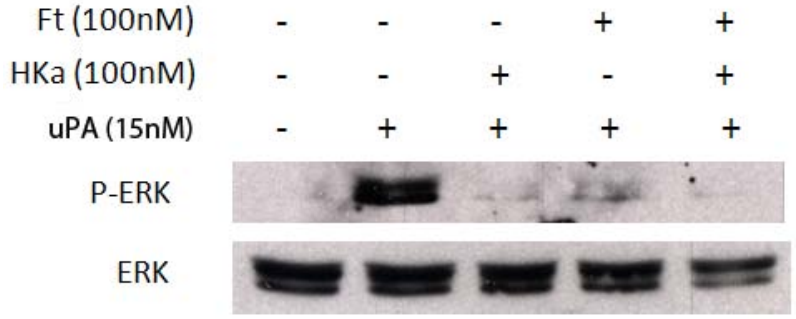


Figure 8. HKa and ferritin's effect on uPA-induced PERK. 80-90% confluent HUVECs were treated without (control) or with 100nM HKa, 100nM Ft, 100nM HKa plus 100nM Ft for 1 hour and then stimulated 15nM HMW uPA for 30 minutes. Cell lysates collected from these cells with these treatments were probed for detection of Phosphorylated ERK and total ERK using western blot. 40ug protein with each treatment was loaded into each well before running gel.

HKa's effect on phosphorylation of FAK (P-FAK) and paxillin (P-paxillin). FAK and paxillin are two important focal adhesion-associated proteins that can transmit signals downstream of integrins. These signals mediate cell migration, adhesion and survival. Phosphorylation of Tyr-397 (P-FAK) is a critical step in the activation of FAK. Paxillin is a substrate of FAK and can be phosphorylated by FAK at Tyr-118. HKa was shown to inhibit the adhesion of endothelial cells to ECM proteins including vitronectin and gelatin. We determined here if FAK and paxillin play a role in HKa-inhibited endothelial cell adhesion to vitronectin. 150mm dishes were first coated with vitronectin for 1 hour. HUVECs treated without or with 100nM HKa were seeded and incubated for 30 minutes, 60 minutes and 3 hours. At these time points, cell lysates were collected to detect P-FAK, total FAK, P-paxillin and total paxillin using western blot. As seen from Figure 9, HKa did not inhibit the expression of P-FAK and P-paxillin. Total FAK and paxillin level was not affected by HKa as well.

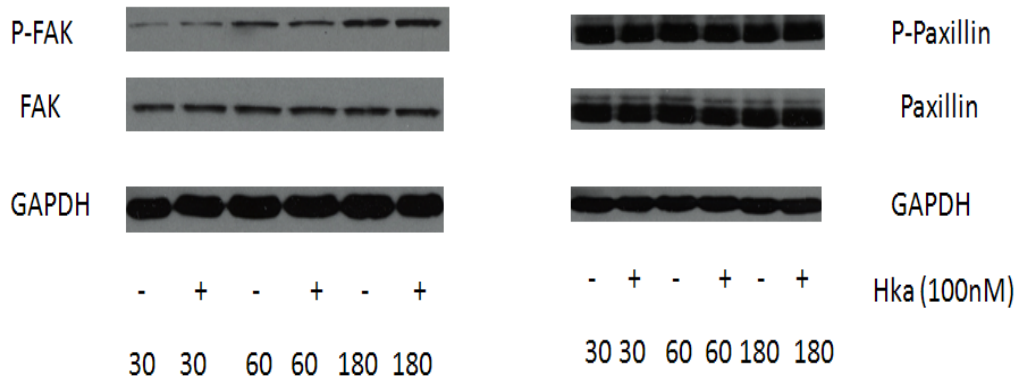


Figure 9. HKa's effect on P-FAK and P-paxillin. Suspended HUVECs were seeded onto vitronectin-coated dishes and treated without or with 100nM HKa for 30 minutes, 60 minutes and 3 hours. Cell lysates were collected for western blot to detect the expression of Phosphorylated and total FAK and paxillin. Equal amount of protein (40ug/ml) loaded in each well before running gel.

HKa and ferritin's effect on VEGF-induced phosphorylated Akt (P-Akt). VEGF binding to VEGFR2 stimulates endothelial cell survival, proliferation and migration through PI-3Kinase-Akt pathway. Our Akt experiment was designed to assess the effect of HKa and ferritin on VEGF-induced P-Akt. We treated HUVECs with 100nM HKa for 30 minutes and then stimulated cells with 20ng/ml VEGF for 5 minutes. Cell lysates were collected for western blot to detect P-Akt and total Akt. We can see from Figure 10 that VEGF increased the level of P-Akt. However, neither HKa nor ferritin affected the level of P-Akt. The level of total Akt was comparable between each treatment and control cells as well.

