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Abnormal angiogenesis in blood outgrowth endothelial cells derived from von Willebrand disease patients

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Abstract

Bleeding associated with angiodysplasia is a common, often intractable complication in patients with von Willebrand disease (VWD). Von Willebrand factor (VWF), the protein deficient or defective in VWD, is a negative regulator of angiogenesis, which may explain the pathologic blood vessel growth in VWD.

OBJECTIVE—This study explores the normal range of angiogenesis in blood outgrowth endothelial cells (BOECs) derived from healthy donors and compares this to angiogenesis in BOECs from VWD patients of all types and subtypes.

METHODS—BOECs were assessed for VWF and angiopoietin-2 (Ang-2) gene expression, secretion, and storage. To explore angiogenic potential we characterized cellular proliferation, matrix protein adhesion, migration, and tubule formation.

RESULTS—We found great angiogenic variability in VWD BOECs with respect to each of the angiogenesis parameters. However, type 1 and 3 VWD BOECs had higher Ang-2 secretion associated with impaired endothelial cell migration velocity and enhanced directionality. Type 2A and 2B BOECs were the most proliferative and multiple VWD BOECs had impaired tubule formation in Matrigel.

CONCLUSION—This study highlights the angiogenic variability in BOECs derived from VWD patients. Abnormal cell proliferation, migration, and increased Ang-2 secretion are common features of VWD BOECs. Despite the many abnormalities of VWD BOECs, significant

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SS, LC MB, LH, AL, JM, MO, SA, and DM: None Declared.

Author Contributions

S. N. Selvam and L. J. Casey made equally important contributions to this manuscript. S. N. Selvam performed research, data analysis and interpretation, and wrote the manuscript. L. J. Casey performed research, data analysis and interpretation, and contributed to the writing of the manuscript. M. Bowman, L. Hawke, and A. J. Longmore performed research and contributed to the writing of the manuscript. J. Mewburn and S. Archer helped obtain high resolution confocal images and reviewed the manuscript. M. O. helped with flow cytometry data acquisition and analysis. D. H. Maurice helped with the design of the study and reviewed the manuscript. P. D. James designed the study, supervised research, and reviewed the manuscript. All authors approved the final version of the manuscript.

heterogeneity amongst individual VWD phenotypes precludes a simple description of relationship between VWD type and in vitro surrogates for angiodyplasia.

Keywords

Angiogenesis; Angiopoietin-2; Endothelial cells; von Willebrand disease; von Willebrand factor

Introduction

von Willebrand factor (VWF), a multimeric glycoprotein, is required for normal hemostasis. VWF forms adhesive strings that mediate platelet adhesion and aggregation to sites of vascular damage [1]. VWF is primarily produced by endothelial cells (ECs) and is either constitutively secreted or stored in rod-shaped Weibel-Palade bodies (WPBs) [2]. Deficiency or dysfunction of VWF can cause von Willebrand disease (VWD) – the most commonly inherited bleeding disorder in humans affecting 0.1 to 1% of the population [3,4]. The International Society on Thrombosis and Haemostasis (ISTH) currently recognizes three types of VWD: type 1 – the least severe form of the disease resulting from a partial quantitative VWF deficiency; type 2 – qualitative VWF abnormalities; and type 3 – the most severe form of the disease resulting from negligible VWF levels [5]. Type 2 VWD has 4 subtypes: type 2A results from absence of the most haemostatically active high molecular weight multimers (HMWM); type 2B occurs through increased VWF-platelet interactions causing accelerated clearance of HMWM and thrombocytopenia; type 2M results from a loss of platelet-binding function; and type 2N is characterized by reduced factor VIII (FVIII) binding ability [5].

Gastrointestinal (GI) bleeding is a severe complication of VWD often stemming from angiodyplasia of the small bowel. In up to 10% of cases, VWD is associated with angiodyplasia. While the incidence of angiodyplasia varies across VWD subtypes, it is most frequently reported in types 2 and 3 patients [6]. Angiodyplasia is the tortuous growth of dilated, thin-walled mucosal and submucosal veins, venules and capillaries. This pathological manifestation of unregulated angiogenesis creates a fragile vascular network susceptible to rupture. Angiodyplasia can lead to severe epistaxis and hematuria in VWD patients [7,8]. Disruption of vascular architecture and the resulting intractable bleeding may result in anemia and often requires hospitalization for transfusion of packed red blood cells, with an attendant decrease in quality of life [9].

Recently, VWF has been described as a negative regulator of angiogenesis [10]. Functional blood vessel formation involves EC migration, proliferation, stabilization, and maturation [11]. A delicate balance of interactions between pro- and anti-angiogenic molecules maintains the development of normal, stable vessel structures. During the early stages of angiogenesis, vascular endothelial growth factor (VEGF)-A binds to VEGF receptor-2 (VEGFR-2) on ECs activating intracellular signalling cascades promoting EC proliferation and migration [12]. Blood vessel integrity is controlled by angiopoietin (Ang)-1 signalling through Tie-2 receptors [13]. Ang-2 destabilizes the vascular network, working synergistically with VEGF to promote angiogenesis by competitively inhibiting Ang-1 binding to Tie-2 [13]. The extracellular matrix (ECM) influences blood vessel development

using cell surface adhesion molecules, such as integrin receptor $\alpha v\beta 3$: the best-characterized EC receptor for VWF. Signalling through integrin $\alpha v\beta 3$ can be pro- or anti-angiogenic depending on the ligand bound [14,15].

The study of VWD and angiodysplasia has been advanced by the use of an *ex vivo*, late blood outgrowth endothelial cell (BOEC) model [10,16]. BOECs are an accessible source of vascular endothelium that maintain their differentiated phenotype through multiple passages. Like native ECs, BOECs contain WPBs and express EC surface proteins [17]. BOECs model the pathogenic effects of *VWF* mutations on VWF synthesis, storage, secretion, and string formation [18]. This study aimed to use BOECs to determine baseline angiogenic capacities in healthy controls and investigate the role of quantitative and qualitative VWF abnormalities on in vitro surrogates for the development of angiodysplasia.

