The growth factor effect on endothelial cell dysfunction in the presence of glycated collagen and Aβ peptide: implications for decreased angiogenesis in diabetes and Alzheimer’s disease.

A Thesis
Submitted to the Faculty
Of Drexel University
By
Justin George Mathew
In partial fulfillment of the Requirements for the degree Of Doctorate of Philosophy in Mechanical Engineering
May 2014
Acknowledgements

This work was made possible by viewers like you. I will certainly not be able to thank everyone who has been helpful to me throughout my academic career, but to those not explicitly named after please do know you were instrumental in my development and my future success.

I would first and foremost like to thank my advisor, Dr. Alisa Morss Clyne, who has motivated me both academically and personally. Alisa, was a unique advisor in that she herself was open to development as a researcher and person, which allowed me to be open as well. A characteristic I deeply admire and will carry with me. Her personal time was invaluable as she offered continuous guidance, being available at all times for questions and support. I truly appreciate most of all that she pulled the best out of me, when others would have been satisfied with just average results.

I would also like to thank the committee, Dr. Alan Lau, Dr. Aleister Saunders, Dr. Moses Noh and Dr. Margaret Wheatley for their technical support in developing my research and development in presenting it in the most effective manner.

I would like to thank my lab mates, friends and family. The support you have given for me cannot be expressed in words. If they were, it would add a chapter to this thesis…I would title it, the growth factor effect on a PhD candidate. I will avoid naming any names since it is a slippery slope but instead express my deepest gratitude to all of you that are here on earth and praying over me from heaven.

Last but not least thanking God for making all things possible through Him (I know I’m a scientists…).
1 Background ................................................................. 1
  1.1 Clinical Motivation – Diseases of disordered angiogenesis .......... 1
    1.1.1 Angiogenesis in Disease ..................................... 1
    1.1.2 Diseases of increased/decreased angiogenesis .............. 1
      a) Diabetes .................................................................. 1
      b) Alzheimer’s Disease ................................................. 4
  1.2 Angiogenesis in Endothelial Cells ..................................... 6
    1.2.1 Endothelial cell function ....................................... 6
    1.2.2 Angiogenic Process ................................................ 7
      a) ECM degradation .................................................... 7
      b) Cell Migration ........................................................ 8
      c) Cell Proliferation .................................................... 8
      d) Tube Formation ....................................................... 9
  1.3 Plasminogen Activating System ....................................... 10
    1.3.1 Plasminogen Activating System Overview .................. 10
    1.3.2 Plasminogen activating system components ................ 11
      a) Urokinase Plasminogen Activator ................................ 11
      b) Urokinase Plasminogen Activator Receptor .................. 11
      c) Plasminogen Activating Inhibitor – 1 ........................ 12
      d) Vitronectin ............................................................ 13
    1.3.3 Plasminogen activating system in angiogenesis ............. 14
      a) Extracellular Matrix Breakdown ............................... 14
      b) Cell Motility .......................................................... 15
  1.4 Glycated Collagen ...................................................... 16
    1.4.1 Glycation Process ................................................ 16
    1.4.2 Effect of collagen glycation on endothelial cells .......... 17
      a) Growth Factors ...................................................... 17
      b) Plasminogen Activators .......................................... 17
  1.5 Aβ Peptide .................................................................. 19
    1.5.1 Formation ............................................................ 19
    1.5.2 Growth Factors ..................................................... 19
    1.5.3 Plasminogen Activators ......................................... 20
  1.6 Thesis Summary .......................................................... 21
    1.6.1 Objective and Hypothesis ..................................... 21
    1.6.2 Thesis Organization .............................................. 21
  2 Glycated collagen influences on the plasminogen activating system .... 23
    2.1 Introduction ............................................................ 23
    2.2 Methods .................................................................. 27
      2.2.1 Cell Culture, collagen glycation and vitronectin multimerization .......... 27
      2.2.2 Plasmin Activity: Chromozym PL ............................ 28
      2.2.3 3D Migration: Cell Invasion Assay ......................... 29
      2.2.4 Tube Formation: Cell Extension Assay ..................... 29
Figure 2.1: Glycated collagen decreased endothelial cell plasmin activity, 3D migration, and tube length compared to native collagen. FGF-2 only partially abrogated this effect. A) Cellular plasmin activity was measured using the Chromozym PL assay. HUVEC were seeded on native and glycated collagen (50 µg/ml) coated substrates for 48 hours, after which 50 ng/ml FGF-2 was added for 24 hours. Cell extracts were collected and assayed immediately. B) 3D cell migration was measured using a Boyden chamber assay. 150,000 HUVEC +/- 50 ng/ml FGF-2 were added to the top of an 8.0 µm pore size Transwell insert coated with 100 µg/ml native or glycated collagen. After 24 hours, cells that migrated to the chamber bottom were labeled with Hoechst, imaged by fluorescent microscopy, and quantified with ImageJ. Normalized to native collagen without FGF-2. C) For the tube formation assay, 15,000 HUVEC were added to native and glycated collagen gels (4 mg/ml) +/- FGF-2 (50 ng/ml). After 18 hours, tubes were imaged by phase contrast microscopy and tube length was analyzed with ImageJ. D) Sample images of tube formation assay, with tubes indicated by black arrows. * p < 0.01; ** p < 0.01 compared to native collagen null condition and to FGF-2 (indicated with brackets)....

Figure 2.2: Glycated collagen increased PAI-1 protein level, and FGF-2 treatment returned PAI-1 back to the native collagen level. Glycated collagen did not significantly affect uPA or uPAR protein levels. A) HUVEC were seeded on native and glycated collagen (50 µg/ml) coated substrates for 48 hours, after which 50 ng/ml FGF-2 was added for 24 hours. Cell extracts were collected and normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. B, C, D) Band intensity for PAI-1, uPA, and uPAR, respectively, was quantified and normalized to GAPDH using AlphaEase FC software. * p < 0.01; ** p < 0.01 compared to native collagen null condition and to FGF-2 (indicated with brackets)....

Figure 2.3: Both PAI-1 and FGF-2 bound to glycated collagen and vitronectin more than to native collagen, likely at the αvβ3 binding site. A) Plates coated with native collagen (25 µg/ml), glycated collagen (25 µg/ml), or vitronectin (25 µg/ml) were incubated overnight at 4°C. After washing, plates were incubated with 5 µg/ml PAI-1 for 1 hour at 37°C; then washed to remove unbound PAI-1. Primary and secondary antibodies were incubated in succession for 1 hour at 37°C each to detect bound PAI-1. The solid phase binding was developed with 2,2-azinobis and read at an absorbance of 405nm. *p<0.01 compared to native collagen. B) Plates coated with native collagen (50 µg/ml), glycated collagen (50 µg/ml), or vitronectin (3 µg/ml) were incubated with 50 ng FGF-2 overnight at 4°C. After thorough washing, 250,000 HUVEC were added to the plates and incubated for 2 hours at 37°C. After removing unbound cells, attached cells were trypsinized and counted using a Coulter counter. * p < 0.01; ** p < 0.01 compared to native collagen null condition and to FGF-2 (indicated with brackets)....
Figure 2.4: Exogenous uPA increased endothelial cell 3D migration and tube length on both native and glycated collagen. Exogenous PAI-1 decreased 3D migration on both substrates, but only decreased tube length on glycated collagen. A) 150,000 HUVEC +/- 12 IU/ml uPA or 1 ng/ml PAI-1 were added to the top of an 8.0 µm pore size Transwell insert coated with 100 µg/ml native or glycated collagen. After 24 hours, cells that migrated to the chamber bottom were labeled with Hoechst, images by fluorescent microscopy, and quantified with ImageJ. B) 15,000 HUVEC were added to native and glycated collagen gels (4 mg/ml) +/- FGF-2 (50 ng/ml). After 18 hours, tubes were imaged by phase contrast microscopy and tube length was analyzed with ImageJ. Samples with uPA or PAI-1 are normalized to samples without uPA or PAI-1 on the respective substrate to best compare exogenous protein effects. * p < 0.01 compared to samples without uPA or PAI-1 on the respective substrate.

Figure 2.5: FGF-2 increased vitronectin protein levels in cells on native and glycated collagen. However, soluble vitronectin had different effects for plasmin activity, 3D migration, and tube formation. A) HUVEC were seeded on native and glycated collagen (50 µg/ml) for 48 hours, after which 50 ng/ml FGF-2 was added for 24 hours. Cell extracts were collected and normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. *p<0.01; #p<0.05. B) Soluble vitronectin decreased plasmin activity in cells on native but not glycated collagen. HUVEC were seeded on native and glycated collagen (50 µg/ml) coated substrates for 48 hours, after which 3 µg/ml vitronectin was added for 24 hours. Cell extracts were collected and assayed immediately via Chromozym PL. C) Soluble vitronectin increased cell 3D migration on glycated but not native collagen. 150,000 HUVEC +/- 3 µg/ml vitronectin were added to the top of an 8.0 µm pore size Transwell insert coated with 100 µg/ml native or glycated collagen. After 24 hours, cells that migrated to the chamber bottom were labeled with Hoechst, imaged by fluorescent microscopy, and quantified with ImageJ. D) Soluble vitronectin increased tube length on both native and glycated collagen. 15,000 HUVEC were added to native and glycated collagen gels (4 mg/ml) +/- 3 µg/ml vitronectin. After 18 hours, tubes were imaged by phase contrast microscopy and tube length was analyzed with ImageJ. * p < 0.01 compared to native collagen null condition and to Vn (indicated with brackets).

Figure 3.1: FGF-2 increased vitronectin in human umbilical vein endothelial cells (HUVEC, A-B), bovine brain microvascular endothelial cells (BBmVEC, C-D), porcine aortic endothelial cells (PAEC, E-F), and porcine vascular smooth muscle cells (PSMC, G-H). Cells seeded on native collagen (50 µg/ml) coated substrates for 48 hours were stimulated with 50 ng/ml FGF-2 for 24, 48 and 72 hours. Cell extracts were collected and normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. mVTN and nVTN band intensity was quantified and normalized to GAPDH or β-actin and then normalized to null nVTN levels at 24 hours. # p < 0.05; * p < 0.01 compared to null. .......
Figure 3.2: HUVEC produced vitronectin, but FGF-2 did not increase vitronectin binding in a serum free environment. HUVEC were treated with 50 ng/ml FGF-2 +/- 3 µg/ml nVTN or mVTN for 24 hours in EBM-2 medium with 0% FBS, 1% PSG. A) RNA was isolated reverse transcriptase PCR was performed to qualitatively determine vitronectin mRNA. Vitronectin protein levels were analyzed by Western blot for HUVEC treated with B) mVTN and C) nVTN. D) Fixed samples were labeled with a vitronectin primary antibody coupled with an AF488 secondary antibody and Hoechst (nuclei). Samples were imaged in a z-stack (1 µm depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. E) Mean intensity values were quantified with ImageJ; vitronectin labeled with AF488 (green) and nuclei labeled with Hoechst (blue). All samples were normalized to -FGF2, -nVTN, -mVTN conditions. # p < 0.05; * p < 0.01 compared to null.

Figure 3.3: Immunofluorescence imaging showed little vitronectin inside the cell (acid wash) and FGF-2 primarily increased extracellular vitronectin (un-permeabilized membrane). HUVEC were cultured and exposed to FGF-2 as described. A) Immunofluorescence images of cells labeled for vitronectin (AF488, green) and nuclei with (Hoescht, blue). Specific samples were acid washed to remove primary antibody bound to vitronectin on the cell membrane, and thereby label just intracellular vitronectin. B) Samples were imaged in a z-stack (1 µm depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. C) Immunofluorescence images of cells labeled for vitronectin and nuclei as described. Specific samples were permeabilized with Triton X-100 (membrane and cytoplasmic) or not permeabilized (membrane only). D) Mean intensity values were quantified with ImageJ. All samples were normalized to -FGF2 condition. # p < 0.05 null vs. FGF-2 treated.

Figure 3.4: FGF-2 induced vitronectin binding to HUVEC was abrogated when FAK phosphorylation was inhibited using a protein tyrosine kinase inhibitor. HUVEC were exposed to 30 µg/ml genistein for 2 hours prior to stimulation with 50 ng/ml FGF-2 for 24 hours. A) Samples were fixed and permeabilized with 0.1% Triton X-100 in PBS for 4 minutes at room temperature or treated with PBS alone. Samples were then incubated with a vitronectin primary antibody along with Hoechst. Samples were imaged in a z-stack (1 µM depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. (vitronectin = green, nuclei = blue) B) Mean intensity values were quantified with ImageJ. Total vitronectin was quantified in permeabilized samples, and extracellular vitronectin was quantified in unpermeabilized samples. C) Cell extracts were collected and normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. Vitronectin band intensity was quantified and normalized to GAPDH and then normalized to null levels. D) Samples were lysed in T/T buffer and prepared for the Chromozym assay. Absorbance (405 nm) was measured for 24 hours. Plasmin enzymatic activity was calculated from the change in 4-nitroaniline absorbance at 405 nm. All samples were normalized to -FGF2, -Genistein, Membrane (IF) conditions. # p < 0.05 compared to null.
Figure 3.5: FGF-2 induced cell binding of vitronectin was removed when the αvβ5 integrin was blocked. HUVEC were treated with 1 µg/ml anti-αvβ5 blocking antibody concurrently with 50 ng/ml FGF-2 for 24 hours. A) Samples were fixed and some samples were permeabilized with Triton X-100 (total vitronectin) while some samples were unpermeabilized (membrane vitronectin). Cells were labeled for vitronectin (AlexaFluor 488, green) and nuclei (Hoechst, blue). B) Samples were imaged in a z-stack (1 µm depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. Mean intensity values were quantified with ImageJ. Total vitronectin was quantified in permeabilized samples, and extracellular vitronectin was quantified in unpermeabilized samples. C) Following anti-αvβ5 blocking antibody and FGF-2 treatment, cell extracts were collected and normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. Vitronectin band intensity was quantified and normalized to GAPDH and then normalized to null levels. D) Samples were lysed in T/T buffer and prepared for the Chromozym assay. Absorbance (405 nm) was measured for 24 hours. Plasmin enzymatic activity was calculated from the change in 4-nitroaniline absorbance at 405 nm. All samples were normalized to -FGF2, -anti-αvβ5, Membrane (IF) conditions. # p < 0.05 compared to null. ..........................................................72

Figure 3.6: FGF-2 induced cell binding of vitronectin was abrogated by silencing the β5 integrin with siRNA. 250,000 cells/well were seeded on native collagen (50 µg/ml) coated substrates for 24 hours after which lipofectamine was used to transfect the cells with β5 siRNA for 72 hours. Samples were then treated with 50 ng/ml FGF-2 for 24 hours. A) Samples were fixed and permeabilized with 0.1% Triton X-100 in PBS for 4 minutes at room temperature or treated with PBS alone. Samples were incubated with a vitronectin antibody along with Hoechst. Samples were imaged in a z-stack (1 µM depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. (Vitronectin = green, nuclei = blue). B) Mean intensity values were quantified with ImageJ. Total vitronectin was quantified in permeabilized samples, and extracellular vitronectin was quantified in unpermeabilized samples. C) Cell extracts were collected and normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. Vitronectin band intensity was quantified and normalized to GAPDH and then normalized to null levels. D) Samples were lysed in T/T buffer and prepared for the Chromozym assay. Absorbance (405 nm) was measured for 24 hours. Plasmin enzymatic activity was calculated from the change in 4-nitroaniline absorbance at 405 nm. All samples were normalized to -FGF2, -β5 siRNA, Membrane (IF) conditions. # p < 0.05 compared to null. ............................................................................................................75
Figure 4.1: Aβ42-O did not affect FGF-2 induced endothelial cell proliferation or ERK phosphorylation. A) BBMVEC were seeded at 4,000 cells/cm$^2$ on native collagen coated substrates and allowed to attach for 24 hours. Cells were then treated with 5 µM Aβ42-O +/- 50 ng/ml FGF-2 on days 1 and 3, and cells were counted on days 1, 3, and 5. # p < 0.05, * p < 0.01 compared to null day 3 and day 5. B) 125,000 cells/cm$^2$ were cultured for 48 hours on native collagen coated substrates in experimental DMEM. Cells were then subjected to 24 hours of 1 µM, 5 µM, and 10 µM Aβ42-O, and cell death was measured using a Live/Dead assay (green = live, red = dead). Mean fluorescence intensity per channel was determined with Image J. ** p < 0.001 compared to null. C) BBmVEC were seeded at 25,000 cells/cm$^2$ on native collagen coated substrates and cultured for 48 hours. Samples were then stimulated with 50 ng/ml FGF-2 for 15 minutes in experimental medium. Cell extracts were collected and normalized protein samples were analyzed by Western blot. D) Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. pERK band intensity was quantified and normalized to total ERK. * p < 0.01 compared to null. # p < 0.05 compared to Aβ42-O (indicated with brackets).

Figure 4.2: Aβ42-O did not change FGF-2 stimulated plasmin activity or 3D migration. BBmVEC were seeded at 25,000 cells/cm$^2$ on native collagen coated substrates and cultured for 48 hours. Samples were then stimulated with 50 ng/ml FGF-2 for 24 hours in experimental medium. Cell lysates were collected and assessed for plasminogen system activity using the Chromozym PL assay. A) Chromozym PL absorbance at 405nm over 4 hours in slope region. B) Change in absorbance was multiplied by volume, absorbance and light path constants to quantify plasmin activity. * p < 0.01 compared to null and to Aβ42-O (when indicated with brackets). 3D cell migration was measured using a Boyden chamber assay. 150,000 BBmVEC with 5 µM Aβ42-O +/- 50 ng/ml FGF-2 were added to the top of an 8.0 µm pore Transwell insert coated with 100 µg/ml native collagen. After 24 hours, cells that migrated to the chamber bottom were labeled with Hoechst. C) Sample fluorescent microscopy images of migrated cells. D) Cell migration as quantified with ImageJ. All samples were normalized to FGF2, -Aβ42-O condition. # p < 0.05 compared to null and to Aβ42-O (indicated with brackets).

Figure 4.3: Aβ42-O did not change FGF-2 stimulated tube formation. A) Sample phase contrast images of the tube formation assay. Endothelial network formation was determined with a tube formation assay. BBmVEC were seeded at 15,000 cells/well in a 96 well plate on native collagen (4mg/ml) gels for 18 hours dosed with 5 µM Aβ42-O +/- 50 ng/ml FGF-2 in experimental medium. B) Tube length and C) number of tubes per image was manually analyzed using ImageJ. Samples were normalized to FGF2, -Aβ42-O condition. * p < 0.05 compared to null and to Aβ42-O (indicated with brackets).
Figure 4.4: Aβ42-O did not change the influence of VEGF on cell proliferation or Akt phosphorylation. A) BBMVEC were seeded at 10,000 cells/well on native collagen coated substrates and allowed to attach for 24 hours. Cells were then stimulated with 50 ng/ml VEGF on days 1 and 3 in experimental medium and counted on days 1, 3, and 5. # p < 0.05, * p < 0.01 compared to null day 3 and day 5. B) BBmVEC were seeded at 25,000 cells/cm² on native collagen coated substrates for 48 hours and then stimulated with 50 ng/ml VEGF for 15 minutes in experimental medium. Cell extracts were collected and normalized protein samples analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. pAkt band intensity was quantified and normalized to total Akt. # p < 0.05 compared to null and to Aβ42-O (indicated with brackets).

Figure 4.5: Aβ42-O did not affect VEGF dependent plasmin activity but decreased growth factor dependent tube length. A) BBmVEC were seeded at 25,000 cells/cm² on native collagen coated substrates for 48 hours and then stimulated with 50 ng/ml VEGF for 24 hours in experimental medium. Plasminogen system activity was measured in cell extracts by the Chromozym PL assay. ** p < 0.001 compared to null and to Aβ42-O (when indicated with brackets). B) Tube length was determined with a tube formation assay. BBmVEC were seeded at 15,000 cells/well in a 96 well plate on native collagen (4 mg/ml) gels for 18 hours and then stimulated with 5 μM Aβ42-O +/- 50 ng/ml VEGF in experimental medium. Samples were imaged by phase contrast microscopy (5 images per well). C) Tube length and D) number of tubes per image was manually analyzed using ImageJ. Normalized to null conditions. # p < 0.05, * p < 0.01 compared to null and to Aβ42-O (when indicated with brackets).

