NEURAL REGENERATION RESEARCH
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Research Article

Integrin binding peptides facilitate growth and interconnected vascular-like network formation of rat primary cortical vascular endothelial cells *in vitro*

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https://doi.org/10.4103/1673-5374.355760

Date of submission: March 30, 2022

Date of decision: June 28, 2022

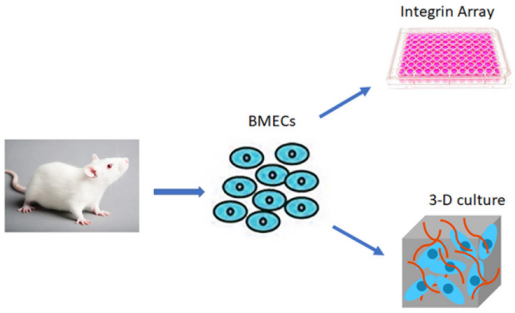
Date of acceptance: July 11, 2022

Date of web publication: October 10, 2022

From the Contents

Graphical Abstract

Primary rat brain vascular endothelial cells have improved growth with interconnected vascular-like network formation at the culture condition of appropriate integrin binding peptides



Abstract

Neovascularization and angiogenesis in the brain are important physiological processes for normal brain development and repair/regeneration following insults. Integrins are cell surface adhesion receptors mediating important function of cells such as survival, growth and development during tissue organization, differentiation and organogenesis. In this study, we used an integrin-binding array platform to identify the important types of integrins and their binding peptides that facilitate adhesion, growth, development, and vascular-like network formation of rat primary brain microvascular endothelial cells. Brain microvascular endothelial cells were isolated from rat brain on post-natal day 7. Cells were cultured in a custom-designed integrin array system containing short synthetic peptides binding to 16 types of integrins commonly expressed on cells in vertebrates. After 7 days of culture, the brain microvascular endothelial cells were processed for immunostaining with markers for endothelial cells including von Willibrand factor and platelet endothelial cell adhesion molecule. 5-Bromo-2′-dexoyuridine was added to the culture at 48 hours prior to fixation to assess cell proliferation. Among 16 integrins tested, we found that $\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_8$ greatly promoted proliferation of endothelial cells in culture. To investigate the effect of integrin-binding peptides in promoting neovascularization and angiogenesis, the binding peptides to the above three types of integrins were immobilized to our custom-designed hydrogel in three-dimensional (3D) culture of brain microvascular endothelial cells with the addition of vascular endothelial growth factor. Following a 7-day 3D culture, the culture was fixed and processed for double labeling of phalloidin with von Willibrand factor or platelet endothelial cell adhesion molecule and assessed under confocal microscopy. In the 3D culture in hydrogels conjugated with the integrin-binding peptide, brain microvascular endothelial cells formed interconnected vascular-like network with clearly discernable lumens, which is reminiscent of brain microvascular network *in vivo*. With the novel integrin-binding array system, we identified the specific types of integrins on brain microvascular endothelial cells that mediate cell adhesion and growth followed by functionalizing a 3D hydrogel culture system using the binding peptides that specifically bind to the identified integrins, leading to robust growth and lumenized microvascular-like network formation of brain microvascular endothelial cells in 3D culture. This technology can be used for *in vitro* and *in vivo* vascularization of transplants or brain lesions to promote brain tissue regeneration following neurological insults.

Key Words: 3D culture; angiogenesis; brain microvascular endothelial cells; hydrogel; integrins; platelet endothelial cell adhesion molecule (PECAM-1); vascular endothelial growth factor (VEGF); vascularization

Introduction

Neurovascular dysfunction resulting from cerebrovascular and neurological disorders or brain trauma features complicated pathological mechanisms. Angiogenesis plays a critical role in regeneration process in neurological diseases. Neovascularization and angiogenesis are characterized by remodeling of the extracellular matrix (ECM), proliferation and migration of vascular endothelial cells (ECs) followed by lumen formation and functional maturation, and these processes require adhesive molecules such as integrins (Pulous and Petrich, 2019). Studies have shown that ECM maintains adhesive interactions with integrins that are expressed on the surface of ECs facilitating neovascularization (Kant et al., 2019; Edwards et al., 2020; Mo, 2021). Integrins are the major class of ECM receptors that are present on cell surfaces as αβ heterodimers (Hynes et al., 1999). To date, 18 types of α subunits and eight types of β subunits have been recognized, giving rise to 20 recognized αβ dimers with different specificity (Hynes et al., 1999). The instructive role of integrins in angiogenesis at both developmental and pathological conditions has been demonstrated in studies of genetic ablation and conditional deletion of integrin related genes (Gustafsson and Fassler, 2000; Hou et al., 2020; Zhang et al., 2022). Because of their crucial role in angiogenesis, integrins are often targeted for the treatment of malignant growth using antagonists (Rocha et al., 2018; Hou et al., 2020). Among integrin family members, several of them are particularly associated with angiogenesis. Studies have reported that ECs express higher levels of integrins αvβ3 and α5β1 following stimulation with growth factors or proangiogenic signaling molecules (Brooks et al., 1994; Kim et al., 2000a,