Methods

Controls and Patients

Eligible subjects were recruited during clinic visits. Inclusion criteria included a history of excessive bleeding as documented by a bleeding score (BS) ≥ 4 using the Condensed MCMDM-1 VWD Bleeding Assessment Tool [19], and for type 1 VWD, normal VWF multimers and VWF:Ag and/or VWF:RCO between 0.05–0.50 IU/mL. Type 2 patients had VWF:RCO or FVIII:C of <0.50 IU/mL. Type 3 subjects had plasma VWF:Ag and/or VWF:RCO <0.05 IU/mL, and FVIII:C of <0.10 IU/mL. Healthy, non-VWD controls were included with VWF:Ag, VWF:RCO, and FVIII:C >0.50 IU/mL, a BS of <4 and no *VWF* mutations. Controls with a previous diagnosis of an inherited bleeding disorder, or those with an acquired cause of bleeding (low platelets, renal or liver disease) were excluded.

Upon enrollment, BS were calculated and VWF and FVIII levels were measured. Data presented in this paper represents factor levels measured at the time of BOEC isolation. This study was approved by the Research Ethics Board of Queen's University, Kingston, Canada and all participants gave written informed consent.

BOEC Isolations

BOEC isolations were performed according to published methods with modifications [17]. Mononuclear cells (MNCs) were isolated using BD Vacutainer Cell Preparation Tubes (CPT; BD Biosciences), washed and re-suspended in EBM-2 (Lonza) supplemented with 10% FBS, 1% antibiotics (Invitrogen), and the EGM-2 bullet kit (cEGM-2). 4×10^7 cells/well were seeded on collagen-coated 6-well tissue culture plates. After 9–21 days, BOECs appeared with characteristic cobblestone morphology. BOECs were used between passages 3 and 9 after flow cytometry characterization for CD31+, CD14-, CD45-, CD144+ and/or CD146+. All antibodies were purchased from eBiosciences (San Diego, CA).

VWF and Ang-2 expression and secretion

BOECs were seeded, 1×10^6 cells/well, in EGM-2 on a six-well collagen-coated plate. After 24 hours, media was changed to serum free Opti-MEM[®] (Life Technologies) supplemented with 100 mg/L calcium chloride. After 24-hours, media and lysates were collected and

levels of intracellular and secreted VWF and Ang-2 were quantified by ELISA, using polyclonal antibodies (VWF- A0082 and P226, DAKO Mississauga, ON) and the Human Angiopoietin-2 DuoSet ELISA kit (R&D Systems Minneapolis, MN), respectively.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNA was isolated from confluent BOECs using the QIAamp RNA Blood Mini Kit (QIAGEN). qRT-PCR was carried out using the following primers to *VWF* (5'-CCCCTGAAGCCCCTCCTCCTA-3', 5'-ACGAACGCCACATCCAGAACC-3'), *Ang-2* (5'-CAGCATCAGCCAACCAGGAA-3', 5'-CAAACCACCAGCCTCCTGTTA-3'), *integrin β 3* (5'-TGACGAAAATACCTGCAACCG-3', 5'-GCATCCTTGCCAGTGTCTTAA-3'), and *glyceraldehyde-3-phosphate dehydrogenase* (5'-CAAGGTCATCCATGACAACCTTG-3', 5'-GGGCCATCCACAGTCTTCTG-3'). One healthy control was used to establish normal mean and range; each VWD sample from single isolations was tested in double. PCR reaction were run using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) on a ViiATM 7 Real-Time PCR System with a 96-Well Block.

Confocal immunofluorescence microscopy

Intracellular VWF and Ang-2 were visualized with rabbit anti-human VWF polyclonal antibody (DAKO, A0082) and goat anti-human Ang-2 polyclonal antibody (R&D Systems, AF623). The secondary antibodies were FITC-mouse anti-rabbit immunoglobulin (DAKO, F0054) and rhodamine-donkey anti-goat immunoglobulin (Santa Cruz, 2094). Actin filaments were stained with phalloidin-TRITC (Sigma, P1951) and nuclei with a DAPI stain (Sigma, D9542). VE-Cadherin staining was performed using mouse anti-human CD144 (DAKO, 555661) and Donkey anti-mouse (Alexa Fluor 568) antibodies. A Widefield Super-Resolution System Leica SR GSD microscope was used to obtain images and Image J software was used to generate composite images.

Adhesion assay

Black-bottom 96-well plates (Nunc) were coated with 100 mg/mL rat-tail type 1 collagen, 1% gelatin, 30 μ g/mL ultra-pure VWF (Biotest), or 1% bovine serum albumin (BSA). The assays were performed as published [10].

Proliferation Assay

BOECs were seeded, 4000 cells/well, in cEGM-2 onto three separate collagen-coated black-bottomed 96-well plates. At each of 48, 96, and 144 hours, media was removed from one plate and the plate was frozen at -80°C to initiate lysis of the cells. All three plates were thawed within four weeks of freezing and the cell number at 48, 96, and 144 hours was quantified separately with the fluorescence-based CyQuant® Cell Proliferation Assay Kit (Life Technologies).

Scratch-wound healing assay

BOECs were seeded, 3.5×10^4 cells/well, onto collagen-coated ImageLock 96-well plates (Essen Biosciences, Inc.) in cEGM-2. After 12 hours, confluent monolayers were scratched

using the WoundMaker™ tool (Essen Biosciences, Inc.). The wells were washed and cEBM-2 was added. Images were captured at 30 minute intervals using the Incucyte ZOOM 4X objective (Essen Biosciences, Inc.). The migration paths of 10 random cells at the upper border of the wound were recorded using the Manual Tracking plugin for Fiji. Data from the first 15 hours was used to quantify Euclidean distance, migration velocity, and directionality using the stand-alone Chemotaxis and Migration tool (Ibidi).

Matrigel tubule formation assay

Forty-eight well plates were coated with Matrigel (Corning) and polymerized for at least 30 min before use. BOECs were seeded (5×10^4 cells/well) in duplicate onto the Matrigel. After 8 and 20 hours at 37°C, cells were imaged at 4X magnification on a Nikon TE-2000U inverted microscope. Tubules were characterized using the online software program WimTube (Wimasis Image Analysis, Ibidi) [20].

Statistical Analysis

Graphing and statistical analyses were performed with Prism 6 for Mac (version 6.0c). The data was collected from at least three distinct experiments and presented as means $\pm$ standard deviation (SD) or standard error of the mean (SEM). Data sets were analyzed using the Kruskal-Wallis test followed by post-hoc Dunn's multiple comparison test and non-parametric Mann-Whitney tests. Sample size for each experiment is stated in the figure legends.

