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Fractal analysis of extra-embryonic vessels of chick embryos under the effect of glucosamine and chondroitin sulfates

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ABSTRACT

Like heparan sulfate proteoglycans, some monosaccharides and glycosaminoglycans, such as sulfated glucosamine (GS) and chondroitin (CS), integrate the vascular extracellular matrix and may influence vascular endothelial cell growth. To assess the effects of these substances on blood vessel formation, we used the chick yolk sac membrane (YSM) model and fractal geometry quantification, which provided an objective in vivo method for testing potential agents that promote vasculogenesis and angiogenesis. An image processing method was developed to evaluate YSM capillary vessels after they were implanted in a methylcellulose disk of GS or CS at a concentration between 0.001–0.1 mg/disk (performed on 2-day old embryos). This method resulted in a binary image of the microvascular network (white vessels on a black background). Fractal box-counting (D_{BC}) and information (D_{INF}) dimensions were used to quantify the activity of GS and CS in vasculogenesis and angiogenesis. YSM treated with GS (0.001–0.1 mg) and CS (0.03–0.1 mg) showed an increase in fractal dimensions that corresponded to vitelline vessel growth compared to the control group (vehicle), with GS displaying higher fractal dimension values.

Introduction

Vasculogenesis is the formation of a primary vascular plexus through the differentiation of endothelial cells from hemangioblasts (Czirok and Little, 2012; Liu et al., 2014). Angiogenesis can be understood to be the development of the primary vascular plexus into a branched vascular network (Liu et al., 2014). Both vasculogenesis and angiogenesis are controlled by growth factors, such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) (Flamme et al., 1997; Tabibiazar and Rockson, 2001; Folkman, 2007; Katoh, 2013; Wong and Crawford, 2013). However, some biomolecules can influence growth factors, stimulating vascular growth. Glycosaminoglycans (GAGs) are key biomolecules in the regulation of growth factor signaling, angiogenesis and inflammation (Yip et al., 2006). GAGs are polysaccharides consisting of a disaccharide with repeating units of alternating uronic acids (D-glucoronic acid and L-iduronic acid) and amino sugars (D-galactosamine or D-glucosamine) (Yip et al., 2006; Gandhi and Mancera, 2008). GAGs are present on cell surfaces in the extracellular matrix (ECM), and some can bind and regulate a number of distinct proteins, including chemokines, cytokines, growth factors, morphogens, enzymes and adhesion molecules (Gandhi and Mancera, 2008). Depending on the presence of uronic acids, amino sugars and sulfation, GAGs can be classified as hyaluronan, heparin and heparan sulfate (HS), keratan sulfate, dermatan sulfate or chondroitin sulfate (CS) (Afratis et al., 2012). HS is among the GAGs that may stimulate vascular development, as it can increase the interaction affinity between FGF and its receptor (FGFR). It is also important to the signaling pathway of VEGF, which is involved in blood vessel formation (Iozzo and San Antonio, 2001; Tabibiazar and Rockson, 2001; Coombe, 2008; Dreyfuss et al., 2009; Afratis et al., 2012). One or more HS chains can also covalently attach to a core protein, generating heparan sulfate proteoglycans (HSPGs), which can interact with angiogenic growth factors and thus participate in the regulation of angiogenesis (Fuster and Wang, 2010). Similar to HS, CS may be attached to a core protein, generating a proteoglycan that is able to regulate growth factor activities, such as VEGF and transforming growth factor β (TGF-β) (Le Jan et al., 2012). Therefore, CS can be a potential regulator of angiogenesis. In addition to GAGs, such as HS and CS, glucosamine, an amino sugar that is a component of some GAGs, regulates angiogenic activity (Vournakis et al., 2008; Scherer et al., 2011).

Vasculogenesis and angiogenesis have been studied through various models. An effective study model is the vascular network of the yolk sac membrane (YSM) of chick embryos (Dias et al., 2008a; 2008b). In the analysis of these two processes, some studies have utilized morphometric methods, including fractal geometry. Fractal geometry is used to measure the dimension of fractal objects, and the blood vascular

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network has been well-established as a fractal object due to the following characteristics: self-similarity, irregularity, fractional dimension, and dependence on the scale used to measure the vessel distributions (Mandelbrot, 1983; Grizzi et al., 2005; Costa et al., 2013; Costa and Nogueira, 2015).

The action of glucosamine sulfate on angiogenesis as well as the action of glucosamine and chondroitin sulfates on vasculogenesis has not been well studied. Therefore, we propose the use of fractal geometry to evaluate the two processes of blood vascular development under the action of both glucosamine and chondroitin sulfates.

Materials and methods

Materials

The CS and GS used in these experiments have a purity above 90% and were provided by the Phytomare Company (Governador Celso Ramos, SC, Brazil).

Animals

Pathogen-free fertilized chicken eggs (Ross strain, n = 6 per experimental group, n = 72 eggs in total) were supplied by local poultry producers (Tyson S.A., Florianopolis, SC, Brazil).

