Application Note

Cancer Biology

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Quantitative Microscopy of Angiogenesis in Zebrafish Embryos

Using the Agilent BioTek Gen5 microplate reader and imager software to study the inhibition of angiogenesis

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Abstract

Perturbed angiogenesis has implications for numerous conditions, including cancer and ischemic heart disease. This study uses transgenic zebrafish to analyze vascular development over 24 hours of VEGF inhibitor treatment. This was accomplished using time lapse image stacks of control and treated embryos combined with the masking function of Agilent BioTek Gen5 microplate reader and imager software to determine changes in vascular area over time. This application note demonstrates that vascular growth is repressed by VEGF inhibitors in a dose-dependent manner.

Introduction

The circulatory system is one of the first systems to become functional during vertebrate development, and its accurate assembly is essential for proper embryo development and patterning. Blood vessel development is characterized by two fundamental features: the generation of new vessels to supply blood to under-perfused regions and the elimination of vessels that do not sustain proper blood flow to tissues of the body.[1] Blood vessels affect all other tissues, supplying them with oxygen and nutrients necessary to sustain life, and thus it is crucial to understand how they function in both normal and perturbed conditions.

Angiogenesis is the process by which new blood vessels are formed from the pre-existing vasculature. Angiogenesis is a complex and controlled process, and all aspects of it, including endothelial cell migration, proliferation, and activation are under tight regulation of factors that either promote or inhibit angiogenesis.

Angiogenesis plays a crucial role in many conditions including embryonic development, tissue repair, and disease. While inadequate vessel growth leads to tissue ischemia, excessive vascular growth promotes cancer and inflammatory disorders.[2] Enhanced angiogenesis as a reparative therapy is a major goal of regenerative medicine, especially as pertaining to ischemic diseases such as myocardial infarction. On the other hand, inhibition of angiogenesis is an important area of interest for the field of cancer biology. Previous research has shown that tumor angiogenesis is required for the growth and metastasis of solid tumors.[3] As such, inhibition of vessel formation is a potential target for cancer therapy.

Many of the pathways involved in embryonic development are implicated in adult human disease. Zebrafish vascular develpment is an excellent example of this. Zebrafish have a closed circulatory system, and the form of the developing vasculature, the processes used to assemble vessels, and the molecular mechanisms underlying vessel formation are very similar to those in humans.[4] Furthermore, the zebrafish offers many unique advantages for studying vascular development *in vivo*. Zebrafish embryos develop externally, making them easily accessible to manipulation. Also, their optical transparency facilitates high-resolution imaging of blood vessels in the developing embryo.

Circulation begins in the zebrafish embryo at approximately 24 hours post fertilization (hpf). Initially, blood flows through a simple single circulatory loop. The intersegmental vessels (ISVs) of the trunk are among the first angiogenic vessels to form in vertebrate animals. The multistep process for intersegmental vessel formation begins with a set of sprouts emerging from the dorsal aorta and growing along somite boundaries. As they reach the dorsal-lateral surface of the neural tube, the growing vascular segments branch and interconnect to form the dorsal longitudinal anastomotic vessel (DLAV) (Figure 1).[1]

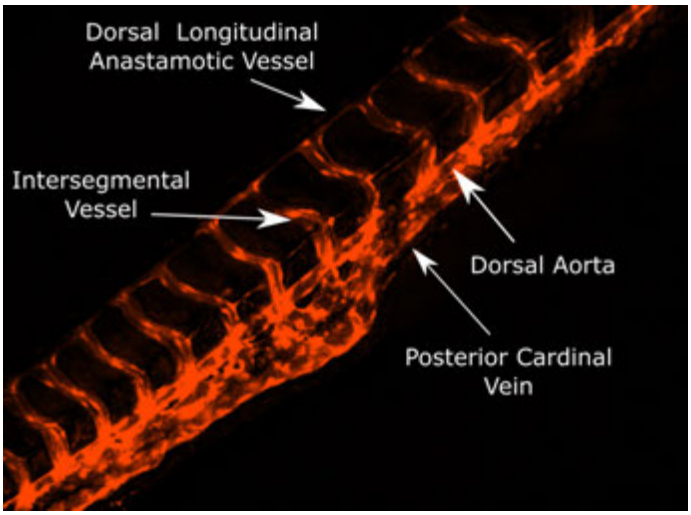

Figure 1. Vascular anatomy of the zebrafish tail at 2 days post fertilization (dpf). Image shown is 10x stack of kdrl:mcherry transgenic zebrafish.

