Hyaluronic acid alters vessel behavior in CXCL12-treated HUVECs

Honors Undergraduate Research Thesis in Mechanical Engineering

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April 2018

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Abstract

Hyaluronic acid (HA) is a key component of the extracellular matrix known for absorbing water, swelling, and altering solid stress of tumors. HA's anionic behavior may provide important biochemical effects toward tumor progression as well. Tumors obtain nutrients by relying on signaling molecules such as CXCL12 to recruit blood vessels and promote vessel leakage. Recent work suggests that additional positively-charged residues on CXCL12's β and γ isoforms cause different biochemical functionality compared to the well-studied α isoform. These studies aimed to determine whether the presence of HA in a tumor's microenvironment could alter the relative response strength of CXCL12's various isoforms on blood vessel sprouting and apparent vascular permeability. The vessel microenvironment was modeled using a 3-channel microfluidic device with Human Umbilical Vein Endothelial Cells (HUVECs) in the outer channels forming monolayers against a 3D collagen or collagen/HA matrix in the center channel. HUVECs were cultured with media containing recombinant CXCL12 (α, β or γ). Results show that total HUVEC sprouting area follows an α>β>γ trend in isoform-treated HUVECs within a collagen matrix, matching the binding affinity order of CXCL12 to endothelial CXCR4 receptors. The presence of HA decreased overall sprouting response but shifted pro-angiogenic potency towards CXCL12's γ isoform. Vascular permeability studies also showed an α> β>γ trend for HUVECs in collagen. With HA added, control and α-treated HUVECs became less permeable while γ-treated HUVECs became more permeable. Overall results suggest that an HA-infused collagen matrix facilitates γ isoform binding, leading to a stronger isoform-specific vessel response. Knowing how HA impacts CXCL12 isoform potency on vessels will help in the future design of CXCL12-targeted cancer therapies.

Acknowledgements

I would like to acknowledge and thank …

Dr. Jonathon Song, my research PI and advisor, for providing guidance on this project and sitting on my defense committee.

Dr. Jeffrey Chalmers, for agreeing to be a member of my defense committee.

Chia-Wen Chang, for contributing equally to this project and mentoring me along the way.

Dr. Robert Siston and fellow MAE 4999H classmates for helping me prepare my final thesis paper and oral defense presentation.

The **American Heart Association, Institute for Materials Research at OSU, Lumley Engineering Fund**, and **Pelotonia** for providing the necessary funding for me to complete this research project.

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1 Introduction

1.1 Literature Overview

1.1.1 Tumor microenvironment and reliance on blood vessels

In the early 1970s, a medical scientist by the name of Judah Folkman noticed that tumors struggled to grow to a meaningful size when in the absence of an external blood supply[1],[2]. This observation prompted his idea to combat cancer progression by preventing new blood vessel formation. Folkman's insight pushed for a new movement in cancer research focusing on how tumors interact with vessels and other factors in the microenvironment around them.

The tumor microenvironment (TME), depicted in Figure 1.1[3], refers to all the other cells and molecules surrounding the tumor at the micro scale, most notably the extracellular matrix (ECM) and blood vessels. The ECM is composed mainly of collagen, a fibrous protein secreted by stromal cells that serves as structural support for neighboring cells. Other components include the stromal cells themselves as well as various glycosaminoglycans such as hyaluronic acid. Blood microvessels at this scale are essentially single-layer tubes of endothelial cells. In tumors, these vessels become highly disorganized and endothelial cells become separated by large gaps[4].

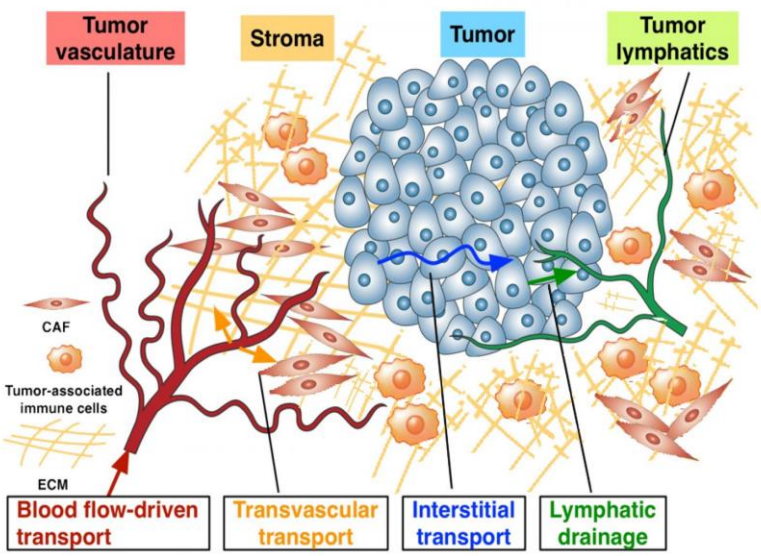

Figure 1.1: Tumor Microenvironment[3]
The tumor microenvironment includes three main components: the tumor, the blood/lymphatic vessels, and the extracellular matrix (ECM) or tissue stroma.

One way tumor cells manipulate blood vessels is by recruiting them in an effort to obtain enough nutrients to rapidly grow and proliferate[5]. In a process called angiogenesis, new sprouts can emerge from pre-existing blood vessels. Angiogenesis is upregulated in the TME, resulting in highly irregular blood vessel networks that infiltrate the entire tumor, shown in Figure 1.2[6].

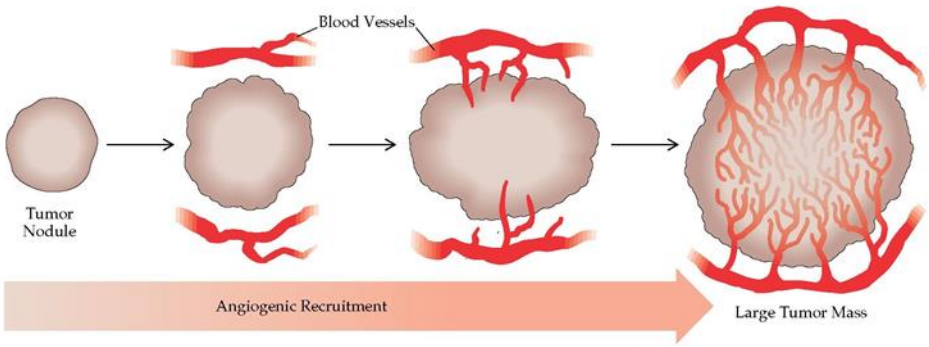

Figure 1.2: Angiogenic Vessel Recruitment in Solid Tumors[6]
Tumors are capable of inducing angiogenic behavior in vessels in order to receive sufficient nutrients.

