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Characterization of Vascular Endothelial Growth Factor (VEGF) Receptors on the Endothelial Cell Surface During Hypoxia Using Whole Cell Binding Arrays

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Abstract

Angiogenesis plays a central role in a variety of important biological processes such as reproduction, tissue development, and wound healing, as well as being critical to tumor formation in cancer. The development of chromosomal substitution (consomic) rat strains has permitted the chromosomal localization of genetic factors critical to angiogenesis but many questions remain as to the mechanisms involved. We utilize here a novel cell capture assay to assess changes in the functional expression of Vascular Endothelial Growth Factor (VEGF) receptors on the surface of vascular endothelial cells isolated from rat strains that are normal or impaired in angiogenesis. It is shown that functional VEGF receptor expression is increased under hypoxic conditions in rat strains that exhibit normal angiogenesis, but not in a strain impaired in angiogenesis. This result implicates the dysregulation of VEGF receptor expression levels on the endothelial cell surface as a key factor in impaired angiogenesis.

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vessels, plays a critical role in reproduction, tissue development, and wound healing [1]. Angiogenesis is also implicated in a variety of disorders such as ischemic heart disease, stroke, and hypertension [1–3]. There have been many efforts to explore how genetic factors contribute to the physiology of angiogenic pathways and their diseases. Specifically, consomic rats generated from chromosome substitution enable the identification of regions of the genome responsible for angiogenic traits and their diseases without the effects of heterogeneous genomic backgrounds in which phenotypic noise may make detection difficult [4,5]. In consomic rats, one chromosome at a time is substituted from a disease-resistant strain into a rat with a disease-susceptible genetic background, and the contribution of genes from each chromosome is assessed by phenotyping for the traits of interest [6,7]. Consomic rat strains recently derived from the normotensive Brown Norway rat strain (BN/NHsdMCWi, referred to hereafter as BN) and the Dahl salt-sensitive rat strain (SS/JrHsdMCWi, referred to hereafter as SS) [8],

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which develops hypertension rapidly when exposed to a high-salt diet, have provided increased understanding not only of the disease of hypertension but also of the physiology of angiogenesis [9]. The SS-13^{BN} rat, in which chromosome 13 from a BN rat is substituted into the genetic background of the hypertensive SS rat, exhibits dramatic reductions in blood pressure compared with the SS rat, indicating that genes on chromosome 13 may be important in hypertension [10,11]. Furthermore, the consomic SS-13^{BN} rat exhibits a different physiological response to an angiogenic stimulus than the SS rat. When electrical stimulation is applied to the skeletal muscles of BN, SS, and SS-13^{BN} rats, increased vessel density is observed in the BN and the SS-13^{BN} rats, but not in the SS rat, suggesting that chromosome 13 genes also play a critical role in angiogenesis [7]. In the present study the mechanism underlying these differences is investigated.

It is known that angiogenesis is a compensatory response to prolonged imbalances between the metabolic requirements of the tissues and the perfusion capabilities of the blood vessels [12]. It has been shown that reduced oxygen tension (hypoxia) along with electrical stimulation increases the metabolic demands of the tissues and leads to growth of new blood vessels promoting oxygen delivery to the tissues [13,14]. Under hypoxic conditions, the key component, which responds to hypoxia and forms new blood vessels, is the vascular endothelial cells located at the inner surface of blood vessels [15,16]. In quiescent vessels, the endothelial cells form a tight barrier between flowing blood and the tissues. In contrast, under hypoxic conditions, the endothelial cells are activated to release proteases which degrade the surrounding basement membrane, allowing cells to escape from their original position in the vessel walls [17]. The endothelial cells then migrate towards the angiogenic stimulus and proliferate, finally forming new vessels [18]. While several angiogenic factors such as fibroblast growth factor (FGF), angiopoietin-2, and platelet-derived growth factor (PDGF) are implicated in the activation of endothelial cells [19], vascular endothelial growth factor (VEGF) is considered the most potent and direct regulator of the process [12,15]. VEGF produced by mediation of the hypoxia-inducible factor (HIF) under hypoxic conditions increases vascular permeability, which is a crucial step for growth of new vessels, and promotes endothelial cell migration and proliferation [15,18–25].

