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Differential effect of hypoxia on early endothelial–mesenchymal transition response to transforming growth beta isoforms 1 and 2

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ABSTRACT

Angiogenesis is essential for mammalian development and tissue homeostasis, and is involved in several pathological processes, including tumor growth and dissemination. Many factors within the tissue microenvironment are known to modulate angiogenesis, including cytokines, such as transforming growth factor beta (TGFβ), and oxygen level. TGFβ exists in three different isoforms (1, 2 and 3), all of which (albeit in different contexts) might mediate angiogenesis and are able to induce endothelial–mesenchymal transition (EndoMT), a process involved in heart development, pathologic fibrosis and, as recently reported, in angiogenesis. Low oxygen level, referred to as hypoxia, has been independently shown to induce angiogenesis, modulate TGFβ signalling and promote EndoMT. However, how these phenomena might be interconnected to drive angiogenesis is rather unexplored. To begin addressing the potential contribution of TGFβ-induced EndoMT to angiogenesis, and to explore how microenvironmental hypoxia might influence these processes, we investigated the effect of TGFβ isoforms 1 and 2 on early EndoMT response in cultured adult endothelium under standard (21 %) and hypoxic (1 %) culture conditions. Our data indicates that EndoMT-like changes, such as an increase in expression and nuclear translocation of Snail, Slug and Zeb1, and reduction of VE-cadherin expression, occur in response to TGFβ1 and/or TGFβ2 as early as 6 h after stimulation and might be enhanced by hypoxia in an isoform-specific manner. Further, hypoxia enhances canonical TGFβ signalling, and appears to be a key determinant of Snail's differential involvement in endothelial cell responses to TGFβ1 versus TGFβ2.

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1. Introduction

Angiogenesis, the formation of new blood vessels by sprouting from pre-existing ones, is a crucial process that regulates the level of oxygen and nutrients delivered to tissues. It is a highly complex phenomenon, orchestrated by the interaction of many growth factors, cytokines and environmental factors, including vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ) and hypoxia, among many others (Carmeliet and Jain, 2011).

Judah Folkman was the first to observe that in order for solid tumors to grow past 1 mm^3 they need to trigger angiogenesis to acquire a blood supply (Folkman, 1971). This facilitates tumor growth and metastasis by providing the cancer cells with more nutrients and oxygen, as well as means for cancer cells to escape and colonize distant sites (Carmeliet and Jain, 2011). Despite these broadly accepted pivotal roles of angiogenesis in tumor progression, the clinical effectiveness of anti-angiogenic therapy in cancer has been rather modest with virtually no impact on overall survival (McIntyre and Harris, 2015), highlighting the need for improved anti-angiogenic targeting. Based on evidence from our group (Kuczynski et al., 2011a; Kuczynski et al., 2011b) and others (reviewed in (Hawinkels et al., 2013)), we believe TGFβ signaling targeting might prove beneficial in improving response to specific anti-angiogenic therapies, such as VEGF-targeted modalities (Hawinkels et al., 2013). However, a better understanding of TGFβ signaling in adult endothelium, in particular in the complex and multifactorial tumor environment, is imperative to validate this concept.

TGFβ is part of a 33-member superfamily that includes activins, nodal and BMPs, and TGFβ itself can exist as 3 different isoforms (1, 2 and 3), which are expressed in nearly all cell types and tissues. Signaling by TGFβ family members is mediated through canonical (SMAD-dependent) and non-canonical (SMAD-independent) pathways that regulate transcription, translation, protein synthesis, and post-translational modifications (reviewed in (Viloria-Petit et al., 2013)). In the canonical signaling pathway, TGFβ ligands bind to the type II TGFβ receptor (TβRII) with high affinity, and this in turn recruits the type I TGFβ receptor (TβRI). Both TGFβ receptors contain transmembrane serine/threonine kinase domains. TβRII's kinase domain is constitutively active but TβRI kinase activation depends on TβRII. Once in close proximity,