Vessel leakiness or permeability tends to be significantly increased in the TME as well. VE-cadherin, a type of cell adhesion molecule that holds endothelial cells together at gap

junctions, becomes degraded in the presence of some tumors[4]. Reorganization of VE-cadherin can result in the widening of these junctions, and more fluid is allowed to leak or permeate through the vessel wall. Widened junctions between adjacent endothelial cells facilitate the invasion of primary tumor cells into the vasculature[7]. Once in the bloodstream, tumor cells can extravasate elsewhere to form secondary tumors. That process, known as metastasis, is shown in Figure 1.3[8]. Metastatic cancer often proves much deadlier than cancers involving just the primary tumor[9], so preventing invasion through leaky vessels becomes an important issue to address.

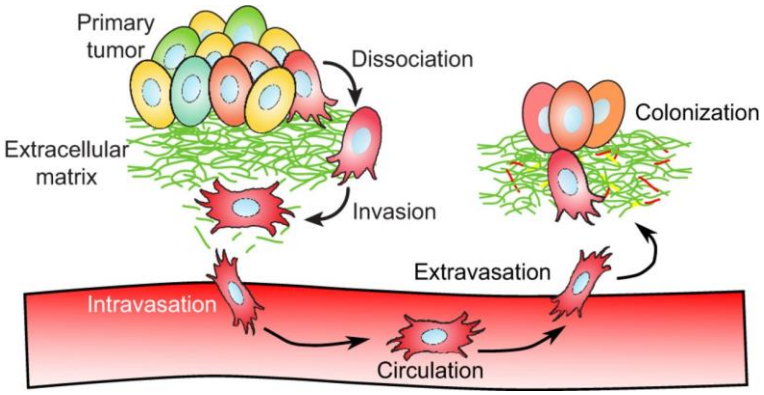

Figure 1.3: Primary Tumor Metastasis to Secondary Site[8]
Tumor cells can invade blood vessels at leaky junctions, travel through the blood stream, and exit elsewhere in the body to form secondary tumors.

It has been well studied that vessel angiogenesis and vascular permeability are mediated by growth factors and other signaling molecules expressed in the TME[4],[5]. Vascular endothelial growth factor (VEGF), for example, is a signaling protein that cells secrete in response to hypoxia to induce blood vessel growth and enhance oxygen supply. Endothelial cells express the VEGF-activated receptor VEGFR. Most tumor cells have been shown to overexpress growth factors such as VEGF, allowing them to promote angiogenesis. Chemokines are other signaling molecules that can induce a chemotactic migratory response (movement in the direction of a chemical gradient) in cells that

express the appropriate receptors. Chemokine (C-X-C motif) ligand 12 (CXCL12), for example, can bind to its receptor CXCR4 on endothelial cells to induce cell motility[10].

1.1.2 CXCL12 signaling and binding affinities

Chemokine (C-X-C motif) Ligand 12 (CXCL12) is a type of chemotactic signaling protein that promotes gradient-directed migratory behavior in cells that contain its receptor CXCR4[10]. Both cancer cells and endothelial cells have been found to express this receptor, and CXCL12 is known to be heavily expressed in the TME. Given its migratory influence, CXCL12 is often studied in the context of tumor cell metastasis. Focusing instead on endothelial cells, CXCL12 treatment has been known to contribute to the formation of new vessel sprouts and disruption of vessel integrity[10]. Provided CXCL12's further impact on vessel behavior, it becomes a multi-faceted target for stopping tumor progression.

Like many signaling proteins, CXCL12 exists as a number of different isoforms[10], which are similar proteins with slight differences in amino acid sequences. Three main isoforms (α, β and γ) are expressed by rats, mice, and humans, and these isoforms are known to be elevated in breast tumors[10]. Humans also express three other isoforms in lesser quantities (δ, ε, and φ) that are not as well-studied. CXCL12-α has been shown to activate CXCR4 receptors with a significantly greater potency than the β and γ isoforms (binding affinity follows an α>β>γ trend), leading several studies to focus only on the α isoform. However, recent work suggests that the β and γ isoforms can elicit meaningful responses in cancer and endothelial cells depending on the cancer type and composition of the

TME[10]. Paramita et al. demonstrate that CXCL12-β and γ increase angiogenesis in HUVECs (endothelial cells) compared to CXCL12-α, while CXCL12-γ also promotes metastasis in CXCR4-containing breast cancer cells[10].

An important difference between the CXCL12-α, β, and γ isoforms is that additional positively-charged amino acid residues are present on the carboxy-terminal side of the β and γ isoforms[10]. Therefore, a more negatively-charged matrix may provide a binding surface for positively-charged CXCL12 molecules to be sequestered within the matrix, augmenting the potencies of the matrix-bound isoforms. It is known that the binding affinity strengths of CXCL12 isoforms to negatively-charged ECM follows the trend of γ>β>α. Amplifying this trend could cause a shift in CXCL12 isoform potency toward the γ direction and could be a factor for why the CXCL12-β and γ isoforms become more active in certain tissues. As a result, an interesting question to explore is how the ECM binding affinities of CXCL12 isoforms impact CXCR4-mediated responses.