Since the biological effects of VEGF are mediated through the interaction of VEGF with receptors located on the endothelial cell surface, characterization of these VEGF receptors is expected to provide increased understanding of blood vessel formation. We have recently developed a system to investigate ligand-receptor binding interactions on cell surfaces without prior isolation or purification of the receptors [26]. This system utilizes aldehyde-terminated self-assembled monolayers (SAMs) on gold surfaces to react with amine-bearing biomolecules [27]. Protein ligands can be attached to the SAMs through their lysine residues and then capture whole cells through a ligand-receptor interaction. In the present study, VEGF was immobilized on the aldehyde-terminated SAMs and the binding patterns of vascular endothelial cells from three rat strains, SS, BN, and consomic SS-13^{BN}, were monitored by phase contrast microscopy. Different levels of VEGF receptor-mediated binding were observed from endothelial cells of the three rat strains under the effect of hypoxic (2% O₂) compared to normoxic conditions (21% O₂). There was little change in binding for the endothelial cells from the SS rats which do not undergo normal angiogenesis, whereas increased binding was observed for endothelial cells from the BN and the consomic SS-13^{BN} rats, which do undergo normal angiogenesis in response to appropriate stimuli. This observation provides strong evidence that the impaired angiogenic response in the SS rat is associated with a defect in regulation of the expression or accessibility of VEGF receptor on endothelial cell surfaces.

Materials and methods

Materials

Recombinant rat VEGF, fetal bovine serum (heat-inactivated) and gentamicin were obtained from Invitrogen (Carlsbad, CA). Cellgro RPMI 1640 with L-glutamine and Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium were from Fisher Scientific (Pittsburgh, PA). Heparitinase was purchased from Associates of Cape Cod, Inc. (East Falmouth, MA). Bovine serum albumin (BSA), trypsin-EDTA solution, antibiotic-antimycotic solution, and all the chemicals used for synthesis of di(11-undecanal) sulfide were purchased from Sigma-Aldrich (Saint Louis, MO). 7.6 cm × 2.5 cm gold-coated glass substrates (1 nm chromium and 30 nm gold) were obtained from GenTel BioSciences, Inc. (Madison, WI). The gold substrates were cut into 0.9 cm × 1.25 cm pieces for cell-binding experiments.

Synthesis of di(11-undecanal) disulfide

The synthesis of di(10-decanal) disulfide and its use in the creation of gold-thiol self-assembled monolayers (SAMs) for the fabrication of DNA and protein arrays has been previously reported [26,27]. In the present study, a similar molecule, di(11-undecanal) disulfide, was prepared using an improved synthetic scheme and employed as described previously. The new synthesis reduces the number of synthetic steps required while increasing the overall product yield by 48% to give a final reaction yield of 74%. 2.0 grams (97% purity, Aldrich, 9.8 mmol) of 11-mercapto-1-undecanol was dissolved in 15.0 mL of anhydrous dichloromethane (99.8% purity) and placed in an ice bath. 0.27 mL of bromine (99.99% purity, Aldrich, 5.4 mmol) was added with stirring. The formation of di(11-undecanol) disulfide was monitored by thin-layer chromatography (TLC) on silica (9:1 hexanes:EtOAc). The product was washed with a saturated solution of sodium bisulfite to remove any excess bromine and then dried to yield a white solid (1.9 g, 4.7 mmol, 96% yield). ^1H NMR (CDCl_3) δ : 4.80 (s, 1H), 3.52 (t, 2H), 2.56 (t, 2H), 1.54 (m, 4H), 1.41 (m, 4H), 1.40–1.25 (m, 10H).

The di(11-undecanol) disulfide was redissolved in 15.0 mL of anhydrous dichloromethane and slowly added to a round bottom flask containing 4.8 g of Dess-Martin periodane (97% purity, Aldrich, 11.3 mmol) in 10.0 mL anhydrous dichloromethane and stirred for 4 hours under nitrogen. The reaction progress was monitored via TLC on silica (9:1 hexanes:EtOAc). Upon completion the product was washed with a saturated bicarbonate solution, and purified using column chromatography (silica gel, 9:1 hexanes:EtOAc). This yielded a white solid (1.51 g, 3.8 mmol, 80% yield) that was identified as di(11-undecanal) disulfide using ^1H NMR. ^1H NMR (CDCl_3) δ : 9.75 (t, 1H), 2.56 (t, 2H), 2.40 (t, 2H), 1.56 (m, 4H), 1.42 (m, 2H), 1.40–1.25 (m, 10H).