All animal studies were carried out in accordance with procedures approved by the Local Committee for the Care and Ethical Use of Animals in Research (CEUA/UFSC, Florianopolis, SC, Brazil), outlined in protocol number PP00586/2011/CEUA/UFSC.

Chicken yolk sac membrane assay

The ability of SC and SG to stimulate vasculogenesis and angiogenesis was determined using YSM as described by Dias et al. (2008a). Pathogen-free fertilized chicken eggs (Ross strain) were supplied by a local commercial incubatory and incubated at 37.5 °C and 70% humidity. After 48 h, the eggs were removed from the incubator and a window (Ø: 10 mm) was opened in the eggshell at a position adjacent to the embryo. The treatment in ovo was performed by implanting methylcellulose disks (7.5 μl volume, 3 mm diameter, one disk per embryo) on the blood islands of 2-day (48 h) old YSM near the cephalic region of the embryos (Fig. 1A). The disks contained CS or GS diluted in phosphate buffered saline (PBS) that ranged from 0.001 to 0.1 mg/disk or PBS only (negative control). The concentrations used were based on the work of Calamia et al. (2014). FGF-2 was used as a positive control (50 ng/disk). The shell-windows were then closed with black binding cellophane tape and the eggs were returned to the incubator until the fourth day (96 h). For each egg, images were captured with a Motic 1000 1.3 MP camera (Motic, Gloucester Road Causeway Bay, Hong Kong, China) attached to a stereomicroscope (20×). The vessels in the region around the limit of the methylcellulose disk were quantified by calculating the fractal dimensions.

Image segmentation

The digital images (1240 × 1024 pixels) were processed to calculate the fractal dimensions of the vascular network as follows. First, the images were manually segmented using Microsoft Paint to differentiate blood vessels from the rest of the images. Each vessel was traced by a line that was 1 pixel thick; then, the images were binarized (i.e., white vessels on black background) using Microsoft Paint (Costa et al., 2013). In this scale, each pixel was equivalent to 7.25 μm.

Fractal analysis

Version 1.3 Fractal Analysis System Benoit software (Trusoft, St. Petersburg, FL, USA) was used to calculate the fractal dimensions of

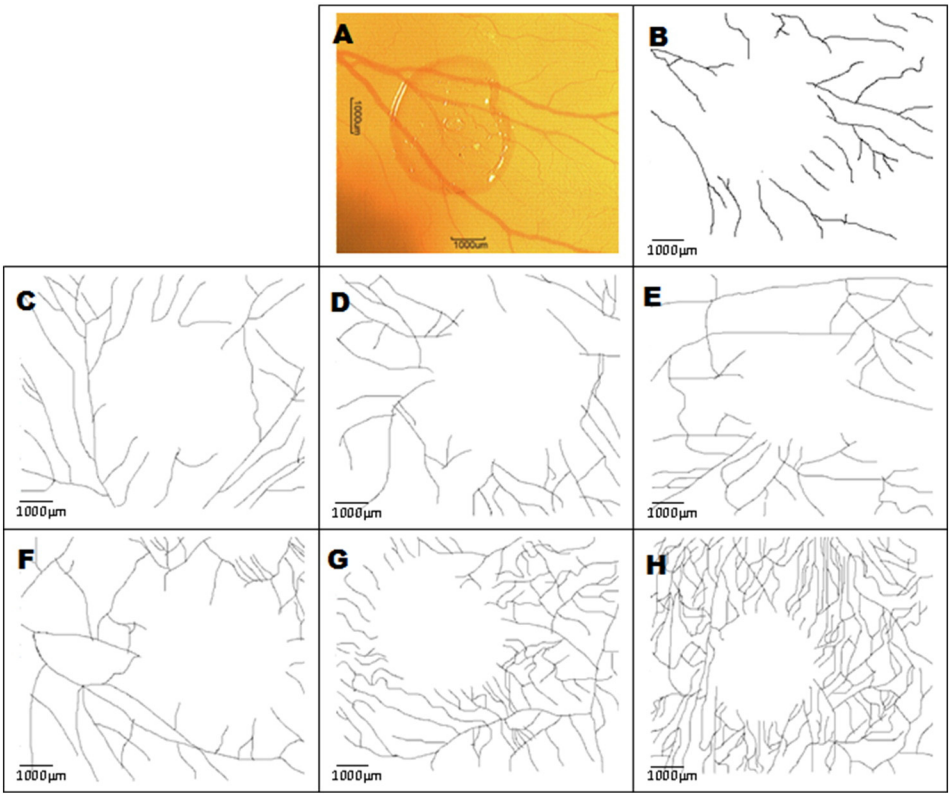

Fig. 1. Methylcellulose disk on YSM vessels and skeletonized images (black vessels on a white background) of the YSM blood vascular network after the implantation of methylcellulose disks with different compounds. A: methylcellulose disk supports on YSM vessels; B: vehicle (PBS, negative control); C: SC, 0.001 mg/disk; D: SC, 0.003 mg/disk; E: SC, 0.01 mg/disk; F: SC, 0.03 mg/disk; G: SC, 0.1 mg/disk; H: FGF2 (positive control). Final magnification: ×20 (bars: 1000 μm).