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Funding: *This study was supported by NIH grant RO1 NS093985 (to DS, NZ, XW) and RO1 NS101955 (to DS). Confocal microscope images were performed at the VCU Microscopy Facility, supported, in part, by funding from NIH-NCI Cancer Center Support Grant P30 CA016059.*

How to cite this article: *Kuwar R, Wen X, Zhang N, Sun D (2023) Integrin binding peptides facilitate growth and interconnected vascular-like network formation of rat primary cortical vascular endothelial cells in vitro. Neural Regen Res 18(5):1052-1056.*

b; Dudvarski Stankovic et al., 2018). Moreover, the binding of integrin with its ligands has been shown to trigger the intracellular cascades that lead to neovascularization and angiogenesis (Karimi et al., 2018), suggesting binding with the proper ligands is crucial for the generation of cell signaling for angiogenesis. Additionally, recent studies have shown that short peptides, called integrin-binding peptides, bind specifically to the integrin, initiating the signaling for angiogenesis (Conibear et al., 2017; Jiang et al., 2019).

As the initial step toward brain tissue regeneration following neurological disorders, reconstructing the vascular network of primary brain microvascular endothelial cells (BMECs) is the key. Available strategies to engineer vascular network of BMECs are heavily relied upon Matrigel, a solubilized basement membrane matrix with heterogeneous composition derived from mouse sarcoma cells. Matrigel supports complex cellular behavior that is otherwise difficult to observe under regular laboratory conditions. For examples, ECs grown on Matrigel coated surfaces can form intricate network structure typical of microvascular network seen *in vivo* (Kastana et al., 2019). However, mouse tumor origin and heterogeneous composition of Matrigel make it impossible for clinical translation. As an alternative, we have previously developed an integrin-binding array system which allows scholars to recognize the specific types of integrins and their binding peptides that can best support the growth of cell types of interest (Jiang et al., 2019). The integrin-binding array is created by immobilizing short synthetic peptides that exclusively bind to 16 types of integrins commonly expressed on cells of vertebrates on a 48-well cell culture plate (Jiang et al., 2019). This array system can help to identify the key types of integrins and their binding peptides that mediate cell adhesion and growth. Using this array, we have identified integrin peptides that are supportive for optimal growth of several types of primary cells from human and animals that are known difficult to culture on regular culture wares, including human induced pluripotent stem cells (hiPSCs), hiPSC-derived neuroepithelial progenitors (NEPs), and rat primary cortical neurons (Jiang et al., 2019; Kuwar et al., 2020).

Vascularization is the key step for initiation of brain regeneration following neurological injures. To facilitate this process, it is necessary to identify factors that can stimulate the optimal growth of BMECs. In this study, we assessed the growth of primary rat BMECs isolated from the cortical region of 7-day-old rat pups on the integrin-binding array. To further confirm the supporting effect of integrin binding peptide on BMEC growth, we also immobilized the identified integrin-binding peptides and vascular endothelial growth factor (VEGF) peptides in a synthetic hydrogel for three-dimensional (3D) culture of BMECs.

Methods

Animals

All the experiments involving animals were approved by the Institutional Animals Care and Use Committee at Virginia Commonwealth University on March 27, 2019 (#AM10132) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th ed, National Research Council, 2011). Fisher 344 female rats approximately 3 months old at the 15 days of pregnancy stage (240–260 g) were purchased from the vendor (Charles River Laboratory, Wilmington, MA, USA). Animals were housed in an American Association for Laboratory Animal Care (AAALAC) credited pathogen-free facility at 21 ± 2°C and 45% humidity with a 12-hour light/dark cycle, water and food provided *ad libitum*.

Isolation of brain microvascular endothelial cells

The post-natal day 7 rat pups were used for isolation of primary BMECs following a published protocol with modification (Teng et al., 2008). Briefly, the rat pups were deeply anaesthetized via inhalation of isoflurane in a glass desiccator. The brains were dissected and pooled in the RPMI 1640 medium (Invitrogen, Waltham, MA, USA.) supplemented with 1% penicillin and streptomycin. The cortical tissues were dissected out, cut into 1 mm pieces and homogenized using loose-fitting wheaton dounce tissue grinder, then suspended with 15% dextran (Sigma, St. Louis, MO, USA). After spinning down, the pellet was collected, digested in 0.1% collagenase/dispase (Sigma) and incubated for 2–3 hours. The pellet was then suspended in 45% percoll gradient (Sigma). The microvessels, which floated to the top of the Percoll gradient after centrifugation at 20,000 × *g* for 10 minutes at 4°C, were collected and seeded in rat tail type I collagen coated flasks with the Complete Rat Endothelial Cell Medium (Cat# M1266, Cell Biologics, Chicago, IL, USA). Cells were passaged when they were 80–90% confluent. Rat primary ECs at passages 2–4 were used for this study.