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TβRII is able to phosphorylate TβRI (Wrana et al., 1994). There are 2 different types of TβRI: activin-like kinase-5 (ALK5) and activin-like kinase-1 (ALK1). ALK5 is widely expressed across all cell types, whereas ALK1 expression is restricted to the endothelium. Further, the level of activation of these Type I receptors is modulated by Endoglin, a type III co-receptor that is primarily expressed by endothelial cells (Goumans et al., 2003). Upon TβRI phosphorylation, nuclear effector proteins called receptor-SMADs (R-SMADs) bind to TβRI. This enables their activation via phosphorylation of SMAD2/3 by ALK5 or SMAD1/5 by ALK1. The phosphorylated SMAD proteins dissociate from the SMAD Anchor for Receptor Activation (SARA) protein and hetero-oligomerize with co-SMAD4. This enables nuclear translocation and interaction with different transcriptional modulators, which facilitate either activation or suppression of gene transcription (Massague, 2012; Viloria-Petit et al., 2013).

The essential role played by TGFβ in angiogenesis has been extensively demonstrated *in vitro* and *in vivo*, most dramatically through knockout studies (Jakobsson and van Meeteren, 2013). Mice deficient in TGFβ1 die in utero due to vascular defects, including the formation of weak, aberrantly formed capillaries (Dickson et al., 1995), while TGFβ2-null embryos display, among other developmental defects, severe cardiac malformations, which were found to result from defective endothelial-to-mesenchymal transition (EndoMT) (Bartram et al., 2001). TGFβ3-null mice have delayed pulmonary development and cleft palate but lack an angiogenesis-associated phenotype (Kaartinen et al., 1995). Of note, the specific contribution of each individual TGFβ isoform and TGFβ-induced EndoMT to adult physiologic and tumor angiogenesis is unclear, in part due to the complexity of TGFβ signaling in endothelium.

TGFβ signalling outcome in endothelial cells can be modulated by many factors, including the relative abundance of its different isoforms and of other molecular members of its superfamily, the TGFβ receptor type I that gets incorporated into the signaling pathway, and the level of expression of co-receptors (van Meeteren et al., 2011; Viloria-Petit et al., 2013). For instance, TGFβ has been shown to exert a biphasic effect on endothelial cells, whereby low doses promote endothelial cell migration and proliferation but high doses inhibit these processes (Goumans et al., 2002; Pepper et al., 1993). This can be attributed to the presence of two TGFβ type I receptors in endothelial cells, ALK5 and ALK1, with the first one favoring inhibitory signals at higher doses, and the later one favoring stimulatory signals at lower doses (Goumans et al., 2002). To add to this complexity, ALK5 is activated by TGFβ whereas the physiological ligands for ALK1 also include BMP9 and BMP10 (Chen et al., 2013), and the level of Endoglin further influences the relative activation of ALK1 versus ALK5. Endoglin cytoplasmic and extracellular domains were shown to interact specifically with ALK1, and *in vitro* studies with embryonic stem cells that lacked one or both endoglin alleles demonstrated that endoglin expression mediates TGFβ1 signalling through ALK1 and this promotes cell proliferation (Blanco et al., 2008; Lebrin et al., 2004; Lopez-Novoa and Bernabeu, 2010).

HIF-1, the main effector of the hypoxia pathway, is responsible for driving the expression of VEGF-A to promote angiogenesis. Previous research has identified an active hypoxia response element (HRE) within VEGF promoter and implicated that TGFβ can cooperate with hypoxia to enhance VEGF transcription (Sanchez-Elsner et al., 2001), providing some of the first evidence that these two factors can work together to drive angiogenesis. In addition, several studies suggest that hypoxia regulates the expression of TGFβ1, 2 and 3 (Caniggia et al., 2000; Hung et al., 2013; Zhang et al., 2003), and promotes TGFβ signalling in endothelium via enhancement of endoglin and ALK1-mediated signalling (Sanchez-Elsner et al., 2002; Tian et al., 2010). Finally, hypoxia also induces the expression of EndoMT-associated transcription factor Snail and Slug (Ehsan et al., 2014; Xu et al., 2015). Both of these factors are induced by TGFβ in endothelial cells (Zhang et al., 2014), and were recently demonstrated to mediate the sprouting phase of angiogenesis (Welch-Reardon et al., 2014), thus providing the first evidence that EndoMT drives angiogenesis.