Results

Healthy Controls and VWD Patients

BOECs were isolated from 10 healthy controls (C-1 to C-10) and 13 VWD patients: four type 1 (T1-1 to T1-4), one each of type 2A, 2B, 2M, type 2N, and five type 3 (T3-1 to T3-5). Laboratory characteristics and *VWF* mutations pertaining to each individual are displayed in Table 1.

BOEC Phenotypic Characterization

BOECs isolated from the 10 healthy controls and 13 VWD patients appeared 9–21 days after isolation with the cobblestone morphology typical of ECs (Fig. 1a). Flow cytometry of BOECs indicated that all BOECs were highly positive for EC markers CD31, CD144, and/or CD146 with no indication of leukocyte antigens CD45 and CD14 (Supplemental Table. S1). BOECs also express the endothelial marker, VE-cadherin, as shown in Figure 1.

VWF expression and storage in BOECs

BOECs were assessed for intracellular and constitutively released VWF protein from cell lysates and culture supernatants. While intracellular and basally released VWF levels varied within the control BOEC group (range, 353–841 mU/mL and 11–51 mU/mL respectively; Supplemental Fig. S2a and b), there was a significant positive linear correlation between plasma VWF:Ag and released BOEC VWF ($R^2 = 0.22$, $P = 0.0062$; Supplemental Fig. S3a). On average, the 10 control BOECs released 31 ± 11 mU/mL VWF, about 5% of their total

VWF content of 558 ± 154 mU/mL. Confocal IF microscopy of control BOECs showed VWF staining that was localized to WPBs with minimal diffuse staining (Fig. 1c).

As with the control BOECs, there was a significant positive correlation between type 1 patient plasma VWF:Ag and released VWF protein levels from BOECs ($R^2=0.38$, $P=0.032$; Supplemental Fig. S3b). qRT-PCR analysis of BOEC mRNA indicated that three of the four type 1 BOECs (T1-1, T1-3, and T1-4) had reduced *VWF* expression when compared to a healthy control (Fig. 2b). In the fourth type 1 VWD patient, T1-2, *VWF* mRNA levels were three times as high as the control and did not correspond with elevated VWF in the patient's plasma or BOECs. While the four type 1 patient BOECs (T1-1 to T1-4) had variable constitutive VWF release (Fig. 2c), these differences are reflective of varying patient plasma VWF:Ag levels (Table 1). BOECs from patients T1-1 and T1-2, who had low plasma VWF:Ag (0.27 IU/mL and 0.14 IU/mL, respectively), displayed significantly reduced VWF release when compared to controls (T1-1 = 14 ± 2 mU/mL, $P<0.001$ and T1-2 = 5 ± 4 mU/mL, $P<0.0001$). T1-3 and T1-4 who had normal and mildly reduced plasma VWF levels (0.53 IU/mL and 0.44 IU/mL, respectively) had VWF release that was comparable to the healthy controls (35 ± 14 mU/mL and 44 ± 4 mU/mL, respectively). Significant intracellular VWF retention was observed in T1-1 (902 ± 281 mU/mL, $P<0.001$) and T1-4 (871 ± 105 mU/mL, $P<0.0001$) BOECs, while T1-2 had significantly decreased intracellular protein (19 ± 12 mU/mL, $P<0.0001$) and T1-3 had VWF levels similar to controls (603 ± 148 mU/mL). Confocal IF microscopy shows the heterogeneity of type 1 VWD BOECs with respect to WPBs formation in line with previously published reports [18,21] (Fig. 2a). T1-1 and T1-2 BOECs displayed diffuse VWF staining in addition to some VWF localized to WPBs consistent with heterozygosity for *VWF* propeptide mutations [21] whereas T1-3 and T1-4, had intracellular VWF staining that closely resembled the controls.

Type 2 VWD is a diverse subtype encompassing a wide range of qualitative *VWF* defects. BOECs from all four type 2 VWD subtypes displayed increased *VWF* expression when compared to a healthy control (Fig. 3b). While, intracellular VWF levels of type 2A BOECs (500 ± 104 mU/mL; Fig 3c) were comparable to those in control BOECs, type 2A BOECs manifested significantly impaired basal VWF release (11 ± 1 mU/mL, $P<0.0001$) compared to control BOECs, consistent with very low plasma VWF (VWF:Ag = 0.07 IU/mL). In type 2A BOECs, VWF staining localized to rounded WPBs and was diffuse with a perinuclear dispersion (Fig. 3a). While, the type 2B VWD patient has low plasma VWF levels (0.40 IU/mL), this was not reflected in the BOEC model, due to the absence of platelets and ADAMTS13. In vivo, the presence of platelets and ADAMTS13 would result in increased clearance from the blood due to the formation of platelet-VWF complexes. Hence, there were no observable differences with respect to VWF release of the type 2B BOECs (36 ± 15 mU/mL) relative to the controls (Fig. 3c). VWF storage appeared normal in the type 2B BOECs, with the protein being stored in WPBs with little diffuse staining (Fig. 3a). In the type 2M BOECs, despite low plasma VWF:Ag of 0.11 IU/mL, constitutive VWF release (33 ± 3 mU/mL) was equivalent to the controls (Fig. 3c). Nonetheless, significant VWF retention was also observed in these cells (1321 ± 138 mU/mL, $P<0.0001$) and VWF staining indicated the presence of dispersed cytoplasmic distribution (Fig. 3a). Type 2N BOECs had normal VWF release (33 ± 7 mU/mL) associated with significantly increased VWF retention

(829 ± 138 mU/mL, $P < 0.001$) and VWF storage visualized through confocal IF microscopy appeared normal in type 2N BOECs with little diffuse staining (Fig. 3a).

VWF expression was reduced in 4 of 5 type 3 VWD BOEC (Fig. 4b). Correspondingly, in the type 3 VWD BOEC with reduced VWF expression, VWF release was negligible (T3-1 = 1.17 ± 0.21 mU/mL, T3-2 = 0.86 ± 0.49 mU/mL, and T3-4 = 1.27 ± 0.32 mU/mL) as was intracellular retention (T3-1 = 2.30 ± 2.29 mU/mL, T3-2 = 7.70 ± 1.59 mU/mL, and T3-4 = 29.36 ± 6.04 mU/mL; $P < 0.0001$ for all; Fig. 4c). Despite these negligible VWF protein levels, confocal images of T3-1, T3-3, and T3-4 BOECs show the presence of VWF (Fig. 4a). T3-1 BOECs have no WPBs and contain only diffuse perinuclear VWF staining. T3-3 and T3-4 have mostly diffuse VWF staining alongside a limited number of WPBs. In contrast to the other type 3 VWD BOECs, T3-2 VWF mRNA levels are 25 times greater than normal (Fig. 4b) and although this high gene expression does not reflect the observed virtual lack of plasma VWF (VWF:Ag = 0.01) or BOEC VWF release (3.40 ± 1.75 mU/mL, $P < 0.0001$; Fig. 4c), BOECs from this individual have significantly increased levels of intracellular VWF (1208 ± 190 mU/mL; $P < 0.0001$; Fig. 4c). The confocal IF of cells from this patient show a lack of WPB formation and diffuse perinuclear staining (Fig. 4a).