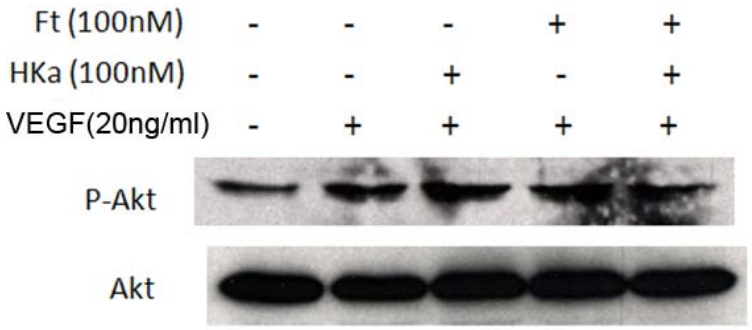


Figure 10. Effect of HKa and ferritin on VEGF- induced phosphorylated Akt. Suspended HUVECs were seeded onto vitronectin-coated 100mm dishes (1 hour) and untreated (control) or treated with 100nM HKa, 100nM Ft, 100nM HKa plus 100nM Ft for 30 minutes. Then cells were stimulated with 20ng/ml VEGF for 5 minutes except for the control. Cell lysates were collected for western blot to detect phosphorylated Akt and total Akt. Equal amount of protein (40ug/ml) loaded in each well before running gel.

Mechanism by which how HKa and ferritin interact in endothelial cells

HKa binds to endothelial cells through uPAR and other binding sites. Ferritin has been shown to antagonize HKa's inhibitory effects on endothelial cells. We used flow cytometry to determine how HKa and ferritin interact on endothelial cell surface. Suspended HUVECs were untreated (control) or treated with 1uM biotinylated HKa (bio-

HKa), 1uM biotinylated ferritin (bio-Ft), 1uM bio-HKa plus unlabeled ferritin for 1 hour. Then cells were treated with streptavidin-APC for 30 minutes before running through a flow cytometer. As shown in Figure 11, both HKa and ferritin could bind to endothelial cell. Ferritin slightly blocked HKa's binding to endothelial cell.

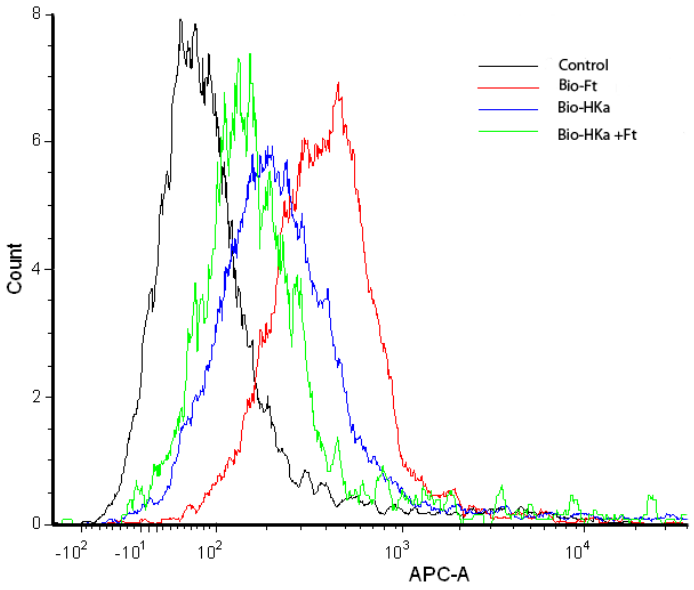


Figure 11. HKa and ferritin bind to endothelial cells. 1 million suspended HUVECs were treated without (control) and with Bio-HKa, Bio-Ft, Bio-HKa plus Ft all at 1uM for 1 hour at 37°C. After washed with 0.5% BSA/PBS for 3 times, cells were treated with strepavidin-APC for 30 minutes. Treated cells were dissolved in 0.1% BSA/PBS in flow tubes and running through a flow cytometer for fluorescence detection.