Figure 4.6: Aβ42 structure impacted VEGF effects on cell proliferation. BBmVEC were seeded at 10,000 cells/well on native collagen coated substrates and allowed to attach for 24 hours. Cells were then stimulated with +/- 10, 25, or 50 ng/ml VEGF, Aβ42 oligomers, or Aβ42 monomers on days 1 and 3 in experimental medium, and cells were counted on days 1, 3, and 5. A) Day 3 and B) Day 5. Two-way ANOVA shows a significant difference between Aβ42 and VEGF gradient. Post hoc Student’s t-test corrected with Bonferroni’s method between groups showed # p < 0.05; * p < 0.01 compared to null. # p < 0.05 Aβ42-M vs. Aβ42-O at respective VEGF concentrations (when indicated with brackets) on respective days.
Figure 4.7: VEGF restored endothelial cell tube length in a concentration dependent manner in the presence of Aβ42-monomers only, without any significant influence on plasmin activity. A) Tube length was determined in a tube formation assay. BBmVEC were seeded at 15,000 cells/well in a 96 well plate on native collagen (4mg/ml) gels for 18 hours in the presence of +/- 10, 25, 50 ng/ml VEGF, 5 µM Aβ42 oligomers, or 5 µM Aβ42 monomers in experimental medium. Samples were imaged by phase contract microscopy (5 images per well). Tube length was manually analyzed using ImageJ. Two-way ANOVA shows a significant difference between Aβ42 and VEGF gradient. Post hoc Student’s t-test corrected with Bonferroni’s method between groups showed # p < 0.05 compared to null. # p < 0.05 Aβ42-M vs. Aβ42-O at respective VEGF concentrations (indicated with brackets). B) BBmVEC were seeded at 250,000 cells/cm² on native collagen coated substrates for 48 hours and then stimulated with +/- 10, 25, 50 ng/ml VEGF, 5 µM Aβ42 oligomers, or Aβ42 monomers for 24 hours in experimental medium. Plasminogen system activity was measured by Chromozym PL assay. Two-way ANOVA shows no significant difference between Aβ42 and VEGF gradient. Post hoc Student’s t-test corrected with Bonferroni’s method between groups showed # p < 0.05 compared to null. # p < 0.05 Aβ42-M vs. Aβ42-O at respective VEGF concentrations (indicated with brackets).
List of Schematics

**Schematic 2.1: AIM 1 Hypothesis.** FGF-2 binds to its receptor, FGFR, and influences transcription of uPA, uPAR, and PAI-1. Specifically, FGF-2 decreases the amount of PAI-1 in cells on glycated collagen so that uPA can bind to uPAR and promote plasmin activity in the presence of low concentrations of PAI-1.................................................................26

**Schematic 2.2: AIM 1 Summary.** At short times, plasmin activity decreased in cells on glycated collagen due to PAI-1 binding. A) PAI-1 is able to bind to glycated collagen at cryptic binding sites. B) PAI-1 then is able to inhibit plasmin activity at localized sites which prevents ECM degradation, 3D migrations and tube formation. FGF-2 has no influence on the ability of PAI-1 to bind to glycated collagen. ........................................................................................................................................45

**Schematic 3.1: AIM 2 Hypothesis.** FGF-2 activates the αvβ5 integrin internally, signified by FAK phosphorylation. This integrin activation increases vitronectin uptake and thereby prevents vitronectin from stabilizing and/or localizing PAI-1. Vitronectin bound to the αvβ5 integrin and uPAR promote plasmin activity and lead to ECM degradation, 3D migration and tube formation................................................................................................................................53

**Schematic 3.2: AIM 2 Summary.** FGF-2 avtivates αvβ5 and the αvβ3 integrin, signified by FAK phosphorylation. However, only the αvβ5 integrin binds vitronectin from the serum, leading to increased plasmin activity, ECM degradation, 3D migration and tube formation....................................................................................................................................53

**Schematic 4.1: AIM 3 Hypothesis.** Aβ inhibit endothelial cell angiogenic processes in response to growth factors by competing for VEGFR and inhibiting VEGF binding. Leading decreased plasmin activity, ECM degradation, 3D migration, and tube formation. ............84

**Schematic 4.2: AIM 3 Summary.** Aβ42 monomers only inhibit endothelial cell angiogenic processes in response to low growth factor concentration. A) Low concentrations of VEGF inhibit plasmin activity, ECM degradation, 3D migration and tube formation, B) high concentrations of VEGF are able to compete with Aβ42 and promote angiogenic functions.................................................................106
Abstract

The growth factor effect on endothelial cell dysfunction in the presence of glycated collagen and Aβ peptide: implications for decreased angiogenesis in diabetes and Alzheimer’s disease.

Reduced angiogenesis, the growth of new blood vessels from existing vessels, is important in disease states including diabetes and Alzheimer’s disease. People with diabetes experience morbidity and mortality from unregulated micro-vascular remodeling, which may be linked to hyperglycemia. People with Alzheimer’s disease experience diminished memory and motor function from a buildup of Aβ plaques and a reduction in microvascular density. The primary focus of this research is to determine if growth factors can rescue endothelial cell angiogenic processes from altered extracellular proteins, specifically glycated collagen and Aβ peptide. Assays were developed to discover how these altered extracellular proteins affect growth factor induced cell plasminogen system activity. The resultant data would then add additional insight into growth factor therapy for patients with diabetes and Alzheimer’s disease.

Endothelial cells on glycated collagen displayed decreased plasminogen system activity, which impacted angiogenic functions such as tube formation and 3D migration. FGF-2 did not fully rescue the negative effects of glycated collagen. uPA, uPAR and PAI-1 protein levels did not significantly change at short time points. However, PAI-1 bound to glycated collagen at higher levels than native collagen. Since even cells on native collagen did not show a short term response in uPA, PAI-1, or uPAR protein levels in response to FGF-2 stimulation, FGF-2 effects on vitronectin were explored. FGF-2 increased cell-associated vitronectin by 24 hours in varied endothelial cell types and vascular smooth muscle cells.
The vitronectin appeared to be taken up on from the serum and remained on the endothelial cell membrane, as shown by immunofluorescence microscopy with and without permeabilizing cells. FGF-2 signaled intracellularly to activate the αvβ5 integrin, a vitronectin binding site. Blocking focal adhesion kinase phosphorylation or the αvβ5 integrin, or knocking down the β5 integrin with siRNA, abrogated this FGF-2 induced effect. Since FGF-2 had limited influence on cells on glycated collagen, we then examined if a similar effect would be seen in the presence of Aβ42. Endothelial cells in the presence of Aβ42 did not alter plasminogen system activity; however specific secondary structure of the peptide attenuated growth factor induced effects. Aβ42 oligomers were non-toxic and did not affect FGF-2 or VEGF induced cell proliferation, ERK phosphorylation, 3D migration and had limited effect on endothelial cell tube formation. Monomeric Aβ42 impacted VEGF effects on cell proliferation and tube length, although with limited influence on plasmin activity.

These data show that endothelial cell response to growth factors is dependent on the protein interactions of PAI-1 with glycated collagen, vitronectin with αvβ5 and Aβ42 secondary structure. Demonstrating a new way in which growth factors impacts the plasminogen system and subsequent angiogenesis is a fundamental step in growth factor therapy. This research highlights the use of growth factor therapy in both diabetes and Alzheimer’s patients where there is a reduction of angiogenic function.
1 Background

1.1 Clinical Motivation – Diseases of disordered angiogenesis

1.1.1 Angiogenesis in Disease

Angiogenesis, the growth of new blood vessels from existing vessels, relies on regulated interactions among endothelial cells; soluble biochemical factors such as cytokines, growth factors, enzymes, and proteases; and extracellular matrix [1]. The importance of new blood vessel formation is especially highlighted in diseases such as cancer, diabetes, and Alzheimer’s disease. In cancer, excessive angiogenesis at the tumor site enables the tumor to grow and metastasize [2]. In diabetes, excessive angiogenesis in the eye leads to diabetic retinopathy and blindness, whereas inadequate peripheral angiogenesis contributes to diabetic neuropathy and poor wound healing [3]. Patients with Alzheimer’s disease have decreased capillary density in the brain, which may relate to capillary regression and/or decreased angiogenesis [4]. In each case, disordered angiogenesis contributes to disease progression. This thesis focuses on diabetes and Alzheimer’s disease, both diseases in which altered proteins or peptides impact growth factor-stimulated angiogenesis.

1.1.2 Diseases of increased/decreased angiogenesis

a) Diabetes

According the American Diabetes Association, in 2013 nearly 26 million Americans suffered from diabetes. If diabetes prevalence continues to increase at the present rate, in 2050 1 in 3 Americans will suffer from the disease. The present estimated yearly cost of diabetes is $245 billion. Diabetes results in a higher yearly mortality rate than AIDS and breast cancer combined [5].
Diabetes is a metabolic disease that results from a combination of factors, including autoimmune dysfunction, genetics, environment, and lifestyle. There are two types of diabetes. Type 1 diabetes, which affects 5-10% of all people with diabetes, is characterized by insulin deficiency [6]. An autoimmune attack destroys pancreatic β cells, which regulate insulin flux based on blood glucose levels, thus leaving the patient without insulin production capacity [7]. Specific genes that control immune response, specifically the HLA locus and insulin-VNTR and CTLA-4, predispose people to the disease [8]. Exposure to environmental factors including viruses, toxins and food may also increase susceptibility. Early introduction of certain proteins found in cow milk, cereals or gluten can contribute to disease onset [9-11]. The remaining 90% of people with diabetes have non-insulin dependent Type 2 diabetes. Insulin is produced, often at high rates, but it is ineffective in catalyzing glucose metabolism because cells are insulin resistant. Lifestyle and obesity are the leading causes of the disease. However, genetic predisposition adds a 2-4 fold risk increase [12]. In both types of diabetes, improper glucose metabolism leads to high blood glucose levels and protein glycation [13].

Cardiovascular disease is the leading cause of mortality in patients with diabetes [12]. Hyperglycemia affects both the macro- and microvasculature. In the macrovasculature, hyperglycemia leads to atherosclerosis and associated cardiovascular diseases such as heart attack, stroke, and peripheral artery disease. Atherosclerosis is the hardening and thickening of the arterial wall. Endothelial cell dysfunction, indicated by decreased nitric oxide production, is thought to be a primary stimulus for atherosclerosis. Nitric oxide (NO) maintains the vascular tone by inhibiting angiotensin II, a vasoconstrictor. NO also inhibits platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation [14]. Low-density lipoprotein (LDL) oxidation by angiotensin II is thought to be a major contributor to
atherosclerosis development. Oxidized LDL is taken up by tissue macrophages, which were activated and allowed to penetrate the arterial wall by dysfunctional endothelial cells. These lipid-laden macrophages, called foam cells, then recruit T-lymphocytes, enable smooth muscle cell infiltration, and enhance collagen accumulation [15]. There is a strong correlation between hyperglycemia and atherosclerosis [16]. Patients with diabetes and no history of myocardial infarction (MI) have the same risk of MI as patients that have suffered a previous MI [17]. Molnar et al showed a diabetes disease progression is directly related to an individual’s blood glucose level. Unstable hyperglycemia leads to an overall elevated mean amplitude of glycemic excursions compared to a stable diabetic. Nathan et al displayed through a population sample size of 1441 patients with type 1 diabetes with a target to reversing hyperglycemia reduces the risk the of microvascular complications of diabetes and a 42% decrease in risk of cardiovascular disease. [18, 19].

Diabetes also leads to microvascular complications including diabetic retinopathy and poor wound healing. Diabetic retinopathy causes 10,000 new cases of blindness each year [20]. In diabetic retinopathy, unstable neovascularization in the retina results in hemorrhage and eventual blindness. Advanced glycated end products (AGEs) [20], oxidative stress [21], and even growth factors including vascular endothelial growth factor (VEGF) contribute to diabetic retinopathy [22]. Hyperglycemia duration and severity are associated with increasing susceptibility to retinopathy [21]. Poor wound healing in the periphery results party from inadequate angiogenesis. More than 80% of diabetic ulcers result in amputation because of a reduced wound healing capability [23]. Wound healing is divided into 4 phases: coagulation, inflammation, migration-proliferation and remodeling. Complications arise in diabetes because wound healing progression does not follow a linear order. Instead cells in chronic
wounds remain in the coagulation and inflammation phases of wound healing, with limited progression to proliferation. Loots et al examined punch biopsies from chronic diabetic wounds, venous ulcers and acute wound healing. They highlighted a reduction of angiogenesis due to differences in ECM and cell infiltrate [24]. In an animal model, Tsuboi et al administered 6mm punch biopsy wounds to diabetic mice and allowed to heal for 8 days after which mice were sacrificed and histological samples were collected. The mice showed decreases in the degree of wound closure, capillary number and granulation tissue thickness with increases in matrix density [25].

b) Alzheimer’s Disease

Alzheimer’s disease (AD) has no cure; the neurodegenerative disorder progresses with age ultimately resulting in mortality. In 2013, approximately 5.2 million Americans suffered from the disease. Of these, 200,000 people were under the age of 65 and 450,000 people died. By 2050, the number of people with Alzheimer’s disease age 65 and older is expected to reach 13.8 million. This indicates the growing importance of research in the area. AD also has a direct impact on the patient’s family. Care giving is associated with a tremendous cost, and many families have difficulty paying for Alzheimer’s care. Care giving costs are estimated to rise from $203 billion in 2013 to $1.2 trillion in 2050 [26-28].

AD is the most prevalent form of dementia. AD causes patients to lose cognitive capabilities, leading to early death. The hallmark of the disease is amyloid beta (Aβ) peptide aggregation in extracellular plaques [29]. The peptide can aggregate in a number of forms; however monomers and oligomers have been shown to be the most toxic [30]. Aβ leads to formation of neurofibrillary tangles of hyper-phosphorylated tau protein, which cause synaptic dysfunction and eventual neuron death [31]. These cellular events are responsible
for the cognitive deficits associated with AD. The Aβ hypothesis implicates that the peptide plays an essential role in AD progression. There are 2 forms of the Aβ peptide: Aβ40 and Aβ42. Aβ40 is most common however Aβ42 has been associated with increase plaques and fibril formation. In AD, Aβ formation occurs due to proteolytic cleavage of amyloid precursor protein by β-secretase and γ-secretase, instead of by α-secretase [32]. In a clinical study, Aβ40-42 production and clearance rates were studied in patients with and without disease. Peptide production rates were unchanged; however clearance rates were significantly lower in AD patients [33]. Peptide clearance is largely regulated by low-density lipoprotein receptor-related protein and the receptor for advanced glycation end products [34, 35].

Aβ accumulation also can decrease blood flow in the brain, resulting in functional impairments [36]. AD patients showed regional cerebral hypoperfusion, a decrease in brain blood flow, by performing magnetic resonance imaging. Compared to the control group, the AD patients showed decreased blood flow in the inferior parietal cortex, bilateral superior, middle frontal gyri and the left inferior parietal lobe. These regions of the brain are important for the perception of emotions, interpretation of sensory information, language and mathematical operations [37]. Wu et al collected frontal cortex sections from 36 patients with AD and found that total cortical capillary length was reduced [38]. Patel et al showed that the Aβ peptide directly decreases the ability of endothelial cells to form capillary networks, which could contribute to decreased blood flow in AD [36].
1.2 Angiogenesis in Endothelial Cells

1.2.1 Endothelial cell function

Endothelial cells (EC) line all blood contacting surfaces in the body. In large blood vessels, the EC lining is separated from smooth muscle cells and connective tissue by a basal lamina [27]. In contrast, capillaries are composed of a single EC layer with a basal lamina. EC function varies depending on blood vessel location and size. However, overall EC maintain cardiovascular homeostasis through control of permeability, vascular tone, inflammation, injury repair, and the growth and regression of blood vessels. EC can regulate the permeability of the lumen through the distribution of adherens and tight junction proteins. These proteins themselves are regulated by cadherins, occludin, focal adhesions and matrix metalloproteinase (MMP) activation [39]. The contraction and relaxation of smooth muscle cells set the vascular tone. EC can secrete proteins like prostacyclin (PGI$_2$) [40] endothelial nitric oxide synthase (eNOS) [41], and acetylcholine (ACh) [42] to influence smooth muscle cell behavior directly. Injury repair processes including inflammation and the subsequent recruitment of leukocytes are controlled by EC [43]. The process of injury repair also includes neovascularization; EC secrete proteins responsible for initiating steps of extracellular matrix (ECM) degradation, proliferation, migration and ultimately lumen formation [1, 44].
1.2.2 Angiogenic Process

Endothelial cells (EC) are the primary cell associated with angiogenesis, formation of new capillaries form from pre-existing vessels in response to a mechanical or biochemical cue. In general, EC in a blood vessel are stimulated by tissue hypoxia or a growth factor cue. These EC breakdown the nearby ECM. The leading cell, termed a “tip cell,” migrates through the degraded ECM. The cells behind the tip cell begin to proliferate and are termed “stalk cells.” Finally this new tube is called a sprout and can either continue growing towards the attractant or connect with an adjacent capillary [45]. The major angiogenic processes—basement membrane degradation, cell migration, cell proliferation, and tube formation—are now described in greater detail [46].

a) ECM degradation

The first step of angiogenesis is ECM degradation, which allows cells to invade the surrounding tissue. Endothelial cells secrete plasminogen and matrix metalloproteinases (MMPs) in response to a biochemical or biomechanical cue. Plasminogen is converted to the active enzyme plasmin by enzymes including tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), kallikrein and factor XII that cleave the peptide between Arg561 and Val562 [47]. Plasmin is a primary initiator of ECM degradation because it can either directly degrade matrix proteins or activate MMPs that then degrade the matrix [48]. As the ECM is degraded by plasmin and MMPs, cryptic binding sites are exposed which can enhance EC migration [49].
b) Cell Migration

Once the ECM is degraded, EC migrate out of the existing vessel into the surrounding tissue. During migration, EC dynamically reorganize their cell-matrix contacts via integrin internalization at the trailing edge and redistribution to the leading edge [50]. Integrin activation via focal adhesion kinase (FAK) phosphorylation initiates the PKC and Rac pathways that are essential to enhance migration [51]. Angiogenic growth factors, including VEGF and FGF-2, drive EC migration by intracellularly activating integrins [52]. Growth factors drive endothelial cell migration in 4 steps: sensing, extension, attachment and contraction. EC sense the growth factor gradient, and following receptor signaling, PI3K is activated and the cell extends its leading edge. In the attachment phase, RhoA and Src signaling leads to focal adhesion assembly and subsequent actin stress fiber reorganization by p38 and MLC signaling. The cell then contracts and moves forward [53].

c) Cell Proliferation

Once the ECM has been degraded and the EC have invaded, EC proliferate to support EC’s to build the new blood vessel. Growth factors, specifically VEGF and fibroblast growth factor-2 (FGF-2) increase cell proliferation through the PLCγ, PKC and Ras-MAPK pathways which directly upregulate proliferative transcription factors [54] including Etd-1, NF-ATm Id1, Id3, and Vexf1 [55]. Presta et al showed that long lasting protein kinase C (PKC) activation (> 12 hours) is required for FGF-2 to have a complete proliferative response in bovine aortic endothelial cells [56].
d) Tube Formation

Tubule formation is the final step of angiogenesis. This process involves at least two cells that together develop an intercellular vacant space. The vacant space is formed through the accumulation of intracellular vacuoles [57]. Yang et al showed that VEGF increases tube formation through binding specifically to the kinase insert domatin-containing receptor (KDR) preferentially over the fms-like tyrosine kinase (Flt-1). They also demonstrated that p38 and MAPK activation is essential in tube formation [58]. Bates et al propose that VEGF induced vesiculovacuolar organelle formation follows the same signaling pathway as VEGF induced permeabilization. VEGF is able to interact with KDR then signal to increase calcium stores, PLC-γ, PKC and ERK 1/2 [59]. Xiaohua et al support the previous research, showing an increase in vacuole accumulation in VEGF and FGF-2 treated human umbilical vein endothelial cells (HUVEC) [60].
1.3 Plasminogen Activating System

1.3.1 Plasminogen Activating System

The plasminogen activating system is a proteolytic cascade critical for matrix protein degradation and cell invasion in angiogenesis. The primary plasminogen activating system components are urokinase plasminogen activator (uPA), urokinase plasminogen activator receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1), and vitronectin. Tissue type plasminogen activator (tPA) can also activate plasmin; however its primary role is to degrade fibrin. uPA is the dominant plasminogen activator in angiogenesis that drives matrix turnover and cell motility [32, 61]. pro-uPA is secreted from the cell and binds to its receptor, uPAR. Pro-uPA bound to uPAR is then primarily cleaved by plasmin, or other less often other enzymes including kallikrein, factor XIIa, and MMPs, to form uPA [62]. This uPA/uPAR complex cleaves inactive plasminogen into the active form, plasmin. Plasmin then activates more uPA and MMPs, as well as directly degrades the ECM. PAI-1 can bind the uPA/uPAR complex to prevent plasmin generation and pro-angiogenic signaling. Once PAI-1 is bound, lipoprotein receptor-related protein 1 (LRP-1) binds to the complex forcing internalization and degradation[63]. Interestingly, like integrins, uPAR upon internalization is redistributed to the leading edge during EC migration. Vitronectin stabilizes PAI-1 in its active form and also promotes uPA localization to uPAR [64]. uPA, uPAR, PAI-1, and vitronectin levels determine whether the system is pro-angiogenic or anti-angiogenic.
1.3.2 Plasminogen activating system components

a) Urokinase Plasminogen Activator (uPA)

The primary function of uPA is to convert plasminogen to plasmin and increase proteolytic activity. Pro-uPA is secreted as a single chain protein called pro-uPA, the inactive form of uPA. Cells that secrete pro-uPA include vascular endothelial cells, smooth muscle cells, epithelial cells, and in malignant squamous-cell carcinomas [65-68]. uPA is a 55kDa protein consisting of 411 amino acids. Plasmin, cathespin B or kallikrein cleave the K158I159 peptide bridge, resulting in a two chain peptide held together by a single disulfide bond [69]. uPA is composed of three domains: the growth factor domain (GFD; 1-46), a kringle domain (KD; 47-135) and a serine protease domain (PD; 159-411). uPAR binds to the GFD, PAI-1 interacts with the kringle domain, and the PD is the active catalytic site [70-72].

b) Urokinase Plasminogen Activator Receptor (uPAR)

uPAR is an essential regulator of proteolysis and cell attachment when it is bound to its ligand pro-uPA or active uPA. Pro-uPA is readily converted to the active form uPA, when the N-terminal domain binds to its receptor uPAR. Kinetic studies have shown that uPA bound to uPAR has a 40-fold lower $K_m$ (Michaelis constant; substrate concentration at which the reaction rate is half of the maximum reaction rate) than soluble uPA in the system. Unbound uPA has a 6-fold reduction in $K_{cat}$ (number of substrate molecules converted to product per second) compared to bound uPA, leading to decreases in catalytic activity [73]. uPAR is a glycophsophatidylinositol (GPI) anchored receptor that lacks intracellular domains to send signals through the cell. Instead it works cooperatively with transmembrane receptors like integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ to activate signaling [74]. uPAR consist of three domains (D1,
D2 and D3) which form a concave structure that binds to the GFD of uPA. The N-terminal Somatomedin B (SMB) domain of vitronectin can bind to the D1 and the D1-D2 linker regions of uPAR [75, 76]. uPA and vitronectin can bind to uPAR at the same time. In fact, when uPA is bound to uPAR it enhances vitronectin binding to uPAR [77]. This has the potential to decrease the amount of available vitronectin to stabilize PAI-1.

c) **Plasminogen Activating Inhibitor – 1**

PAI-1, a 48 kDa protein consisting of 379 amino acids, is part of the SERAPIN family, a superfamily of serine-protease inhibitors [78]. PAI-1 is produced by endothelial cells, smooth muscle cells, fibroblasts, monocytes and macrophages [79]. PAI-1 is secreted into the blood, where it is stored in platelets, or it is deposited in the ECM with a half-life of 2 hours. PAI-1 can increase its half-life to 145 hours by forming a complex with vitronectin at its N-terminal somatomedin B domain. Vitronectin binding slows the transition of PAI-1 to the latent form by inhibiting strands 1 and 2 of the main β-sheet from folding into the gap between helices E and F, hiding the enzymatic site for inhibition [80, 81]. PAI-1 is the primary uPA inhibitor in endothelial cells. Other uPA inhibitors include PAI-2, PAI-3 (protein C inhibitor), α2-antiplasmin, thrombin, and factor Xa [82, 83]. Although PAI-1 has historically been thought to decrease angiogenesis via uPA inhibition, there is growing research that says that PAI-1 is beneficial for the angiogenic process. This is known as the “PAI-1 paradox” [84]. PAI-1 stabilizes ECM degradation, thereby providing a supportive and adaptive ECM on which cells can migrate [85].
d) Vitronectin

Vitronectin is an RGD-type adhesive glycoprotein, which is essential in cell attachment, migration, proliferation and vascular remodeling [86]. The protein is produced by the liver and can be found circulating in plasma, associated with the cell surface or in the ECM [87]. Other cell types also produce vitronectin in much smaller amounts including endothelial, smooth muscle and retinal cells.

