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Differential effects of Heparitinase I and Heparitinase III on endothelial tube formation *in vitro*

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Abstract

Heparan sulfate proteoglycans (HSPGs) play vital roles in many steps of angiogenesis under physiological and pathological conditions. HSPGs on endothelial cell surfaces act as coreceptors for a variety of pro-angiogenic growth factors such as FGF and VEGF and anti-angiogenic factors such as endostatin. However, the fine structural requirements of these binding interactions are dependent on the sulfation patterns of HSPGs. Previous studies have shown that Heparitinases, heparin lyases isolated from *flavobacterium heparinum*, can cleave heparan sulfate chains. These enzymes have been shown to reduce tumor—derived neovascularization *in vivo* in mice. However, the results from these experiments could not conclusively pinpoint the origin of the HS fragments. Thus, in this study we utilized an *in vitro* assay to assess the differential effects of Heparitinase I (Hep I) and Heparitinase III (Hep III) on endothelial tube formation. Hep III was found to be a more potent inhibitor of tube formation than Hep I. In conclusion, differential cleavage of endothelial cell surface bound HS can affect the extent of inhibition of tube formation.

Keywords

Heparan sulfate; Angiogenesis; Heps; Heparin lyases

Introduction

It is well known that solid tumors require extensive angiogenesis in order to facilitate their aberrant growth.[1] Targeting angiogenesis has been proven to be an effective strategy to starve tumors and increase patient survival. Heparan sulfate proteoglycans, present on cell surfaces and within the extracellular matrix, are intimately involved with endothelial cell function and have been shown to be important in angiogenesis and tumor progression.[2;3] By binding to growth factors such as FGF, HBEGF, and VEGF, heparan sulfates act as co-receptors that are integral to signal transduction.[4] However, in order to bind growth factors, HS chains need certain sulfation patterns. A well-studied example, FGF2-binding-HS chains contain *N*-Sulfated glucosamine units and 2-*O* sulfated iduronic acid.[5]

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Additionally, only HS chains containing 6-*O* sulfated glucosamine residues and 2-*O* sulfated iduronic acid along with *N*-sulfated glucosamine can bind to FGF receptor.[6;7] Thus, a combination of unique sulfation codes is necessary to potentiate FGF/FGFR mediated signaling. Tumors tailor their sulfation patterns using aberrant expression of HS biosynthetic and catabolic enzymes to improve their survivability.[3;8]

Heparitinases, isolated from *Flavobacterium Heparinum*, have been found to cleave heparan sulfate chains at defined locations (Figure 1). Heparitinase I (Hep I, EC 4.2.2.8) cleaves HS chains containing glucuronic acid residues adjacent to glucosamine residues containing either *N*-acetyl or *N*-sulfate groups.[9;10] Heparitinase III (Hep III, EC 4.2.2.7) cleaves near sulfated iduronic acid residues.[9;10;11]

It has been known for a long time that low molecular weight heparins, formed by treatment of unfractionated heparin with heparin lyases, have anti-angiogenic properties.[12] Previously, it has also been found that cell surface HS, digested with Hep I and III, reduces VEGF₁₆₅ binding and activity in endothelial cells.[13;14] Hep I and III also reduced neovascularization in an ex vivo CAM assay by reducing bFGF binding and subsequent signaling.[15] Additionally, both direct Hep I injections and Hep I-generated fragments have been found to inhibit myeloma growth in vivo in SCID mice.[16] Hep I and III have also shown differential effects in reducing tumor growth and angiogenesis in vivo.[17]

Past results have shown the anti-angiogenic activity of Hep I and III. However, several of these results are muddled due to digestion of tumor derived GAGs or due to digestion of heparan sulfate chains from surrounding tissue as well as from endothelial cell surfaces. The effects of direct Heparitinase digestion of endothelial GAGs on angiogenesis have not yet been examined. Thus, in this study we examined the differential effects of Hep I and III on tube formation of BLMVEC on RGF-BME *in vitro*.