Enhanced Ang-2 secretion in type 1 and 3 BOECs

BOECs were also evaluated for Ang-2 expression, storage, and release to determine if the specific mutations imparting aberrant storage of VWF also affect Ang-2. Intracellular Ang-2 levels fluctuated greatly between the 10 controls from 6.8 ± 2.9 ng/mL to 85 ± 30 ng/mL (mean = 37 ± 25 ng/mL; Supplemental Fig. S2c). Additionally, the basal release of Ang-2 was also variable, ranging from 2.4 ± 2.6 ng/mL to 16.3 ± 2.3 ng/mL (mean = 6.5 ± 4.2 ng/mL; Supplemental Fig. S2d). Co-staining of Ang-2 with VWF in control BOECs demonstrated that these two molecules co-localize together in WPBs however, not every WPB had Ang-2 associated with it (Fig. 1c).

qRT-PCR results demonstrated increased Ang-2 expression (Fig. 2b) compared to control BOECs in three of the four type 1 VWD BOECs (T1-1, T1-2, and T1-4). Correspondingly, T1-1, T1-2, and T1-4 also had significantly higher levels of basally secreted Ang-2 (23 ± 6 ng/mL, $P < 0.0001$; 13 ± 2 ng/mL, $P < 0.01$; and 23 ± 6 ng/mL, $P < 0.0001$, respectively; Fig. 2d). Conversely, BOECs from T1-3 had comparatively less basally secreted Ang-2 (4 ± 2 ng/mL, $P < 0.05$; Fig. 2d) associated with reduced gene expression. Confocal IF identified more discrete Ang-2 staining in T1-1, T1-2, and T1-4 than in T1-3 BOECs which had diffuse Ang-2 staining (Fig. 2a). Furthermore, the confocal images show higher amounts of Ang-2 staining in T1-1 and T1-4 which support that despite very high Ang-2 release, T1-1 and T1-4 also had significantly higher retention of the protein when compared to the controls (176 ± 47 ng/ml and 127 ± 26 ng/mL, respectively; $P < 0.0001$ for both; Fig. 2d).

Ang-2 expression was significantly increased in all type 2 BOECs with the greatest expression in type 2A and 2B BOECs (Fig. 3b). Correspondingly, 2A and 2B BOECs exhibited approximately a two-fold increase in Ang-2 secretion compared to controls (12 ± 3 ng/mL, $P < 0.01$ for both; Fig. 3d). This enhanced Ang-2 secretion was associated with normal levels of intracellular Ang-2 in the 2A BOECs (27 ± 7 ng/mL) and significantly higher levels in the 2B BOECs (118 ± 15 ng/mL, $P < 0.0001$). In line with this, confocal IF data

indicated that the Ang-2 staining in the 2A BOECs was discrete and more closely resembled the control BOECs, while in the 2B BOECs, Ang-2 was diffuse throughout the cell (Fig. 3a). In the type 2M BOECs, normal basal Ang-2 release (9 ± 2 ng/mL) was associated with significantly increased retention of the protein (72 ± 21 ng/mL, $P < 0.0001$) and IF staining was similar to the 2B BOEC with diffuse Ang-2 staining alongside some limited WPBs. Although, the type 2N BOECs also had normal Ang-2 secretion (6 ± 1 ng/mL), this was associated with increased retention of the protein (56 ± 10 ng/mL, $P < 0.05$) despite confocal images indicating that most of the Ang-2 colocalized with VWF.

Ang-2 expression was elevated in all the type 3 BOECs (Fig. 4b). However, basal Ang-2 secretion was only increased in T3-1 and T3-2 (10 ± 3 ng/mL and 16 ± 6 ng/mL; Fig. 4d) which show very little intracellular Ang-2 staining (Fig. 4a). Protein data (Fig. 4d) shows that secretion and intracellular stores of Ang-2 were reduced in the T3-3 (2.8 ± 0.4 ng/mL and 0.4 ± 0.2 ng/mL, respectively) and T3-4 BOECs (3 ± 2 ng/mL and 8 ± 7 ng/mL, respectively) despite confocal images that show an abundance of intracellular Ang-2 (Fig. 4a).

Improved Integrin Function in type 1 and 2N VWD BOECs

To examine the relationship between *VWF* mutation and integrin function, BOEC binding to collagen-, gelatin-, or VWF-coated surfaces was measured (Fig. 5). Adhesion of control BOECs was greater to the $\beta 1$ integrin ligand collagen type I ($14 \pm 6\%$ of cells), then to gelatin ($11 \pm 5\%$ of cells) or VWF ($7 \pm 6\%$ of cells; Fig. 5a–c).

EC binding to collagen is mediated in part by integrin $\alpha V \beta 1$ and as expected similar number of control versus VWD BOECs were retained to the collagen-coated surface. One type 1 BOEC, T1-1, and the 2N BOECs had significantly increased adherence to the $\alpha V \beta 3$ -dependant substrates VWF (18 ± 8 and $15 \pm 3\%$ of cells, respectively, $P < 0.05$ for both) and gelatin (18 ± 7 and $19 \pm 4\%$ of cells, respectively, $P < 0.05$ for both). A functional defect of $\alpha V \beta 1$ integrin was observed in one type 3 VWD BOECs, T3-2, since reduced binding to gelatin was observed ($5 \pm 3\%$ of cells, $P < 0.05$).

BOEC Proliferation

To assess BOEC proliferation, cell numbers were assessed at 48, 96, and 144 hours (Fig. 6). Rates of proliferation were variable between normal controls but on average, BOECs showed a 5-fold increase in cell numbers over 48 hours and a further doubling by 144 hours (Supplemental Fig. S4). Type 2A (52208 ± 2661 cells; $P < 0.001$) and 2B (55143 ± 2132 cells, $P < 0.001$) were significantly more proliferative after 144 hours but one type 1 BOEC (T1-3 = 11376 ± 3167 cells; $P < 0.01$) and two of the type 3 BOECs (T3-3 = 17860 ± 4056 cells, $P < 0.01$ and T3-4 = 4974 ± 1822 cells, $P < 0.001$) were significantly less proliferative compared to control BOECs.