Vascular endothelial growth factor (VEGF) is the master regulator of angiogenesis. VEGF is expressed in the vicinity of sprouting vessels. Endothelial cells express VEGF receptor 2 (VEGFR2) (also known as kdrl), a tyrosine kinase receptor that drives the mitogenic and chemotactic responses of endothelial cells to VEGF.[5] VEGF is important for the formation of ISVs. It is produced and secreted by the somites between which the ISV sprouts migrate, and loss of VEGFR2 function inhibits ISV formation. VEGFR2 and VEGFR1 are expressed primarily on precursors and mature endothelial cells and have been strongly implicated to play a direct role in angiogenesis. For example, VEGFR2 is required for development of mature endothelial cells and mitogenesis.[5]

Blood vessel patterning is highly characteristic in the developing zebrafish embryo and the ISVs can be visualized microscopically as a primary screen for compounds that affect angiogenesis. Small molecules added directly to the fish water diffuse into the embryo and induce observable, dose-dependent effects that can be quantified by image analysis algorithms.[6]

This study uses Tg(kdrl:mcherry)[7] zebrafish embryos, in combination with time-lapse imaging and Gen5 microplate reader and imager software to analyze the decrease in vasculature area over 24 hours of VEGF inhibitor treatment. This study takes time lapse image stacks of control and treated embryos and uses the masking function of Gen5 to determine vasculature area.

Materials and methods

Zebrafish maintenance

Zebrafish were maintained essentially as described in Westerfield.[8] Adult zebrafish, both male and female, were mixed and maintained at 28 °C with a 14/10 hour light/dark cycle. To collect embryos, male and female zebrafish were put into a breeder basket the night before and embryos were collected in the morning. Zebrafish embryos were kept in a 28 °C incubator in E3 media (5 mM NaCl, 0.33 mM $MgSO_4$, 0.33 mM $CaCl_2$, 0.17 mM KCl, and 0.1% methylene blue).

VEGF inhibitor treatment

Embryos were mounted in 1% low melt agarose and positioned in clear bottom black sided 96-well plates (Corning, Corning, NY) in a dorsal orientation. Zebrafish embryos were then treated with the VEGF receptor inhibitors SU5416 or sunitinib (Tocris, Minneapolis, MN). In one experiment, 28 hpf embryos were treated with 2.5 µg/mL, 1.25 µg/mL, 0.65 µg/mL, and 0 µg/mL SU5416. In another experiment, 25 hpf embryos were treated with 100 µg/mL, 25 µg/mL, 6.25 µg/mL, and 0 µg/mL sunitinib. All treatments were diluted in E3 media and 100 µL was added to each well of the 96-well plate.

Imaging

Images were acquired using a 10x objective on an Agilent BioTek Lionheart FX automated microscope configured with a Texas Red light cube. In each well, a beacon was placed at the end of the yolk sac (Figure 2). Stacks of images were taken in both the brightfield and Texas Red channels around the beacon. For each embryo, 10 stacks were taken with a height of 17 µm per stack.

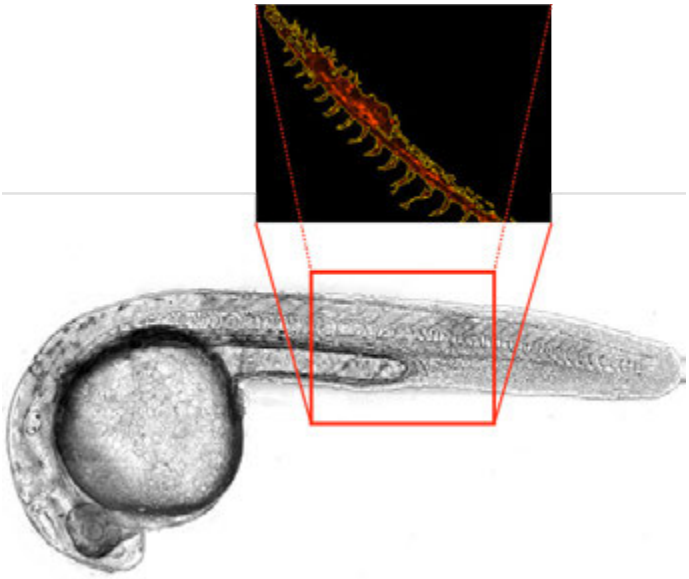

Figure 2. Position of 10x images relative to 1 dpf zebrafish embryo. Beacon is created at the end of the yolk sac so that the resulting image is set around the trunk of the embryo.