Vitronectin exists in two conformational states: native and multimeric. Native vitronectin is a single chain 75 kDa protein composed of 459 amino acids and constitutes 95% of the total plasma vitronectin concentration. Native vitronectin has a folded conformation that has limited activity with its ligands. Multimeric vitronectin is a double chain 65 kDa protein, which arises as a result of cleavage by PAI-1, thrombin-serpin complexes or heparin [88].

The protein is composed of 3 domains. The Somatomedin B (SMB) domain is made up of 40 amino acids and is the main PAI-1 binding site [89]. When PAI-1 binds to the SMB domain, vitronectin can no longer bind to uPAR. This forces PAI-1 and uPAR to compete for binding and the proteolytic direction. PAI-1 binding to vitronectin would decrease proteolytic activity, while vitronectin binding to uPAR would promote proteolytic activity. The RGD domain is adjacent to the SMB domain and is responsible for ligation to the αvβ3 integrin family, through which it controls cell adhesion and migration [90]. When PAI-1 binds to vitronectin, it also overlaps the αvβ3 integrin binding domain. This interaction may prevent vitronectin dependent angiogenic activity, including cell migration [91].

Following the RGD domain is the region that binds thrombin, antithrombin, or Hka. This area is followed by the last protein region that binds collagen and heparan sulfate proteoglycans (HSPGs) [92].
1.3.3 Plasminogen activating system in angiogenesis

The plasminogen activating system has two primary roles in the angiogenic process: ECM breakdown and cell motility. ECM breakdown is driven by the uPA cleavage of plasminogen to plasmin, which both directly breaks down matrix proteins and activates MMPs such as collagenases [93]. The uPA/uPAR complex can also re-distribute focal adhesions sites to the leading edge of endothelial cells, thereby increasing cell migration in a specific direction [64].

a) Extracellular Matrix Breakdown

VEGF and FGF-2 have been shown to up-regulate the expression of uPA, uPAR and PAI-1, leading to increased ECM breakdown and increased tube formation. Growth factors can modulate the plasminogen system components based on cell type and growth factor dose. FGF-2 and VEGF can individually increase uPA expression in BME cells, however only FGF-2 can produce the same increase in BAE cells [94, 95]. This increase in uPA expression leads to elevated plasmin activity in the system resulting in increased ECM breakdown [96]. On the other hand, growth factors increase PAI-1 expression and inhibit ECM breakdown [97]. Preventing complete uncontrolled degradation of the ECM creates a versatile matrix for endothelial cells to be motile.
b) **Cell Motility**

Initiating cell motility is a process that is governed in part by the plasminogen system activity and largely with the integrins. Integrins transmit intercellular signals by binding to the ECM and uPAR. An integrin consists of two subunits (an α unit and a β unit). These two non-covalently associated glycoprotein subunits associate with each other to bind to specific ECM proteins. Collagen, vitronectin and fibronectin play an important role in the plasminogen activating system for their ability to attach to integrins and induce motility and invasion [98]. Collagen can bind to α1β1 and α2β1 integrins; fibronectin can bind to α4β1, α5β1, αⅢβ3, αvβ3, αvβ6 and αvβ8 integrins; vitronectin can bind to αvβ3 and αvβ5 integrins [99-101]. Inside the cell, integrins are coupled with actin filaments (F-actin) by actin binding proteins (ABP). When integrins are activated, for example by growth factor signaling, focal adhesion kinase (FAK) is phosphorylated. pFAK leads to Src activation which then induces RhoA and Rac 1 which are directly responsible for F-actin reorganization and cytoskeletal remodeling and cell movement [102]. uPAR does not contain a transmembrane domain and cannot transmit intracellular signals alone. Therefore, uPAR must associate with other proteins such as integrins and caveolin [103, 104]. uPA/uPAR and integrin association lead to a protein phosphorylation cascade which include but are not limited to FAK, p38, ERK and PKC leading to reorganization of cell attachment sites and increased migration response [105, 106].
1.4 Glycated Collagen

1.4.1 Glycation Process

Protein glycation (non-enzymatic glycosylation) occurs when a protein’s amino group binds to the carbonyl group of a reducing sugar. Glycation is glucose concentration dependent, therefore it is enhanced by disease states like diabetes in which glucose is chronically elevated [107, 108]. Sugars such as glucose-6-phosphate and fructose glycate proteins much more readily than glucose [109]. Collagen glycation occurs through a progressive series of reactions known as the Maillard reaction. First, early glycation products occur from Schiff base formation, in which a double bond forms between the carbon atom of a sugar and an amino group’s nitrogen atom. The next step is the intermediate Amadori product. Amadori products are formed when the Schiff base rearranges its carbon-nitrogen double bond into a nitrogen single bond with both carbon and hydrogen, resulting in ketone formation- a reversible reaction. The final, irreversible step occurs through an oxidative pathway. The Amadori product is oxidized by a transition metal, and advanced glycation end productions (AGE) are formed [110-112]. AGEs accumulation alter the ECM. Collagen is especially susceptible to modification because of its slow turnover rate. Collagen glycation leads to elevated intermolecular cross linking, which then increases matrix stiffness and degradation resistance [113]. Collagen glycation also changes the way endothelial cells can bind to the substrate as binding sites are altered [113]. Endothelial cells may use different integrins to bind to glycated collagen which has been shown to change adhesion strength [114].
1.4.2 Effect of collagen glycation on endothelial cells – Proliferation, Migration, Tube Formation

a) Growth Factors

Angiogenic endothelial cells are stimulated by growth factors in an ECM-adhesion dependent manner. Growth factors such as VEGF and FGF-2 induce endothelial cell proliferation, migration, and collagenase production [115-117]. Growth factors also increase expression and activation of certain integrins essential to cell-ECM interaction. In dermal microvasculature, VEGF increased the expression of $\alpha_1\beta_1$ and $\alpha_1\beta_2$ integrins responsible for collagen binding [118]. Similarly, antibodies to block the $\alpha_v\beta_3$ integrin abrogated vessel formation induced by FGF-2 [119]. Collagen glycation can influence endothelial cell response to growth factors. Kuo et al showed that HUVEC cultured on glycated collagen substrates had a full fold lower response to 24 hour epidermal growth factor (EGF) induced prostacyclin production as measured by radio immunoassay for the presence of a PGI$_2$ metabolite [120].

b) Plasminogen Activators

Endothelial cell branching on glycated collagen is delayed compared to cells on native collagen. HUVEC seeded on glycated collagen gels decreased the number of tubes by 40% at 24 hours. This delayed branching resulted from a 4-fold increase in PAI-1 expression at 24 hours. A neutralizing PAI-1 antibody restored branching density in cells on glycated collagen in vitro. Aortic ring explants on glycated collagen from PAI-1 knockout mice showed no change in capillary sprouting; however sprouting was inhibited in wild-type [121]. There has been evidence of glycated albumin and fibrinogen increasing PAI-1 expression, there is
however no specific research that looks into the pathway that increases PAI-1 expression in cells on glycated collagen [122, 123].
1.5 Aβ Peptide – Proliferation, Migration, Tube Formation

1.5.1 Formation

Aβ peptide size and structure are critical to its cellular effects. First, Aβ42 monomers aggregate into intermediates called oligomers which are globular structures. The oligomers then combine to form protofibril structures (6 – 10 nm in diameter) with a curvilinear morphology. Protofibrils then assemble into protofilaments and finally fibrils. Although this pathway is still under intense research, it is hypothesized that the oligomers are the most toxic structure [124, 125]. Walsh et al injected mice with low nM concentrations of Aβ oligomers for 2 minutes and showed a decrease in hippocampal long term potentiation for 3 hours as determined by high frequency stimulation. Monomeric Aβ did not produce any effect [126]. Likewise, low nano-molar concentration of Aβ oligomers inhibited cognition [127], caused dendritic spine loss [128] and tau hyperphosphorylation [129, 130]. Extracellular peptide degradation is carried out by MMPs and plasmin [131]. Therefore, the plasminogen activating system may affect Aβ peptide clearance in Alzheimer’s disease.

1.5.2 Growth Factors

Aβ42 inhibits VEGF induced tube formation in brain endothelial cells. Patel et al cultured human brain microvascular endothelial cells on a layer of Matrigel and added 10 μM Aβ42 peptide along with a VEGF gradient (1-5 nM). Aβ peptide decreased endothelial cell tube formation, and VEGF rescued tube formation in a dose-dependent manner. Aβ peptide likely competitively binds to the VEGFR-2 and thereby inhibits VEGF binding, as confirmed by co-immunoprecipitation assays and molecular modeling [36].

In contrast, Aβ may promote angiogenic activity due to FGF-2. Donnini et al cultured post-capillary venular endothelial cells (CVEC) and exposed them to 0.5-50 μM Aβ40. At
low concentrations, Aβ increased FGF-2 and FGFR proteins determined by Western blot. Elevated peptide levels (5-50μM) caused apoptosis (Tunnel assay), down regulated FGF-2 production, inhibited FGF-2 binding to heparin and decreased FGFR1 phosphorylation (Western blot). FGF-2 overexpression restored angiogenic activity [132] via phosphorylation of Akt.

1.5.3 Plasminogen Activators

Aβ can impact cellular production of plasminogen activating system components, and the plasminogen activating system can impact Aβ degradation. Tucker et al cultured primary rat cortical neuron cells with Aβ40 peptide over 72 hours and examined uPA, tPA and MMP expression by RT-PCR. Aggregated Aβ increased tPA and uPA mRNA. Plasmin cleaved Aβ at multiple sites, causing degradation at 1/10th the rate that it degrades fibrin. Furthermore, plasmin inhibited the neurotoxic and negative cytoskeletal effects of Aβ, shown through chromatin condensation scoring; neurons with condensed chromatin were determined to be apoptotic [133]. Conversely, Medina et al showed elevated levels of tPA activate Erk 1/2 and PKC independent of plasmin in rat hippocampal neuron cells treated with Aβ40 for 24 hours [134].
1.6 Thesis Summary

1.6.1 Objective and Hypothesis

Patients with either diabetes or Alzheimer’s disease accumulate altered extracellular proteins (glycated collagen and Aβ peptide, respectively) and display decreased angiogenesis in critical vascular beds. The objective of this thesis was to examine how angiogenic growth factors impact endothelial cell angiogenic processes in the presence of glycated collagen and Aβ peptide. My overarching hypothesis was that growth factors could not fully rescue endothelial cell angiogenic processes from altered extracellular proteins. This hypothesis was explored through three primary studies, which are described in Chapters 2, 3, and 4 of this thesis.

1.6.2 Thesis Organization

Chapter 2: In this chapter, I investigated whether FGF-2 could counteract glycated collagen effects on the plasminogen system and thereby rescue endothelial cell angiogenic processes. I hypothesized that FGF-2 would decrease endothelial cell PAI-1 production in cells on glycated collagen, and therefore restore plasminogen system activity. Endothelial cells were cultured on native or glycated collagen with or without FGF-2. Plasminogen activity was measured, along with uPA, PAI-1, and uPAR protein and mRNA levels. Cell 3D migration and capillary-like tube formation were quantified in the varied conditions.

Chapter 3: In this chapter, I investigated the effect of FGF-2 has on vitronectin in the plasminogen activating system. I hypothesized that FGF-2 would increase vitronectin uptake by activating integrins, and thereby enhance plasminogen system activity. Vitronectin uptake and spatial location were determined in endothelial cells treated with and
without FGF-2. Integrins were inhibited using blocking antibodies, inhibiting integrin activation, and siRNA integrin knockdown. The impact on vitronectin binding and the plasminogen system were then quantified.

**Chapter 4:** In this chapter, I investigated how the Aβ peptide affected endothelial cell response to both FGF-2 and VEGF. *I hypothesized that Aβ would inhibit endothelial cell angiogenic processes in response to these growth factors by competing for growth factor receptor binding.* Bovine brain microvascular cell proliferation, 3D migration, and tube formation were measured with and without growth factor stimulation. Various forms of the Aβ peptide were also studied to determine their effects.

**Chapter 5:** The final chapter provides conclusions, research significance, and contributions to the field. In addition, future research directions are identified.
2 Glycated collagen influences on the plasminogen activating system

2.1 Introduction

Angiogenesis, the growth of new blood vessels from existing vessels, relies on regulated interactions between endothelial cells and extracellular matrix [1]. Endothelial cells degrade the vascular basement membrane, migrate and invade into the surrounding tissue, proliferate, and form capillary tubes. These processes rely on both extracellular matrix proteolysis via the plasminogen system and matrix metalloproteinases, as well as cell-matrix connections via integrins at focal adhesions [135]. The plasminogen system is particularly interesting because it plays an important role in both matrix proteolysis and cell adhesion.

Like many biological systems, the plasminogen system is a balance of activators and inhibitors. Urokinase plasminogen activator (uPA), a serine protease, is the primary activator in pericellular matrix proteolysis [136]. Cells secrete uPA as a single chain pro-enzyme (scuPA) [137]. Secreted scuPA binds to its cell surface receptor (uPAR), where plasmin cleaves it to the active two-chain uPA form. uPA then cleaves plasminogen to plasmin, which either directly breaks down matrix components or activates matrix metalloproteinases [138]. Plasminogen activator inhibitor-1 (PAI-1) is the primary uPA inhibitor [139]. Cells secrete PAI-1 in active form, but it rapidly decays into its latent form unless it binds to vitronectin in plasma, platelets, or extracellular matrix [140]. Active PAI-1 binds to and inactivates receptor bound uPA, preventing proteolysis [141]. The entire uPAR-uPA-PAI-1 complex is then internalized, after which free uPAR returns to the cell surface [142, 143]. The uPA:PAI-1 balance is postulated to break down enough of the extracellular matrix to enable cell invasion yet still maintain adequate matrix stability so that cells can adhere to and migrate along the matrix.
Plasminogen system components, specifically uPAR and PAI-1, are also important in cell adhesion through interactions with vitronectin. uPA alters uPAR conformation upon binding, which increases uPAR affinity for the vitronectin somatomedin B domain and enhances cell adhesion [144, 145]. uPA bound to uPAR also increases cell migration without proteolysis, perhaps by associating with and activating intracellular integrin signalling pathways [106]. PAI-1 similarly binds to the somatomedin B domain of both plasma and matrix-bound vitronectin, where it is stabilized in its active conformation. PAI-1 inhibits both uPAR and integrin interactions with vitronectin [91]. Active PAI-1 decreases cell adhesion and migration independent of plasminogen activation specifically on vitronectin coated surfaces [91, 146, 147]. Thus plasminogen system interactions with the extracellular matrix via vitronectin control angiogenesis by localizing proteolytic activity at the cell surface and by modulating cell adhesion and migration.

Angiogenesis is critical to both physiological and pathological processes [1]. In diabetes, unregulated microvascular remodelling is a primary cause of morbidity and mortality. Glomerular capillary hypertrophy impairs renal filtration in diabetic nephropathy, and fragile new vessel formation in the eye leads to diabetic retinopathy [148]. In contrast, reduced angiogenesis in the extremities contributes to diabetic neuropathy and poor wound healing [149]. While many biochemical changes occur in diabetes, uncontrolled plasma glucose is strongly implicated in diabetic vascular disease including disordered angiogenesis [18, 150]. Glucose affects endothelial cells directly through mitochondrial superoxide overproduction and indirectly via extracellular matrix modifications [3, 148].

Diabetic hyperglycemia modifies both structural and biochemical functions of the extracellular matrix in angiogenesis. Glucose reacts with amino protein groups to glycate
collagen and other structural matrix proteins via the Maillard reaction. Glycation enhances collagen cross-linking, which makes the matrix stiffer and more resistant to proteolysis [151-153]. Glycation also changes collagen type I fiber assembly and arginine residues in both RGD (arg-gly-asp) and GFOGER (gly-phe-hyp-gly-glu-arg) integrin binding sites, thereby decreasing cell adhesion [154]. Glycated collagen inhibited angiogenesis in in vitro studies [121, 155]. In addition to the structural role, extracellular matrix components such as collagen and heparan sulfate proteoglycans bind and release growth factors to biochemically support angiogenesis. We previously showed that endothelial cells release more FGF-2 in high glucose conditions, and this excess FGF-2 is stored in the extracellular matrix due to a glucose-induced increase in endothelial cell permeability [156]. In vivo, human retinal tissue from patients with proliferative retinopathy showed increased FGF-2 [157]. FGF-2, together with other growth factors such as vascular endothelial growth factor, promotes angiogenesis [158].

Both collagen glycation and elevated FGF-2 storage are glucose-induced extracellular matrix changes that could impact the plasminogen system, albeit in opposite directions. Chen et al showed delayed capillary cord branching in human umbilical vein endothelial cells grown on glycated as compared to native collagen gels, which was ascribed to elevated PAI-1 protein and mRNA levels [121]. In fact, elevated PAI-1 is prevalent in patients with metabolic syndrome, and both diabetic animals and humans showed increased PAI-1 in the arterial wall [159-161]. Thus glycated collagen shifts the plasminogen system balance in the anti-angiogenic direction by increasing PAI-1. FGF-2 increases uPA and PAI-1 at both the transcriptional and translational levels [162, 163]. However, FGF-2 stimulates more uPA than PAI-1. Thus FGF-2 moves the plasminogen system in a pro-angiogenic direction.
In this study, we investigated whether FGF-2 could counteract glycated collagen effects on the plasminogen system and thereby rescue endothelial cell angiogenic processes (Schematic 2.1). Endothelial cells were cultured on native or glycated collagen with or without FGF-2. Plasminogen activity was measured, along with uPA, PAI-1, uPAR, and vitronectin protein and mRNA levels. Cell 3D migration and capillary-like tube formation were quantified in the varied conditions. We now show that PAI-1 protein increased in endothelial cells on glycated collagen, which may relate to increased PAI-1 binding to glycated collagen. FGF-2 could not fully rescue endothelial cell angiogenic function on glycated collagen. These data show that growth factor efficacy is partially determined by the extracellular matrix state, which will help inform growth factor therapies for modulating angiogenesis in people with diabetes.

**Schematic 2.1: AIM 1 Hypothesis.** FGF-2 binds to its receptor, FGFR, and influences transcription of uPA, uPAR, and PAI-1. Specifically, FGF-2 decreases the amount of PAI-1 in cells on glycated collagen so that uPA can bind to uPAR and promote plasmin activity in the presence of low concentrations of PAI-1.
2.2 Methods

2.2.1 Cell Culture, collagen glycation and vitronectin multimerization

Human umbilical endothelial cells (HUVEC, passages 4–9; Lonza) were maintained in Endothelium Growth Medium (EGM-2; Lonza) supplemented with 5% fetal bovine serum (FBS; Hyclone), 1% penicillin–streptomycin (Gibco), and 1% glutamine (Gibco). HUVEC were selected because they are widely used for in vitro endothelial cell studies, show a robust response to FGF-2, and react with human plasminogen system proteins and antibodies. In addition, HUVEC were used in the original paper by Chen et al in which glycated collagen delayed endothelial cell capillary-like cord branching [121]. Collagen was glycated by incubating 100 µg/mL collagen type I (BD Biosciences) with 500 mM D-glucose-6-phosphate (G6P; Sigma) in phosphate buffered saline (PBS) at 37°C for 4 weeks. Collagen glycation was validated via autofluorescence and decreased collagenase digestion as described previously (Figueroa, 2011). Cell culture substrates were coated with 50 µg/ml native or glycated collagen (BD Biosciences), or 3 µg/ml vitronectin (Invitrogen) overnight at 4°C. After washing, 25,000 cells/cm² were seeded on these substrates in Endothelial Basal Medium (EBM-2; Lonza) supplemented with 5% FBS and allowed to attach for 48 hours. EBM-2, which is EGM-2 without growth factors and cytokines, was used for all experiments to enable measurement of FGF-2 stimulation.
2.2.2 Plasmin Activity: Chromozym PL

Chromozym PL was used to determine plasmin activity in cell extracts. In our assay, uPA cleaved exogenous plasminogen to plasmin, which then cleaved Chromozym PL into a residual peptide and 4-nitroaniline (405 nm). Confluent HUVEC were cultured and treated with FGF-2 as described. HUVEC were lysed in T/T buffer (60 mM Tris hydrochloride, 0.5% Triton X-100) for 5 minutes, after which cell extracts were centrifuged at 10,000 g for 10 minutes to remove insoluble material. A final solution of cell extract, 127 ng/mL Chromozym PL (Roche), and 67 µU/mL plasminogen (Roche) was mixed in a 96 well plate. Absorbance (405 nm) was measured for 24 hours in an Infinite 200 PRO microplate reader (TECAN) maintained at 37°C. The change in 4-nitroaniline absorbance at 405 nm is directly proportional to uPA enzymatic activity. Absorbance was plotted vs. time, and the linear region slope (ΔA/min) was used to calculate plasmin activity via the following equation:

\[
Plasmin\ Activity \left( \frac{U}{ml} \right) = \left[ \left( \frac{V}{v} \right) * \epsilon * d \right] * \left( \frac{\Delta A}{\text{min}} \right)
\]

where \( V \) is total volume (300 µl), \( v \) is cell extract volume (33 µl), \( \epsilon \) is absorbance coefficient for 4-nitroaniline (10.4 mmol-1·cm-1), and \( d \) is light path (1 cm).
2.2.3 3D Migration: Cell Invasion Assay

HUVEC 3D migration was assessed using a Boyden chamber assay. The bottom side of a Transwell chamber (6.5 mm diameter, 8 µm pore size; Corning Costar) was coated with 100 µg/ml native or glycated collagen, which was allowed to gel for 1 hour at room temperature. 150,000 cells were then added to the top of each chamber in EBM-2 with 5% FBS. In specific experiments, FGF-2 (50 ng/ml), uPA (12 U/ml; American Diagnostica), PAI-1(1 ng/ml; American Diagnostica), and vitronectin (3 μg/ml) were added to the upper chamber. EGM-2 with 10% FBS was added to the bottom chamber. Samples were incubated for 24 hours at 37°C, after which the cells remaining in the upper Transwell chamber were removed by gentle cotton swabbing. Cells that migrated to the chamber bottom were labeled with Hoechst (10 ng/ml; Invitrogen) for 30 minutes. 5 randomly selected areas per sample were imaged using an Olympus IX81 inverted fluorescent microscope. Cell number was quantified with ImageJ.