1.1.3 Hyaluronic acid in the extracellular matrix

Hyaluronic acid (HA) is a highly negatively-charged glycosaminoglycan and major component of the ECM[11]. HA's negative charge causes it to readily absorb water and swell, often leading to stiffer matrices in tissues. Apart from its structural role, HA plays a role in mediating cell migration and proliferation as well as maintaining blood vessel integrity[12]. The ECM tends to overexpress HA production in several cancer types (i.e. pancreatic and breast), and increased HA concentration around tumors is a marker for worse survival in patients[13]. One reason for this is that HA contributes to increased solid

stress in the tumor microenvironment[14], impairing vessel function leading to leakier vessels. Increased solid stress by HA compresses blood vessels as well, blocking drug perfusion[15]. HA in the TME is depicted in Figure 1.4[16] below.

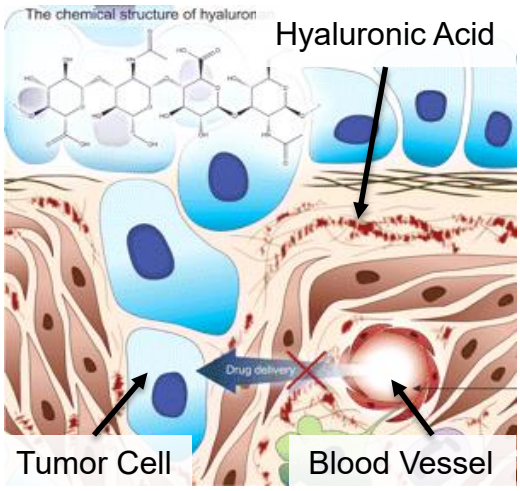

Figure 1.4: Hyaluronic Acid's Role in the Tumor Microenvironment[16]
Hyaluronic Acid upregulation in tumors has been shown to disrupt vessel integrity, increase transvascular permeability through solid stress, and block drug perfusion through vessels.

Hyaluronic Acid (HA) exists in the body at varying sizes and molecular weights. Intact, high molecular weight HA (HMW-HA) is attributed with maintaining vessel integrity through interactions with CD44 receptors on endothelial cells[17]. HMW-HA can get degraded by hyaluronidase and other reactive oxygen species into lower molecular weight HA (LMW-HA). Interestingly, studies show that LMW-HA leads to degraded vessel integrity while HMW-HA strengthens vessel integrity[18].

The biomechanical properties of HA play a large role in governing how blood vessels behave in the TME. HA's negative charge distribution could affect signaling-induced vessel response as well by biochemically altering signaling protein binding behavior. However, few studies have looked at how HA's negative charge could influence chemokine interaction with blood vessels. The chemokine CXCL12, with its three major

isoforms having different electrochemical properties, serves as a good target for studying how HA's negative charge can influence blood vessel response to signaling proteins.

1.2 Objectives and research significance

The objective of this research is to investigate whether hyaluronic acid's biochemical properties could affect signaling protein stimulation of blood vessels in the tumor microenvironment. Most research involving hyaluronic acid (HA) and its influence on blood vessels focuses on the additional mechanical forces expressed when HA-rich matrices swell[18]. HA has a strong inherent negative charge, and some studies have suggested that HA can affect cell signaling[19]. These notions suggest that HA can alter the potency of signaling proteins such as CXCL12 in the extracellular matrix (ECM). CXCL12's existence as multiple isoforms with different charge distributions makes it an ideal molecule to study the influence of HA's biochemical properties on chemokine-mediated vessel behavior.

Vessel behavior will be analyzed by designing microfluidic devices to study angiogenic sprouting and vascular permeability in response to chemotactic CXCL12 isoforms. Both vessel properties will first be studied against a collagen-only matrix to study how each CXCL12 isoform affects vessels in the absence of HA. Experiments will be repeated with the addition of HA to the matrix, and vessel behavior will be compared to that of collagen-only samples. It is hypothesized that the addition of HA will reverse the CXCL12 isoform potency on vessel behavior due to its dense negative charge. CXCL12 isoform rank order binding affinity to endothelial CXCR4 receptors (α>β>γ) is opposite the affinity for binding

to the ECM (γ>β>α). The addition of negatively-charged HA to the matrix may increase the instance of matrix-bound CXCL12, shifting vessel signaling response potency to better resemble the matrix binding affinity of CXCL12. A schematic of the hypothesized results is shown in Figure 1.5. The addition of HA may bind more positively-charged CXCL12-β and γ isoforms to the ECM, allowing them to induce a migratory vessel response more potently than the normally prominent CXCL12-α isoform.

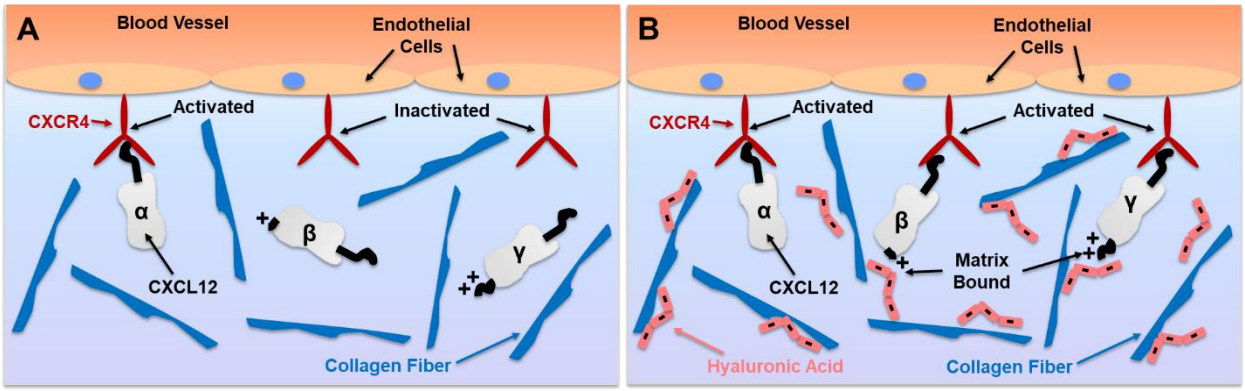

Figure 1.5: Hypothesized Effect of HA on Endothelial Cell Activation
(A) With collagen-only matrices, CXCL12-α has been shown to have the greatest affinity for activating endothelial CXCR4 receptors to induce movement. (B) Adding negatively-charged HA to the matrix may increase matrix-bound CXCL12, especially for more positively-charged β and γ isoforms, which would result in intensified signal activation.