To begin addressing the potential contribution of TGFβ-induced EndoMT to angiogenesis, and to explore how microenvironmental hypoxia influences these processes, here we investigate the effect of TGFβ isoforms 1 and 2 on early EndoMT response in adult endothelium under standard and low oxygen culture conditions. Taken together, our data indicate that early EndoMT-like changes occur in response to either TGFβ1 or TGFβ2 and are enhanced by hypoxia. Further, hypoxia appears to be a key determinant of Snail's differential involvement in endothelial cell responses to TGFβ1 versus TGFβ2.

2. Material and methods

2.1. Antibodies and growth factors

The source, catalog number and dilutions for each specific antibody were as follow: from Cell Signaling Technology: SMAD2 (#3103) 1:1000, phospho-SMAD2 (S465/467, #3101) 1:500, SMAD1 (#6944) 1:1000, phospho-SMAD1/5 (Ser463/465, #9516) 1:1000, Snail (#3879) 1:1000, Slug (#9585) 1:1000, Zeb1 (#3396) 1:1000, Lamin A/C (#2032) 1:2000, VEGFR2 (#2472) 1:1000, and Cleaved Caspase-3 (#9664) 1:1000; from Santa Cruz Biotechnologies: ZO-1 (sc-33725) 1:4000, Zeb2 (sc-48789) 1:2000, ALK5 (sc-398) 1:500, PECAM (sc-1505) 1:200, VE-cadherin (sc-6458) 1:2000; from Sigma-Aldrich: α-tubulin (T5168) 1:1,000,000. Secondary antibodies used were goat-anti mouse HRP (A0168) 1:10,000, mouse anti-rabbit HRP (A0545) 1:10,000, rabbit anti-goat (A5420) 1:25,000 or 1:50,000, all from Sigma-Aldrich; and goat anti-rat HRP (AP136P) 1:4000 (Millipore). Growth factors included: rhTGFβ1, rhTGFβ2, rhVEGFA, and rhBMP-9 (all from Invitrogen, Life Technologies).

2.2. Cell lines and culture conditions

Primary Bovine Aortic Endothelial Cells (BAEC) were isolated and characterized as previously described (Coomber, 1995) from the aorta of a single donor at the Ontario Veterinary College, and were grown and maintained as monolayers in high glucose DMEM (Thermoscientific) supplemented with 5% fetal bovine serum (FBS; Invitrogen) and 1 mM Sodium Pyruvate (Sigma). Media was changed every 3–4 days. Cells were used in experiments between passages 5 and 10. Once confluency was reached, cells were either passaged or plated into an experiment. To passage BAEC, monolayers were washed once with PBS and then trypsinized using a 0.05 % Trypsin and 0.53 mM EDTA (Invitrogen) solution. Cells were collected through centrifugation at 350 ×*g* for 4 min, and resuspended in fresh complete media. Cells were re-plated with a split ratio of 1:4. Cells to be used for experiments were serum starved for 24 h prior to treatment, by washing the monolayer with PBS and then adding reduced serum media (DMEM supplemented with 0.2% FBS, 1 mM sodium pyruvate). Cells were maintained in a humidified incubator at 37 °C in the presence of 5% CO_2 and 95% atmospheric air (21% O_2). The representative normoxia oxygen concentration of 21% was chosen based on this standard tissue culture condition, which is used for most *in vitro* studies worldwide; although normal tissue oxygenation *in vivo* is estimated to be between 3 and 5% O_2 (Brizel et al., 1996; Hockel et al., 1996). The oxygen level to represent hypoxia was chosen because 1% oxygen (equivalent to 7 mm Hg) is within the range of what has been previously documented within solid tumors (Vaupel et al., 2004). To achieve hypoxic culture conditions, cells were incubated in a hypoxia modulator chamber (Stemcell Technologies) purged with gas (1% oxygen, 5% CO_2, 94% N_2) at a rate of 20 L/min for 10 min. At 10 min, the chamber was sealed and placed in the incubator.

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