Image analysis

Focus stacking was used to process the 10-image stack into one focused image. Next, automatic background flattening parameters were used to remove background fluorescence from the Texas Red channel. No processing was needed in the brightfield channel. Object masking thresholds were then set to identify the vascular area. Images were analyzed by masking on the Texas Red channel. Image preprocessing and analysis settings are presented in detail in Table 1.

Table 1. Agilent BioTek Gen5 microplate reader and imager software settings for Z projection, image preprocessing, and cellular analysis. A Z projection creates a focused stack of the images. Image preprocessing removes background from the stacked imaged to facilitate masking of the vasculature. Cellular analysis creates a mask around the vasculature to calculate the vascular area.

Z Projection	
Image Set	Brightfield/Texas Red
Method	Focus stacking
Size of Maximum Filter	11 px
Top Slice	10
Bottom Slice	1
Image Preprocessing	
Image Set	ZProj[Texas Red 586]
Background	Dark
Rolling Bar Diameter	Auto
Priority	Fast speed
Image Smoothing Strength	0
Cellular Analysis	
Detection Channel: Primary Mask and Count	Tsf[ZProj[Texas Red]]
Threshold	2,000
Background	Dark
Split Touching Objects	Unchecked
Fill Holes in Masks	Unchecked
Minimum Object Size	10 µm
Maximum Object Size	100,000 µm
Include Primary Edge Objects	Checked
Advanced Detection Options	
Image Smoothing Strength	1

Results and discussion

Transgenic zebrafish embryos were treated with SU5416 to determine the effect of the drug on angiogenesis during 1 dpf of zebrafish development. Embryos were treated later in the day, at about 28 to 30 hpf. SU5416 is a VEGFR inhibitor, which is selective for the catalytic activity of VEGFR2.[9] By 1 dpf a simple circulatory system has formed which consists of the dorsal aorta and axial vein. Sprouting of the ISVs has also begun by this point.

Findings show that treatment with SU5416 inhibits vascular growth compared to control embryos that is dose dependent (Figure 3). This results in changes in vascular area where the untreated vascular area increases to about 1.8 times that of the initial area, and embryos treated with the highest dose of 2.5 ug/mL only increase their area to 1.2 times that of the initial area.

Zebrafish were also treated with sunitinib, which is a small molecule that inhibits members of the receptor tyrosine kinase family including VEGF receptors 1 and 2 and platelet derived growth factor receptors (PDGFR). Inhibition of these receptor tyrosine kinases block signal transduction affecting processes involved in angiogenesis.[10] These embryos were treated slightly earlier than the sunitinib treated embryos, at the very beginning of 1 dpf or approximately 25 hpf. Findings show that treatment with sunitinib, similar to SU5416, inhibits vascular growth compared to control embryos that is dose dependent (Figure 4). This results in changes in vascular area where the untreated vascular area increases to about 3 times that of the initial area, and embryos treated with the highest doses of 100 ug/mL only increase their area to 1.5 times the initial area.