2.2.4 Tube Formation: Cell Extension Assay

HUVEC tube formation was assessed on native or glycated collagen gels. Native collagen gels were prepared by incubating 50 µl 4 mg/ml collagen I at 37°C for 1 hour. Glycated collagen gels were prepared by incubating native collagen gels with 500 mM G6P for 4 weeks at 37°C. 15,000 HUVEC were added to each gel in EBM-2 with 5%FBS. FGF-2 (50 ng/ml), uPA (12 U/ml), PAI-1 (1 ng/ml), and vitronectin (3 μg/ml) were added during cell seeding to determine their effects on tube formation. Cells were incubated for 18 hours at 37°C, after which samples were imaged using a Nikon Eclipse TS100 phase contrast microscope with a Nikon DS-Fi1 CCD camera (5 images per well). Tube length was manually analyzed using ImageJ by an objective technician with no knowledge of the coded images [164].
2.2.5 uPA, PAI-1, uPAR, and vitronectin protein and mRNA levels

Key plasminogen system component (uPA, PAI-1, uPAR, vitronectin) protein and mRNA levels were quantified by Western blot and real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR), respectively. HUVEC were cultured for 48 hours on native and glycated collagen coated substrates as described, after which they were treated with 50 ng/ml FGF-2 for 24 hours. For Western blot, cells were scraped off the surface in ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 2 mM NaVO₄, 2 mM PMSF, 50 mM NaF, 10% glycerol, complete protease inhibitor, pH 7.4). Cell lysates were normalized for protein content, separated by SDS-PAGE on a 4–12% Bis-Tris gel (Invitrogen), and transferred to nitrocellulose membranes using the Invitrogen i-Blot system. Membranes were incubated overnight at 4°C with primary antibodies to uPA, PAI-1, uPAR or vitronectin (Santa Cruz), followed by a secondary horseradish peroxidase-conjugated antibody (Promega) for 2 hours at room temperature. Protein bands were detected using an enhanced chemiluminescence kit (Western Lightning, PerkinElmer) and visualized with a Fluorchem digital imager (Alpha Innotech). Band intensity was quantified using AlphaEase FC software.

For RT-PCR, RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer protocol. RNA concentration was quantified by measuring sample absorbance (260 nm) using a NanoDrop 1000 (Thermo Scientific), and RNA purity (1.8-1.99 for all samples) was determined using the 260/280 nm ratio. RNA samples were stored at -80°C until use. Primers for uPA (forward: 5’-AATGACTGTGTGTAAGCTGATTTC-3’; reverse: 5’-AGGGGTCCCCCTGAGTCTTTCTCTTCGATTTC-3’) and PAI-1 (forward: 5’-TCCAGCAGCTGAATTCCTG-3’; reverse: 5’-GCTGGAGACATCTGCATCCT -3’) were from Integrated DNA Technologies. PCR was performed on a LightCycler 480 Real-Time PCR System using
LightCycler 480 RNAMaster Hydrolysis probes (Roche) according to the manufacturer protocol. Each 20 μl reaction contained 150 ng total RNA, 0.4 μM Universal Probe #66 (Roche), 0.5 μM uPA or PAI-1 primer, 3.25 mM Mn(OAc)$_2$, 1x Master Hydrolysis Probes and 1x Enhancer (Roche). Thermal cycling conditions were set as follows: 1 cycle reverse transcription at 63°C for 20 min; 1 cycle denaturation at 95°C for 30 sec; and 45 cycles amplification and annealing at 95°C for 10 sec with a second amplification at 60°C for 45 sec with single acquisition mode. The protocol was selected to allow primers to anneal at a greater efficiency. mRNA level was quantified using both the standard curve and the second derivative maximum method. All standard curves and samples were determined to have a maximum error of 0.05 and doubling rate greater than 1.85. All reactions were performed in triplicate. Reactions without cell sample were the negative control.
2.2.6 PAI-1 and FGF-2 binding to extracellular matrix proteins

PAI-1 binding to native collagen, glycated collagen and vitronectin was assessed using a solid phase binding assay [165]. 96 well plates were coated with the appropriate extracellular matrix protein as described and blocked using PBS with 3% bovine serum albumin (BSA) and 0.05% Tween. 5 µg/ml PAI-1 (in tris-buffered saline with 1% BSA, 0.01% Tween, and 1 mM CaCl$_2$) was added and incubated for 2 hours at 37°C. After thorough washing, bound PAI-1 was detected by incubating samples with a PAI-1 primary antibody (Santa Cruz) followed by a horse radish peroxidase-conjugated secondary antibody (Promega). The assay was developed using a two-step process to decrease background absorbance. First, 1.8 nM 2,2-azinobis(3-ethylbenzthiazidine-6-sulfonic) acid (Sigma) in 0.1 M sodium citrate, pH 4.5 was added to samples for 30 min at 37°C. This was then replaced with 1.8 mM 2,2-azinobis(3-ethylbenzthiazidine-6-sulfonic) acid and incubated for 30 min at 37°C. Absorbance (405nm) was quantified using a microplate reader.

FGF-2:integrin competitive binding to extracellular matrix proteins was determined using a cell adhesion assay. 50 ng/ml FGF-2 was added to native collagen, glycated collagen and vitronectin-coated substrates for 24 hours. This assay was used to simulate matrix-bound FGF-2, since FGF-2 binds to and releases from tissue culture polystyrene with similar binding kinetics as matrix heparan sulfate proteoglycans [166]. 250,000 cells in EBM with 1% FBS were added to each sample and incubated for 2 hours at 37°C to allow cells to attach [167]. Substrates were thoroughly washed to remove unbound cells, after which attached cells were trypsinized and counted using a Coulter counter (Beckman).
2.2.7 Statistical Analysis

Samples were collected in triplicate and experiments performed at least two times. Data are graphed as mean ± standard deviation. Significance between two groups were compared using Student’s t-test. Comparisons among multiple groups were analyzed by two-way ANOVA with a Bonferroni post-hoc test. p-values are indicated in the figures by # p < 0.05, * p < 0.01, ** p < 0.001, unless otherwise indicated.
2.3 Results

2.3.1 Endothelial cell response to FGF-2 on native and glycated collagen

Glycated collagen was previously shown to delay endothelial cell capillary cord branching, perhaps due to elevated PAI-1 [27]. We therefore measured whether FGF-2 could reverse glycated collagen-induced changes in plasminogen activity and cellular angiogenic processes. HUVEC attachment and spreading were statistically similar on both native and glycated collagen coated substrates (data not shown). Glycated collagen and FGF-2 had a statistically significant effect on endothelial cell plasmin activity, 3D migration, and tube length compared to native collagen (p < 0.05 by two-way ANOVA). Indicating that FGF-2 can only partially abrogate the effect of glycated collagen. Plasmin activity was 35% lower in cells on glycated compared to native collagen (Figure 2.1A). While FGF-2 increased plasmin activity by 51% in cells on native collagen, it only increased plasmin activity by 22% in cells on glycated collagen. Cell proliferation (assessed by cell counts) and 2D migration (assessed by cage assay) increased with FGF-2 on both native and glycated collagen, but there were no differences based on substrate composition (data not shown). In contrast, collagen glycation did change endothelial cell 3D migration through a collagen coated Boyden chamber. 27% fewer cells migrated through the Boyden chamber on glycated as compared to native collagen substrates. FGF-2 stimulation restored cell migration on glycated collagen back to native collagen levels (Figure 2.1B). Endothelial cell tube length was 48% lower for HUVEC on glycated collagen gels as compared to native collagen gels. FGF-2 more than doubled tube length on native collagen gels, but only increased tube length 46% on glycated collagen gels (Figure 2.1C; sample images in Figure 2.1D). Thus FGF-2 fully rescued 3D migration but not plasmin activity or capillary-like tube formation from glycated collagen effects.
Figure 2.1: Glycated collagen decreased endothelial cell plasmin activity, 3D migration, and tube length compared to native collagen. FGF-2 only partially abrogated this effect.

A) Cellular plasmin activity was measured using the Chromozym PL assay. HUVEC were seeded on native and glycated collagen (50 µg/ml) coated substrates for 48 hours, after which 50 ng/ml FGF-2 was added for 24 hours. Cell extracts were collected and assayed immediately.

B) 3D cell migration was measured using a Boyden chamber assay. 150,000 HUVEC +/- 50 ng/ml FGF-2 were added to the top of an 8.0 µm pore size Transwell insert coated with 100 µg/ml native or glycated collagen. After 24 hours, cells that migrated to the chamber bottom were labeled with Hoechst, imaged by fluorescent microscopy, and quantified with ImageJ. Normalized to native collagen without FGF-2.

C) For the tube formation assay, 15,000 HUVEC were added to native and glycated collagen gels (4 mg/ml) +/- FGF-2 (50 ng/ml).
After 18 hours, tubes were imaged by phase contrast microscopy and tube length was analyzed with ImageJ. D) Sample images of tube formation assay, with tubed indicated by black arrows.

* p < 0.01; ** p < 0.01 compared to native collagen null condition and to FGF-2 (indicated with brackets) with Bonferroni post hoc.
2.3.2 Glycated collagen and FGF-2 effects on plasminogen system components

Plasmin activity derives from uPAR binding and the uPA:PAI-1 balance. We therefore measured uPA, uPAR, and PAI-1 protein levels by Western blot (Figure 2.2A) to determine which proteins contributed to decreased plasmin activity in cells on glycated collagen. At the selected time point (48 hours on glycated collagen, 24 hours of FGF-2 stimulation), glycated collagen and FGF-2 had a statistically significant effect on PAI-1 protein level (p < 0.05 by two-way ANOVA). Indicating that FGF-2 treatment can return PAI-1 back to the native collagen level. HUVEC on glycated collagen showed more than double the PAI-1 protein as cells on native collagen, and FGF-2 brought the PAI-1 level back down to that of cells on native collagen (Figure 2.2B). FGF-2 had no effect on cellular PAI-1 level on native collagen, and neither glycated collagen nor FGF-2 affected uPA or uPAR levels (Figures 2.2C and 2.2D).

We next measured uPA and PAI-1 mRNA levels to determine if changes in PAI-1 protein levels were related to PAI-1 production. Neither glycated collagen nor FGF-2 affected uPA mRNA, which was consistent with the Western blot data (Figure 2.3A). Interestingly, glycated collagen did not increase PAI-1 mRNA even though it did increase PAI-1 protein. FGF-2 decreased PAI-1 mRNA by around 30% in cells on both native and glycated collagen (Figure 2.3B). These data suggest that the PAI-1 increase on glycated collagen at this time point may not be due to changes in PAI-1 transcription.
Figure 2.2: Glycated collagen increased PAI-1 protein level, and FGF-2 treatment returned PAI-1 back to the native collagen level. Glycated collagen did not significantly affect uPA or uPAR protein levels. A) HUVEC were seeded on native and glycated collagen (50 µg/ml) coated substrates for 48 hours, after which 50 ng/ml FGF-2 was added for 24 hours. Cell extracts were collected and normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. B, C, D) Band intensity for PAI-1, uPA, and uPAR, respectively, was quantified and normalized to GAPDH using AlphaEase FC software. * p < 0.01; ** p < 0.01 compared to native collagen null condition and to FGF-2 (indicated with brackets) with Bonferroni post hoc.
2.3.3 PAI-1 binding to glycated collagen

We and others previously showed that denaturation and glycation-induced structural changes reveal cryptic collagen binding sites, including an RGD site [25, 40, 41]. We therefore hypothesized that PAI-1 levels increased in cells on glycated collagen partially due to PAI-1 binding. Glycated collagen had a statistically significant effect on PAI-1 binding (p < 0.01 by Student’s t-test). Glycated collagen and bound FGF-2 had a significant effect on attached number of cells (p < 0.05 by two-way ANOVA). Indicating that the PAI-1 bound to both glycated collagen and vitronectin significantly compared to native collagen, likely at the αVβ3 binding site. Indeed, more than three times as much PAI-1 bound to glycated collagen as compared to native collagen in an equilibrium binding assay (Figure 2.3A). We next hypothesized that the available cryptic binding sites on glycated collagen might cause FGF-2 to affect cell adhesion as well as plasminogen system activity. Surface bound FGF-2 promotes cell adhesion and spreading by interacting with αvβ3 integrins, and soluble FGF-2 decreases cell adhesion to RGD sites [39]. We therefore measured the number of cells adhered to native or glycated collagen (or vitronectin as the RGD positive control) in the presence of FGF-2. FGF-2 decreased HUVEC bound to both glycated collagen and vitronectin by around 22%, whereas FGF-2 increased HUVEC bound to native collagen slightly (Figure 2.3B). These data suggest that the increase in PAI-1 protein may relate to protein binding, and that FGF-2 may affect cell adhesion and hence cell migration on glycated collagen.
Figure 2.3: Both PAI-1 and FGF-2 bound to glycated collagen and vitronectin more than to native collagen, likely at the α3β3 binding site. A) Plates coated with native collagen (25 µg/ml), glycated collagen (25 µg/ml), or vitronectin (25 µg/ml) were incubated overnight at 4°C. After washing, plates were incubated with 5 µg/ml PAI-1 for 1 hour at 37°C; then washed to remove unbound PAI-1. Primary and secondary antibodies were incubated in succession for 1 hour at 37°C each to detect bound PAI-1. The solid phase binding was developed with 2,2-azinobis and read at an absorbance of 405nm. *p<0.01 compared to native collagen. B) Plates coated with native collagen (50 µg/ml), glycated collagen (50 µg/ml), or vitronectin (3 µg/ml) were incubated with 50 ng FGF-2 overnight at 4°C. After thorough washing, 250,000 HUVEC were added to the plates and incubated for 2 hours at 37°C. After removing unbound cells, attached cells were trypsinized and counted using a Coulter counter. * p < 0.01; ** p < 0.01 compared to native collagen null condition and to FGF-2 (indicated with brackets) with Bonferroni post hoc.
2.3.4 Exogenous uPA and PAI-1 effects

We next added exogenous uPA and PAI-1 to cells on native and glycated collagen and measured 3D migration and tube formation to determine how varying the plasminogen system balance would affect these key endothelial cell functions. Exogenous protein but not glycated collagen have a statistically significant effect on number of migrated cells and tube length (p < 0.05 by two-way ANOVA with Bonferroni post hoc). Indicating exogenous uPA increased endothelial cell 3D migration and tube length on both native and glycated collagen. Exogenous uPA had minimal effect on HUVEC 3D migration, but it increased endothelial tube length on both native and glycated collagen gels by around 70%. Exogenous PAI-1 decreased 3D migration on both native and glycated collagen by around 18%, but it only decreased endothelial tube length on glycated collagen gels (Figure 2.4A, B). Thus plasminogen system shifts may have different effects depending on whether those shifts occur due to changes in uPA or PAI-1.
Figure 2.4: Exogenous uPA increased endothelial cell 3D migration and tube length on both native and glycated collagen. Exogenous PAI-1 decreased 3D migration on both substrates, but only decreased tube length on glycated collagen. A) 150,000 HUVEC +/- 12 IU/ml uPA or 1 ng/ml PAI-1 were added to the top of an 8.0 µm pore size Transwell insert coated with 100 µg/ml native or glycated collagen. After 24 hours, cells that migrated to the chamber bottom were labeled with Hoechst, images by fluorescent microscopy, and quantified with ImageJ. B) 15,000 HUVEC were added to native and glycated collagen gels (4 mg/ml). After 18 hours, tubes were imaged by phase contrast microscopy and tube length was analyzed with ImageJ. Samples with uPA or PAI-1 are normalized to samples without uPA or PAI-1 on the respective substrate to best compare exogenous protein effects. * p < 0.01 compared to samples without uPA or PAI-1 on the respective substrate with Bonferroni post hoc.
2.3.5 Vitronectin role in FGF-2 effects

In our experiments, FGF-2 more than doubled vitronectin levels in HUVEC on both native and glycated collagen, as measured by Western blot (Figure 2.5A). We therefore added vitronectin to HUVEC cultures and measured plasmin activity, 3D cell migration, and tube formation on native and glycated collagen gels. FGF-2 but not glycated collagen had a statistically significant effect on vitronectin protein level, uPA activity, number of migrated cells or tube length (p < 0.05 by two-way ANOVA). Indicating that FGF-2 increased vitronectin protein levels in cells on native and glycated collagen. However, soluble vitronectin had different effects for plasmin activity, 3D migration, and tube formation. Vitronectin decreased plasmin activity in HUVEC on native collagen by 11%, but had no effect in cells on glycated collagen (Figure 2.5B). In contrast, vitronectin slightly increased HUVEC 3D migration on glycated collagen only (Figure 2.5C). Vitronectin had the greatest effect on endothelial tube formation. Exogenous vitronectin added into the culture medium more than doubled endothelial tube length for cells on both native and glycated collagen gels (Figure 2.5D).
Figure 2.5: FGF-2 increased vitronectin protein levels in cells on native and glycated collagen. However, soluble vitronectin had different effects for plasmin activity, 3D migration, and tube formation. A) HUVEC were seeded on native and glycated collagen (50 µg/ml) for 48 hours, after which 50 ng/ml FGF-2 was added for 24 hours. Cell extracts were collected and normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. *p<0.01; #p<0.05. B) Soluble vitronectin decreased plasmin activity in cells on native but not glycated collagen. HUVEC were seeded on native and glycated collagen (50 µg/ml) coated substrates for 48 hours, after which 3 µg/ml vitronectin was added for 24 hours. Cell extracts were collected and assayed immediately via Chromozym PL. C) Soluble vitronectin increased cell 3D migration on glycated but not native collagen. 150,000 HUVEC +/- 3 µg/ml vitronectin were added to the top of an 8.0 µm pore size Transwell insert coated with 100 µg/ml native or glycated collagen. After 24 hours, cells that migrated to the chamber bottom were
labeled with Hoechst, imaged by fluorescent microscopy, and quantified with ImageJ. D) Soluble vitronectin increased tube length on both native and glycated collagen. 15,000 HUVEC were added to native and glycated collagen gels (4 mg/ml) +/- 3 µg/ml vitronectin. After 18 hours, tubes were imaged by phase contrast microscopy and tube length was analyzed with ImageJ. * p < 0.01 compared to native collagen null condition and to Vn (indicated with brackets) with Bonferroni post hoc.

**Schematic 2.2: AIM 1 Summary.** At short times, plasmin activity decreased in cells on glycated collagen due to PAI-1 binding. A) PAI-1 is able to bind to glycated collagen at cryptic binding sites. B) PAI-1 then is able to inhibit plasmin activity at localized sites which prevents ECM degradation, 3D migrations and tube formation. FGF-2 has no influence on the ability of PAI-1 to bind to glycated collagen.
2.4 Discussion

2.4.1 Glycated collagen changes plasminogen activity and protein levels

Glucose induced changes in the extracellular matrix, such as collagen glycation, decrease endothelial cell capillary-like tube formation in vitro [27]. We previously showed that glucose also increases FGF-2 extracellular matrix storage [28]. Therefore in this study we determined whether FGF-2 could rescue endothelial cell angiogenic function in cells cultured on glycated collagen. We now show that glycated collagen decreased plasminogen system activity, increased PAI-1 protein levels, and decreased endothelial cell migration and capillary-like tube length. FGF-2 only partially restored plasminogen system activity in cells on glycated collagen, even though the growth factor significantly decreased PAI-1 protein levels. Overall, our study highlights the complexity of growth factor-plasminogen system interactions in angiogenesis, since both impact matrix proteolysis and cell adhesion. A thorough understanding of these interactions is needed before growth factor therapy can be used to restore or inhibit blood vessel growth in diseases of protein glycation (e.g., diabetes).

