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Effects of normal and high circulating concentrations of activin A on vascular endothelial cell functions and vasoactive factor production

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ABSTRACT

Objectives: Activin A, a TGFβ family member, circulates in the maternal blood at increasing concentrations throughout gestation during a healthy pregnancy. The circulating concentration of activin A is further increased in pre-eclampsia (PE), a hypertensive disorder of pregnancy that is marked by systemic maternal vascular endothelial cell dysfunction. The effect of increasing activin A concentrations on the maternal vascular endothelium is unknown. The study aim was to investigate the effect of physiological and pathological activin A concentrations observed in normotensive and PE pregnancies respectively, on vascular endothelial cell function.

Methods and results: Immunostaining demonstrated the presence of the activin A receptor, ACVR2A, in SGHEC-7 cells used to model the vascular endothelium. SGHEC-7 cells were treated with activin A concentrations representative of concentrations throughout gestation in normotensive (0–10 ng/ml) and PE (50 ng/ml) pregnancies. xCELLigence functional assays revealed that normotensive activin A concentrations increased SGHEC-7 proliferation and migration, which was inhibited by PE concentrations. Additionally, fluorescence based assays showed that PE concentrations increased endothelial permeability. None of the tested activin A concentrations affected cell apoptosis. PE concentrations also resulted in an imbalance of the vasoactive factors *eNOS*, *PTGIS* and *EDN1*, as determined by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assays.

Conclusion: Compared with normotensive activin A concentrations, the higher PE activin A concentrations resulted in abnormal endothelial functions, which may contribute to the systemic maternal vascular endothelial cell dysfunction observed in the disorder.

Introduction

Activin A is a transforming growth factor (TGF) β superfamily glycoprotein hormone. TGF β superfamily members are involved in cellular processes of proliferation, differentiation and apoptosis [1]. The regulation of these cellular processes in female reproduction leads to controlled cycles of menstruation, decidualisation and pregnancy establishment [2]. The well-characterised function of activin is to stimulate follicle stimulating hormone release from the pituitary gland [3]. Throughout pregnancy, circulating maternal concentrations of activin A gradually rise until parturition [4–6].

The fetal placenta is the main source of activin A in the maternal circulation [7], as placental delivery results in significant decreases of circulating maternal activin A [8]. The fetal membranes and maternal decidua are additional sources of activin A [9]. Activin receptors are abundantly present at the maternal-fetal interface and expressed by various cell types such as maternal decidual stromal and vascular endothelial cells, and fetal syncytiotrophoblast and extravillous trophoblast cells [10–12]. Activin A regulates activin receptor expression [13], adhesion [14] and invasion of trophoblast cells [15,16]. Activin A levels rise in endometrial stromal cells during the decidualisation process, which further enhances decidualisation [17], increases matrix metalloproteinase production [18] and regulates pro-inflammatory and angiogenic cytokine secretion [19]. Activin A also regulates decidualised endometrial stromal cell apoptosis through caspase 3 activity

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[20]. Therefore, activin A is clearly a key player at the maternal-fetal interface during placentation.

Pre-eclampsia (PE) is a proteinuric hypertensive disorder of pregnancy, which is associated with systemic maternal vascular endothelial cell dysfunction. Abnormal placentation, characterised by shallow trophoblast invasion into the maternal uterine wall and deficient spiral artery remodelling, is commonly observed in PE [21]. The dysfunctional PE placenta releases many factors into the maternal circulation that damage the maternal vascular endothelium, leading to the clinical syndrome of PE [22]. Increased placental activin A production in PE [10,11] correlates with increased maternal serum concentrations [8,23–25]. Circulating maternal activin A concentrations increase to 50 ng/ml or higher in PE, well above the baseline concentrations of up to 10 ng/ml in a normotensive pregnancy [8,23–25]. Maternal blood activin A concentrations are also significantly increased weeks before the onset of clinical disease [26–29]. Additionally, administration of activin A into pregnant mice induces a PE-like phenotype that includes hypertension, proteinuria and endothelial dysfunction [30]. Collectively, these studies support a role for activin A in the development of PE.

However, no study has yet examined the impact of changing activin A concentrations on the maternal vascular endothelium in normotensive and PE pregnancies. We hypothesised that pathologically high activin A concentrations observed in PE contribute to maternal vascular endothelial cell dysfunction by adverse effects on key endothelial cell functions. Therefore, the study aim was to examine the effect of physiological and pathological circulating concentrations of activin A found in normotensive and PE pregnancies on endothelial cell functions, using an *in vitro* cell line model of the maternal endothelium.