Type 1 and type 3 VWD BOECs display increased directionality and reduced velocity

BOEC migration was assessed using a scratch-wound healing assay (Fig. 7). The Euclidean distance (Fig. 7a) is a measure of the direct linear distance between the start and end point of a cell's trajectory. This measure of cellular migration did not vary significantly between the 10 control BOECs and averaged 219 ± 50 μ m (Supplemental Fig. S4d top). All VWD patient

BOECs except type 2N BOECs had similar Euclidean distance to the control BOECs. Type 2N BOECs had significantly shorter Euclidean distance ($157 \pm 23 \mu\text{m}$, $P < 0.05$) indicative of a shorter cell migration path.

Mean cell front migration velocity (Fig. 7b) was consistent across control BOECs ($0.50 \pm 0.07 \mu\text{m/min}$; Supplemental Fig. S4d middle) and did not differ from one type 1 (T1-4), type 2B, 2M, and 2N BOECs. However, BOECs from the other type 1 (T1-1 = $0.30 \pm 0.02 \mu\text{m/min}$, $P < 0.001$), type 2A patient ($0.41 \pm 0.07 \mu\text{m/min}$, $P < 0.05$), and type 3 (T31-1 = $0.33 \pm 0.06 \mu\text{m/min}$ and T3-2 = 0.28 ± 0.02 , $P < 0.0001$ for both) patients displayed significantly reduced cell front migration velocity than the control BOECs.

Directionality (Fig. 7c) is calculated by dividing the mean Euclidean distance of the tracked cells by the mean accumulated distance of the same cells. It is a measurement of the directness of cell trajectories and a value approaching 1 indicates straight-line migration. All control BOECs had similar directionality averaging 0.50 ± 0.06 (Supplemental Fig. S4d bottom). Significantly greater straight-line migration was observed in T1-1 (0.73 ± 0.01 , $P < 0.0001$), 2B (0.55 ± 0.03 , $P < 0.05$), 2N (0.40 ± 0.05 , $P < 0.01$), T3-1 (0.65 ± 0.03 , $P < 0.001$), and T3-2 (0.65 ± 0.10 , $P < 0.001$) when compared to the control BOECs. The migration of these BOECs had increased directionality toward wound closure. T1-4, 2A, and 2M BOECs did not differ from control BOECs with respect to cell directionality.

Figure 7d displays cell trajectory plots for 10 randomly chosen individual cells from the upper border of the wound for control and patient BOECs. These plots demonstrate that the average BOEC migration in all BOECs was towards the wound (in the negative y direction). Ultimately, the migration data reveal that the type 1 and 3 BOECs had reduced mean cell front migration velocity and increased directionality than the control and type 2 VWD BOECs.

Impaired tubule formation in 1 type 1, 2B and type 3 BOECs

Vessel formation was evaluated through three-dimensional BOEC cultures in Matrigel by characterizing total tubule length eight hours after seeding (Table 2 and Supplemental Fig. S1). There was great variability in vessel formation in the 10 controls ranging from 33mm to 100mm (mean = $59 \pm 23\text{mm}$). When the individual control BOECs were compared, tubule formation was the greatest in two young (ages 26 and 31) female donors, and four of the six female donors were above the average tubule length (Supplemental Fig. S4b).

With respect to the BOECs from VWD patients, T1-1 ($25 \pm 8\text{mm}$, $P < 0.05$), 2B ($22 \pm 6\text{mm}$, $P < 0.05$), T3-1 ($13 \pm 3\text{mm}$, $P < 0.01$), and T3-2 ($27 \pm 7\text{mm}$, $P < 0.05$) BOECs formed significantly shorter tubules than the control BOECs eight hours after seeding. These BOECs also had significantly less branching points, loops, and tubes (Table 2). Interestingly, despite variations in total tube lengths, mean tube length was mostly conserved across all BOECs. Unlike the control BOECs, we did not observe a difference in tube formation between females and males as BOEC from half of the female patients had above average tubule length and the other half had below average tubule formation.

Discussion

VWF has been characterized as a negative regulator of angiogenesis and is suggested to act through two pathways involving Ang-2, integrin $\alpha v\beta 3$, and VEGFR-2 signalling [10]. The intracellular pathway involves sequestering Ang-2 to WPBs, preventing its interactions with Tie-2 receptors and thereby reducing cellular proliferation. Secondly, extracellular interactions between VWF and its integrin $\alpha v\beta 3$ inhibit proliferative signalling through VEGFR-2 [10]. The current study examining the angiogenesis of BOECs derived from control and VWD patients is the largest to date. Overall, our results indicate that there is great variability in the angiogenic properties of both control and VWD BOECs. Abnormal cell proliferation, migration, and increased Ang-2 secretion are common features of endothelial cells derived from the BOECs of VWF patients. Type 1 and 3 BOECs had reduced mean cell front migration velocity and increased directionality as compared to control BOECs. However, even within a single VWD type, BOECs from different patients presented with diverse angiogenic characteristics. These findings are consistent with recent studies of BOECs from VWD patients which also showed great inter-individual variability of angiogenic capacity [16].

Ang-2 promotes VEGF-dependent stimulation of EC sprouting and migration [22] and induces degradation of integrin $\beta 3$ [23]. In siVWF-treated HUVECs, Ang-2 storage and release were dysfunctional with the loss of VWF [10]. In our study, Ang-2 storage was defective across all VWD BOECs with half of the type 1s, 2A, 2B, and two type 3 VWD BOECs displaying increased secretion. Interestingly, our highest Ang-2 levels were observed in two type 1 BOECs and not in type 3 BOECs where VWF levels were negligible, suggesting that defective Ang-2 storage due to mutations in the *VWF* gene are not primarily responsible for the regulation of endothelial Ang-2 release. In fact, these BOECs also had elevated expression of the Ang-2 gene which would have contributed to the increased secretion; the reason for this is not clear.