Formation of aldehyde-terminated SAMs

Gold-coated glass substrates, briefly washed using deionized water and ethanol, were soaked in 1–10 mM di(11-undecanal) disulfide ($(\text{CHO}-(\text{CH}_2)_{10}\text{-S})_2$)/ethanol solution overnight. The aldehyde-terminated substrates were washed thoroughly with ethanol and deionized water, and dried gently with pure N_2 gas.

Immobilization of proteins on the aldehyde-terminated SAMs

VEGF and BSA were covalently attached to the aldehyde-terminated gold substrates by depositing 0.7 μL by hand-spotting using a micropipette and incubation in a humid chamber overnight. Proteins were dissolved in HEPES buffer (10 mM HEPES, 1 mM CaCl_2 , 0.1% NaN_3 , pH 8.5) to 26 μM . NaBH_3CN was added to the protein solutions (50 mM final concentration) right before spotting on the gold substrate to reduce the imine product of reaction between the primary amine groups on the proteins and the aldehyde groups on the gold substrate.

to a more stable secondary amine (Figure 1). After incubation overnight in a humid chamber at room temperature, the protein-modified substrates were soaked in 1% BSA/Dulbecco's phosphate buffered saline (DPBS, without calcium and magnesium) for 30 minutes to block the remaining amino-reactive surface sites on the substrate. Typical spots were about 1.3 mm in diameter. The substrates were kept on ice until use in cell-binding experiments.

Cell culture

Vascular endothelial cells were derived from different rat strains including Sprague-Dawley (SD), Dahl salt-sensitive (SS/JrHsdMCWi, referred to as SS), Brown Norway (BN/NHsdMCWi, referred to as BN), and the consomic SS-13^{BN}. Rats used in the present study were housed in an AAALAC-accredited animal care facility at the Medical College of Wisconsin, and all procedures received prior IACUC approval. Cells were isolated as described in Pellitteri-Hahn *et al.*'s recent work [28]. The endothelial cells were cultured in RPMI 1640 with L-glutamine supplemented with 20% fetal bovine serum (FBS), 1% 100x antibiotic-antimycotic solution, and 0.4% gentamicin at 37°C in a humidified incubator containing 5% CO₂. Hypoxia was created by placing the confluent cells in a hypoxia chamber (2% O₂, 5% CO₂, balance with N₂, 37°C) for 18 hours.

Cell binding to immobilized protein substrates

Endothelial cells were treated under normoxic or hypoxic conditions as described above. The medium was removed and the cells were washed twice with DPBS. The cells were trypsinized using 1 mL of trypsin-EDTA solution and 3 mL of fresh medium was added to the cells to block the trypsin activity. The cell suspension was transferred to a centrifuge tube and centrifuged at $1000 \times g$ for 10 minutes. The supernatant was removed and the cell pellet was resuspended in DPBS followed by another 10 minute centrifugation. The supernatant was removed and the cell pellet was resuspended in RPMI 1640 with L-glutamine for the binding experiment. The cell concentrations were between 3.7×10^5 cells/mL and 1.2×10^6 cells/mL and 3 mL of the cell suspension was added to each protein-modified substrate placed in the wells of a 6-well tissue culture plate. The plate was then incubated for 1 hour at 37°C in a humidified incubator containing 5% CO₂. After incubation, the substrates were dipped into 6 mL of DPBS solution in a well of a 6-well plate and promptly removed in order to wash off non-specifically bound cells. After 4 such washes, the cells bound to the protein-modified substrates were observed and imaged using an Olympus IX81 phase contrast microscope or a Nikon Eclipse 600 microscope.

Heparitinase treatment

Endothelial cells (5.5×10^6 cells/mL) isolated from the SD rat strain were treated with different concentrations (0, 2.5 and 5 μ L of a 0.1 unit/100 μ L stock) of heparitinase for 30 minutes at 37°C. The treated cells were added to VEGF-modified substrates. After a 1 hour incubation, the substrates were washed with DPBS and the binding was monitored using an Olympus IX81 phase contrast microscope.