Materials and methods

Cell culture

The well-characterised SGHEC-7 vascular endothelial cell line was derived from primary human umbilical vein endothelial cells transfected with the early region of SV40 and has an extended lifespan of up to 24 passages [31]. Cells were maintained in RPMI 1640 medium and Medium 199 in a 1:1 ratio with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin under humidified conditions (5% CO_2/95% air) at 37 °C. All tissue culture reagents except Medium 199 and FCS were obtained from Murdoch Children's Research Institute (Melbourne, Australia). Medium 199 and FCS were obtained from Thermo Fisher Scientific Corp. (Waltham, MA, USA).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde solution for 10 min at room temperature. Blocking was performed with 10% goat serum/PBS for 1 h at room temperature. After a 4 °C overnight incubation with rabbit polyclonal activin receptor type IIA ab71521 antibody (Abcam, Cambridge, UK) at 5 μg/ml, staining was performed using Zymed Laboratories Histostain®-Plus Broad Spectrum Kit and Zymed Laboratories AEC Red Substrate Kit (Thermo Fisher Scientific Corp.) as described previously [32]. Cells were visualised at 200× magnification with an Axioskop 2 light microscope (Zeiss Gruppe, Oberkochen, Germany) and a Nikon DXM1200C camera (Nikon Corp., Tokyo, Japan).

Activin A treatment

Cells were treated with recombinant human activin A (R&D Systems, Minneapolis, MN, USA) diluted in serum-free media at concentrations of 0.1–10 ng/ml and 50 ng/ml, which are reflective of concentrations detected throughout gestation in normotensive and PE pregnancies respectively [4,23]. Cells not treated with activin A served as baseline controls in all experiments. Medium supplemented with 10% FCS was used as a positive control in the proliferation and migration assays, while 400 ng/ml of actinomycin D (Sigma–Aldrich, St. Louis, MO, USA) was used as a positive control in the permeability and time-lapse microscopy apoptosis assays.

Proliferation and migration assays

Proliferation and migration assays were performed using the xCELLigence RTCA DP Analyzer (ACEA Biosciences Inc., San Diego, CA, USA) with E-16 plates and CIM-16 plates for proliferation and migration respectively, according to the manufacturer's protocol. The xCELLigence RTCA DP Analyzer was housed in a humidified incubator (5% CO_2/95% air) at 37 °C. xCELLigence assays are produce data consistent with conventional endpoint assays for proliferation and migration [33]. xCELLigence results are expressed as an arbitrary cell index. The cell index was recorded every 5 min for 24 h. For analyses, the cell index of each timepoint was normalised to the starting cell index of each well after cells were added, so as to maintain consistency between experiments.

Permeability assay

Permeability was determined using the fluorescence-based *In Vitro* Vascular Permeability Assay (Millipore Corporation, Billerica, MA, USA), according to the manufacturer's protocol. Fluorescence was measured at 485 nm excitation and 520 nm emission using FLUOstar OPTIMA fluorescence plate reader (BMG LABTECH, Offenburg, Germany). After treatment, cell monolayers were stained with Cell Stain solution provided in the kit and viewed by light microscopy on a Zeiss Axiovert 100 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with a DP200 camera (DeltaPix, Maalov, Denmark).

Apoptosis assays: time-lapse microscopy and annexin V flow cytometry

Apoptosis was monitored by time-lapse microscopy over 24 h using the Cell-R microscope (Olympus Corporation, Tokyo, Japan) with a motorised stage in a humidified (5% CO_2/95% air) chamber at 37 °C. After addition of treatment medium, each well was photographed every 15 min using the inbuilt software. For analyses, the frame at which apoptotic morphology first appeared was recorded for 40 cells chosen at random from each treatment group. Apoptotic morphology was defined as visible cell condensation, membrane blebbing and apoptotic body formation.

Apoptosis was also determined after 24 h using TACS® Annexin V Kit (Trevigen Inc., Gaithersburg, MD, USA), according to the manufacturer's protocol. Samples were processed within 1 h by flow cytometry in a LSR II Flow Cytometer (BD Biosciences, San Jose, CA, USA).

RNA extraction, cDNA synthesis and quantitative real-time polymerase chain reaction

Total RNA was extracted from treated cells using the Ambion Mini RNA kit (Thermo Fisher Scientific Corp.) according to the manufacturer's protocol. Quality and quantity of extracted RNA was determined using a NanoDrop 2000 (Thermo Fisher Scientific Corp.).

Subsequently, cDNA was synthesised using Superscript III (Life Technologies Corp., Carlsbad, CA, USA) according to manufacturer's instructions on a Veriti® 96-well Thermal Cycler (Life Technologies Corp.).