Our results indicate that type 1 and 3 VWD BOECs with significantly increased Ang-2 basal release have increased cell migration directionality and decreased velocity. In contrast, type 2 BOECs with both increased and normal Ang-2 secretion migrated with increased velocity and impaired directionality towards to wound, as observed in siVWF-treated HUVECs [10]. Groeneveld *et al*, 2015 found that while VWD BOECs did not differ from control BOECs with respect to migration velocities, they all displayed a loss of directionality when compared to control BOECs. Variations in multiple angiogenic factors and VWF mutations in these BOECs could differentially influence the migration characteristics of these cells. VEGF and fibroblast growth factor have been known to coordinate and promote the chemotaxis of ECs [24], and thus it may be of interest to monitor the levels of these agents in the BOECs to determine whether their expression is reduced and could be contributing to the reduced migration velocity. Physiologically, the shear stress of fluid flowing through blood vessels also functions to mediate EC migration [24]. The lack of this modulation in the migration assay *in vitro* could have also impacted the migration properties of these BOECs.

Starke *et al*, 2011 showed that BOECs from VWD patients have increased VEGF-dependent proliferation and this was the case for the type 2A and 2B BOECs in our study. In fact, BOECs with significantly reduced Ang-2 release compared to controls displayed significantly reduced cell numbers 144 hours after seeding.

Given that internalization of the $\beta 3$ subunit of integrin $\alpha v\beta 3$ is augmented in VWF deficiency [10] and $\beta 3$ subunit loss corresponds with increased angiogenesis in mice [25], decreased adhesion of BOECs to integrin $\alpha v\beta 3$ -dependent substrates gelatin [26] and VWF was expected. While adhesion to the different integrin mediators was comparable across BOECs, one type 3 VWD BOEC displayed reduced binding to gelatin suggesting impaired integrin function. We also observed that one type 1 and the type 2N BOECs were more adhesive to gelatin and VWF indicating improved integrin function. This unexpected result may be further explored through measurement of gene expression and surface levels of integrin $\beta 3$ to assess inter-individual variation in integrin abundance on adhesion. Additionally, adhesion to integrin $\alpha v\beta 3$, using vitronectin, could be assessed to better recapitulate the *in vitro* integrin $\alpha v\beta 3$ -mediated adhesion of ECs.

Finally, the ability of BOECs to form tubules in three dimensional Matrigel which combines multiple aspects of EC angiogenesis [20] was observed. In control BOECs, there was great variability in terms of total tubule length which did not correlate significantly with VWF level, Ang-2 release, or donor age. Yet, BOECs isolated from females formed more tubules than those from males. This finding compliments previous studies that report that BOECs from females exhibit greater proliferative, adhesive, and capillarogenic tendencies than BOECs from men [27] possibly due to the role of estrogen in promoting cyclical vascularization [28]. Starke *et al*, 2011 showed significant increases in tubule formation of siVWF-treated HUVECS and VWD BOECs in Matrigel but Groeneveld *et al*, 2015 show that while some VWD BOECs do have increased total tube length, BOECs from their type 1 and 2B patients actually have significantly decreased total tubule length. Our results are consistent with the latter findings as BOECs isolated from one type 1, the 2B, and two type 3 patients had significantly reduced total tubule formation. The variability in tubule formation may be attributed to a number of different factors including cell passage, as higher passage BOECs have declining abilities to form tubules [16].

BOECs are a relatively new model for studying EC-dependent processes *ex vivo* and the range of what constitutes “normal” VWF expression and angiogenesis for these cells is not yet well established. Absent *in vitro* is ADAMTS13, which results in higher degrees of multimerization in BOEC VWF than plasma. Additionally, variances in BOEC VWF levels may be attributed to inter-individual differences in the *VWF* gene and other modifiers. It is difficult to isolate the effects of VWF on angiogenesis in BOECs because their inter-individual variability affects all expression profiles including many other angiogenic regulators. Even between siblings C-9 and C-10, Ang-2 expression/release, adhesion and permeability diverged greatly, despite similarities in VWF expression and tubule formation. Additionally, variability of BOEC Ang-2 release may be attributed to factors other than VWF. For instance, Ang-2 expression is elevated by many diseases with known endothelial dysfunction including hypertension and atherosclerosis [29]. The isolation and culturing process of BOECs may contribute to the varied angiogenic responses between donors. The

quality of the BOEC cultures, cell size, time to confluence, cell density at confluence, and/or variation in BOEC passages may have influenced cellular performance on the angiogenesis and VWF-processing assays [16].

Our study included relatively small numbers of BOECs. Indeed, with a single BOEC line for each type 2 VWD subtype, we are unable to generalize our findings to all type 2 VWD. This, plus the variability observed limits generalization of the results. While a larger sample size might help overcome this concern, access to larger numbers of patients, and the resource-intensive nature of endothelial cell experiments are barriers. Additionally, given that the stages of angiogenesis do not function in isolation from each other, assays measuring only one aspect of angiogenesis are artificial. Angiogenesis is a complex process relying on delicate interplay between hundreds of molecules [30]. An imbalance of any of these molecules in the pro- or anti-angiogenic direction can cause abnormal angiodyplastic vessel growth. It is unlikely that the pathogenesis of angiodyplasia in VWD can be characterized by a change in BOEC behavior in one assay. With these limits in mind, BOECs do provide access to patient vascular endothelium and enable the study of cellular interactions of VWF with other angiogenic mediators. The results of this study further validate the use of BOECs as investigative models of patient- and individual-specific differences in VWF expression patterns and angiogenesis. The future use of physiologically representative assays, such as the fibrin bead model of sprouting angiogenesis, could help to further define the aberrant qualities of angiogenesis occurring in VWD patients [31] and complement studies in BOECs. Perhaps the most valuable use of the BOEC model is in their therapeutic applications for VWD. BOECs could be used for the optimization of patient-specific treatment regimens in VWD patients with the goal of lessening the considerable burden of GI bleeding in VWD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

(a) Phase contrast microscopy at 4X magnification of confluent BOEC monolayer depicts the characteristic cobble-stone morphology of these cells. (b) Type 1 VWD BOEC, T1-3 was stained for VE-Cadherin (red) and DAPI (blue). Images were taken at 10X and 60X magnification. (c) Confocal Immunofluorescence co-staining of VWF (green) and Ang-2 (red) with DAPI nuclear stain (blue) of representative control BOEC. Areas of co-localization of VWF and Ang-2 appear yellow in merge images. Punctate staining indicates storage of VWF in WPBs, whereas diffuse staining indicates improper WPB formation. All images were taken by a Widefield Super-Resolution System Leica SR GSD microscope.

Fig. 2.