Materials and Methods

Cell culture

Bovine lung microvascular endothelial cells of passage 4-8 (a kind gift from Dr. Randal Dull) were cultured in MCDB-131 Complete media (Vec Technologies). Hep I and Hep III from *flavobacterium heparinum* were expressed and purified as previously described.[18]

Tube formation assay

Reduced growth factor basement membrane matrix (RGF-BME, Trevigen) was thawed overnight for 16 hrs at 4 °C. 50 µl of RGF-BME was then plated out in each well of a 96 well plate. Plates were then incubated in a humidified incubator at 37 °C for 1 hr. BLMVEC were then suspended by incubation with Tryp LE Express (Invitrogen). 1×10^5 cells were then added to each well along with MCDB-131 Complete media and different amounts of Hep I and Hep III. Plates were then incubated in the incubator for 16 hrs prior to calcein staining and imaging.

Calcein staining

Media was removed from wells in the 96 well plate by gentle dabbing with a paper towel. Cells were then washed twice with PBS and incubated in 100 µl of 2 µM Calcein AM for 30 min in the humidified incubator at 37 °C. After incubation in the Calcein AM working solution, the cells were washed once again with PBS and imaged in a Olympus IX81 microscope with an attached color CCD Filter and a GFP emission filter using 485 nm excitation/520 nm emission.

Results and discussion

Amounts of Hep I and Hep III needed to have comparable enzymatic activity were added to BLMVEC as described in the methods section. Since BLMVEC form tubes spontaneously on the basement membrane extract in the culture media, wells without any additives were used as positive controls. Sulforaphane (included with the tube formation assay) at 20 μ M final concentration was used as a negative control. 30 mU and 100 mU of either enzyme were added to 200 μ l of total volume including cells and media. Hep III was found to be a much stronger inhibitor of angiogenesis compared to Hep I as shown in Figure 2. Tube formation in the well containing 100 mU Hep I is comparable to that in the well containing 30 mU Hep III in terms of the qualitative extent of tube formation and the number of tubes. The 100 mU Hep III well is comparable to the inhibition shown in the negative control. Additional tests performed at 60 mU of activity also led to the same conclusion (data not provided).

Angiogenesis is critical to tumor growth and heparan sulfates play key roles in this process by acting as co-receptors for growth factors among other functions. Thus, altering heparan sulfate interactions using drugs or enzymes can be a potent method of mitigating tumor growth and survival. Several studies have already shown the efficacy of this treatment strategy. Heparin, an antagonist of P-Selectin mediated interactions with platelets, has been known to reduce tumor metastasis and increase tumor-monocyte interactions by inhibiting the formation of a platelet 'cloak'. [19] Additionally, PI-88, a highly charged heparin analog, has entered human clinical trials because it prevents angiogenesis mediated by FGF and VEGF among other effects. [20] By targeting heparan sulfates, it is possible to reduce cancer growth and increase patient survival.

Heparan sulfates represent a potent target for angiogenesis treatments because of their numerous interactions with growth factors on cell surfaces and within the extracellular matrix. In this study we found that Hep treatment of endothelial cell heparan sulfates reduces their capability to form capillary tubes *in vitro*. Hep III was a more potent inhibitor of tube formation when compared with Hep I at equal activity. We attribute this difference to the substrate specificities of the two enzymes. Hep I cleaves at undersulfated, NA, regions of HS chains whereas Hep III cleaves at highly sulfated, NS, regions. Hep III therefore may disrupt the growth factor binding domains that are important for angiogenesis. Hep I, on the other hand, still generates fragments that retain their growth factor binding capability and therefore still potentiate tube formation.

This conclusion is also supported by previous studies that show that heparan sulfate proteoglycans on endothelial cells are essential to VEGF and FGF binding (potent proangiogenesis factors). Future studies will probe the relative importance of the different sulfate groups in the highly sulfated HS regions with the aid of specific, reversible biosynthetic inhibitors that are currently under development in our lab. Utilizing such knowledge will allow researchers to further understand the structure-function relationships of heparan sulfate proteoglycans in angiogenesis. In conclusion, we have discovered that the NS domains of heparan sulfates are more important to endothelial tube formation than the non-sulfated domains.

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Figure 1. Site of action of **a)** Hep I and **b)** Hep III. Hep I fragments NA domains so that only NS domains are left intact. Hep III fragments NS domains and leaves NA domains intact.

Figure 2.

Hep III and Hep I were added at equal activities onto BLMVEC on GFR matrigel. **a)** Control without enzymes **b)** –Control with 20 μ M Sulforaphane **c)** Hep I 30 mU **d)** Hep I 100 mU **e)** Hep III 30 mU **f)** Hep III 100 mU. Results are from three independent experiments performed in triplicate.