This research will provide insight as to whether or not the presence of HA in the tumor microenvironment can biochemically affect vessel behavior in addition to its known mechanical effects. Understanding how HA composition affects CXCL12 isoform-specific activation of endothelial cells will contribute to the design of cancer therapies targeting CXCL12-CXCR4 signaling in the TME. An increase in CXCL12-β or γ activation of endothelial response with HA present would support recent research suggesting that those two isoforms are just as significant as CXCL12-α in some tissues. The composition of the TME is very heterogeneous and varies between tumor to tumor, and the importance of targeting CXCL12 for therapy may vary based on the tissue composition.

2 Methodology

2.1 Cell culture

Human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured with endothelial growth medium EGM-2. Cells were passaged every 4 to 5 days. All experiments were run with HUVECs at passage 6 to 12.

2.2 Microfluidic device design and fabrication

A microfluidic system was designed to mimic the vessel microenvironment *in vitro*. Devices were fabricated with polydimethylsiloxane (PDMS) based on a well-documented technique utilizing photolithography and soft lithography[20]. The fabrication process is outlined in Figure 2.1[21].

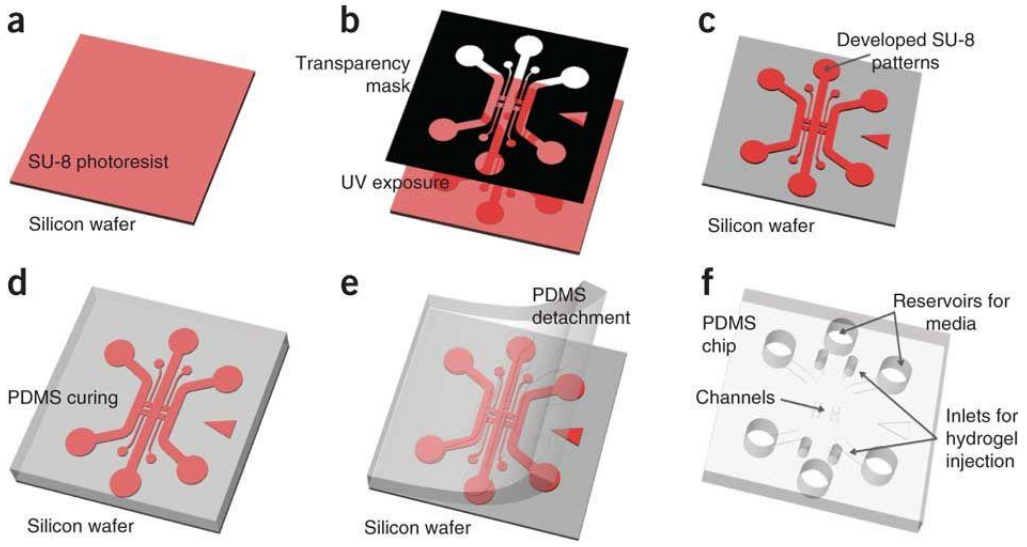

Figure 2.1: Microfluidic Device Fabrication
(a-c) Photolithography: UV light is shown through a transparent mask onto a silicon wafer, creating a raised surface in the shape of the designed device. (d-f) Soft lithography: PDMS is poured over the silicon mold, cured, and detached to form a device with microchannels.

For this particular study, a previously designed 3-channel device was used in which a collagen matrix could fill the center channel while endothelial cells (HUVECs) lined the

two outer channels. Six apertures were present on either side of the central channel at which HUVECs would form monolayers against the matrix. A schematic of the design is shown in Figure 2.2.

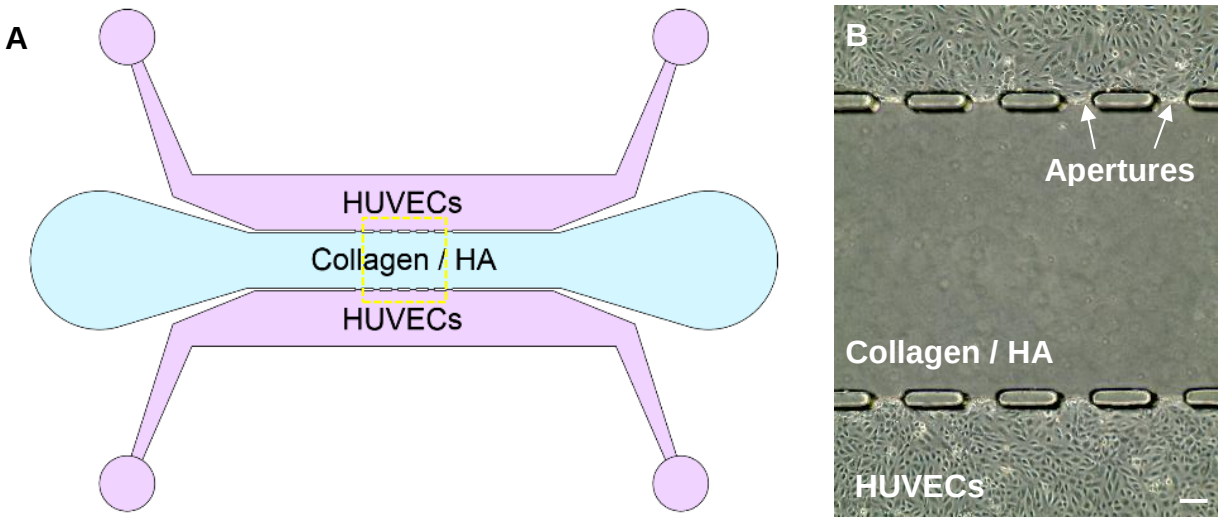

Figure 2.2: Three-channel Device Schematic
(A) AutoCAD drawing of the microfluidic device. HUVECs were seeded in the outer channels against a collagen or collagen/HA matrix formed in the center channel. (B) Microscope image of yellow boxed-in region of the device (Scale bar 100 µm). Seeded HUVECs have formed monolayers against the collagen matrix at each of the apertures.