Storage, gene expression, and constitutive release of Ang-2 and VWF in BOECs from four type 1 VWD patients. (a) Confocal Immunofluorescence co-staining of VWF in green (column 1) and Ang-2 in red (column 2) with blue DAPI nuclear stain in the BOECs. Areas of co-localization of VWF and Ang-2 appear yellow in merge images (column 3). Punctate staining indicates storage of VWF in WPBs, whereas diffuse staining indicates improper WPB formation. All images were taken by a Widefield Super-Resolution System Leica SR GSD microscope. (b) qRT-PCR with primers specific for *VWF* and *Ang-2* were performed on RNA isolated from BOECs from one healthy control and VWD patients. *VWF* and *Ang-2* expression was normalized to *GAPDH*, and then all VWD samples were normalized to one healthy control. Intracellular and constitutively released VWF (c) and Ang-2 (d) of 10 control BOECs (data averaged) and VWD patients. Protein levels were normalized to cell counts. Each value represents the mean $\pm$ standard deviation of triplicate or quintuplicate measurements as determined by ELISA. Black bars, controls; dark grey bars, type 1 VWD BOECs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs Controls as indicated by the Mann-Whitney U test.

Fig. 3.

Storage, gene expression, and constitutive release of Ang-2 and VWF in BOECs from four type 2 VWD patients (one type 2A, one type 2B, one type 2M, and one type 2N). (a) Confocal Immunofluorescence co-staining of VWF in green (column 1) and Ang-2 in red (column 2) with blue DAPI nuclear stain in the BOECs. Areas of co-localization of VWF and Ang-2 appear yellow in merge images (column 3). Punctate staining indicates storage of VWF in WPBs, whereas diffuse staining indicates improper WPB formation. All images were taken by a Widefield Super-Resolution System Leica SR GSD microscope. (b) qRT-PCR with primers specific for *VWF* and *Ang-2* were performed on RNA isolated from BOECs from one healthy control and VWD patients. *VWF* and *Ang-2* expression was normalized to *GAPDH*, and then all VWD samples were normalized to one healthy control. Intracellular and constitutively released VWF (c) and Ang-2 (d) of 10 control BOECs (data averaged) and VWD patients. Protein levels were normalized to cell counts. Each value represents the mean $\pm$ standard deviation of triplicate or quintuplicate measurements as determined by ELISA. Black bars, controls; light grey bars, type 2 VWD BOECs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs Controls as indicated by the Mann-Whitney U test.

Fig. 4.

Storage, gene expression, and constitutive release of Ang-2 and VWF in BOECs from five type 3 VWD patients. (a) Confocal Immunofluorescence co-staining of VWF in green (column 1) and Ang-2 in red (column 2) with blue DAPI nuclear stain in the BOECs. Areas of co-localization of VWF and Ang-2 appear yellow in merge images (column 3). Punctate staining indicates storage of VWF in WPBs, whereas diffuse staining indicates improper WPB formation. All images were taken by a Widefield Super-Resolution System Leica SR GSD microscope. (b) qRT-PCR with primers specific for *VWF* and *Ang-2* were performed on RNA isolated from BOECs from one healthy control and VWD patients. *VWF* and *Ang-2* expression was normalized to *GAPDH*, and then all VWD samples were normalized to one healthy control. Intracellular and constitutively released VWF (c) and Ang-2 (d) of 10 control BOECs (data averaged) and VWD patients. Protein levels were normalized to cell counts. Each value represents the mean $\pm$ standard deviation of triplicate or quintuplicate measurements as determined by ELISA. Black bars, controls; light grey bars, type 2 VWD BOECs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs Controls as indicated by the Mann-Whitney U test.

Fig. 5.

Integrin-dependent adhesion function of healthy and VWD patient BOECs. BOECs were seeded onto type 1 collagen-, gelatin-, and VWF-coated substrates and allowed to adhere for 40 minutes after which non-adherent cells were removed by gentle washing. Percentage of cells remaining bound to (a) type 1 collagen, (b) gelatin, and (c) VWF were measured in 10 control and 12 VWD BOECs. Black bars, controls; dark grey bars, type 1 VWD BOECs; light grey bars, type 2 VWD BOECs; white bars, type 3 VWD BOECs. Each value represents the mean $\pm$ standard deviation of triplicate measurements. * $P < 0.05$ vs Controls as indicated by the Mann-Whitney U test.

Fig. 6.

Proliferative capacity of BOECs derived from healthy controls and VWD patients. Cells were initially plated at 4000 cells per well and cell number was measured over 144 hours using a CyQuant proliferation assay kit (Invitrogen). Data shown is cell number at 144 hours. Black bars, controls; dark grey bars, type 1 VWD BOECs; light grey bars, type 2 VWD BOECs; white bars, type 3 VWD BOECs. Each value represents the mean $\pm$ standard deviation of triplicate measurements. ** $P < 0.01$ and *** $P < 0.001$ vs Controls as indicated by the Mann-Whitney U test.

Fig. 7.

Characteristics of wound-healing migration in healthy control and VWD patient BOECs. Ten random cells from the upper border of the wound were recorded for 30-minute intervals over 15 hours and analyzed using the stand-alone Chemotaxis and Migration tool (Ibidi). (a) Euclidean distance, (b) velocity, and (c) directionality (Euclidean distance/accumulated distance of BOEC motion, describing the “straightness” of the migration path) during these first 15 hours after endothelial monolayer wounding are shown here. Black bars, controls; dark grey bars, type 1 VWD BOECs; light great bars, type 2 VWD BOECs; white bars, type 3 VWD BOECs. Each value represents the mean $\pm$ standard deviation of triplicate measurements. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ vs Controls as indicated by the Mann-Whitney U test. (d) Cell trajectory plots of one representative control (C-1), type 1 VWD (T1-1 and T1-4), type 2 (2A, 2B, 2M, and 2N), and type 3 VWD (T3-1, T3-2, and T3-5) BOECs are shown. Blue ellipses represent the center of mass which identifies the averaged point of all cell migration endpoints. The coordinates of the center of mass represent the direction in which the BOECs migrated.