We measured decreased plasmin activity likely related to elevated PAI-1 in HUVEC on glycated collagen, similar to previously published reports [27]. However, in our experiments glycated collagen did not increase PAI-1 mRNA. This could be because PAI-1 mRNA levels had already returned to baseline by our measurement point (24 hours). However, PAI-1 protein production may be less important than PAI-1 binding and stabilization since capillary-like tube changes occur within 18-24 hours of glycated collagen exposure. From our data, PAI-1 appeared to bind to cryptic collagen sites that are revealed by glycation, which may stabilize and localize PAI-1 to the extracellular matrix [25, 40-42]. This could help explain the early
elevated PAI-1 protein levels, as well as decreased cell migration and tube formation in cells on glycated collagen.

### 2.4.2 FGF-2 is not able to fully rescue endothelial cells from glycated collagen influences

FGF-2 fully rescued cell migration on glycated collagen perhaps due to a combination of its proteolytic and adhesive effects. The plasmin activity increase with FGF-2 stimulation would enhance cell migration on glycated collagen due to matrix proteolysis. Then, FGF-2 could further enhance cell migration by interacting with α3β3 integrins to alter cell adhesion [39]. Cells that weakly adhere to matrix proteins are unable to generate adequate traction forces and therefore cell migration is low. Cells that strongly adhere to the substrate are unable to dynamically dissociate and reorganize adhesion sites and therefore are also relatively immobile. Efficient cell migration requires an intermediate adhesion strength [43]. We previously showed that glycated collagen increases endothelial cell adhesion strength, since cells use α3β3 integrins to attach to glycated collagen rather than the α2β1 integrins they use to attach to native collagen [41]. Therefore FGF-2 could return endothelial cell adhesion strength back down to the native collagen level by decreasing α3β3 integrin binding to revealed RGD sites on glycated collagen. This intermediate adhesion strength could enable more cell migration [44].

FGF-2 did not fully restore plasmin activity or capillary-like tube formation in cells on glycated collagen. FGF-2 decreased PAI-1 in cells on glycated collagen, which should have restored plasmin activity. However, our data show that PAI-1 binds to glycated collagen. Thus more of the remaining PAI-1 may have been active and localized to the uPA:uPAR site in cells on glycated collagen, where it would inhibit plasmin activity. Capillary lumen formation occurs later in the angiogenic process than proteolysis and cell migration, representing a bridge
between the activation and resolution angiogenic phases [2]. The diminished FGF-2 effect on tube formation in cells on glycated collagen could be related to a complex interaction of PAI-1 protease and adhesive functions as well as FGF-2 stimulation and adhesive functions (Schematic 2.2). However, tube formation is also a more complex process that requires signaling from multiple growth factors and integrins to initiate specific cytoskeletal and morphological changes. Thus it is more likely that glycated collagen affects an additional aspect of tube formation (perhaps cell response to another growth factor like vascular endothelial growth factor), and therefore adding FGF-2 alone is insufficient to restore tube formation.

Some of the FGF-2 effects on the plasminogen system may also be mediated through increased vitronectin levels. When we added exogenous vitronectin, cells on native and glycated collagen responded differently. Vitronectin binds to native collagen, but shows reduced binding to denatured collagen, so it may not bind with the same affinity to glycated collagen [45]. Therefore more vitronectin may remain soluble rather than be incorporated into the matrix in cells on glycated collagen. Interestingly, vitronectin dramatically increased capillary-like tube length in cells on both native and glycated collagen. Since vitronectin is part of the provisional matrix near angiogenic sites, it may have pro-angiogenic effects that reach beyond the plasminogen system [46].

When we added exogenous uPA and PAI-1 to endothelial cells on both native and glycated collagen, the proteins affected cell migration and tube formation differently. uPA had a larger effect on tube formation, whereas PAI-1 had a larger effect on cell migration. This could be related to the overall plasminogen system activity and adhesion induced by adding these specific uPA and PAI-1 amounts, or it could be because the two proteins affect these similar
but distinct processes in different ways. Our data do show that the plasminogen system is not saturated, even for situations in which a particular component increases (e.g., PAI-1 in cells on glycated collagen). Thus both uPA and PAI-1 are potential targets for pro- and anti-angiogenic therapies in disease states. However which protein to target and at what level must be carefully considered in light of the desired effect.

2.4.3 Importance of plasminogen activating system

While these studies produced interesting new findings in growth factor and glycated collagen effects on the plasminogen system, they are not without limitations. We used HUVEC in our studies to relate to previous work in the literature and because they form capillary-like tubes in in vitro culture. However, microvascular endothelial cells may be a better model for future work. In our experiments, FGF-2 only affected PAI-1 protein and mRNA levels. This was initially surprising, since others had previously shown that FGF-2 regulates both uPA and PAI-1 [34, 35]. However, continuous FGF-2 stimulation may be required for these effects, mediator proteins such as fibrinogen may be needed to enhance FGF-2 effects, and typically FGF-2 has less of an effect on the plasminogen system in human as compared to animal cells [2, 34, 47]. Finally, additional PAI-1 and FGF-2 binding effects on glycated collagen confounded our efforts to uncover plasminogen system proteolytic mechanisms. Additional studies with recombinant proteins that separate proteolytic and adhesion effects are needed to clarify mechanisms behind our observations.
2.5 Conclusion

Our data show that FGF-2 cannot completely rescue endothelial cell capillary-like tube formation in cells on glycated collagen. Thus single growth factor therapy is unlikely to be an effective treatment to enhance angiogenesis, especially in disease models. Perhaps more importantly, our data highlight the complexity of the interactions among glycated collagen, the plasminogen system, and growth factors. Such complexities are critical to unravel, especially since the plasminogen system and angiogenesis play key roles in metabolic syndrome, atherosclerosis, and cancer growth and metastasis.
3 FGF-2 effects on the plasminogen activating system mediated by vitronectin

3.1 Introduction

In Chapter 2, we showed that FGF-2 increased plasminogen system activity in human endothelial cells cultured on native collagen. However, this increase occurred without significant changes in mRNA or protein levels of the key plasminogen system components: uPA, uPAR, or PAI-1. In this chapter, we therefore investigated whether FGF-2 impacts another key plasminogen system component: vitronectin.

Vitronectin is a glycoprotein primarily produced by liver hepatocytes that circulates in the serum at 0.25-0.45 mg/ml [175]. Native vitronectin (nVTN) is initially synthesized as a single chain polypeptide having a molecular weight of 75kDa [176, 177]. In its native form, vitronectin is folded and therefore has limited ligand binding activity. Multimeric vitronectin (mVTN) is produced when native vitronectin is cleaved by PAI-1, thrombin-serpin complexes, or heparin. The two-chain polypeptide has a molecular weight of 65kDa [178]. mVTN interacts with multiple ligands, including PAI-1, uPAR, and integrins.

Vitronectin impacts the plasminogen system through its interactions with PAI-1. PAI-1 binds to the somatomedin B vitronectin domain. When latent PAI-1 binds to vitronectin, its half-life is significantly increased due to PAI-1 stabilization in the active form. PAI-1 binding also inhibits vitronectin binding to αvβ3 and αvβ5 integrins, since the somatomedin B domain is adjacent to the vitronectin RGD (Arginine-Glycine-Aspartic acid) sequence. Multimeric and native vitronectin bind to both αvβ3 and αvβ5 integrins. The αvβ5 integrin can induce multimeric vitronectin endocytosis. Once multimeric vitronectin is endocytosed, it can either be degraded or deposited in the ECM [179, 180].
FGF-2 is an important angiogenic growth factor that increases plasminogen system activity that is able to increase uPA and PAI-1 expression [181]. However, FGF-2 also increases integrin activity either by increasing β subunit expression or by internally activating integrins though cytoskeletal remodelling and FAK activation [182]. Thus FGF-2 could also increase plasminogen system activity by binding vitronectin, and thereby preventing PAI-1 stabilization and/or localization.

We hypothesized that FGF-2 activates the αvβ5 integrin, which increases vitronectin uptake and thereby prevents vitronectin from stabilizing and/or localizing PAI-1 (Schematic 3.1). FGF-2 induced vitronectin uptake was measured in various cell types by Western blot to determine if the growth factor could in fact increase cell uptake of vitronectin. Immunofluorescence microscopy was used to view whether vitronectin was internalized or on the cell membrane. Integrins were blocked, their activation was inhibited, or their protein levels were knocked down using silencing RNA to determine how this growth factor induced integrin activation impacted vitronectin binding. Finally, plasminogen system activity in response to FGF-2 with and without vitronectin binding was measured using the Chromozym PL assay.
**Schematic 3.1: AIM 2 Hypothesis.** FGF-2 activates the \( \alpha_v\beta_5 \) integrin internally, signified by FAK phosphorylation. This integrin activation increases vitronectin uptake and thereby prevents vitronectin from stabilizing and/or localizing PAI-1. Vitronectin bound to the \( \alpha_v\beta_5 \) integrin and uPAR promote plasmin activity and lead to ECM degradation, 3D migration and tube formation.
3.2 Methods

3.2.1 Cell culture and materials

HUVEC were cultured as previously described. Bovine brain microvascular endothelial cells (BBmVEC, passages 5-9; Cell Applications INC.) were maintained in Dulbecco’s modified eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone), 1% penicillin–streptomycin (Gibco), 3 ng/ml FGF-2, and 3 μg/ml heparin (J.T.Baker). Porcine aortic endothelial cells (PAEC, passages 5-9) were maintained in DMEM supplemented with 5% FBS, 1% penicillin–streptomycin and 1% glutamine (Gibco). Porcine vascular smooth muscle cells (PvSMC, passages 5-9) were maintained in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% glutamine.

Multimeric vitronectin was created by incubating 1 mg/ml native vitronectin (Molecular Innovations) with 6M urea (Sigma) in 1x tris-buffered saline (TBS) for 1 hour at 37°C. After dialysis in 1x TBS for 18 hours, multimeric vitronectin was collected and stored at -80°C until use. Cell culture substrates were coated with 50 µg/ml native collagen (BD Biosciences) overnight at 4°C. After washing, 25,000 cells/cm² were seeded on these substrates in the respective cell media and were allowed to attach for 48 hours. Samples were then treated with FGF-2 in the respective experimental media for 24 hours.

3.2.2 Vitronectin uptake levels: Western blot

Cell vitronectin protein uptake with FGF-2 was quantified by Western blot. HUVEC, BBmVEC, PAEC and PvSMC were cultured for 48 hours on native collagen coated substrates as described, after which they were treated with 50 ng/ml FGF-2 for 24 hours. Cells were scraped off the surface in ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 2 mM NaVO₄, 2 mM PMSF, 50 mM NaF, 10%
glycerol, complete protease inhibitor, pH 7.4). Cell lysates were normalized for protein content, separated by SDS-PAGE on a 4–12% Bis-Tris gel (Invitrogen), and transferred to nitrocellulose membranes using the Invitrogen i-Blot system. Membranes were incubated overnight at 4°C with vitronectin primary antibody (Santa Cruz), followed by an anti-rabbit secondary horseradish peroxidase-conjugated antibody (Promega) for 2 hours at room temperature. Protein bands were detected using an enhanced chemiluminescence kit (Western Lightning, PerkinElmer) and visualized with a Fluorchem digital imager (Alpha Innotech). Band intensity was quantified using AlphaEase FC software.
3.2.3 Vitronectin expression: RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to assess the ability of HUVEC to produce vitronectin. mRNA was isolated using the PureLink RNA mini kit (Ambion) as per manufacturer protocol. A cDNA library was created by combining 500 ng isolated mRNA with 50 µM oligo(dT)$_{20}$ primers and annealing buffer in a 0.2 ml thin walled PCR tube. The sample was then incubated in a thermal cycler at 65°C for 5 minutes and placed on ice for 1 minute. Contents were collected and briefly centrifuged. 2X First-Strand Reaction Mix and SuperScript III/RNaseOUT enzyme mix was combined and added to the tube. The mixture was incubated in a thermal cycler for 50 min at 50°C. Reaction was terminated at 85°C for 5 min. cDNA was stored at -20°C. PCR was performed by preheating the thermal cycler to 95°C and preparing master mix: 10 µM Forward Primer, 10 µM Reverse Primer, Taq 2X Master Mix (New England BioLabs), nuclease-free water. 5 µl cDNA sample was then added into the reaction tube. Thermal cycler was set to the following settings: Initial denaturation at 95°C for 30 seconds; 30 cycles at 95°C for 30 seconds, 65°C for 30 seconds, 68°C for 1 minute; final extension at 68°C for 5 minutes and held at 4-10°C. Amplification were then stored at -20°C. Samples were then combined with TBE Hi-Density Sample Buffer (5x) (Invitrogen) and diH$_2$O and then loaded onto a 6% TBE polyacrylamide gel (Invitrogen). The gel was run at 200V for 120min with an expected current of 10-18 mA/gel (start); 4-6 mA/gel (end). The gel was stained with 0.5 µg/ml Ethidium bromide (EtBr) (Biorad) by soaking for 15 minutes with gentle agitation. The gel was rinsed and then imaged using a 302 nm UV transilluminator and protein bands were quantified by ImageJ.
3.2.4 Vitronectin localization: Immunofluorescence microscopy

Vitronectin localization (extracellular or intracellular) was determined by confocal immunofluorescence microscopy with and without cell permeabilization (cell membrane vitronectin) and acid wash (internalized vitronectin). HUVEC were cultured and treated with FGF-2 as described. Cells were then rinsed, fixed with 4% paraformaldehyde for 20 minutes at room temperature and washed 3 times for 5 minutes each in phosphate buffered saline (PBS).

Permeabilization: cell membrane bound vitronectin

Cell membrane permeabilization was used to determine the relative levels of extracellular and total vitronectin. After fixing, samples were either permeabilized with 0.1% Triton X-100 in PBS for 4 minutes at room temperature or treated with PBS alone. After washing 3 times with PBS for 5 minutes each, samples were blocked for 30 minutes with 1% BSA in PBS. After thorough washing, samples were incubated with a vitronectin primary rabbit polyclonal antibody (1:100, Santa Cruz) at room temperature for 30 minutes in 0.1% BSA. Unbound antibody was removed by rinsing with PBS. Samples were then incubated with an AlexaFluor 488 conjugated anti-rabbit secondary antibody (1:200, Life Technologies) along with Hoechst (1:2000, Invitrogen) for 1 hour at room temperature. Samples were washed in PBS, mounted by inverting cover slips onto a microscope slide with 50:50 deionized water-glycerol, and sealed with nail polish. Samples were imaged in a z-stack (1 µm depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. Mean intensity values were quantified with ImageJ. Total vitronectin was quantified in permeabilized samples, and extracellular vitronectin was quantified in unpermeabilized samples.
Acid Wash: Internalized vitronectin

Acid wash was used to remove bound antibody on the cell membrane to determine the comparative internal and total vitronectin. Live samples were incubated with a vitronectin primary rabbit polyclonal antibody at 4°C for 30 minutes in 0.1% BSA. Low temperature was used to slow metabolic processes of live cells when labeling. Unbound antibody was removed by rinsing with ice-cold PBS. To remove membrane bound antibody, cells were acid washed for 5 minutes in PBS, pH 2.7, 25 mM glycine and 3% BSA. Cells were then rinsed, fixed with 4% paraformaldehyde for 8 minutes on ice and washed 3 times for 5 minutes each in PBS [183]. Then an AlexaFluor 488 conjugated anti-rabbit secondary antibody was added with Hoechst for 1 hour at room temperature. After incubation, samples were washed and mounted as described. Samples were imaged in a z-stack by confocal microscopy. Basal and apical regions were defined as below and above the cell nucleus, respectively. Mean intensity values for the entire image stack were quantified with ImageJ.
3.2.5 Plasminogen system activity: Chromozym PL

Chromozym PL was used to determine plasmin activity in cell extracts. In our assay, uPA cleaved exogenous plasminogen to plasmin, which then cleaved Chromozym PL into a residual peptide and 4-nitroaniline (405 nm). Confluent HUVEC were cultured and treated with FGF-2 as described. HUVEC were lysed in T/T buffer (60 mM Tris hydrochloride, 0.5% Triton X-100) for 5 minutes, after which cell extracts were centrifuged at 10,000 g for 10 minutes to remove insoluble material. A final solution of cell extract, 127 ng/mL Chromozym PL (Roche), and 67 µU/mL plasminogen (Roche) was mixed in a 96 well plate. Absorbance (405 nm) was measured for 24 hours in an Infinite 200 PRO microplate reader (TECAN) maintained at 37°C. The change in 4-nitroaniline absorbance at 405 nm is directly proportional to uPA enzymatic activity. Absorbance was plotted vs. time, and the linear region slope (ΔA/min) was used to calculate plasmin activity via the following equation:

\[
\text{Plasmin Activity} \left( \frac{U}{ml} \right) = \left[ \left( \frac{V}{v} \right) \times \epsilon \times d \right] \times \left( \frac{\Delta A}{\text{min}} \right)
\]

where V is total volume (300 µl), v is cell extract volume (33 µl), ε is absorbance coefficient for 4-nitroaniline (10.4 mmol·l⁻¹·cm⁻¹), and d is light path (1 cm).
3.2.6 Integrin inhibition: blocking antibody, β5 siRNA transfection, and genistein

The role of FGF-2 induced integrin activation on vitronectin uptake was assessed using integrin blocking antibodies, downregulating β5 via siRNA, and using genistein to inhibit protein tyrosine kinase activation.

To block the αvβ5 integrin using an antibody, HUVEC were treated with 1 µg/ml anti-αvβ5 antibody (Millipore) at the same time as FGF-2 treatment. For β5 downregulation via siRNA, HUVEC were seeded at 60,000 cells/well in 1 ml of supplemented medium and cultured at 37°C for 24 hours. Lipofectamine (3 µl/well) and 10 µM β5 siRNA were added to each sample for 72 hours. Decreased β5 protein levels were confirmed by Western blot. Genistein, a protein tyrosine kinase inhibitor, was used to decrease integrin activation. Samples were pre-treated with 30 µg/ml Genistein (Sigma) for 2 hours in supplemented EBM-2. Genistein was then removed and cells were treated with FGF-2 as described.

3.2.7 Statistical Analysis

Samples were collected in triplicate and experiments performed at least two times. Data are graphed as mean ± standard deviation. Significance between two groups were compared using Student’s t-test. Comparisons among multiple groups were analyzed by two-way ANOVA with a Bonferroni post-hoc test. p-values are indicated in the figures by # p < 0.05, * p < 0.01, ** p < 0.001, unless otherwise indicated.
3.3 Results

3.3.1 FGF-2 increased vascular cell vitronectin levels in different cell lines over time.

In our previous experiments, plasminogen system activity increased with FGF-2 stimulation without any measurable increases in uPA, uPAR or PAI-1 protein. In human microvascular endothelial cells (hMVEC), uPA localization to focal adhesion sites requires vitronectin to bind to αvβ5 and to uPAR [64]. This uPA/uPAR/vitronectin/integrin complex then leads to elevated plasminogen cleavage and cell migration [75]. We therefore measured whether FGF-2 could increase the amount of vitronectin bound to the cell membrane. Both native vitronectin (nVTN) and multimeric vitronectin (mVTN) protein levels were quantified, as both proteins bind to the cell surface and form the uPA/uPAR/vitronectin/integrin complex. No significant changes were observed at 12 hours of FGF-2 treatment (data not shown). Time course and FGF-2 had a statistically significant effect on vitronectin protein level, (p < 0.05 by two-way ANOVA). Indicating that FGF-2 increased vitronectin in all cells types. nVTN and mVTN increased 1.8 and 2.2-fold compared to null in HUVEC, respectively, when treated with FGF-2 for 24 hours. By 48 hours, only mVTN was significantly increased 1.7-fold compared to null. 72 hours of treatment resulted in only a small change in mVTN compared to null (Figure 3.1 A-B). A similar peak in cell-associated vitronectin with FGF-2 treatment was observed in BBmVEC (Figure 3.1C-D; 1.4 and 1.5-fold increase in nVTN and mVTN, respectively); in PAEC (Figure 3.1E-F; 1.2 and 1.6-fold increase in nVTN and mVTN, respectively); and in PSMC (Figure 3.1G-H; 1.5 and 2.1-fold increase in nVTN and mVTN, respectively). Thus FGF-2 increased both native and multimeric vitronectin binding to different vascular cell types and across various species. All subsequent experiments were performed in HUVEC.
Figure 3.1: FGF-2 increased vitronectin in human umbilical vein endothelial cells (HUVEC, A-B), bovine brain microvascular endothelial cells (BBmVEC, C-D), porcine aortic endothelial cells (PAEC, E-F), and porcine vascular smooth muscle cells (PSMC, G-H). Cells seeded on native collagen (50 µg/ml) coated substrates for 48 hours were stimulated with 50 ng/ml FGF-2 for 24, 48 and 72 hours. Cell extracts were collected and
normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. mVTN and nVTN band intensity was quantified and normalized to GAPDH or β-actin and then normalized to null nVTN levels at 24 hours. # p < 0.05; * p < 0.01 compared to null with Bonferroni post hoc.
3.3.2 Endothelial cells produced VTN mRNA, but FGF-2 did not increase VTN binding in serum-free conditions.

This elevated vitronectin could occur through increased protein expression or binding existing vitronectin the extracellular fluid. Vitronectin is abundantly available in the serum and can range from 0.14 mg/l to 0.6 mg/l [184]. Only a limited number of cell types are able to produce vitronectin, with the majority produced by hepatocytes and then circulated throughout the body. Vitronectin is also produced in smaller quantities by endothelial, smooth muscle cell and retinal cells [87]. We therefore measured HUVEC vitronectin production with and without FGF-2 stimulation, as well as whether FGF-2 could increase vitronectin levels in the absence of external vitronectin. mVTN and FGF-2 had a statistically significant effect on vitronectin protein level, (p < 0.05 by two-way ANOVA with Bonferroni post hoc). The interaction effect was not significant, indicating that FGF-2 did not increase vitronectin binding in a serum free environment.