2.3 Assay preparation

The 3-channel system was used to analyze the potency of isolated human recombinant CXCL12 isoforms (α, β, or γ) on sprouting angiogenesis and apparent vascular permeability into a collagen or collagen/HA matrix. PDMS devices were plasma oxidized, adhered to glass slides, and placed in an oven (65˚C) for at least 24 hours to ensure suitable attachment. Type 1 rat-tail collagen (3 mg/mL, Corning) with or without HMW HA (1 mg/mL, Sigma) was injected into the central channel and allowed to polymerize at 37 ˚C in an incubator for 12 hours. A 6 mg/mL collagen gel was used instead for the apparent vascular permeability assay to prevent sprouting into the matrix. Outer channels

were lined with the adherent ECM protein fibronectin (100 µg/mL, Sigma) before filling them with a 7.0 million cells/mL HUVEC solution. The apparent vascular permeability assay only required one of the outer channels to be loaded with cells. Devices were incubated for 12 hours with EGM2 media to allow proper cell attachment and monolayer formation against the collagen matrix at each of the apertures. Cells within the device were then cultured with EGM2 media conditioned with specific CXCL12 isoforms (α, β, or γ, 100 ng/mL, Peprotech). Fresh media was administered every 24 hours.

2.4 Sprouting angiogenesis assay

For the sprouting angiogenesis assay, HUVECs were cultured *in vitro* for an additional 3 days after treatment with conditioned media (CXCL12-α, β, or γ, 100 ng/mL). Initial treatment was considered to be day 0. The HUVECs would typically sprout into the collagen matrix, and images were taken each day to track how much the sprouting area increased over time. Sprout area could be traced using ImageJ software. A 'Sprouting Ratio' percentage was calculated for each day (N = 1, 2, 3) by taking the total area difference since day 0 and dividing that value by a reference area of 80,000 pixels, as shown in Equation 2.1. The procedure for analyzing sprouting area is shown in Figure 2.3.

$$\text{Sprouting Ratio } (\%) = \frac{Area\ Day\ N - Area\ Day\ 0}{Ref.\ Area} * 100\% \qquad \textbf{Equation 2.1}$$

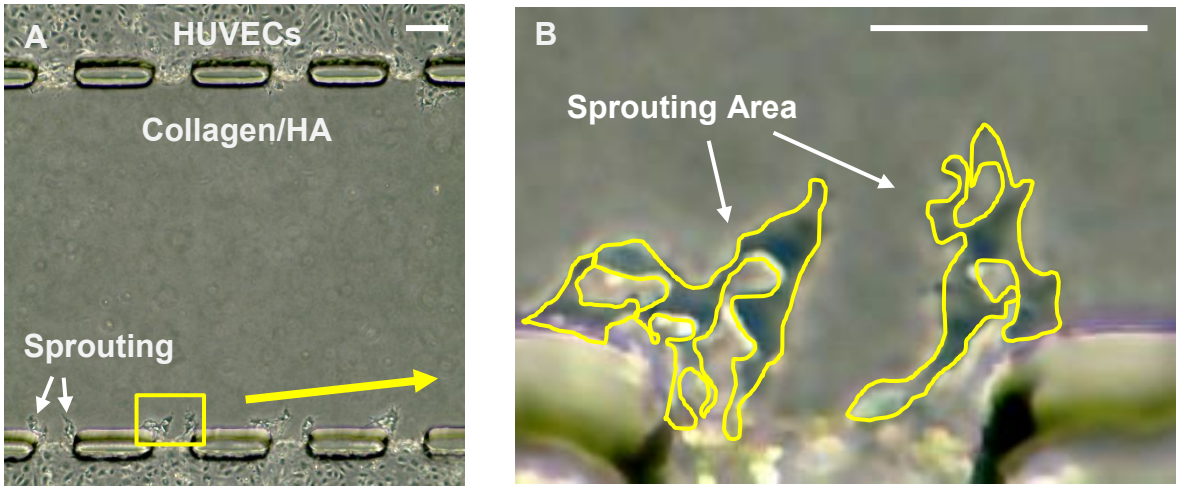

Figure 2.3: Analyzing HUVEC Sprouting Area in Sprouting Angiogenesis Assay
(A) HUVECs sprouting into collagen matrix. (B) Zoomed-in image of yellow box region showing area tracing of HUVEC sprouts (Scale bars 100 μm).

2.5 Apparent vascular permeability assay

HUVECs were cultured for 2 days *in vitro* with conditioned media (CXCL12-α, β, or γ, 100 ng/mL). 6 mg/mL collagen replaced the 3 mg/mL collagen for the apparent vascular permeability assay to reduce spontaneous sprouting occurrence. To test permeability, a red fluorescent conjugated dye (70 kDa Texas Red-labeled Dextran, 2.0 μL, 0.05 mM) was passively pumped into the HUVEC channel and allowed to diffuse across the endothelial monolayer into the collagen matrix. Pumping was performed by holding a droplet of dye above one of the HUVEC channel ports until the droplet made contact with the fluid in the channel. The surface energy in the droplet propelled the dye through. It was assumed that there was no pressure gradient within the microfluidic channel, meaning only diffusion and not convection would affect mass transfer of the dye. If sprouting occurred at one of the apertures, that assumption would not hold (fluid would flow into the collagen matrix), and results could not be taken for that device.

A time-lapse epifluorescent microscope was used to track the intensity of the Texas Red dye as it diffused transvascularly into the collagen matrix, shown in Figure 2.4. ImageJ (NIH) software was used to identify the source intensity at each aperture and track the intensity change over time within specified regions. Knowing those values, an apparent vascular permeability value (P_{App}) was calculated for the system using Equation 2.2[22]. ΔI represents source intensity, dI/dt represents a slope value for intensity change over time, and V_v/S_v is a volume to surface area ratio of the device's dye-loading channel.