Monolayer ECs culture in the integrin-binding peptide array

A total of 30,000 cells/well were seeded in the 48-well integrin-binding array platform according to our published protocol (Jiang et al., 2019), and cultured in the endothelial cell culture medium with supplement of fetal bovine serum, epidermal growth factor and VEGF for a 7-day culture with the media changed every 48 hours. To assess the proliferation rate, thymidine analog 5-bromo-2′-dexoyuridine (BrdU, 10 μM) was added to the complete rat endothelial cell medium (Cat# M1266, Cell Biologics) at day 5 for 48 hours. Cells that grew in collagen coated wells served as control. After a 7-day culture, the ECs were fixed for 30 minutes with 4% paraformaldehyde followed by immunostaining.

Immunocytochemistry

After 30 minutes of fixation, ECs cells were thoroughly washed with PBS followed by a 1-hour serum blocking with 10% normal horse serum (Thermo Fisher Scientific, Waltham, MA, USA) before incubation with the primary antibodies. The primary antibodies used included platelet endothelial cell adhesion molecule (PECAM-1) mouse anti-rat monoclonal antibody (1:500, Cat# MCA1334, Bio-Rad, Hercules, CA, USA) and von Willebrand factor (VWF) polyclonal antibody (rabbit) (1:1000, Cat# bs-0586R, Bioss, Boston, MA, USA) to label EC cells, and mouse anti-rat BrdU (1:200, Cat# MCA6144, Bio-Rad) to labeling proliferating cells. Following overnight incubation at 4°C with agitation, the ECs were washed with PBS to remove primary antibodies and then incubated with the secondary antibodies including Alexa Fluor 488 anti-mouse IgG (Cat# A11001) or Alexa Fluor 568 anti-rabbit IgG (Cat# A11036; both 1:200, Thermo Fisher Scientific) for 1 hour at room temperature on a shaker. After that, cells were washed with PBS and stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000, Thermo Fisher Scientific) for 10 minutes, followed by PBS washes and quick rinse with dH_2O before coverslipped with Vectashield (Vestor Laboratories, Newark, CA, USA).

Three-dimensional cell culture

After assessing cell proliferation in our customer-designed integrin-binding array platform, the peptides that better supported for EC growth were added to a polyethylene glycol-based injectable hydrogel for tubule formation of ECs in a 3D culture according to previously reported method (Li et al., 2014; Kuwar et al., 2020). The experiment was done in triplicates using 48-well plates. Briefly, the wells were first coated with 30 μL of hydrogel and incubated for 30 minutes at 37°C before seeding at a density of 80,000/well. A 0.8% hydrogel was diluted to 50% with the selected integrin-binding peptides plus ECs in EC culture medium (Cell Biologics). The integrin-binding peptides stock solution (Sigma) was at 4 mg/mL in PBS and was diluted to 0.5 mg/mL as the final concentration in the culture. The components for 3D culture included ECs, selected integrin binding peptide, varying concentrations of recombinant VEGF (final concentration in each well: 0, 0.2, 0.5, 1, and 1.5 μg/μL), EC culture medium and hydrogel as above described. All components were thoroughly mixed up before being adding into the wells of the hydrogel pre-coated plate, 50 μL/well, and then incubated for 30 minutes at 37°C. Once the gel was fully set, 200 μL of complete endothelial culture medium described above was gently added. The culture was maintained for 7 days with medium changed every 48 hours. After the 7-day culture, the ECs were fixed with 4% paraformaldehyde before processed for immunostaining for PECAM-1 mouse anti-rat monoclonal antibody (1:500, Cat# MCA1334, Bio-Rad), incubated overnight at 4°C with agitation, washed with PBS, and incubated with Alexa Fluor 568 anti-rabbit IgG (1:200, Cat# A11036, Thermo Fisher Scientific) and Phalloidin Dylight 488 (phalloidin stains F-actin of cell cytoskeleton) (1:50, Cat# 21833, Thermo Fisher Scientific) for 1 hour at room temperature on a shaker. After that, cells were washed, stained with DAPI, and coverslipped with Vectashield as described above.

Cell quantification

To assess the percentage of PECAM-1 or VWF stained cells, five fields per well were randomly selected and imaged with an inverted fluorescent microscope (1X71 Olympus, Olympus, Tokyo, Japan) at 20× objective. The number of PECAM-1$^+$, VWF$^+$, BrdU$^+$ or DAPI$^+$ cells in the culture wells of control condition and 7 integrin-binding peptides, including $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_7\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_v\beta_8$, $\alpha_M\beta_2$ and $\alpha_{IIb}\beta_3$, that have the best cell growth were counted using ImageJ software version 1.53 (NIH, Bethesda, MD, USA). For cell proliferation, the ratio of VWF$^+$/BrdU$^+$ was calculated using the number of VMF/BrdU double-labeled cells against the total number of DAPI$^+$ cells. For 3D culture, images were taken using LSM 710 Zeiss confocal microscope (Carl Zeiss, Stuttgart, Germany).

Statistical analysis

For this *in vitro* study, no statistical methods were used to predetermine sample sizes; however, our sample sizes were similar to those reported in previous publications (Jiang et al., 2019; Kuwar et al., 2020). All experiments were performed in triplicate. For data analysis with multiple group comparison, data were analyzed with one-way analysis of variance followed by *post hoc* test with Tukey's *post hoc* test. GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA, www.graphpad.com) was used and data were presented as the mean ± SEM. A level of $P < 0.05$ was considered statistically significant.