Through PCR, we showed that in fact HUVEC did produce vitronectin mRNA both with and without FGF-2 stimulation (Figure 3.2A). However, the growth factor’s ability to increase vitronectin mRNA was not quantifiable since we did not use real time PCR. Instead, we measured whether the FGF-2-induced increase in vitronectin was from cell-produced or serum-derived vitronectin by removing the serum from the medium. When HUVEC were stimulated with FGF-2 in EBM-2 medium with 0% FBS, Western blot showed no change in vitronectin binding with and without FGF-2. 3 µg/ml mVTN was added back into the system without serum, which increased cell-associated vitronectin by 68%. However, FGF-2 stimulation did not further enhance vitronectin uptake when serum was not present (Figure 3.2B). Similarly addition of 3 µg/ml nVTN increased cell-associated vitronectin 2-fold increase, and again
FGF-2 stimulation had no effect (Figure 3.2C). Immunofluorescence data support the Western blots, showing a similar 2-fold increase in bound vitronectin with added vitronectin but without any influence of FGF-2 in the absence of serum (Figure 3.2D, E).

**Figure 3.2:** HUVEC produced vitronectin, but FGF-2 did not increase vitronectin binding in a serum free environment. HUVEC were treated with 50 ng/ml FGF-2 +/- 3 µg/ml nVTN or mVTN for 24 hours in EBM-2 medium with 0% FBS, 1% PSG. A) RNA was isolated reverse transcriptase PCR was performed to qualitatively determine vitronectin mRNA. Vitronectin protein levels were analyzed by Western blot for HUVEC treated with B) mVTN and C) nVTN. D) Fixed samples were labeled with a vitronectin primary antibody coupled with an AF488 secondary antibody and Hoechst (nuclei). Samples were imaged in a z-stack (1 µm depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. E) Mean intensity values were quantified with ImageJ; vitronectin labeled with AF488 (green) and nuclei labeled with Hoechst (blue). All samples were normalized to -FGF2, -nVTN, -mVTN conditions. # p < 0.05; * p < 0.01 compared to null with Bonferroni post hoc.
3.3.3  FGF-2 increased vitronectin bound to the cell membrane.

Vitronectin can influence angiogenic activity in two ways. It can bind to the $\alpha_v\beta_3$ or the $\alpha_v\beta_5$ integrin and couple with uPAR/uPA complex to create the uPA/uPAR/vitronectin/integrin complex that increases plasmin activity by increasing plasminogen cleavage. Alternatively, vitronectin can bind to the $\alpha_v\beta_5$ integrin and become endocytosed to prevent PAI-1 binding and stabilization [180, 185]. To determine whether the FGF-2 induced vitronectin increase was on the cell membrane or intracellular, we performed immunofluorescence assays using an acid wash and cell permeabilization. Intracellular vitronectin was determined by an acid wash assay to remove primary antibody bound to vitronectin on the cell membrane (Figure 3.3A-B). Immunofluorescence imaging showed little vitronectin inside the cell (acid wash) and FGF-2 primarily increased extracellular vitronectin (un-permeabilized membrane) ($p < 0.05$), indicating that FGF-2 increased vitronectin protein levels on the cell membrane. Of the total amount of vitronectin associated with the cell, only about 15% was intracellular. FGF-2 increased total cellular vitronectin by 25%, but no change was seen in intracellular vitronectin. HUVEC were then labeled for vitronectin without permeabilizing the cell membrane to determine how much vitronectin was bound to the cell membrane (Figure 3.3C-D). These data show FGF-2 increased cell membrane vitronectin by 75%, which was similar to the change in total vitronectin. We therefore determined that FGF-2 increased cell membrane bound vitronectin without a significant effect on vitronectin internalization.
Figure 3.3: Immunofluorescence imaging showed little vitronectin inside the cell (acid wash) and FGF-2 primarily increased extracellular vitronectin (un-permeabilized membrane). HUVEC were cultured and exposed to FGF-2 as described. A) Immunofluorescence images of cells labeled for vitronectin (AF488, green) and nuclei with (Hoescht, blue). Specific samples were acid washed to remove primary antibody bound to vitronectin on the cell membrane, and thereby label just intracellular vitronectin. B) Samples were imaged in a z-stack (1 µm depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. C) Immunofluorescence images of cells labeled for vitronectin and nuclei as described. Specific samples were permeabilized with Triton X-100 (membrane and cytoplasmic) or not permeabilized (membrane only). D) Mean intensity values were quantified with ImageJ. All samples were normalized to -FGF2 condition. # p < 0.05 null vs. FGF-2 treated with Bonferroni post hoc.
3.3.4 Inhibiting FAK phosphorylation with Genistein abrogated FGF-2 induced increase in extracellular vitronectin and plasminogen system activity.

Growth factors such as FGF-2 activate integrins, which can bind vitronectin [186]. Integrin activation via inside-out signaling, as occurs with growth factor stimulation, occurs through focal adhesion kinase (FAK) [51]. We blocked integrin activation via FAK phosphorylation using the general protein tyrosine kinase (PTK) inhibitor, genistein [187]. Genistein and FGF-2 had a statistically significant effect on vitronectin protein level and plasmin activity, (p < 0.05 by two-way ANOVA with Bonferroni post hoc). Indicating that FGF-2 induced vitronectin binding to HUVEC was abrogated when FAK phosphorylation was inhibited using a protein tyrosine kinase inhibitor. Genistein abolished the ability of FGF-2 to increase vitronectin binding to the cell membrane. By immunofluorescence, genistein decreased vitronectin bound to the cell membrane by 28%, with no increased vitronectin bound with added FGF-2 (Figure 3.4A-B). By Western blot, genistein decreased cell associated vitronectin (with or without FGF-2) by more than 70% (Figure 3.4C). Plasminogen system activity decreased by 34% in the presence of genistein, and FGF-2 was not able to restore this effect (Figure 3.4D).
Figure 3.4: FGF-2 induced vitronectin binding to HUVEC was abrogated when FAK phosphorylation was inhibited using a protein tyrosine kinase inhibitor. HUVEC were exposed to 30 µg/ml genistein for 2 hours prior to stimulation with 50 ng/ml FGF-2 for 24 hours. A) Samples were fixed and permeabilized with 0.1% Triton X-100 in PBS for 4 minutes at room temperature or treated with PBS alone. Samples were then incubated with a vitronectin primary antibody along with Hoechst. Samples were imaged in a z-stack (1 µM depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. (vitronectin = green, nuclei = blue) B) Mean intensity values were quantified with ImageJ. Total vitronectin was quantified in permeabilized samples, and extracellular vitronectin was quantified in unpermeabilized samples. C) Cell extracts were collected and normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence
kit and visualized with a Fluorchem digital imager. Vitronectin band intensity was quantified and normalized to GAPDH and then normalized to null levels. D) Samples were lysed in T/T buffer and prepared for the Chromozym assay. Absorbance (405 nm) was measured for 24 hours. Plasmin enzymatic activity was calculated from the change in 4-nitroaniline absorbance at 405 nm. All samples were normalized to -FGF2, -Genistein, Membrane (IF) conditions. # p < 0.05 compared to null with Bonferroni post hoc.
3.3.5 Blocking the αvβ5 integrin inhibited vitronectin uptake and decreased plasminogen system activity.

Vitronectin has two integrin ligands on the cell membrane, αvβ3 and αvβ5, and preferential binding to either integrin has not been determined [188]. We therefore decided to block the αvβ5 integrin using a blocking antibody to determine if this integrin was important in FGF-2 induced increased in cell vitronectin binding. We first performed an immunofluorescence assay to examine cell membrane vitronectin by not permeabilizing the cells (Figure 3.5A). The blocking antibody did not completely inhibit vitronectin bound to the cell membrane; however, it did remove any increase in vitronectin binding with FGF-2 (Figure 3.5B). These data were confirmed by Western blot performed under the same conditions. anti-αvβ5 integrin and FGF-2 had a statistically significant effect on vitronectin protein level and plasmin activity, (p < 0.05 by two-way ANOVA with Bonferroni post hoc). Indicating that FGF-2 induced cell binding of vitronectin was removed when the αvβ5 integrin was blocked. Vitronectin increased by 50% with the FGF-2 addition; however this increase was blocked when the anti-αvβ5 antibody was used (Figure 3.5C). To assess if preventing vitronectin binding to the cell membrane by blocking the integrin impacted plasminogen system activity, we performed a Chromozym PL assay (Figure 3.5D). FGF-2 increased plasminogen system activity by 36%. Blocking the αvβ5 integrin eliminated the increase in plasminogen system activity seen with FGF-2.
Figure 3.5: FGF-2 induced cell binding of vitronectin was removed when the αvβ₅ integrin was blocked. HUVEC were treated with 1 µg/ml anti-αvβ₅ blocking antibody concurrently with 50 ng/ml FGF-2 for 24 hours. A) Samples were fixed and some samples were permeabilized with Triton X-100 (total vitronectin) while some samples were unpermeabilized (membrane vitronectin). Cells were labeled for vitronectin (AlexaFluor 488, green) and nuclei (Hoechst, blue). B) Samples were imaged in a z-stack (1 µm depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. Mean intensity values were quantified with ImageJ. Total vitronectin was quantified in permeabilized samples, and extracellular vitronectin was quantified in unpermeabilized samples. C) Following anti-αvβ₅ blocking antibody and FGF-2 treatment, cell extracts were collected and normalized protein samples
were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. Vitronectin band intensity was quantified and normalized to GAPDH and then normalized to null levels. D) Samples were lysed in T/T buffer and prepared for the Chromozym assay. Absorbance (405 nm) was measured for 24 hours. Plasmin enzymatic activity was calculated from the change in 4-nitroaniline absorbance at 405 nm. All samples were normalized to -FGF2, -anti-αvβ5, Membrane (IF) conditions. # p < 0.05 compared to null with Bonferroni post hoc.
3.3.6 Silencing the β5 integrin inhibited vitronectin uptake and decreased plasmin system activity.

Finally, we silenced the αvβ5 integrin using siRNA to completely remove any activation of this integrin by FGF-2, and vitronectin binding was measured by immunofluorescence and Western blot. β5 siRNA and FGF-2 had a statistically significant effect on vitronectin protein level and plasmin activity, (p < 0.05 by two-way ANOVA). Indicating that FGF-2 induced cell binding of vitronectin was abrogated by silencing the β5 integrin with siRNA. β5 siRNA knockdown completely abolished FGF-2 induced vitronectin uptake. By immunofluorescence, β5 siRNA knockdown decreased total and membrane bound vitronectin (with or without FGF-2) more than 25% (Figure 3.6A-B). Western blot corroborated immunofluorescence data and confirmed that silencing the β5 integrin reduced the capability of the cell to bind vitronectin by 90%. FGF-2 increased vitronectin binding by 2-fold with the αvβ5 integrin intact; however with the β5 integrin silenced, the growth factor had no impact on vitronectin binding (Figure 3.6C). β5 siRNA knockdown eliminated FGF-2 induced plasminogen system activity (Figure 3.6D).
Figure 3.6: FGF-2 induced cell binding of vitronectin was abrogated by silencing the β5 integrin with siRNA. 250,000 cells/well were seeded on native collagen (50 µg/ml) coated substrates for 24 hours after which lipofectamine was used to transfect the cells with β5 siRNA for 72 hours. Samples were then treated with 50 ng/ml FGF-2 for 24 hours. A) Samples were fixed and permeabilized with 0.1% Triton X-100 in PBS for 4 minutes at room temperature or treated with PBS alone. Samples were incubated with a vitronectin antibody along with Hoechst. Samples were imaged in a z-stack (1 µM depth, 12 images per sample) to obtain fluorescence intensity throughout the cell. (Vitronectin = green, nuclei = blue). B) Mean intensity values were quantified with ImageJ. Total vitronectin was quantified in permeabilized samples, and extracellular vitronectin was quantified in unpermeabilized samples. C) Cell
extracts were collected and normalized protein samples were analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. Vitronectin band intensity was quantified and normalized to GAPDH and then normalized to null levels. D) Samples were lysed in T/T buffer and prepared for the Chromozym assay. Absorbance (405 nm) was measured for 24 hours. Plasmin enzymatic activity was calculated from the change in 4-nitroaniline absorbance at 405 nm. All samples were normalized to -FGF2, -β5 siRNA, Membrane (IF) conditions. # p < 0.05 compared to null with Bonferroni post hoc.

Schematic 3.2: AIM 2 Summary. FGF-2 activates αvβ5 and the αvβ3 integrin, signified by FAK phosphorylation. However, only the αvβ3 integrin binds vitronectin from the serum, leading to increased plasmin activity, ECM degradation, 3D migration and tube formation.
3.4 Discussion

3.4.1 FGF-2 pulls vitronectin from the serum to increase plasmin activity

Growth factors increase angiogenic activity via many pathways and targets [181]. Previously we demonstrated that FGF-2 increased plasminogen system activity without any direct increases in uPA or uPAR, or any reduction in PAI-1. We therefore investigated how FGF-2 influenced vitronectin. We now show that FGF-2 is able to increase vitronectin bound to the cell membrane, thereby removing ability of the protein to stabilize PAI-1 and actively cleave plasmin in the complex with uPA/uPAR/integrins.

FGF-2 increased vitronectin levels in various cell types, including HUVEC, BBmVEC, PAEC, and PSMC. The cell-associated vitronectin came from the serum, rather than from cell production. Interestingly, FGF-2 only increased vitronectin binding with serum in the medium. When vitronectin alone was added to serum-free medium, FGF-2 had no effect. This suggests that additional serum proteins may be required to potentiate the FGF-2 effects on vitronectin.

We added fibrinogen in combination with FGF-2 in serum-free conditions to potentiate the growth factor influence [97]; however no change was seen. Since we were unable to determine the specific effect of serum, we used serum-containing medium in the remainder of our experiments.

This growth factor influence is interesting because we highlight the ability of vitronectin to be pulled from the serum to increase angiogenic capabilities (Schematic 3.2). Growth factors could therefore be used in disease states in which there are elevated levels of vitronectin and PAI-1 circulating in the plasma like atherosclerosis [189], type 2 diabetes and Alzheimer’s disease [190], age related macular degeneration, systemic amyloidosis and glomerulonephropathy [191].
3.4.2 The majority of the vitronectin binding induced by FGF-2 occurred at the cell membrane

Vitronectin can either remain bound on the cell surface or become endocytosed [81]. Utilizing two well-known imaging techniques to view vitronectin location throughout the cell, we determined that most FGF-2 induced vitronectin binding occurred at the cell membrane. The cell-associated vitronectin decreased after 24 hours. This could be caused by vitronectin unbinding from the integrin as the growth factor effects decline over time, or vitronectin endocytosis could take place at later times. It would be interesting to examine intracellular vitronectin at later time points. This would help determine the timing of growth factor treatment to realize continuous pro-angiogenic effects.

Vitronectin deposition is elevated in tumor cell ECM. This leads to increased cell adhesion and migration [192]. This research now has potential to explain the reason why this deposition is so high and a target for cancer therapy. Vitronectin bound to the cell membrane can be used as an early biomarker for tumor detection, as the αvβ5 integrin has been shown to be highly expressed in tumor cells [193]. It is likely that growth factors in cancer continually bind vitronectin to the cell membrane in early phases leading to an accumulation of vitronectin in the ECM after time.
3.4.3 αvβ5 is a critical integrin responsible for FGF-2 induced vitronectin binding

FGF-2 increased vitronectin binding via αvβ5 integrin activation. Integrin activation is essential in cell migration and plasmin activity [98]. Blocking, silencing and inhibiting αvβ5 integrin activation eliminated any growth factor based increase in vitronectin binding and plasminogen system activity. We believe that FGF-2 induced integrin activation promoted uPAR/uPA/vitronectin/integrin complex formation to localize plasminogen activation (Schematic 3.2).

More specifically for disease related therapy, the up regulation or down regulation of the αvβ5 integrin has potential to be modulated in the desired angiogenic direction. Silencing the integrin could have the potential to be a very powerful tumor repressor. Activating the integrin would increase neovascularization in areas of need. Another interesting area of research would be to explore the possibility of using a drug delivery vehicle coated with αvβ5 integrins. The vehicle would be able to remove excess vitronectin in the system that promotes PAI-1 stabilization.
3.4.4 Limitations

This research is not without limitations. We focused on growth factor effects at 24 hours, since the greatest vitronectin response occurred at this time point. Assays at later times could highlight the whether vitronectin is released from the integrin or endocytosed, which could be critical to long-term effects.

The amount of vitronectin added to the system in a serum free environment may have reached membrane bound saturation. Therefore growth factor administration would have no further effect on this. Lower concentrations of vitronectin should be added to a serum free system to assess this.

This study was done in cells on a static native collagen substrate with limited protein interaction. The next step would be to view changes in a dynamic environment, specifically in a parallel plate flow chamber. Shear stress has the ability to activate a number of integrins. It would be interesting to see if the mechanical influence of shear stress modulates vitronectin uptake, and to see if disturbed flow has a different effect. After mechanical in vitro assays have been conducted, it would be ideal to move into a mouse model to see if administering growth factor still resulted in an increase in vitronectin.
3.5 Conclusion

FGF-2 increased plasminogen system activity through increased vitronectin binding, which occurred primarily through the $\alpha_v\beta_5$ integrin activation. FGF-2 activated the $\alpha_v\beta_5$ integrins intracellularly. Once the integrins were activated, they bound vitronectin extracellularly. Vitronectin binding to integrins increased plasminogen system activity by: 1. removing circulating vitronectin from the serum, which would have stabilized PAI-1, and 2. localizing uPA to uPAR, which is required for plasminogen cleavage. These data demonstrate a new way in which FGF-2 impacts the plasminogen system and subsequent angiogenesis.
4 Aβ42 peptide effects on growth factor-stimulated angiogenesis

4.1 Introduction

Patients with Alzheimer’s disease (AD) have reduced blood flow in the brain, which may relate to a decline in cognitive function [30]. Amyloid β (Aβ) plaque accumulation is thought to cause much of this dysfunction [36]. In vitro models showed that an elevated concentration of Aβ reduced HUVEC ability to form capillary networks [36]. In vivo research supports the idea that Aβ decreases angiogenesis. Frontal cortex sections from 36 patients with AD displayed total cortical capillary length reduction [38].

Growth factor therapy has been suggested as a therapeutic option for diminished angiogenesis. Growth factors have pro-angiogenic effects. VEGF and FGF-2 increase endothelial cell proliferation, migration, and plasminogen system activity [115-117]. Specifically, growth factors activate the intracellular signalling proteins ERK and Akt. These protein pathways are especially important in cell proliferation, survival and migration [36, 132]. Growth factors increase plasminogen system activity through increased uPA expression, which leads to increased ECM breakdown [96]. Similarly growth factors increase the inhibitor PAI-1 expression to inhibit uncontrolled ECM breakdown. This process creates a dynamic ECM into which endothelial cells invade and form capillary networks [97].

Endothelial cell dysfunction can arise from Aβ aggregation. There are two forms of Aβ that are thought to cause dysfunction in the endothelial cells in patients with AD: Aβ40 and Aβ42 [194]. Aβ42 has been shown to be the most toxic [195]. Each of the peptides has a secondary structure formed through aggregation that also has toxicity implications. The peptide is initially cleaved and released as monomers, which can then aggregate to form oligomers. From there, further aggregation leads to fibril formation [30]. Environment pH can influence the
aggregation of monomers into oligomers. The Aβ N-terminus domain is present as a soluble monomeric α-helical structure at pH 1-4 and 7+; however within the pH range of 4-7 the peptide precipitates and forms the β-sheet seen in oligomers. AD patients have a lower pH than normal patients [196, 197]. As the peptide aggregates to fibril form, β-strands are the dominant structure, with the core of the structure containing 2 or more β-sheets [198]. While the debate on which peptide form is responsible for toxicity continues, there is increasing evidence that the intermediates, oligomers, have increased toxicity compared to monomers and fibrils. Furthermore, elevated aggregation of any secondary structure leads to late stages of AD [194, 199, 200].

Neurotoxicity of specific secondary structures may result from interaction with growth factors. Lindahl et al showed that Aβ fibrils and FGF-2 have a common binding site on heparin sulphate (HS) in AD cerebral cortex samples. Aβ fibril binding to HS increases fibril deposition and neurotoxicity. FGF-2 has a similar binding specificity to HS as Aβ fibrils, which is not shared by Aβ monomers, as determined by nitrocellulose filter trapping experiments quantified through radiolabeling Aβ [201]. VEGF prevented Aβ aggregation through Aβ binding to VEGF heparin-binding domain [202]. Conversely, Aβ monomers block VEGF signalling by binding to VEGFR-2. Aβ decreased HUVEC capillary formation on Matrigel, and this decrease was rescued by increased VEGF concentrations [36]. However, limited research has compared the interaction of different Aβ structures with growth factors directly.

We hypothesized that Aβ would differentially affect endothelial cell response to the growth factors VEGF and FGF-2 depending on Aβ secondary structure (Schematic 4.1). We measured bovine brain microvascular endothelial cell proliferation, capillary network formations, and plasminogen system activity with different forms of Aβ and varying growth factor
concentrations. These data will elucidate growth factor effects and the potential of growth factor therapy in different stages of AD.

**Schematic 4.1: AIM 3 Hypothesis.** Aβ inhibit endothelial cell angiogenic processes in response to growth factors by competing for VEGFR and inhibiting VEGF binding. Leading decreased plasmin activity, ECM degradation, 3D migration, and tube formation.
4.2 Methods

4.2.1 Cell Culture, Aβ preparation/verification, growth factors

Cell Culture

Bovine brain microvascular endothelial cells (BBMEC, passages 5-8; Cell Applications) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Cellgro) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 1% penicillin/streptomycin (PS) (Gibco), 3 ng/ml FGF-2 (Peprotech), and 3 μg/ml heparin (Baker). Cells were seeded on tissue culture plates coated with 10 μg/ml type I rat tail collagen (BD Biosciences) for stable cell attachment. BBmVEC were selected because they are widely used for in vitro brain microvascular endothelial cell studies, show a robust VEGF response, and react with plasminogen system proteins, antibodies and most importantly Aβ42 peptide.