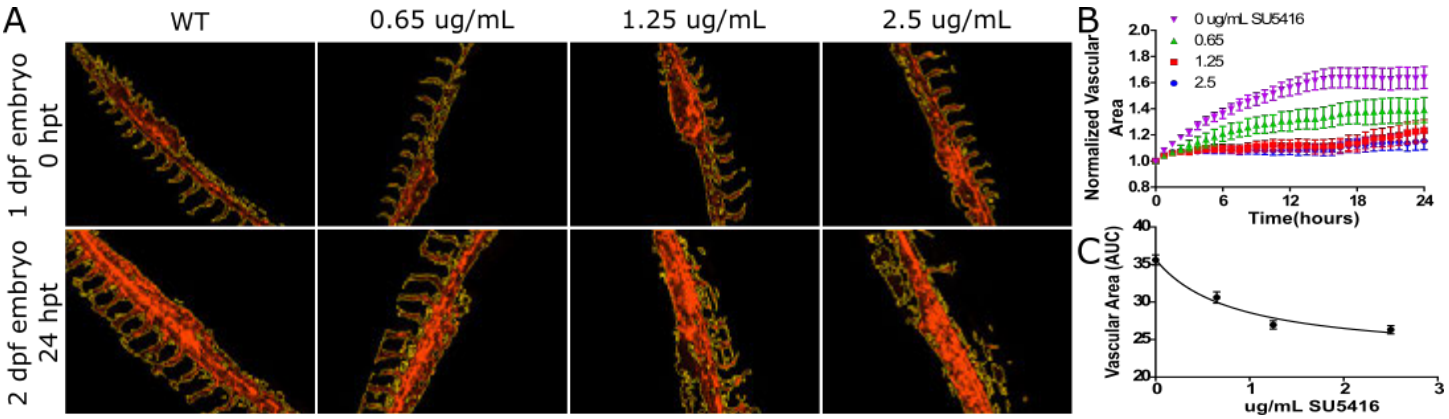

Figure 3. Treatment with SU5416 results in decreased vascular growth in a dose-dependent manner. (A) 10x images of Tg(kdrl:mcherry) zebrafish. Top row is before treatment, bottom row is 24 hours after SU5416 treatment. (B) Graph of vascular area showing area changes in response to sunitinb treatment. (C) Dose response curve at 24 hours showing lower area with higher doses of inhibitor.

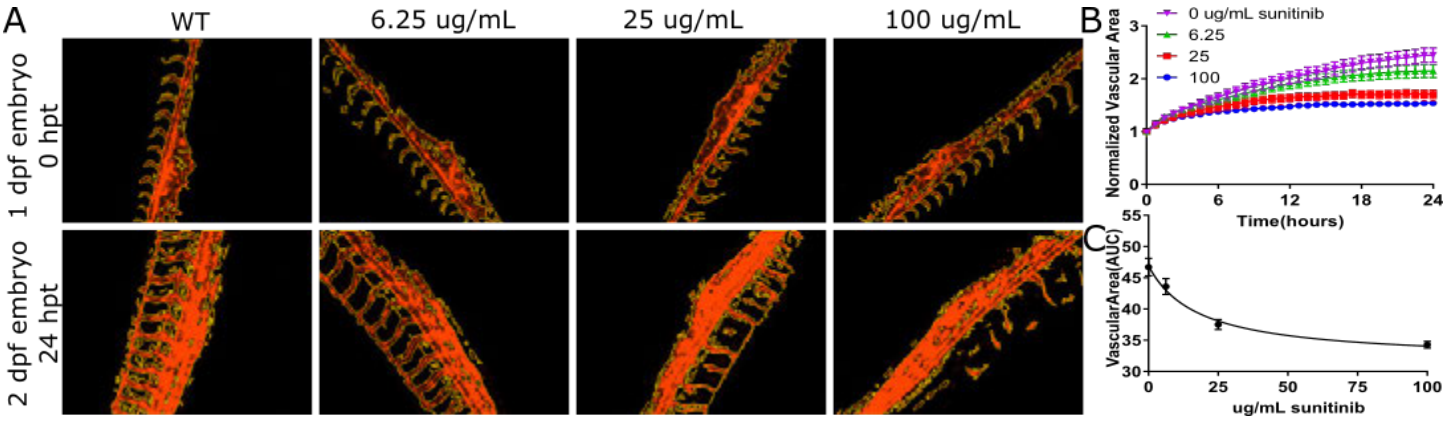

Figure 4. Treatment with sunitinib results in decreased vascular growth in a dose-dependent manner. (A) 10x images of kdrl:mcherry transgenic zebrafish. Top row is before treatment, bottom row is 24 hours after SU5416 treatment. (B) Graph of vascular area showing dose response of sunitinb treatment. (C) Dose response curve at 24 hours.

Conclusion

Angiogenesis is a crucial developmental process, as well as an important therapeutic target for conditions ranging from cancer to heart disease. This study analyzed the decrease in vasculature area over 24 hours of VEGF inhibitor treatment in transgenic zebrafish embryos. As expected, a dose-dependent decrease in vascular area following treatment with SU5416 and sunitinib was seen. Analysis with Agilent BioTek Gen5 microplate reader and imager software allows for consistent and unbiased determination of vascular area.

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