Results

Growth of rat brain-derived primary endothelial cells in the integrin-binding array platform

In this study, we assessed the growth of primary brain endothelial cells in our custom-designed integrin-binding array system. This array platform contained short synthetic peptides binding to 16 types of integrins, including $\alpha_1\beta_1$,

$\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_4$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_{IIb}\beta_3$, which are commonly expressed on almost all cells in vertebrates. BMECs isolated from p7 rat brain cerebral cortex were grown in the integrin-binding array plate for 7 days. Using EC cell markers including VWF and PECAM-1, as well as nuclei marker DAPI, we found about 95% of cultured cells were VWF and PECAM-1 positive, suggesting the purity of ECs with our culture method. The assessment of growth of BMECs on the integrin-binding array platform with VWF and PECAM-1 immunostaining demonstrated that most of the integrin-binding peptides supported the attachment and survival of BMECs (**Figure 1**).

BMECs were cultured with thymidine analogue BrdU for 48 hours to assess their proliferation. Extensive VWF/BrdU double-labeled cells were found in all wells (**Figure 2**). Among 16 integrin-binding peptides, BMECs grown in the wells with several integrin-binding peptides including $\alpha_5\beta_1$, $\alpha_7\beta_1$, $\alpha v\beta 1$, $\alpha_v\beta_5$ or $\alpha_v\beta_8$ had a significantly higher percentage of VWF/BrdU double labeled cells than cells grown in the standard collagen coated cells, among them BMECs grew in $\alpha_5\beta_1$ had the highest proliferation rate (**Figure 3**). Subsequently, $\alpha_5\beta_1$ was used in the 3D culture studies.

$\alpha_5\beta_1$ integrin-binding peptides promote cultured BMECs angiogenic sprouting and vessel formation in 3-D culture

To assess the effect of integrin peptide on promotion of BMECs growth, we tested $\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_8$ integrin-binding peptides for the 3D culture based on the cell proliferation study. We found $\alpha_5\beta_1$ was the best one, and therefore we selected $\alpha_5\beta_1$-binding peptide for the final 3D culture study. In this study, we first assessed different concentrations of $\alpha_5\beta_1$ integrin binding peptide at the concentration of 0.2, 0.5, 1.0 or 1.5 μg/μL was mixed with the hydrogel and BMECs first, then the mixed components were added to the culture wells that were pre-coated with hydrogel. For the 3-D culture, the density of BMECs was at 80,000 cells/well. VEGF (0.2 μg/μL) was also added to the culture. After 7-day culture, we found that BMECs had the best growth in wells with $\alpha_5\beta_1$ integrin binding peptide at 0.2 μg/μL but not in wells with higher concentrations. Taking 0.2 μg/μL as the optimal concentration for $\alpha_5\beta_1$ integrin binding peptide, we then assessed different concentrations of VEGF for the growth of BMECs in 3D culture. VEGF was added to the 3D culture at 0.2, 0.5, 1.0 or 1.5 μg/μL. After 7-day culture, ECs were fixed and processed for immunostaining for PECAM-1, VWF or F-actin plus DAPI. We found that in the presence of VEGF at 1 μg/μL, BMECs had numerous sprouts-like structures from the main trunks spreading in the culture (**Figure 4**). The vascular-like network structures were positive for PECAM-1, F-actin and VWF, and lumen patency was assessed in x-y-z three planes with the Zen 2011 software (**Figure 4**). When BMECs grew in 3-D culture with $\alpha_5\beta_1$-binding peptide but without VEGF or with VEGF but without integrin binding peptide, no robust tubular formation or sprouting was observed. This finding suggests that $\alpha_5\beta_1$-binding peptide and VEGF work synergistically in promoting growth and interconnected and lumenized vascular-like ECs network formation of BMECs in the 3-D culture.

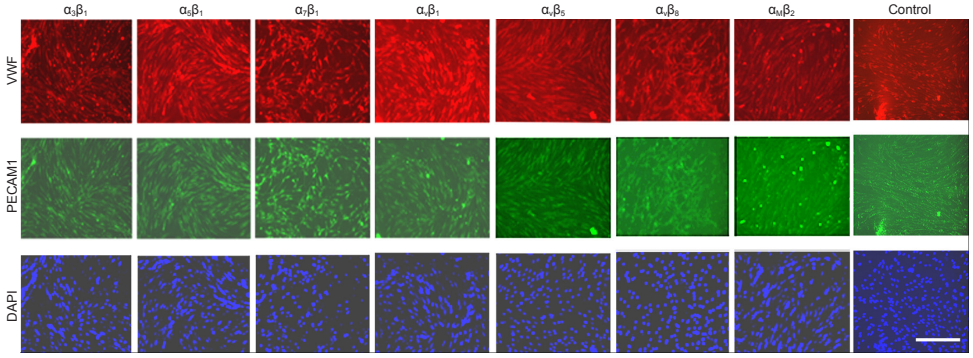

Figure 1 | Growth of primary rat BMECs in the integrin-binding array platform.
BMECs after 7-day culture were identified with EC markers von Willibrand factor (VWF, red), PECAM-1 (green) and neucli marker DAPI (blue). Representative image showed the growth pattern of BMECs in wells of the selected integrin-binding peptide and the control well with standard collagen coating. Note that the majority of cells were ECs expressing VWF and PECAM1. Scale bar: 50 μm. BMECs: Brain microvascular endothelial cells; DAPI: 4′,6-diamidino-2-phenylindole; ECs: endothelial cells; PECAM-1: platelet endothelial cell adhesion molecule 1; VWF: von Willibrand factor.