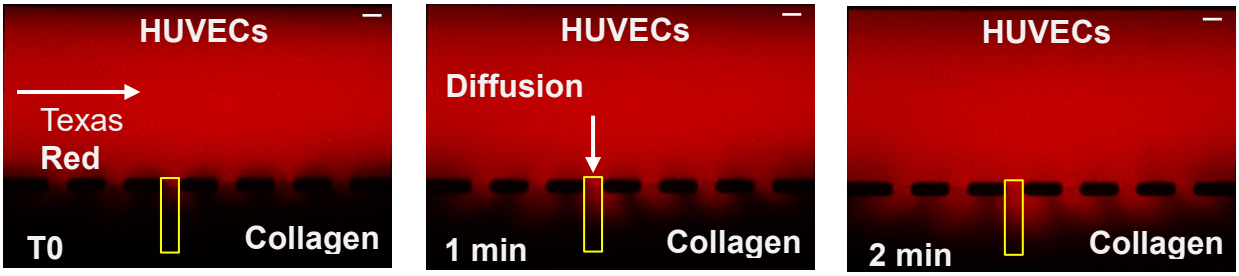

Figure 2.4: Dye Permeating Vessels in Apparent Vascular Permeability Assay
Texas Red-labeled dextran diffuses across HUVEC monolayer into collagen channel over two minute time scale (Scale bars 100 μm). Source intensity was taken for each aperture just above the vessel monolayer. Intensity within the yellow boxed-in region was analyzed over time using ImageJ to determine a dI/dt value.

$$P_{App} = \left(\frac{1}{\Delta I}\right) * \left(\frac{dI}{dt}\right) * \left(\frac{V_v}{S_v}\right) \qquad \textbf{Equation 2.2}$$

3 Results

3.1 Quantifying vessel sprouting

HUVECs were cultured for 3 days after initial treatment with isolated CXCL12 isoforms and observed for sprouting behavior at each aperture. Representative day 3 images for the collagen-only experiments are shown in Figure 3.1.

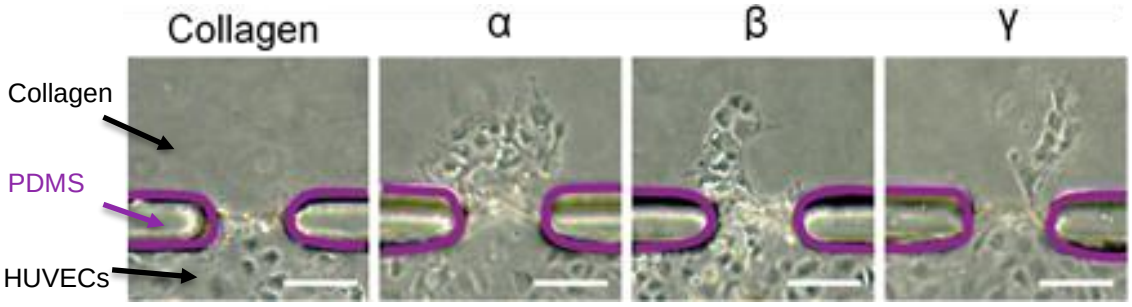

Figure 3.1: Day 3 Images of HUVECs Sprouting into Collagen-only Matrix
HUVECs treated with CXCL12 (α, β, or γ, 100 ng/ml) show α>β>γ trend in sprouting response. CXCL12-α exhibits the most spread-out sprouting behavior. PDMS is outline in purple. Scale bars 100 µm.

CXCL12-α treated HUVECs appeared to elicit the strongest response in terms of sprouting area. The overall sprouting response to CXCL12 seemed to follow an α>β>γ trend, following the reported binding affinity rank order of those isoforms to its cell receptor CXCR4[10]. The CXCL12-α treated HUVECs also exhibited the largest number of individual, highly motile cells that spread away from the initial cell monolayer. Normally, a vessel sprout observed in this type of assay would consist of one to a few cells that form a single branch-like structure, similar to what's seen in the CXCL12-γ treated HUVECs in Figure 3.1. The CXCL12-α treated cells did not show typical sprouting behavior but instead spread out in a fan-like manner as they moved into the collagen matrix. That unusual vessel behavior was partially seen in CXCL12-β treated HUVECs but observed very little in control and CXCL12-γ treated HUVECs. For the purposes of

this study, any cells that moved away from the cell monolayer into the collagen matrix at a particular aperture were considered towards that aperture's sprouting area.

To adjust for how some sprouting could occur in the period before initial treatment, a sprouting ratio term was calculated that negated any sprouting present at day 0 (described in 2.4). Sprouting ratio at each aperture was determined each day for 3 days after treatment with CXCL12-conditioned media and averaged for each device. Average sprouting ratio by device is reported in Figure 3.2 (N = 4 devices each).

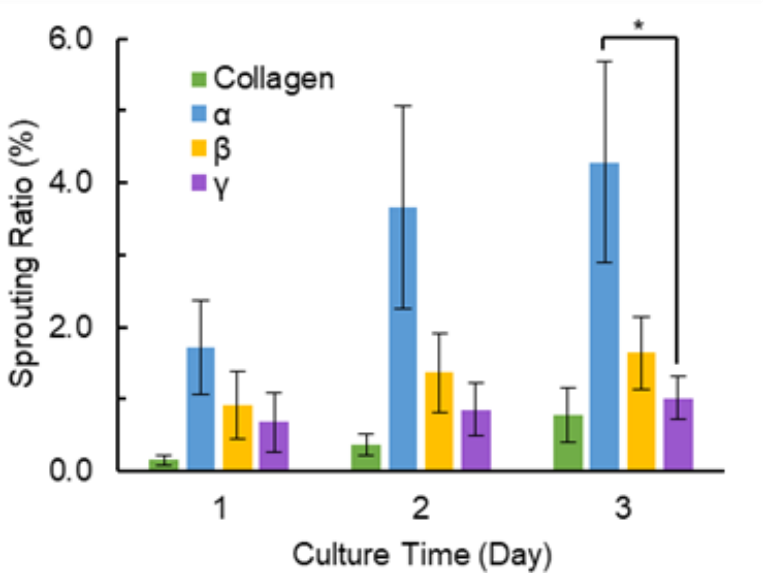

Figure 3.2: Sprouting Ratio of HUVECs into Collagen Matrix over 3-Day Period
CXCL12-α treated HUVECs show the greatest increase in sprouting response. A significant difference was observed between CXCL12-α and γ treated HUVECs. Error bars expressed as SEM. Statistical analysis performed using one-way analysis of variance. N=4. *, P value < 0.05.