Aβ preparation/verification

To obtain monomeric Aβ42 (Aβ42-M) from recombinant protein Aβ42 (Yale), 1 mg of lyophilized peptide was dissolved in 1 mL of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and allowed to air dry in a chemical fume hood for 1 hour, followed by further drying in a SpeedVac centrifuge for 30 min. The clear peptide film was reconstituted in 100% dimethyl sulfoxide (DMSO; Life Technologies) to achieve a stock solution of 1 mM. The reconstituted stock solution was aliquoted for storage at -80°C. To obtain oligomeric Aβ42 (Aβ42-O), peptide was dissolved in 10 mM NaOH to 2 mg/ml. Secondary Aβ peptide structure was verified by native Western blot. 10 μl Aβ42 was loaded with diH₂O and 2.5 μl 4x NativePAGE Sample Buffer (Life Technologies) onto NativePAGE Bis-Tris gel (Life Technologies). XCell SureLock Mini-Cell was prepped with 1X NativePAGE Anode buffer and 1X NativePAGE Cathode buffer. The gel was run at 150V, 12-16 mA/gel (start); 2-4
mA/gel (end) for 90-115 minutes. The gel was then fixed with 40% methanol, 10% acetic acid in diH2O, microwaved for 45 seconds and then placed on orbital shaker for 20 minutes at room temperature. This process was repeated twice. The gel was then stained with Coomassie R-250 (0.02% Coomassie R-250 in 30% methanol and 10% acetic acid in diH2O) in the microwave for 45 seconds and then placed on orbital shaker for 20 minutes at room temperature. Destain solution (8% acetic acid in diH2O) was then added to the gel, microwaved for 45 seconds, and then incubated as long as needed to obtain proper background. Bands were visualized with a Fluorchem digital imager (Alphalnotech). Band intensity was quantified using AlphaEase FC software. Stock peptide solution was diluted in cell culture medium to obtain the appropriate final concentration.

**Growth factor administration**

Cell culture substrates were coated with 50 µg/ml native collagen (BD Biosciences) overnight at 4°C. After washing, 25,000 bovine brain microvascular endothelial cells (BBMvEC)/cm² were seeded on these substrates in supplemented DMEM without FGF-2. This experimental medium was used for all growth factor experiments. Cells were allowed to culture for 48 hours and then treated with 5 µM Aβ42 +/- FGF-2 or VEGF at 10, 25, and 50 ng/ml for 24 hours.
4.2.2 Cell Count: Proliferation and Aβ42 Toxicity Study

Cell Proliferation

The impact of Aβ42 with and without growth factor stimulation ability on endothelial cell proliferation was measured by cell counts. 4,000 BBMvEC/cm² were cultured for 24 hours on native collagen coated substrates for 24 hours in experimental DMEM. Cells were counted 24 hours after seeding using a particle counter (Beckman Coulter). 5 μM Aβ42 +/- FGF-2 or VEGF in experimental DMEM was then added to cells on days 1 and 3. On days 3 and 5, BBMvEC were trypsinized and counted.

Aβ42 Toxicity Study

Aβ42 toxicity was measured to determine if cell proliferation measurements needed to be adjusted for cell death. 125,000 cells/cm² were cultured for 48 hours on native collagen coated substrates in experimental DMEM. Cells were then subjected to 24 hours incubation with 1 μM, 5 μM, and 10 μM of Aβ42; 97% Methanol (MeOH) was added to the cells for 20 minutes for positive control. Samples were then treated with Eth-D and Calcein AM (1:500) for Live/Dead imaging (Novex) for 30 minutes. Samples were imaged by fluorescent microscopy and mean intensity per channel was determined with Image J.

4.2.3 ERK1/2 and Akt protein phosphorylation levels: Western blot

Aβ42 effects on growth factor signaling were measured through the Erk and Akt pathways. 25,000 cells/cm² BBmVEC were cultured for 48 hours on native collagen coated substrates. To view growth factor induced phosphorylation changes in ERK1/2, BBmVEC were dosed with 5μM Aβ42, +/- 50 ng/ml FGF-2 for 15 minutes in experimental medium. Phosphorylation changes in Akt were measured by dosing cells with 5 μM Aβ42 +/- 50 ng/ml VEGF for 60 minutes in experimental medium. Cells were scraped off the surface in
ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 2 mM NaVO₄, 2 mM PMSF, 50 mM NaF, 10% glycerol, complete protease inhibitor, pH 7.4). Cell lysates were normalized for protein content, separated by SDS-PAGE on a 4–12% Bis-Tris gel (Invitrogen), and transferred to nitrocellulose membranes using the Invitrogen i-Blot system. Membranes were incubated overnight at 4°C with phospho-p44/42 MAPK primary antibody (pERK1/2; Cell Signaling) or pAkt primary antibody (Cell Signaling), followed by an anti-rabbit secondary horseradish peroxidase-conjugated antibody (Promega) for 2 hours at room temperature. Protein bands were detected using an enhanced chemiluminescence kit (Western Lightning, PerkinElmer) and visualized with a Fluorchem digital imager (Alpha Innotech). Band intensity was quantified using AlphaEase FC software. Phosphorylated protein was normalized by incubating stripped membranes overnight at 4°C with p44/42 MAPK primary antibody (total ERK1/2; Cell Signaling) or Akt primary antibody (Cell Signaling), followed by an anti-rabbit secondary horseradish peroxidase-conjugated antibody (Promega) for 2 hours at room temperature. Imaging and band intensity were analyzed as previously described.
4.2.4 3D Migration: Cell Invasion Assay

Aβ42 effects on endothelial cell invasion through a native collagen matrix was quantified using a Boyden chamber assay. The bottom side of a Transwell chamber (6.5 mm diameter, 8 μm pore size; Corning Costar) was coated with 100 μg/ml native collagen, which was allowed to gel for 1 hour at room temperature. 150,000 cells were then added to the top of each chamber in DMEM, 5% fetal bovine serum, 1% Pen/Strep), 3 μg/ml heparin treated with +/- 5μM Aβ42, +/- 50 ng/ml FGF-2. DMEM with 10% FBS was added to the bottom chamber to act as a cell chemoattractant. Samples were incubated for 24 hours at 37°C, after which the cells remaining in the upper Transwell chamber were removed by gentle cotton swabbing. Cells that migrated to the chamber bottom were labeled with Hoechst (10 ng/ml; Invitrogen) for 30 minutes. 5 randomly selected areas per sample were imaged using an Olympus IX81 inverted fluorescent microscope. Cell number was quantified with ImageJ.

4.2.5 Tube Formation: Cell Extension Assay

The role of Aβ42 in capillary network formation was determined via a cell extension assay. BBmVEC were seeded on native collagen gels, which were prepared by incubating 50 μl 4 mg/ml collagen I at 37°C for 1 hour. 15,000 BBmVEC were added to the top of each gel in 50 μL of experimental medium (DMEM, 5% fetal bovine serum, 1% Pen/Strep), 3 μg/ml heparin treated with +/- 5μM Aβ42, +/- 50 ng/ml FGF-2 or VEGF. Cells were incubated for 18 hours at 37°C, after which samples were imaged using a Nikon Eclipse TS100 phase contrast microscope with a Nikon DS-Fi1 CCD camera (5 images per well). Tube length and number of tubes/image were manually analyzed using ImageJ [164].
4.2.6 Plasmin Activity: Chromozym PL

Aβ42 effects on plasminogen system activity were evaluated with a Chromozym PL assay. BBmVEC were cultured for 48 hours on native collagen coated substrates then dosed with 5μM Aβ42, +/- 50 ng/ml FGF-2 or VEGF for 24 hours. Samples were rinsed with PBS and collected by incubating with 200 μl of 60 mM Tris hydrochloride with 0.5% Triton X-100 (T/T buffer) for 5 minutes at room temperature on an orbital shaker. Samples were then washed and collected. Then centrifuged at 10,000 g for 10 minutes, after which the supernatant was collected. The supernatant was then used in the Chromozym PL assay, in which exogenous plasminogen was cleaved to plasmin, which then cleaved Chromozym PL into a residual peptide and 4-nitroaniline (405 nm). The change in 4-nitroaniline absorbance at 405 nm is directly proportional to plasmin enzymatic activity. Absorbance was plotted vs. time, and the linear region slope (ΔA/min) was used to calculate plasmin activity via the following equation:

\[
Plasmin\ Activity \left( \frac{U}{ml} \right) = \left( \frac{V}{v} \right) \times \epsilon \times d \times \left( \frac{\Delta A}{\text{min}} \right)
\]

where V is total volume (300 μl), v is cell extract volume (33 μl), ε is absorbance coefficient for 4-nitroaniline (10.4 mmol·1·cm⁻¹), and d is light path (1 cm).

4.2.6 Statistical Analysis

Samples were collected in triplicate and experiments performed at least two times. Data are graphed as mean ± standard deviation. Significance between two groups were compared using Student’s t-test. Comparisons among multiple groups were analyzed by two-way ANOVA with a Bonferroni post-hoc test. p-values are indicated in the figures by # p < 0.05, * p < 0.01, ** p < 0.001, unless otherwise indicated.
4.3 Results

4.3.1 Aβ42-O had limited influence on FGF-2 increases in cell proliferation and ERK activation

Donnini et al have shown that a concentration of Aβ40 from 5-50μM caused apoptosis (Tunnel assay), down regulated FGF-2 production, inhibited FGF-2 binding to heparin and decreased FGFR1 phosphorylation (Western blot). FGF-2 overexpression restored angiogenic activity in the presence of high Aβ40 concentrations [132]. Both Aβ40 and Aβ42 have been shown to have toxic influences on cell systems. We used Aβ42 since preliminary data were more consistent. Therefore we hypothesized that FGF-2 would restore BBmVEC proliferation in the presence of Aβ42-O. Aβ42-O and FGF-2 had a statistically significant effect on cell proliferation ERK phosphorylation level, (by two-way ANOVA). Indicating that Aβ42-O did not affect FGF-2 induced endothelial cell proliferation or ERK phosphorylation. On day 3, Aβ42-O did not affect cell number, but FGF-2 increased cell number by more than 50% either with or without Aβ42-O. By day 5, Aβ42-O decreased cell number by 28% and FGF-2 increased cell number by 27%. In the presence of Aβ42-O, FGF-2 increased cell number by 29% (Figure 4.1A). Aβ42-O was determined not to be toxic. There was only a 0.75%, 2.8% and a 5.9% increase in dead cells when cells were treated with 1 µm, 5 µm and 10 µm Aβ42-O respectively when compared to null (Figure 4.1B). Only FGF-2 increased phosphorylated ERK1/2 by more than 60%, while Aβ42-O had no effect on ERK activation (Figure 4.1C-D). FGF-2 can overcome the influence of Aβ42-O in short and long term assays. We do however see decreased cell number by Aβ42-O at longer time points (after 4 days of exposure).
Figure 4.1: Aβ42-O did not affect FGF-2 induced endothelial cell proliferation or ERK phosphorylation. A) BBMVEC were seeded at 4,000 cells/cm² on native collagen coated substrates and allowed to attach for 24 hours. Cells were then treated with 5 µM Aβ42-O +/- 50 ng/ml FGF-2 on days 1 and 3, and cells were counted on days 1, 3, and 5. # p < 0.05, * p < 0.01 compared to null day 3 and day 5. B) 125,000 cells/cm² were cultured for 48 hours on native collagen coated substrates in experimental DMEM. Cells were then subjected to 24 hours of 1 µM, 5 µM, and 10 µM Aβ42-O, and cell death was measured using a Live/Dead assay (green = live, red = dead). Mean fluorescence intensity per channel was determined with Image J. ** p < 0.001 compared to null. C) BBmVEC were seeded at 25,000 cells/cm² on native collagen coated substrates and cultured for 48 hours. Samples were then stimulated with 50 ng/ml FGF-2 for 15 minutes in experimental medium. Cell extracts were collected and normalized protein samples were analyzed by Western blot. D) Protein bands were detected
using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. pERK band intensity was quantified and normalized to total ERK. * p < 0.01 compared to null. # p < 0.05 compared to Aβ42-O (indicated with brackets) with Bonferroni post hoc.

### 4.3.2 Aβ42-O had limited influence on FGF-2 induced plasmin activity and 3D migration

We next measured whether FGF-2 impacts endothelial cell plasmin activity and 3D migration in the presence of Aβ42-O (Figure 4.2A). Aβ42-O and VEGF had a statistically significant effect on plasmin activity and cell migration, (p < 0.05 by two-way ANOVA). Indicating that Aβ42-O did not change FGF-2 stimulated plasmin activity or 3D migration. Aβ42-O did not affect plasminogen system activity, while FGF-2 increased plasminogen system activity by more than 2-fold with or without Aβ42-O (Figure 4.2B). FGF-2 increased BBmVEC migration through the collagen coated Boyden chamber by 80% with or without Aβ42-O, and Aβ42-O alone did not affect 3D migration (Figure 4.2C-D). These data show that Aβ42-O does not affect FGF-2 induced BBmVEC plasmin activity or 3D migration in collagen gels.
**Figure 4.2: Aβ42-O did not change FGF-2 stimulated plasmin activity or 3D migration.**

BBmVEC were seeded at 25,000 cells/cm² on native collagen coated substrates and cultured for 48 hours. Samples were then stimulated with 50 ng/ml FGF-2 for 24 hours in experimental medium. Cell lysates were collected and assessed for plasminogen system activity using the Chromozym PL assay. A) Chromozym PL absorbance at 405nm over 4 hours in slope region. B) Change in absorbance was multiplied by volume, absorbance and light path constants to quantify plasmin activity. * p < 0.01 compared to null and to Aβ42-O (when indicated with brackets). 3D cell migration was measured using a Boyden chamber assay. 150,000 BBmVEC with 5 µM Aβ42-O +/- 50 ng/ml FGF-2 were added to the top of an 8.0 µm pore Transwell insert coated with 100 µg/ml native collagen. After 24 hours, cells that migrated to the chamber bottom were labeled with Hoechst. C) Sample fluorescent microscopy images of migrated
cells. D) Cell migration as quantified with ImageJ. All samples were normalized to -FGF2, -Aβ42-O condition. #p<0.05 compared to null and to Aβ42-O (indicated with brackets) with Bonferroni post hoc.

4.3.3 Aβ42-O had limited influence on FGF-2 induced tube formation

We next measured whether FGF-2 impacts endothelial cell tube formation in the presence of Aβ42-O. Aβ42-O and FGF-2 had a statistically significant effect on tube length and number of tubes/image, (p < 0.05 by two-way ANOVA). Indicating that Aβ42-O did not change FGF-2 stimulated tube length. Tube network length increased by more than 50% with FGF-2 but was not altered with added Aβ42-O (Figure 4.2B). FGF-2 increased the number of tubes per image by 53% compared to null. Aβ42-O decreased tubes per image by 16% and FGF-2 was not able to change this effect (Figure 4.2C). These data show that Aβ42-O has limited effect on BBmVEC tube formation in collagen gels.
Figure 4.3: Aβ42-O did not change FGF-2 stimulated tube formation. A) Sample phase contrast images of the tube formation assay. Endothelial network formation was determined with a tube formation assay. BBmVEC were seeded at 15,000 cells/well in a 96 well plate on native collagen (4mg/ml) gels for 18 hours dosed with 5 µM Aβ42-O +/- 50 ng/ml FGF-2 in experimental medium. B) Tube length and C) number of tubes per image was manually analyzed using ImageJ. Samples were normalized to -FGF2, -Aβ42-O condition. # p < 0.05 compared to null and to Aβ42-O (indicated with brackets) with Bonferroni post hoc.
4.3.4 Aβ42-O had limited influence on VEGF-induced increases in cell proliferation and Akt activation

VEGF was previously shown to rescue human brain microvascular endothelial cell tube formation on Matrigel in the presence of Aβ42-O in a concentration dependent manner [36]. Therefore we measured the effect of VEGF on BBmVEC proliferation with Aβ42-O. Aβ42-O and VEGF had a statistically significant effect on cell number on day 3 and day 5 and pAkt protein level (p < 0.05 by two-way ANOVA). Indicating that Aβ42-O did not change the influence of VEGF on cell proliferation or Akt phosphorylation. VEGF increased cell number by 45% and 62% on day 3 and 5, respectively. There were no significant changes in cell number with Aβ42-O (Figure 4.3A). Akt phosphorylation increased 37% with VEGF stimulation for 60 minutes. Again, Aβ42-O had no influence on VEGF effects (Figure 4.3B). In these experiments, Aβ42-O did not affect cell proliferation or intracellular signaling with or without VEGF.
Figure 4.4: Aβ42-O did not change the influence of VEGF on cell proliferation or Akt phosphorylation. A) BBMVEC were seeded at 10,000 cells/well on native collagen coated substrates and allowed to attach for 24 hours. Cells were then stimulated with 50 ng/ml VEGF on days 1 and 3 in experimental medium and counted on days 1, 3, and 5. # p < 0.05, * p < 0.01 compared to null day 3 and day 5. B) BBmVEC were seeded at 25,000 cells/cm² on native collagen coated substrates for 48 hours and then stimulated with 50 ng/ml VEGF for 15 minutes in experimental medium. Cell extracts were collected and normalized protein samples analyzed by Western blot. Protein bands were detected using an enhanced chemiluminescence kit and visualized with a Fluorchem digital imager. pAkt band intensity was quantified and normalized to total Akt. # p < 0.05 compared to null and to Aβ42-O (indicated with brackets) with Bonferroni post hoc.
4.3.5 Aβ42-O had limited influence on VEGF induced plasmin activity and tube formation

Aβ42-O and VEGF had a statistically significant effect on plasmin activity, tube length, and number of tubes/image (p < 0.05 by two-way ANOVA). Indicating that Aβ42-O did not affect VEGF dependent plasmin activity but decreased growth factor dependent tube length. VEGF increased tube length by 70%, but tube length was not altered by addition of Aβ42-O alone. Plasminogen system activity more than doubled with VEGF, but the Aβ42-O peptide had no effect (Figure 4.4A). Aβ42-O in combination with VEGF attenuated the growth factor influence (Figure 4.4B). Aβ42-O alone decreased the number of tubes by 50% compared to null and VEGF increased that number by 200% (Figure 4.4C). These data suggest that Aβ42-O reduces the pro-angiogenic VEGF influence without affecting cell proliferation or the plasminogen activation system.
Figure 4.5: Aβ42-O did not affect VEGF dependent plasmin activity but decreased growth factor dependent tube length. A) BBmVEC were seeded at 25,000 cells/cm² on native collagen coated substrates for 48 hours and then stimulated with 50 ng/ml VEGF for 24 hours in experimental medium. Plasminogen system activity was measured in cell extracts by the Chromozym PL assay. ** p < 0.001 compared to null and to Aβ42-O (when indicated with brackets). B) Tube length was determined with a tube formation assay. BBmVEC were seeded at 15,000 cells/well in a 96 well plate on native collagen (4 mg/ml) gels for 18 hours and then stimulated with 5 µM Aβ42-O +/- 50 ng/ml VEGF in experimental medium. Samples were imaged by phase contrast microscopy (5 images per well). C) Tube length and D) number of tubes per image was manually analyzed using ImageJ. Normalized to null conditions. # p < 0.05, * p < 0.01 compared to null and to Aβ42-O (when indicated with brackets) with Bonferroni post hoc.
4.3.6 **VEGF had a concentration dependent influence on Aβ42 monomers in cell proliferation**

We have shown that the oligomeric form of the peptide had limited influence on VEGF induced responses at saturating growth factor levels. However, others have shown a dose dependent effect of VEGF on human brain microvascular endothelial cell response to Aβ42 monomers [36]. We therefore measured whether this dose dependent effect depended on Aβ42 structure. Aβ42 and VEGF had a statistically significant effect on cell number on day 3 and day 5 (p < 0.05 by two-way ANOVA with Bonferroni post hoc). The interaction effect was also significant in specific cases (p < 0.05 by Student’s t-test), indicating that Aβ42-M impacted VEGF effects on cell proliferation. No influence of growth factor gradient was seen on day 3 or day 5 without Aβ42-M or Aβ42-O. In the presence of Aβ42 monomers, VEGF had a concentration dependent effect. On day 3, 10 ng/ml VEGF had no effect on cell proliferation in the presence of Aβ42 monomers; however, 25 and 50 ng/ml increased cell number by 60% and 94% respectively. On day 5, a similar concentration dependent effect was observed. VEGF increased cell number by 82%, 100% and a 170% with 10, 25 and 50ng/ml VEGF, respectively, when Aβ42 monomers were present. These results suggest that Aβ42 structure may differentially affect VEGF signaling.
Figure 4.6: Aβ42 structure impacted VEGF effects on cell proliferation. BBmVEC were seeded at 10,000 cells/well on native collagen coated substrates and allowed to attached for 24 hours. Cells were then stimulated with +/- 10, 25, or 50 ng/ml VEGF, Aβ42 oligomers, or Aβ42 monomers on days 1 and 3 in experimental medium, and cells were counted on days 1, 3, and 5. A) Day 3 and B) Day 5. Two-way ANOVA shows a significant difference between Aβ42 and VEGF gradient. Post hoc Student’s t-test corrected with Bonferroni’s method between groups showed # p < 0.05; * p < 0.01 compared to null. # p < 0.05 Aβ42-M vs. Aβ42-O at respective VEGF concentrations (when indicated with brackets) on respective days with Bonferroni post hoc.
4.3.7 **VEGF has a concentration dependent influence on Aβ42 monomers tube formation but limited influence on plasmin activity**