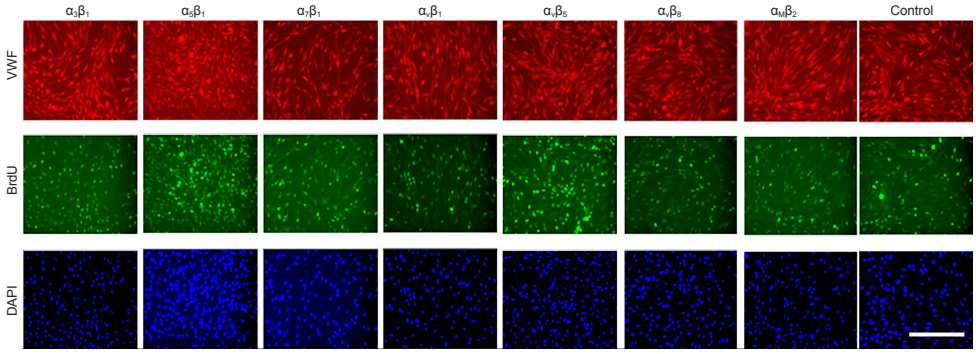

Figure 2 | Proliferation of primary rat BMECs in the integrin-binding array platform.
Proliferation of BMECs in the integrin array platform was assessed by immunofluorecent labeling with EC marker VWF (red), thymidine aanlog BrdU (green) and DAPI (blue) after 7-day culture. Representative images showed the proliferation pattern of BMECs in wells of the selected integrin-binding peptide and the control well with standard collagen coating. Scale bar: 50 μm. BMECs: Brain microvascular endothelial cells; BrdU: 5-bromo-2′-dexoyuridine; DAPI: 4′,6-diamidino-2-phenylindole; PECAM-1: platelet endothelial cell adhesion molecule 1; VWF: von Willibrand factor.

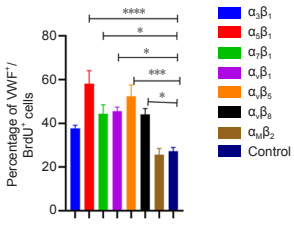

Figure 3 | Quantification analysis of the proliferation rate of the BMECs in the integrin-binding array platform.
The number of VWF and BrdU double-labeled cells were counted against the total number of DAPI$^+$ cells. Cells growing in integrin-binding peptides for $\alpha_5\beta_1$ or $\alpha_v\beta_5$ had significantly higher ratio in comparison to cells growing in control wells. All experiments were performed in triplicate. BMECs: Brain microvascular endothelial cells; BrdU: 5-bromo-2′-dexoyuridine; DAPI: 4′,6-diamidino-2-phenylindole; VWF: von Willibrand factor. *P < 0.05, ***P < 0.001, ****P < 0.0001.

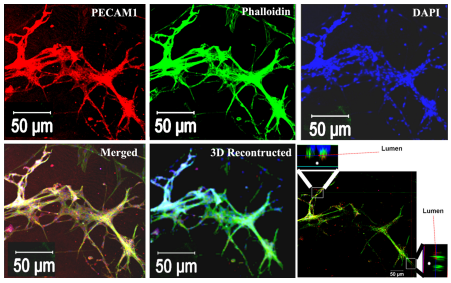

Figure 4 | Growth of primary rat BMECs in three-dimensional culture in integrin binding peptide-conjugated hydrogels.
Immunofluorescent staining with PECAM-1 (red), Phalloidin (green) and DAPI (blue) showed the growth pattern of primary rat BMECs in 3-D culture within selected integrin α5β1-binding peptide-conjugated hydrogels. Formation of interconnected tubular network structures with lumens was clearly seen at the x-y-z plane image (asterisks). Scale bar: 50 μm. BMECs: Brain microvascular endothelial cells; DAPI: 4′,6-diamidino-2-phenylindole; PECAM-1: platelet endothelial cell adhesion molecule 1.