CHAPTER IV

DISCUSSION

Angiogenesis, the new blood vessel formation from existing vasculature, is required for tumors growth over 1-2mm diameter. HKa, a portion of high molecular weight kininogen after cleavage by kallikrein, is an angiogenesis inhibitor besides serving as a cofactor for intrinsic coagulation cascade. Bradykinin (BK), the other molecule cleaved from HK by kallikrein, is an angiogenesis activator. While lots of studies have been focused on BK, relative little work has been done on HKa. In a previous study by Dr. Torti [1], HKa was demonstrated to be a ferritin-binding protein in the plasma. Ferritin, which is mostly known for its iron-storage function, also exists in human serum in small amount. Although serum ferritin is often used as an index for total body iron, the function and source of this ferritin is unknown. Besides, its level was found increased in inflammation and several malignancies [2-6]. To further explore the role of ferritin in HKa-inhibited angiogenesis, Coffman found that ferritin, a HKa-binding protein, antagonized HKa's effects on endothelial cells and enhanced HKa-inhibited endothelial cells proliferation, migration and survival and blocked HKa-inhibited angiogenesis in an *in vivo* mouse model [7]. The angiogenic function of HKa has triggered several groups to look for binding sites for HKa on endothelial cells. While binding sites for HKa on platelets and monocytes have been determined, uPAR, gC1qR and CK1 have been revealed to be receptors for HKa on endothelial cells [8-10]. In these three main receptors for HKa, uPAR is the most relevant to tumorigenesis and its level is often found higher in

tumor tissues than normal tissues in several cancer types such as gastric cancer, prostate cancer, breast cancer and colon cancer, etc. Based on these knowledge about HKa, we hypothesized that HKa might have an effect on tumor cells directly. We chose over 15 different cancer cell lines including ovarian, breast, prostate, cervix, endometrium, lung, stomach and kidney cancer cells which were reported to express more uPAR in tumor tissues or tumor cells than normal tissue and normal cell lines [11]. We performed cell proliferation assay, cell scratch assay, clonogenic assay, cell adhesion assay and cell invasion assay on some of or all of these cells. Our data demonstrated that HKa inhibited the proliferation, clonogenic growth, migration and adhesion of most of the cancer cells we have tested (Figure 1, Figure 3A, Figure 4B and Figure 6A). Unexpectedly, ferritin did not affect HKa's effects on these cancer cells, unlike endothelial cells. This promoted us to think that the angiogenesis mediation by HKa and ferritin in cancer cells might be different with endothelial cells. The first inspiration from these results was that the receptors for HKa on cancer cells might be different with endothelial cells and clearly needs further studies in the future. Although our hypothesis that HKa might affect tumor cells through over-expressed uPAR on cell surface, the correlation analysis between HKa-inhibited cell proliferation and the level of uPAR expression showed that these two factors are not linearly correlated (Figure 7). This suggests that at least in terms of cell proliferation, HKa's effect on cancer cells are not determined by uPAR level or not dependent on uPAR-mediated signaling pathways among different cancer cells. Some other binding sites such as CK1 and gC1qR or even those unknown receptors for HKa on these cancer cells might play a role in determining the sensitivity of cancer cells to HKa. Moreover, to conclude whether uPAR is one of the key roles in determining HKa's

effects on one specific cell line, uPAR knockdown analysis is a better idea. If cells with no uPAR or decreased uPAR showed lower sensitivity to HKa, then it might be concluded that uPAR plays a role in determining this cell line's sensitivity to HKa. All these hypotheses remain further investigation in the future studies.

In addition, in our study, HKa did not inhibit the invasion of AGS and DU145 cells through Matrigel. This was inconsistent with Liu's study [12] which showed that HKa significantly inhibited the invasion of DU145 cells through Matrigel. We used 100nM HKa in this experiment, higher than Liu used. So the difference between our result and their might not be due to the deficient HKa we used. Another possible reason is that the HUVECs we used might be different in terms of source and passage. The other detailed operations involved in this experiment might lead to the different reaction of HUVECs. Anyhow, it is interesting to explore the effect of HKa on other cancer cell lines as well.

Ferritin, as a HKa-binding protein, has been shown to reverse HKa's effects on the proliferation, migration and apoptosis of endothelial cells. Whereas in our study, ferritin did not reverse HKa's effects on the proliferation, clonogenic growth, migration and adhesion of cancer cells (Figure 2, Figure 3B, Figure 4C and Figure 6B). These results indicate that the binding sites for HKa on cancer cells or the signaling pathways mediated by HKa and ferritin in cancer cells may be different with endothelial cells. These studies led to the next part of our study: the signaling pathways initiated by HKa and ferritin in endothelial cells. Here we explored the effect of HKa and ferritin on the ERK, Akt and FAK/paxillin signaling pathways in endothelial cells. ERK1/2, the key molecules in ERK