Since monomeric Aβ42 impacted VEGF effects on cell proliferation at lower VEGF concentrations, we determined if this effect translated to plasmin activity and tube formation. Aβ42 and VEGF had a statistically significant effect on tube length but not on plasmin activity (p < 0.05 by two-way ANOVA). Indicating that VEGF restored endothelial cell tube length in a concentration dependent manner in the presence of Aβ42-monomers only, without any significant influence on plasmin activity. When Aβ42 was not in the system, 10, 25 and 50 ng/ml VEGF similarly increased tube length by around 50%. In the presence of Aβ42 oligomers, VEGF did not increase tube length, as seen in our previous experiments. However, VEGF did increase tube length in the presence of Aβ42 monomers in a concentration dependent manner. While 10 ng/ml VEGF did not significantly increase tube length with Aβ42 monomers, 25 and 50 ng/ml VEGF increased tube length by 200 and 250%, respectively. To see if these changes could be attributed to plasminogen system activity we examined plasmin activity by Chromozym PL. There were no significantly relevant differences that would explain the changes we see in tube formation. These results suggest that Aβ42 structure may differentially affect VEGF tube formation however it is not through the plasminogen activating system.
Figure 4.7: VEGF restored endothelial cell tube length in a concentration dependent manner in the presence of Aβ42-monomers only, without any significant influence on plasmin activity. A) Tube length was determined in a tube formation assay. BBmVEC were
seeded at 15,000 cells/well in a 96 well plate on native collagen (4mg/ml) gels for 18 hours in the presence of +/- 10, 25, 50 ng/ml VEGF, 5 µM Aβ42 oligomers, or 5 µM Aβ42 monomers in experimental medium. Samples were imaged by phase contrast microscopy (5 images per well). Tube length was manually analyzed using ImageJ. Two-way ANOVA shows a significant difference between Aβ42 and VEGF gradient. Post hoc Student’s t-test corrected with Bonferroni’s method between groups showed # p < 0.05 compared to null. # p < 0.05 Aβ42-M vs. Aβ42-O at respective VEGF concentrations (indicated with brackets). B) BBmVEC were seeded at 250,000 cells/cm² on native collagen coated substrates for 48 hours and then stimulated with +/- 10, 25, 50 ng/ml VEGF, 5 µM Aβ42 oligomers, or Aβ42 monomers for 24 hours in experimental medium. Plasminogen system activity was measured by Chromozym PL assay. Two-way ANOVA shows no significant difference between Aβ42 and VEGF gradient. Post hoc Student’s t-test corrected with Bonferroni’s method between groups showed # p < 0.05 compared to null. # p < 0.05 Aβ42-M vs. Aβ42-O at respective VEGF concentrations (indicated with brackets) with Bonferroni post hoc.
**Schematic 4.2: AIM 3 Summary.** Aβ42 monomers only inhibit endothelial cell angiogenic processes in response to low growth factor concentration. A) Low concentrations of VEGF inhibit plasmin activity, ECM degradation, 3D migration and tube formation, B) high concentrations of VEGF are able to compete with Aβ42 and promote angiogenic functions.
4.4 Discussion

We have now shown that Aβ42 oligomeric and monomeric forms have limited influence on endothelial cells stimulated with high concentrations of FGF-2 and VEGF. Unfortunately, endogenous growth factor within the human body will never reach this systemic concentration, and therefore may not be able to counteract the effects of the amyloid beta peptide. Instead the lower concentration of 10 ng/ml VEGF is more comparable to normal plasma growth factor levels (30 pg/ml) [203]. Our data suggest that exogenous growth factors are needed to counteract reduced angiogenesis (Schematic 4.2).
4.4.1 Aβ42 oligomers have limited influence endothelial cells and FGF-2 effects

Toxicity and Proliferation

Aβ42-O was not toxic to endothelial cells in our experiments and did not influence FGF-2 induced endothelial cell proliferation or ERK phosphorylation. Aβ42-O has been shown to be toxic in high concentrations [126]. ERK or MAPK is an essential protein that is central to regulating cell proliferation [134]. Patel et al have previously shown Aβ42 is able to bind to and inhibit VEGFR-2 signaling [36]. We therefore looked to see if other growth factors, namely FGF-2 were involved in this Aβ competitive binding mechanism. However, FGF-2 and Aβ42-O seem to be independent of each other.

Plasmin Activity and Angiogenic Models

Aβ42-O did not change FGF-2 stimulated plasmin activity, 3D migration or tube formation. FGF-2 has been shown to increase plasminogen system activity, through increased expression of uPA, uPAR [97], activation of MMPs [94] and integrins [181]. Plasmin can help degrade and clear Aβ42 [204]. These assays also tested if Aβ42-O created an additional site of cleavage for plasmin, thus diminished the ability of plasmin to degrade the ECM and drive 3D migration and tube formation. These results demonstrate that Aβ42-O has limited influence on angiogenesis in collagen gels, with the exception of reducing the number of tubes increased by FGF-2. Aβ42-O did not appear to compete with ECM proteins to be degraded by plasmin. The implications for these data extend to endothelial cell dysfunction that leads to reduced length of capillary networks. FGF-2 does not increase tube number but therapy would increase length of tubes significantly.
4.4.2 Aβ42 oligomers have limited influence on endothelial cells and VEGF effects

Proliferation

Aβ42-O did not change the influence of high dose VEGF on cell proliferation or Akt phosphorylation. Akt phosphorylation is a key protein needed for cell proliferation and migration [205]. Previously research showed that Aβ42 decreases Akt phosphorylation in HUVEC, and additional VEGF displays a concentration dependent increase [36]. However, this study did not distinguish which form of Aβ42 interacts with VEGFR. We discovered that Aβ42 in the oligomeric structure does not competitively bind with VEGF for VEGFR to influence Akt phosphorylation. This is an important finding that leads to appropriate therapy administration when elevated levels of Aβ42-O are found in the system, since Aβ42-O and VEGF signal independently of each other.

Plasmin Activity and Angiogenic Models

Aβ42-O has no influence on VEGF dependent plasmin activity but decreases growth factor dependent tube length. VEGF is known to increase capillary network length [206] through activation of growth factor receptor expression and binding [45]. Research has shown that Aβ42 can interact with VEGFR-2 to decrease VEGF dependent influence on capillary tube length [36]. We now show a mechanism in which Aβ42-O decreases the influence on VEGF of tube length, but not tube number. The implications for this result extend to areas of endothelial cell dysfunction where there is a reduction in the number of capillary networks. VEGF therapy would have the ability to increase the number of tubes significantly. It also could be used in combination with other growth factors to address multiple areas of dysfunction.
4.4.3 Aβ42 secondary structure impacted VEGF induced endothelial cell responses

Proliferation

Aβ42 monomers decreased VEGF induced endothelial cell proliferation in a growth factor concentration dependent manner. There has been extended debate on which forms of Aβ42 are considered toxic and have the highest anti-angiogenic effect on cells, however monomers and oligomers are considered the most toxic [207]. They are thought to aggregate, bind to growth factor receptor sites and take away the pro-angiogenic induced affect. We have not presented a mechanism for proliferation changes with monomeric Aβ42. However research highlights monomeric interaction with insulin growth factor-1 (IGF-1) receptor signaling that are neuroprotective by activating PI3K, Akt, inhibiting tau phosphorylation [208]. Likewise, many researchers claim a non-toxic role of Aβ42 monomers and aggregation of the monomeric forms result in dysfunction [209]. As a result a proposed mechanism for dysfunction we see in our studies may be due to aggregation over time into intermediates, a potentially more toxic form of the peptide. Implications for growth factor therapy extend to knowledge of peptide structure causing dysfunction. VEGF is able to rescue influence of Aβ42-M in a concentration dependent fashion. This suggests that VEGF is able to counteract the influence of Aβ42-M. This pathway has the potential for increased concentrations of VEGF to combat the competitor/inhibitor influence Aβ42-M has in the system (Schematic 4.2). This is especially interesting because VEGF can act independently of Aβ42-O but not of Aβ42-M.
Plasmin Activity and Angiogenic Models

VEGF restored endothelial cell tube length in a concentration dependent manner in the presence of Aβ42-monomers only, without any significant influence on plasmin activity. We have not proposed a mechanism however it does not seem to be related to plasmin activity. These data imply that in patients with elevated levels of Aβ42-O accumulation, VEGF cannot fully rescue angiogenic function. However, in elevated Aβ42-M, VEGF can be utilized to increase capillary tube length in a concentration dependent manner by reducing the ability of monomers to aggregate into intermediates. Again, Aβ42-M seems to influence endothelial cell function through the same mechanism as VEGF. Increased research needs to be done in order to elucidate this area of growth factor therapy.
4.4.4 Impact and Limitations of Research

Impact

We and others have now shown the different effects Aβ structure can have on angiogenesis. We were able to use growth factors to rescue Aβ monomeric influences, however the oligomeric form of the peptide did not seem to produce any negative influences on endothelial cells, supporting previous data [195, 210, 211]. It is therefore essential to characterize the Aβ in the system prior to growth factor therapy. Administering growth factors to a peptide that will not react with the therapy will result in adverse effects discussed earlier.

One of the challenges of determining structure of peptide is that it is difficult to collect data from patients without laborious resonance imaging techniques or highly invasive biopsies [212]. In most patients sample collection is taken from the cerebral spinal fluid. From these samples, inferences about what is taking place in the brain on a protein level can be made.
**Limitations**

This research is not without limitations. As with any *in vitro* system, we used a simplified model of angiogenesis with only endothelial cells present. Peptide characterization techniques should be improved. Although a native gel was used to compare protein size, mass spectrometry would be the ideal system to differentiate peptide secondary structures. Similarly, sampling the system to view exactly in which conformation the peptide is in throughout the assay would aid in proper understanding of peptide structure over time. Timing of angiogenic assays should be taken into careful consideration. The amyloid beta peptide has the ability to aggregate and degrade based on protein interactions. Viewing how that aggregation and degradation is influenced by the system over time would highlight timing required for growth factor therapy.

Although biochemical analysis was performed at length in this set up, the lack of biomechanical interaction cannot be ignored. The next step would be to introduce stress or strain into the system through fluid flow, with a parallel plate flow chamber. Mechanical forces activate many receptors. It would be interesting to see if the Aβ42 had an influence on these cues. After mechanical *in vitro* assays have been conducted it would be ideal to move into a mouse model to see if discovered interactions still prove true *in vivo*. 
4.5 Conclusion

Aβ42 has limited influence on high concentrations of growth factors FGF-2 and VEGF. Lower concentrations of VEGF indicate Aβ42 is able to impact growth factor dependent influences. Specifically the monomeric form of the peptide can reduce VEGF induced proliferation and tube formation. These data demonstrate the ability of varying forms of Aβ42 to interact and influence angiogenesis in the presence of growth factor therapies.
5 Conclusions and Future Work

5.1 Thesis Summary

Diabetes and Alzheimer’s disease are reaching epidemic proportions. As the population ages, it is essential that a deeper understanding of these diseases be achieved. Both diseases are highly complex and affect many cell types within the body. This thesis focused on endothelial cells, which control the diminished angiogenesis observed in both diabetes and Alzheimer’s disease. I then isolated a specific angiogenic mechanism within endothelial cells to allow deeper understanding of altered intracellular signaling pathways. I explored growth factor response in endothelial cells exposed to altered extracellular proteins—either on glycated collagen in diabetes or in the presence of amyloid β peptide in Alzheimer’s disease.

I showed that FGF-2 does not fully restore plasminogen system activity in endothelial cells cultured on glycated collagen. This short term change appeared to be related to elevated stabilized PAI-1 bound to glycated collagen, rather than changes in uPA, uPAR or PAI-1 mRNA or protein expression levels. FGF-2 also increased cell-associated vitronectin across a variety of vascular cell types, which also impacted plasminogen system activity. FGF-2 enhanced vitronectin binding specifically to the cell membrane through activation of the αvβ5 integrin. Finally, I showed that Aβ42 oligomers do not diminish endothelial cell angiogenic functions with or without FGF-2 or VEGF. However, Aβ42 monomers diminish VEGF induced cell proliferation and tube formation in a concentration dependent manner.

These results bring new understanding to endogenous and exogenous growth factor effects in disease states. Endothelial cell dysfunction, specifically in angiogenesis, contributes to an array of diseases. Growth factor therapy in these diseases should take into account the altered
extracellular protein environment to increase the likelihood of growth factor therapy effectiveness.
5.2 Specific Discoveries

My overarching hypothesis was that growth factors could not fully rescue endothelial cell angiogenic processes from altered extracellular proteins. Assays were developed to discover how these altered extracellular proteins affect growth factor induced cell plasminogen system activity. The resultant data would then add additional insight into growth factor therapy for patients with diabetes and Alzheimer’s disease.

In Chapter 2, I confirmed that endothelial cells on glycated collagen display decreased plasminogen system activity, which impacts angiogenic functions such as tube formation. FGF-2 did not fully rescue the negative effects of glycated collagen. Interestingly, uPA, uPAR and PAI-1 protein levels did not significantly change at short time points. However, PAI-1 bound to glycated collagen at higher levels than native collagen. PAI-1 could be stabilized by this binding, and thereby contribute to decreased plasmin activity and angiogenic functions. Specifically, this section chapter showed for the first time that FGF-2 does not fully restore plasminogen system function in endothelial cells on glycated collagen, and that PAI-1 binds to altered extracellular matrix proteins such as glycated collagen.

In Chapter 3, I explored FGF-2 effects on vitronectin, since even cells on native collagen did not show a short terms response in uPA, PAI-1, or uPAR protein levels in response to FGF-2 stimulation. I showed that FGF-2 increased cell-associated vitronectin by 24 hours in both varied endothelial cell types and vascular smooth muscle cells. The vitronectin appeared to be taken up on from the serum and remained on the endothelial cell membrane, as shown by immunofluorescence microscopy with and without permeabilizing cells. FGF-2 signaled intracellularly to activate the αiβ5 integrin, a vitronectin binding site. Blocking focal adhesion kinase phosphorylation or the αiβ5 integrin, or knocking down the β5 integrin with siRNA,
abrogated this FGF-2 induced effect. These data show for the first time that growth factor-induced vitronectin binding alters plasminogen system activity.

In Chapter 4, I studied the effect of Aβ42 in two different forms – oligomers and monomers - on growth factor-induced angiogenic functions in endothelial cells. I showed that Aβ42 oligomers are non-toxic and did not affect FGF-2 induced cell proliferation, ERK phosphorylation, or 3D migration. Aβ42 oligomers had limited effect on endothelial cell tube formation. This lack of Aβ influence on growth factor induced response was not limited to FGF-2 as endothelial cell stimulated with VEGF in the presence of Aβ42 oligomers had similar results. On the other hand, monomeric Aβ42 impacted VEGF effects on cell proliferation and tube length, although with limited influence on plasmin activity. Our data show that Aβ42 oligomers have no influence on growth factors and have limited influence on the system. Aβ42 monomers attenuate VEGF at lower concentrations. These data show for the first time that Aβ42 structure affects its effect on growth factor activity.
5.3 Contributions to the field

FGF-2 does not fully restore plasminogen system function in endothelial cells on glycated collagen, and PAI-1 binds to altered extracellular matrix proteins such as glycated collagen. This finding is important to proper growth factor therapy in disease states. In disease states, growth factors positively impact angiogenesis. However, the disease state may diminish the impact of growth factor therapy. Therefore, growth factor effects in disease should be measured so that growth factor therapy is not attempted in futile circumstances. Instead of administering growth factors to induce angiogenesis in diabetes, therapies to inhibit PAI-1 stabilization should be explored. This dual targeting of a decreased angiogenesis inhibition through PAI-1 destabilization and pro-angiogenic influence of FGF-2 might accelerate wound healing in patients with diabetes.

These data show for the first time that growth factor-induced vitronectin binding alters plasminogen system activity. This study has now elucidated how the $\alpha_v$-$\beta_5$-vitronectin interaction may be a key factor driving growth factor-induced plasmin activity. Enhancing this interaction has the potential to increase cell migration, tube formation and wound repair in patients with diabetes. It would be especially interesting if a peptide could be formulated that bound to $\alpha_v$-$\beta_5$ and could enhance plasmin activity but in solution would not be able to bind PAI-1. A form of mutated vitronectin would be an exciting therapy for reduced angiogenesis.

These data show for the first time that A$\beta$42 structure affects its effect on growth factor activity. This stresses the importance of characterizing A$\beta$42 structure in patients with disease before administering growth factor therapy. Growth factor therapy could be used in combination with treatment to promote A$\beta$ oligomer formation. Since VEGF competitively
binds to VEGFR with Aβ monomers, growth factors could prevent Aβ monomer toxic effects, if they occur through VEGFR binding.

The clinical motivation of this research was to address decreased angiogenesis in diabetes and Alzheimer’s disease. Targeting the PAI-1 interaction with glycated collagen presents a point of drug therapy. In patients with diabetes, a reduction in angiogenesis is likely caused by this PAI-1 stabilization. Inhibiting this PAI-1-glycated collagen interaction with a peptide would decrease the amount of activated PAI-1 in the system, and thereby restore plasmin activity. Similarly, localized growth factor therapies for patients with reduced angiogenesis can be developed to increase αvβ5-vitronectin ligation and thereby increase plasmin activity. In patients with Alzheimer’s disease a true characterization of the peptide causing dysfunction must be completed. Then growth factor therapy can be administered. In patients that have a reduction in the number of vessels with an accumulation of Aβ42 monomers, VEGF can be used to increase vessel number. In patients with reduced capillary network length, FGF-2 can be administered to restore length to the vessels. The growth factor may also have the potential to be used to prevent monomeric aggregation, a topic that needs further research.

The applications of this research extend far into other diseases. This research has now isolated what we believe to be an alternative pathway for growth factor therapy. Identification of vitronectin interaction with growth factor-activated integrins has potential to be used in cancer therapy as well. The majority of tumor cells have been shown to highly express αvβ5 [193], which may be due to elevated levels of growth factor in the system [213]. Formulating a peptide to bind to the αvβ5 integrin in the place of vitronectin would lead to a reduction of plasmin activity and decreased angiogenesis.
5.4 Future Studies

5.4.1 Role of mechanical shear stress in vitronectin membrane binding

Endothelial cells responded to FGF-2 biochemically to increase vitronectin binding to the cell membrane and thereby increase plasminogen system activity. Biochemical signaling is important in angiogenesis; however mechanical forces may be equally important. Shear stress and strain can similarly activate endothelial cell pathways. Thus, mechanical stimuli could be administered to endothelial cells, and changes in vitronectin binding could be explored. These stimuli should include laminar versus disturbed fluid flow seen in atherosclerosis.

5.4.2 Disease related changes in vitronectin membrane binding

The $\alpha_v\beta_5$ integrin has now been highlighted as the critical integrin involved in growth factor increases in plasminogen activity. Future work should include examining how vitronectin binding to $\alpha_v\beta_5$ is affected by glycated collagen and A$\beta$42. These disease states would be interesting as we have shown glycated collagen decreases plasmin activity and FGF-2 has limited rescue ability. This influence may be due to the fact that vitronectin is being endocytosed at a higher rate than kept at the cell membrane. Also this could be extended into A$\beta$42 studies as reduction of angiogenesis could be due to a vitronectin - $\alpha_v\beta_5$ interaction. Potential therapies for cells on glycated collagen or in the presence of A$\beta$42 would be to prevent endocytosis of vitronectin and keep vitronectin on the cell membrane to enhance plasmin activity. On the other hand, the $\alpha_v\beta_5$ integrin may not be as readily activated by FGF-2 or is down regulated by glycated collagen or in the presence of A$\beta$42. In this instance mechanisms to enhance integrin expression and activation specifically would have to be explored.
5.4.3 Expansion of mechanism to reduce angiogenesis in tumor growth

I have shown that the pro-angiogenic effect of FGF-2 works through the αvβ5 integrin, an important discovery to increase angiogenesis in diabetes and Alzheimer’s disease. This pathway can also be potentially used to suppress tumor growth. Blocking the FGF-2 induced increase in vitronectin binding to integrins may further decreases in angiogenesis in cancer, especially since αvβ5 integrin is highly expressed in many cancer cells. It would be interesting to show a reduction in tumor growth with αvβ5 siRNA knockdown and αvβ5 blocking over time.

5.4.4 Animal models of disease states

Cell based research has many advantages, including the low cost of assay development, the ability to control the microenvironment, and the short turnaround time. Cell based research should then lead to animal and human trials to examine the biological pathways discovered with the more complex physiologic system. *In vitro* animal models of diseases related to angiogenesis, such as diabetes, Alzheimer’s disease, and cancer would strengthen the use of vitronectin-αvβ5 as a therapy. Mouse models would be used with knockouts for αvβ5 integrin or vitronectin then administered FGF-2. Assays could then be developed for immunostaining sections for vitronectin-αvβ5 interaction, quantifying capillary networks in the peripheral regions of the body, wound healing assays and blood plasmin content. I would expect knockout of either αvβ5 integrin or vitronectin to be deleterious to any angiogenic activity induced by FGF-2.
References


Vita

Full Name: Justin George Mathew
Place and Date of Birth: Philadelphia, PA on May 8th, 1987

Education
- PhD Bio-Mechanical Engineering, Drexel University- Philadelphia, PA, June 2014
  - GAANN Fellowship
- BS/MS in Biomedical Engineering, Drexel University- Philadelphia, PA, June 2010
- Central High School, Philadelphia, PA, June 2005

Research Experience
- DAAD RisePRO Scholar, ProQinase GmbH – Freiburg, Germany, July to December 2013
- Validation/Verification Product Specialist, Animas (Johnson & Johnson) – West Chester, PA, May to September 2010
- Medical Device Researcher, Kensey Nash Corporation - Exton, PA, April to September 2008
- Assistant Researcher, Merck and Co. - West Point, PA, April to September 2007

Leadership Experience
- President, Drexel Graduate Student Association, July 2013 to Present
- Vice President, Drexel Mechanical Engineering Graduate Association, January 2013 to June 2013

Poster Presentations


Papers to be Published
Mathew, Justin and Clyne, Alisa Morss, Fibroblast growth factor-2 did not restore endothelial cell plasminogen system activity or capillary-like tube formation on glycated collagen: growth factor angiogenic effects through activation of $\alpha_v\beta_5$ integrin and subsequent vitronectin binding.

Mathew, Justin and Clyne, Alisa Morss, Amyloid beta secondary structure critical for growth factor dependent influence on plasminogen activating system.