Discussion

The current study represents one of the first efforts to identify the specific types of integrins on the surface of BMECs that mediate their adhesion and growth in culture. By immobilizing to synthetic hydrogel in 3D culture the integrin-binding peptides that specifically bind to the identified integrins, we have achieved robust lumenized interconnected ECs network formation of BMECs in 3D culture in the hydrogel. The three types of integrins ($\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_8$) that were identified in the integrin-binding array platform are consistent with previous reports of integrin repertoire on primary ECs and their effect on EC function. In particular, the integrin $\alpha_5\beta_1$ was implicated in endothelial cell chemotaxis and migration during angiogenesis (Li et al., 2006). In integrin α_5-deficient mice, a deficient angiogenesis was exhibited in response to cerebral hypoxia (Li et al., 2012), and triggered neuroinflammation (Kant et al., 2019). β_1-integrins were implicated in vascular lumen formation and vessel patterning (Bayless et al., 2000; Zovein et al., 2010; Yamamoto et al., 2015). Inhibition of β_1-integrins has led to pathological angiogenesis, accompanied by EC junctions weakening and blood leakage (Hynes, 2002; Yamamoto et al., 2015). In CNS angiogenesis, ECM fibronectin plays a strong angiogenic effect on ECs that is mediated by $\alpha_5\beta_1$ and α_v-integrins through the MAP kinase signaling pathway (Wang and Milner, 2006). Similarly, α_v-integrins including $\alpha_v\beta_5$ and $\alpha_v\beta_8$ facilitate the growth and survival of newly forming blood vessels during angiogenesis and tissue remodeling for wound healing (Demircioglu and Hodivala-Dilke, 2016; Duro-Castano et al., 2017). The α_v-integrins belong to a family of RGD-binding integrins promoting distinct pathways of angiogenesis (Hood et al., 2003). For example, $\alpha_v\beta_5$ is required during VEGF-induced angiogenesis to activate focal adhesion kinase and Src kinase (Friedlander et al., 1995), while $\alpha_v\beta_8$ expressed on glial cells regulates cerebrovascular development (Zhu et al., 2002). By conjugating the specific binding peptides of the $\alpha_5\beta_1$ integrin to a 3D synthetic hydrogel substrate, we have seen robust formation of interconnected vascular-like ECs network of BMECs in 3D culture in the hydrogel, suggesting the utilities of the integrin type-specific binding peptides as functionalizing agents for biomaterials to promote the angiogenic phenotype of BMECs. Pairing of integrins on EC cell surface and the ligands that are present within the environment of ECs are important for promoting sprouting and vascular-like network formation of ECs during angiogenesis.

Direct research on characterizing function-specific integrin expression profile on cells is limited. Our integrin-binding array provides a valuable platform for the identification of specific types of integrins on BMEC surfaces that are responsible for mediating the key EC function in angiogenesis. The binding peptides to the identified types of integrins that mediate specific cell functions can be readily conjugated to biomaterial implants or 3D cell culture substrates to promote cell function of interest. Revascularization or angiogenesis in injured/diseased brain is critical for basic research, disease modeling, drug screening, and tissue regeneration in the context of related insults. Prospectively, as a continuation of this study, the binding peptides to the identified types of integrins that mediate angiogenesis of BMECs and neurogenesis of neural stem cells (NSCs) may be co-conjugated to transplantable hydrogel substrates to integrate both angiogenesis and neurogenesis for functional brain tissue regeneration. A 3D hydrogel system that is functionalized with the integrin binding peptides to direct both angiogenesis and neurogenesis of BMECs and NSCs in co-culture or *in vivo* would allow interplay and self-assembly of vascular structures and neuronal cells through cell-cell adhesion, cell-ECM adhesion, and cell-cell signaling, promoting the formation of complete vascular-like structures to support the rewiring of neural networks. Close association of angiogenesis and neurogenesis during brain tissue repair has been documented following brain trauma and different types of neurological insults including cerebral stroke in which angiogenic blood vessels serve as a physical scaffold for neuron migration to the ischemic core (Wang et al., 2019; Yue et al., 2020). In addition, suppression of angiogenesis significantly hindered the migration of neural progenitor cells to the ischemic region (Iadecola, 2017). An accumulating body of evidence reveals the change of integrin expression profile on ECs under pathological conditions (Rocha et al., 2018; Hou et al., 2020) as well as during development (Milner and Campbell, 2002). The unique capability of our integrin-binding array platform to identify the specific types of integrins and their binding peptides that mediate the specific function of cells of interest under the condition of interest (e.g., healthy *vs.* trauma or pathological) at the developmental stage of interest would help to customize biomaterials with key integrin-binding ligands as bioactive cues to orchestrate the interplay of distinct biological processes (e.g., therapeutic angiogenesis, neurogenesis, or tissue repair) for specific biomedical purposes.

In addition to mediating cell interaction with ECM and the environment, integrins directly bind to several growth factors including a number of VEGF isoforms and VEGF receptors. In our 3D culture of BMECs in $\alpha_5\beta_1$-binding peptide-conjugated hydrogel that was supplemented with VEGF, the master regulator of angiogenesis (Hoeben et al., 2004), robust formation of 3D interconnected lumenized vascular-like network of BMECs was observed, which was absent in the control hydrogel groups with $\alpha_5\beta_1$-binding peptide without VEGF or with VEGF but without integrin binding peptide. The $\alpha_5\beta_1$ integrin binding may amplify angiogenic signals in VEGF-induced vascular sprouting and drive lumenized vascular-like network formation (Li et al., 2017). Despite beyond the scope of the present study, a previous study demonstrated that when implanted subcutaneously in mice, $\alpha_5\beta_1$ integrin-modified hydrogels supplemented with control-released VEGF promoted vessel sprouting and non-tortuous uniform vessel network formation typical of the normal mouse skin vasculature within the hydrogels while reducing VEGF-induced vascular permeability (Li et al., 2017). Our observation and the literature suggest the potential of integrin-specific biomaterials in conjunction with VEGF in dictating vascular patterning/morphogenesis and permeability both *in vitro* and *in vivo* for therapeutic angiogenesis.