signaling pathway, play an important role in regulating gene expression and eventually control many biological processes including cell proliferation, cell migration and cell differentiation. Using HUVECs, we demonstrated that HKa inhibited uPA-induced phosphorylation and activation of ERK1/2 (Figure 8), This confirmed the result from another study by Liu etc. who demonstrated that HKa inhibited uPA-induced phosphorylation and activation of ERK1/2 [13]. This suggests that HKa might inhibit angiogenesis *in vitro* through uPA-ERK signaling pathway. Surprisingly, ferritin also inhibited uPA-induced phosphorylation and activation of ERK1/2 and cotreatment of HKa and ferritin did not show much difference with HKa alone treatment (Figure 8). Ferritin alone either did not affect [7] or increased the proliferation endothelial cells (Figure 2). Therefore, it can be concluded that ferritin might affect HKa-inhibited angiogenesis through other signaling pathways than uPA-ERK signaling pathway.

Akt, the key molecule in VEGF-PI3K-Akt signaling pathway, regulates the migration and survival of endothelial cells. To determine the effect of HKa and ferritin on the phosphorylation and activation of Akt, HUVECs were treated with 100nM HKa, 100nM ferritin, 100nM HKa plus 100nM ferritin for 30 minutes then stimulated with VEGF for 5 minutes. Our western blot result demonstrated that neither HKa nor ferritin affect the phosphorylation of Akt (T308). This might suggest that HKa and ferritin did not affect the migration of endothelial cells through Akt signaling pathway. Interestingly, this result contradicted Katkade etc.'s study in which HKa and D5 were shown to inhibit the migration of bovine pulmonary artery endothelial cells (BPAE) and human umbilical vein endothelial cells (HUVEC) toward VEGF through PI3K-Akt signaling pathway [14].

The different time and concentration of HKa we treated HUVECs with might affect phosphorylation of Akt (T308). In addition, they used a different antibody specific against S473 of Akt which could cause cells to respond to HKa differently. These discrepancies might reflect the sensitivity of HUVECs to different treatment.

In Lan Coffman's study [7], ferritin was shown to antagonize HKa-induced endothelial cell apoptosis. Endothelial cells are anchorage-dependent cells and can undergo anoikis, a form of programmed cell death when cells were detached from surrounding ECM. Previous study has showed that HKa inhibited the adhesion of HUVECs to vitronectin, a provisional ECM protein [15]. Whereas our signaling pathway study involving FAK and paxillin showed that HKa did not affect the phosphorylation and activation of FAK and paxillin, two essential focal adhesion proteins (Figure 9). This discrepancy led to speculate that other adhesion molecules such as Src and talin (a focal adhesion adapter protein) might be involved in HKa-inhibited endothelia cell adhesion (Figure 5 in INTRODUCTION). Interestingly, this study contradicted Guo etc's result which demonstrated that HKa and D5 inhibited the phosphorylation and activation FAK and paxillin [15]. This led more mystery and complexity to the signaling pathways initiated by HKa and ferritin in endothelial cells which required further investigation.

We also preliminarily explored the possible mechanism by which HKa and ferritin interact with each other in endothelial cells using flow cytometry analysis. HUVECs were untreated (control) or treated with biotinylated HKa, biotinylated ferritin, biotinylated HKa plus unconjugated ferritin then incubated with streptavidin conjugated

APC. The flow cytometric analysis demonstrated that both HKa and ferritin bind to endothelial cells. In humans, ferritin receptors have not been studied yet. Our study suggests ferritin receptor might exist on endothelial cells. However, ferritin only slightly decreased HKa's binding to endothelial cell. To explore exactly how HKa and ferritin interact on live endothelial cells, other methods such as immunofluoresence and co-immunoprecipitation can be used.

Collectively, our work here preliminarily explored the effects of HKa and ferritin on cancer cells and the signaling pathways involving HKa and ferritin in endothelial cells. Our new finding that HKa inhibited the proliferation, clonogenic growth and adhesion of many cancer cells laid the foundation for further studying HKa's effect or HKa-mediated signaling pathways in these various cancer cell lines. Although ferritin did not antagonize HKa's effects on cancer cells as on endothelial cells, this work triggered our interest in comparing different effects and even different signaling pathways exerted and induced by HKa and ferritin between cancer cells and endothelial cells. Our signaling pathway studies preliminarily explored the main signaling pathways involving ERK, Akt and FAK/paxillin, which regulate endothelial cell proliferation, migration and adhesion. HKa inhibited uPA-induced phosphorylation and activation of ERK confirmed previous study [13] and ferritin showing unexpected effect on uPA-induced P-ERK added the complexity to the signaling pathways initiated by HKa and ferritin. HKa and ferritin showing no effect on P-FAK, P-paxillin and P-Akt might direct our future efforts to other signaling pathways.

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