In assessing the results, the expected α>β>γ sprouting trend of CXCL12-treated HUVECs can easily be seen. Most importantly, CXCL12-α treated HUVECs show a significantly greater sprouting ratio response than CXCL12-γ treated HUVECs ($P<0.05$). These findings suggest that the CXCL12-α isoform has a more potent pro-angiogenic effect on HUVECs than the CXCL12-γ isoform when in the presence of a collagen-only matrix.

Experiments were repeated with hyaluronic acid (1 mg/mL) added to the collagen matrix. Representative day 3 images for the collagen/HA sprouting experiments are shown in Figure 3.3.

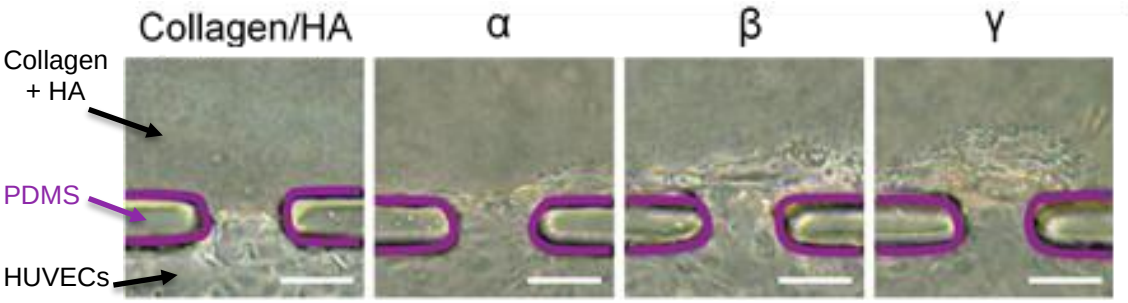

Figure 3.3: Day 3 Images of HUVECs Sprouting into Collagen/HA Matrix
HUVECs treated with CXCL12 (α, β, or γ, 100 ng/ml) show a reversed γ>β>α trend in sprouting area response with the addition of HA to the matrix. HUVEC behavior shows a shift toward pushing against the matrix instead of forming distinct sprouts. PDMS is outline in purple. Scale bars 100 µm.

Upon initial inspection, two observations can be made. First, the area of HUVECs that have moved into the collagen/HA layer reveals an inverted γ>β>α trend compared to sprouting into a collagen-only matrix. Obviously, this no longer follows the rank order binding affinity of CXCL12 isoforms to CXCR4. This does, however, follow the charge-based binding affinity of CXCL12 isoforms to the ECM (γ>β>α)[10]. The CXCL12-α treated HUVECs show a much-diminished sprouting area response, but the CXCL12-γ treated HUVECs seem to be more responsive and now tend to exhibit a fanning out behavior similar to what was seen with CXCL12-α treated HUVECs in collagen-only experiments. A second observation is that instead of cells forming sprouts, the HUVEC monolayer mostly just pushes into the collagen/HA matrix, namely the local expansion of microvessels. This interesting behavior is worthwhile for further investigation but one possible explanation may be related to how HA affects the stiffness of the matrix.

Sprouting ratio was determined for devices with a collagen/HA matrix in the same way as before. Average sprouting ratio for collagen/HA devices are reported in Figure 3.4 (N=3 devices).

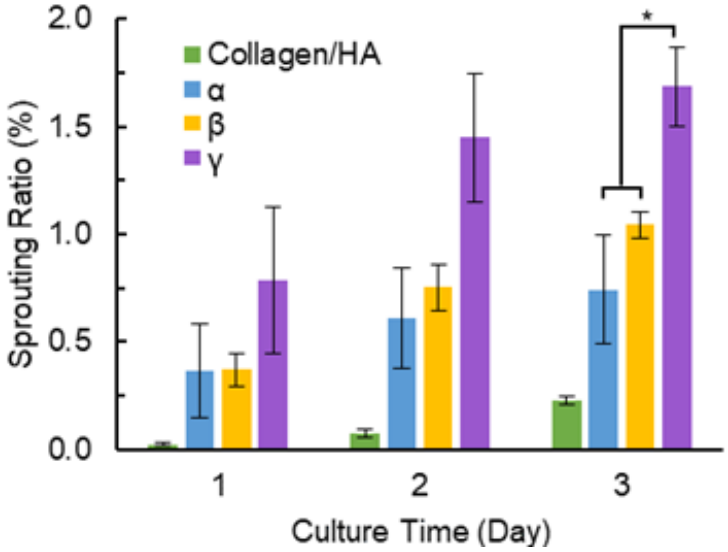

Figure 3.4: Sprouting Ratio of HUVECs into Collagen/HA Matrix over 3-Day Period
CXCL12-γ treated HUVECs in a HA-rich matrix show a significant sprouting response increase compared to CXCL12-α and β. Error bars expressed as SEM. Statistical analysis performed using one-way analysis of variance. N=3. *, P value < 0.05.

As seen in the graph, the sprouting ratio trend in CXCL12-treated HUVECS now follows a γ>β>α order in a collagen/HA matrix. Interestingly, CXCL12-γ treated HUVECs show a significantly greater sprouting ratio than both CXCL12-α and β treated HUVECs ($P<0.05$). This difference suggests that CXCL12-γ gains a more potent effect on HUVEC behavior within a matrix including HA, at least in comparison to CXCL12-α treated HUVECs. One further note is that average sprouting ratio decreased in all cases involving HA except for in CXCL12-γ treated HUVECs. This is likely associated with how the HUVEC monolayers often tended to push into the collagen/HA matrix rather than form clear sprouts into the matrix.

3.2 Quantifying apparent vascular permeability

Apparent vascular permeability was calculated using Equation 2.2 by observing how quickly dye could permeate across the endothelial layer. Average apparent vascular permeability values in CXCL12-treated HUVECs were compared between those lining a collagen-only matrix and those lining a collagen/HA matrix. Those results are shown in Figure 3.5 (N=3-4 devices).