This study is limited to *in vitro*. *In vivo* study is needed to confirm that the identified three specific types of integrins and their binding peptides can stimulate brain endothelial cell growth and new vessel formation to facilitate brain regeneration process.

In conclusion, we assessed the growth of BMECs in different conditions and identified three specific types of integrins (α5β1, αvβ5 and αvβ8) and their binding peptides that are most supportive for adhesion and growth of BMECs in this study. These integrin binding peptides support a robust growth of BMECs and their formation of lumenized interconnected vascular-like network in 3D culture in the hydrogel.

Author contributions: *Methodology, investigation, data curation, formal analysis, writing – original draft: RK. Conceptualization, methodology, resources: XW. Conceptualization, writing – review & editing: NZ. Conceptualization, writing – review & editing, project administration, funding acquisition, supervision: DS. All authors approved the final version of the manuscript.*

Conflicts of interest: *The authors declare no conflict of interest associated with the studies presented in this manuscript.*

Editor note: DS is an Editorial Board member of Neural Regeneration Research. She was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer review handled independently of this Editorial Board member and their research groups.

References

Bayless KJ, Salazar R, Davis GE (2000) RGD-dependent vacuolation and lumen formation observed during endothelial cell morphogenesis in three-dimensional fibrin matrices involves the alpha(v)beta(3) and alpha(5)beta(1) integrins. Am J Pathol 156:1673-1683.

Brooks PC, Clark RA, Cheresh DA (1994) Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science 264:569-571.

Conibear AC, Hager S, Mayr J, Klose MHM, Keppler BK, Kowol CR, Heffeter P, Becker CFW (2017) Multifunctional αvβ6 integrin-specific peptide-Pt(IV) conjugates for cancer cell targeting. Bioconjug Chem. 2017 Sep 20;28(9):2429-2439.

Demircioglu F, Hodivala-Dilke K (2016) αvβ3 Integrin and tumour blood vessels-learning from the past to shape the future. Curr Opin Cell Biol 42:121-127.

Dudvarski Stanković N, Bicker F, Keller S, Jones DT, Harter PN, Kienzle A, Gillmann C, Arnold P, Golebiewska A, Keunen O, Giese A, von Deimling A, Bäuerle T, Niclou SP, Mittelbronn M, Ye W, Pfister SM, Schmidt MHH (2018) EGFL7 enhances surface expression of integrin α5β1 to promote angiogenesis in malignant brain tumors. EMBO Mol Med 10:e8420.

Duro-Castano A, Gallon E, Decker C, Vicent MJ (2017) Modulating angiogenesis with integrin-targeted nanomedicines. Adv Drug Deliv Rev 119:101-119.

Edwards DN, Salmeron K, Lukins DE, Trout AL, Fraser JF, Bix GJ (2020) Integrin α5β1 inhibition by ATN-161 reduces neuroinflammation and is neuroprotective in ischemic stroke. J Cereb Blood Flow Metab 40:1695-1708.

Friedlander M, Brooks PC, Shaffer RW, Kincaid CM, Varner JA, Cheresh DA (1995) Definition of two angiogenic pathways by distinct alpha v integrins. Science 270:1500-1502.

Gustafsson E, Fässler R (2000) Insights into extracellular matrix functions from mutant mouse models. Exp Cell Res 261:52-68.

Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA (2004) Vascular endothelial growth factor and angiogenesis. Pharmacol Rev 56:549-580.

Hood JD, Frausto R, Kiosses WB, Schwartz MA, Cheresh DA (2003) Differential alphav integrin-mediated Ras-ERK signaling during two pathways of angiogenesis. J Cell Biol 162:933-943.

Hou J, Yan D, Liu Y, Huang P, Cui H (2020) The roles of integrin α5β1 in human cancer. Onco Targets Ther 13:13329-13344.

Hynes RO, Bader BL, Hodivala-Dilke K (1999) Integrins in vascular development. Braz J Med Biol Res 32:501-510.

Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. Cell 110:673-687.

Iadecola C (2017) The neurovascular unit coming of age: a journey through neurovascular coupling in health and disease. Neuron 96:17-42.

Jiang C, Zeng X, Xue B, Campbell D, Wang Y, Sun H, Xu Y, Wen X (2019) Screening of pure synthetic coating substrates for induced pluripotent stem cells and iPSC-derived neuroepithelial progenitors with short peptide based integrin array. Exp Cell Res 380:90-99.

Kant R, Halder SK, Bix GJ, Milner R (2019) Absence of endothelial α5β1 integrin triggers early onset of experimental autoimmune encephalomyelitis due to reduced vascular remodeling and compromised vascular integrity. Acta Neuropathol Commun 7:11.