Phenotypic and genotypic characteristics of healthy controls and VWD patients

Table 1

Subject ID	VWD type	Sex:Age (M/F:year)	Blood Type	Bleeding Score	VWF:Ag (IU/mL)	VWF:RCo (IU/mL)	FVIII:C (IU/mL)	Nucleotide Change, HGVS*	Amino Acid Change, HGVS*
C-1	n/a	F/48	A+	2	1.54	1.15	1.45	n/a	n/a
C-2	n/a	M/56	O+	−2	1.62	1.18	1.71	n/a	n/a
C-3	n/a	F/33	O+	1	0.78	0.52	0.88	n/a	n/a
C-4	n/a	F/60	O+	2	0.94	1.13	1.95	n/a	n/a
C-5	n/a	F/31	O	0	0.77	0.81	0.90	n/a	n/a
C-6	n/a	M/62	O−	0	0.68	0.82	1.42	n/a	n/a
C-7	n/a	M/21	O+	0	0.63	0.74	0.79	n/a	n/a
C-8	n/a	F/26	O−	3	0.78	0.63	1.00	n/a	n/a
C-9	n/a	M/19	A	0	1.19	1.46	1.34	n/a	n/a
C-10	n/a	F/17	B	−1	1.21	0.99	1.60	n/a	n/a
T1-1	1	F/33	O+	4	0.27	0.19	0.53	c.1897T>C	p.Cys633Arg
T1-2	1	F/52	O+	12	0.14	0.13	0.46	c.221-977_532+7059del	p.Asp75_Gly178del
T1-3	1	F/36	O+	11	0.53	0.32	0.71	§	§
T1-4	1	M/64	n/d	12	0.44	0.44	0.75	c.6599-20A>T	n/a
2A	2A	F/72	O+	19	0.07	0.06	0.21	c.7130dupC	p.Pro2377Profs*14
2B	2B	M/55	O+	17	0.40	0.13	0.46	c.3946G>A	p.Val1316Met
2M	2M	F/25	A+	7	0.11	0.03	0.39	c.4145T>C	p.Leu1382Pro
2N	2N	F/33	A	8	0.97	1.20	0.44	c.2561G>A, unknown	p.Arg854Gln, unknown
T3-1	3	F/21	O+	24	0.01	0.00	0.01	c.876delC, c.1255C>T	p.Pro293Glnfs*164, p.Gln419*
T3-2	3	F/11	O+	13	0.01	0.03	0.01	c.1897T>C, c.1897T>C	p.Cys633Arg, p.Cys633Arg
T3-3	3	M/27	A+	29	0.02	0.04	0.01	c.3939G>A, c.5842+1 G>C	p.Trp1313*, -
T3-4	3	M/16	A+	24	0.01	0.03	0.01	c.1657dupT, c.8419_8422dupTCCC	p.Trp553Leufs*97, p.Pro2808Leufs*24
T3-5	3	F/19	O+	12	0.01	0.08	0.04	c.1729+3A>C	n/a

n/a, not applicable; n/d, not done

§ unable to determine this patient's mutation using conventional sequencing methods

* Human Genome Variation Society (HGVS)

Table 2

Tubule formation of BOECs from healthy controls and VWD patients in Matrigel

BOEC	Covered Area (%)	Total tube length (mm)	Total branching points (n)	Total loops (n)	Total tubes (n)	Mean tube length (µm)	Mean loop area (mm)	Mean loop perimeter (mm)
Controls	26.53 ± 3.24	56.3 ± 6.88	124.50 ± 29.42	38.13 ± 15.07	275.40 ± 43.62	213.80 ± 9.59	21.15 ± 3.82	0.99 ± 0.07
T1-1	10.43 ± 3.17 *	24.87 ± 7.52 *	31.33 ± 14.97 *	4.83 ± 2.37 **	124.20 ± 29.86 *	181.10 ± 19.04	8.83 ± 4.51 *	0.42 ± 0.20 *
T1-2	38.90 ± 4.30	73.57 ± 0.68	208.50 ± 11.50	76.50 ± 0.50	366.50 ± 23.50	201.30 ± 11.84	31.32 ± 0.21	1.22 ± 0.01
T1-3	21.33 ± 9.98	43.87 ± 19.85	117.30 ± 66.59	46.75 ± 27.03	241.00 ± 96.96	161.10 ± 17.40 *	12.44 ± 7.23	0.54 ± 0.32
T1-4	14.03 ± 3.78 *	30.66 ± 8.22	44.00 ± 20.53	7.00 ± 4.41 *	155.70 ± 37.25	183.30 ± 14.24	13.01 ± 6.16	0.62 ± 0.22
2A	26.23 ± 4.30	57.28 ± 8.59	138.50 ± 36.79	45.50 ± 18.92	269.00 ± 52.72	219.70 ± 11.08	35.47 ± 3.12 *	1.24 ± 0.07
2B	8.700 ± 2.10 *	21.69 ± 5.77 *	26.00 ± 12.00 *	3.000 ± 3.00	114.50 ± 24.50	186.80 ± 10.53	3.63 ± 3.63 *	0.31 ± 0.31 *
2M	15.50 ± 5.10	47.57 ± 14.97	109.80 ± 56.57	34.75 ± 20.81	262.50 ± 83.13	181.50 ± 7.97 *	15.09 ± 8.71	0.64 ± 0.37
2N	13.85 ± 0.15	38.32 ± 1.91	36.50 ± 1.50 *	3.00 ± 0.00	199.50 ± 1.50	192.10 ± 7.90	10.72 ± 1.15	0.78 ± 0.02
T3-1	5.340 ± 1.17 **	13.38 ± 2.51 **	8.80 ± 3.81 **	0.40 ± 0.24 **	74.40 ± 7.34 **	175.20 ± 19.18	27.69 ± 25.48	0.74 ± 0.57
T3-2	14.28 ± 4.41	27.45 ± 6.98 *	44.17 ± 17.36 *	9.17 ± 4.76	126.30 ± 25.99 *	199.10 ± 20.64	19.30 ± 7.11	0.75 ± 0.24
T3-3	42.65 ± 1.05 *	77.72 ± 3.01	220.00 ± 1.00	99.0 ± 10.00	366.00 ± 0.00	213.10 ± 7.89	23.04 ± 0.14	0.96 ± 0.00
T3-4	15.05 ± 5.75	37.05 ± 12.75	56.00 ± 35.00	7.50 ± 7.50	165.00 ± 54.00	222.30 ± 3.95	20.86 ± 20.86	0.72 ± 0.72
T3-5	32.43 ± 3.22	73.62 ± 6.11	206.50 ± 25.97	79.00 ± 14.39	384.50 ± 40.30	193.40 ± 5.53	27.40 ± 1.21	1.20 ± 0.03

Each value represents the mean ± standard error of mean of 2–6 experiments.

* $P < 0.05$;** $P < 0.01$ vs Controls as indicated by the Mann-Whitney U test.