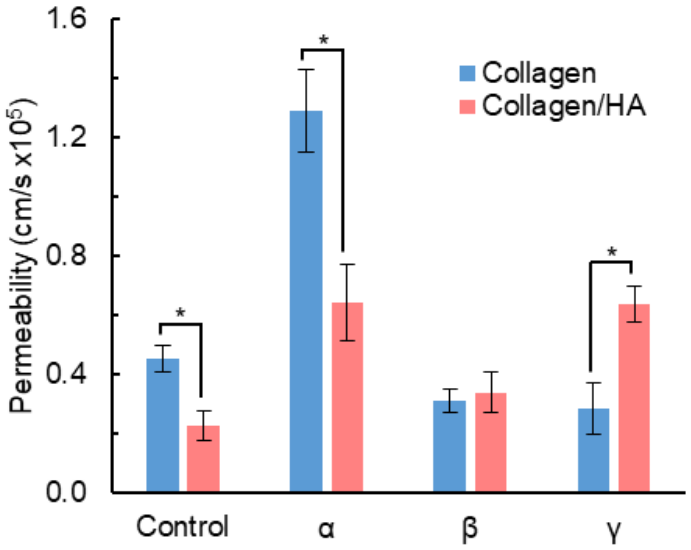

Figure 3.5: Apparent Vascular Permeability Measurements across Endothelial Layer
In collagen-only experiments, HUVECs cultured with CXCL12 (α, β, or γ, 100 ng/ml) were leakiest when treated with CXCL12-α. Addition of HA significantly lowered permeability of control and CXCL12-α treated HUVECs but increased CXCL12-γ treated HUVECs to nearly match CXCL12-α. Error bars express SEM. Statistical analysis performed using one-way analysis of variance. N=3-4. *, P value < 0.05.

In collagen-only samples, CXCL12-α treated HUVECs developed much more permeable monolayers than untreated HUVECs. CXCL12-β and γ treated HUVECs showed no significant difference from control. With HA added to the matrix, permeability of control and CXCL12-α treated HUVECs roughly halved in magnitude while that of CXCL12-γ treated HUVECs more than doubled ($P<0.05$). A decrease in untreated HUVEC permeability against a collagen/HA matrix suggests that HA encourages tighter junctions

between endothelial cells in those monolayers. This could explain why a significant decrease in CXCL12-α treated HUVEC permeability was observed as well. The increase observed in CXCL12-γ treated HUVEC permeability suggests that more isoforms remained bound to the matrix outside the endothelium. HA likely enhanced CXCL12-γ's affinity for the matrix, allowing it to more potently disrupt the vessel monolayer. A summary of trends seen in the sprouting and permeability results is shown in Table 3.1.

Table 3.1: Summary of Sprouting and Permeability Results
In collagen experiments, HUVEC sprouting response followed an α>β>γ trend and CXCL12-α showed the greatest impact on vessel permeability. Adding HA reversed the sprouting response trend, weakened the CXCL12-α mediated response on permeability, and strengthened CXCL12-γ's impact on permeability.

	Sprouting	Permeability
Collagen	α>β>γ trend	α greatest
Collagen/HA	γ>β>α trend	α weakened, γ strengthened

4 Discussion and Conclusions

4.1 HA stabilizes vessels, lowers vascular permeability

HA's role in bodily tissue has been well-documented, but much is still unknown about how the presence of HA impacts blood microvessel function and remodeling. HMW HA has been shown to play a role in supporting vessel integrity through receptors on endothelial cell membranes such as CD44[17],[18]. That role is complex, however, because it has also been shown that as HMW HA gets degraded, the fragmented LMW HA actually disrupts the endothelial layer. Overexpressed HA in the tumor microenvironment is hypothesized to increase solid stress in the tissue, and increased solid stress is correlated with leakier vessels.

This study's results support that HA presence in the extracellular matrix helps maintain more stable vessels. HMW HA was used in this study, making these results unsurprising, but this also implies that no additional solid stress was experienced by the matrix with the addition of HA. A possible explanation is that the cells were seeded against a matrix in which HA was already present. The matrix was already fully swelled, and since it was not trying to expand after the cells were added, no additional tensile stress was applied to the cells that might loosen cell junctions. As a result, the vessel-supporting behavior of HMW HA may have been the main factor at play in this system.

4.2 HA enhances matrix affinity, reverses relative CXCL12 isoform potency

The goal of this study was to determine whether HA's biochemical properties could affect chemokine binding to the ECM and the response potency of endothelial cells. For both

sprouting and permeability assays, the presence of HA in the matrix shifted CXCL12 isoform potency toward a γ>β>α trend, which is unusual given the reverse trend for binding affinity to the endothelial cell receptor CXCR4. Given HA's inherent negative charge, it is reasonable that a stronger matrix binding affinity would be observed in the more positively-charged CXCL12-γ isoforms than in CXCL12-α isoforms. The increased sprouting and permeability response seen in CXCL12-γ treated HUVECs could be explained by the increased concentration of CXCL12-γ remaining bound to the matrix.

4.3 Concluding Remarks

A few important conclusions can come out of these studies. Hyaluronic acid in this system helped stabilize vessels and prevent vessel leakage, supporting previous research that HMW HA benefits vessel integrity. Further studies will investigate whether inhibiting HA's receptor CD44 on HUVECs will return vascular permeability to collagen-only control levels. Results reveal that HA is capable of shifting relative CXCL12 isoform potency due to its dense negative charge. Increased prevalence of matrix-bound CXCL12-γ in collagen/HA matrices likely contributes to the increased sprouting and permeability response in treated HUVECs. Future studies will perform surface plasmon resonance (SPR) techniques on collagen and collagen/HA gels to quantify how much of each specific isoform binds to each matrix. Understanding how different ECM compositions affect CXCL12 signaling can help in the potential novel development of CXCL12-CXCR4 pathway-targeting cancer therapies. Overall, this research reveals how HA's biochemical properties could potentially alter chemokine signaling and vessel behavior in the tumor microenvironment.

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