Karimi F, O'Connor AJ, Qiao GG, Heath DE (2018) Integrin clustering matters: a review of biomaterials functionalized with multivalent integrin-binding ligands to improve cell adhesion, migration, differentiation, angiogenesis, and biomedical device integration. Adv Healthc Mater 7:e1701324.

Kastana P, Zahra FT, Ntenekou D, Katraki-Pavlou S, Beis D, Lionakis MS, Mikelis CM, Papadimitriou E (2019) Matrigel plug assay for in vivo evaluation of angiogenesis. Methods Mol Biol 1952:219-232.

Kim S, Bell K, Mousa SA, Varner JA (2000a) Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. Am J Pathol 156:1345-1362.

Kim S, Harris M, Varner JA (2000b) Regulation of integrin alpha vbeta 3-mediated endothelial cell migration and angiogenesis by integrin alpha5beta1 and protein kinase A. J Biol Chem 275:33920-33928.

Kuwar R, Wen X, Zhang N, Sun D (2020) An integrin-binding array platform identifies αvβ8 and α5β1 integrins on rat primary cortical neurons to support their survival and growth. J Neurosci Methods 339:108729.

Li L, Welser-Alves J, van der Flier A, Boroujerdi A, Hynes RO, Milner R (2012) An angiogenic role for the α5β1 integrin in promoting endothelial cell proliferation during cerebral hypoxia. Exp Neurol 237:46-54.

Li Q, Ford MC, Lavik EB, Madri JA (2006) Modeling the neurovascular niche: VEGF- and BDNF-mediated cross-talk between neural stem cells and endothelial cells: an in vitro study. J Neurosci Res 84:1656-1668.

Li S, Nih LR, Bachman H, Fei P, Li Y, Nam E, Dimatteo R, Carmichael ST, Barker TH, Segura T (2017) Hydrogels with precisely controlled integrin activation dictate vascular patterning and permeability. Nat Mater 16:953-961.

Li X, Liu X, Zhang N, Wen X (2014) Engineering in situ cross-linkable and neurocompatible hydrogels. J Neurotrauma 31:1431-1438.

Milner R, Campbell IL (2002) Developmental regulation of beta1 integrins during angiogenesis in the central nervous system. Mol Cell Neurosci 20:616-626.

Mo FE (2021) Shear-regulated extracellular microenvironments and endothelial cell surface integrin receptors intertwine in atherosclerosis. Front Cell Dev Biol 9:640781.

National Research Council (2011) Guide for the Care and Use of Laboratory Animals, 8th ed. Washington, DC: The National Academies Press.

Pulous FE, Petrich BG (2019) Integrin-dependent regulation of the endothelial barrier. Tissue Barriers 7:1685844.

Rocha LA, Learmonth DA, Sousa RA, Salgado AJ (2018) αvβ3 and α5β1 integrin-specific ligands: From tumor angiogenesis inhibitors to vascularization promoters in regenerative medicine? Biotechnol Adv 36:208-227.

Teng H, Zhang ZG, Wang L, Zhang RL, Zhang L, Morris D, Gregg SR, Wu Z, Jiang A, Lu M, Zlokovic BV, Chopp M (2008) Coupling of angiogenesis and neurogenesis in cultured endothelial cells and neural progenitor cells after stroke. J Cereb Blood Flow Metab 28:764-771.

Wang J, Milner R (2006) Fibronectin promotes brain capillary endothelial cell survival and proliferation through alpha5beta1 and alphavbeta3 integrins via MAP kinase signalling. J Neurochem 96:148-159.

Wang L, Zhang X, Liu X, Feng G, Fu Y, Milner R, Li L (2019) Overexpression of α5β1 integrin and angiopoietin-1 co-operatively promote blood-brain barrier integrity and angiogenesis following ischemic stroke. Exp Neurol 321:113042.

Yamamoto H, Ehling M, Kato K, Kanai K, van Lessen M, Frye M, Zeuschner D, Nakayama M, Vestweber D, Adams RH (2015) Integrin β1 controls VE-cadherin localization and blood vessel stability. Nat Commun 6:6429.

Yue H, Xie K, Ji X, Xu B, Wang C, Shi P (2020) Vascularized neural constructs for ex-vivo reconstitution of blood-brain barrier function. Biomaterials 245:119980.

Zhang Y, Sun L, Li H, Ai L, Ma Q, Qiao X, Yang J, Zhang H, Ou X, Wang Y, Chen G, Xue J, Zhu X, Zhao Y, Yang Y, Liu C (2022) Binding blockade between TLN1 and integrin β1 represses triple-negative breast cancer. Elife 11:e68481.

Zhu J, Motejlek K, Wang D, Zang K, Schmidt A, Reichardt LF (2002) beta8 integrins are required for vascular morphogenesis in mouse embryos. Development 129:2891-2903.

Zovein AC, Luque A, Turlo KA, Hofmann JJ, Yee KM, Becker MS, Fassler R, Mellman I, Lane TF, Iruela-Arispe ML (2010) Beta1 integrin establishes endothelial cell polarity and arteriolar lumen formation via a Par3-dependent mechanism. Dev Cell 18:39-51.

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