Cell counting

The densities of the bound cells on VEGF immobilized substrates were counted using a script written in Matlab (Mathworks, MA). Briefly, each figure was first converted into a binary image by automatic threshold. The binary images were then segmented using the 'WaterShed' image segmentation routines to recognize individual cells [29]. The measured cell density was typically averaged over 3 different areas in the same images, and expressed in units of cells/(100 pixel)².

Statistical data analysis

We present data for cell binding density as mean $\pm$ s.d. from the five experiments, and we determined statistical significance with the Student's t-test (one-tailed) at a confidence level of $P < 0.05$.

Results

Recombinant rat vascular endothelial growth factor (VEGF) and bovine serum albumin (BSA) were deposited on aldehyde-terminated self-assembled monolayers (SAMs) on gold substrates (Fig. 1A) by hand-spotting using a micropipette. The proteins were covalently immobilized on the aldehyde-terminated SAMs by the reaction between the primary amine groups present on the lysine residues of the proteins and the aldehyde groups of the SAMs. The imine (Fig. 1B) formed from the reaction is unstable in air and is therefore reduced to a stable secondary amine using the reducing agent NaBH_3CN (Fig. 1C). BSA served as a negative control to test for non-specific binding. The VEGF- and BSA-modified substrates were incubated with endothelial cells isolated from the Sprague-Dawley (SD) rat strain which exhibits normal angiogenesis. These SD endothelial cells were used to evaluate the effects of VEGF concentration (0.1 $\mu\text{g}/\mu\text{L}$, 0.5 $\mu\text{g}/\mu\text{L}$, 1 $\mu\text{g}/\mu\text{L}$), temperature (0 or 37°C), and incubation time (30 min, 1 h, 2 h) upon cell-binding to the VEGF- or BSA-modified substrates. The best conditions found were 1 $\mu\text{g}/\mu\text{L}$ VEGF concentration, 37°C, and 1 hr incubation time. Higher VEGF concentrations were not evaluated due to the high cost of VEGF and the good performance obtained from the 1 $\mu\text{g}/\mu\text{L}$ concentration. The endothelial cells from the SD rat strain were specifically bound to the VEGF spots (Fig. 2A), whereas no bound cells were observed on spots containing immobilized BSA (Fig. 2B). The specificity of the endothelial cell capture by VEGF spots was further tested using heparitinase treatment. Cell surface-associated heparan sulfate is an essential co-factor in the binding of VEGF to VEGF receptor. It has been previously shown that treatment of endothelial cells with heparitinase to degrade heparan sulfate abolishes VEGF binding [30–32]. This effect was observed in the present study, as manifested by a significant decrease in the binding of endothelial cells which had been treated with heparitinase (data not shown).

Endothelial cells isolated from SS, BN, and SS-13^{BN} rat strains were treated under normoxic (21% O_2) and hypoxic (2% O_2) conditions. The same number of cells from each of the six different conditions, SS_normoxia, SS_hypoxia, BN_normoxia, BN_hypoxia, SS-13^{BN}_normoxia, and SS-13^{BN}_hypoxia, were added to six different VEGF-modified substrates, and incubated for 1 hour at 37°C. After removing non-specifically bound cells, the captured cells on the VEGF spotted substrates were imaged (Fig. 3). The binding images in the top panel of Fig. 3 were obtained from endothelial cells treated under normoxic conditions and those in the bottom panel were obtained from cells treated under hypoxic conditions. There was little change in binding for the endothelial cells of the SS rat strain from normoxic to hypoxic conditions (Fig. 3A), whereas increased binding of the cells was seen for both BN and SS-13^{BN} rats (Fig. 3B, C).

The densities of the bound cells on VEGF-modified substrates were determined using a cell counting program written in Matlab. Fig. 4 summarizes the percent changes in densities of the bound cells to the VEGF modified substrates for the three rat strains, SS, BN, and consomic SS-13^{BN} rats under hypoxic conditions. The binding experiments were repeated five times and three VEGF spots were used in each experiment for every condition of the cells. The endothelial cells of the BN and the consomic SS-13^{BN} rats increased binding by 42% and 29%, respectively, while there was an 8% decrease in the endothelial cell binding of the SS rat strains. According to a pairwise t-test for the measurements, the changes in VEGF receptor-mediated cell binding between the cells of the SS and the BN rats were significantly different ($p = 0.003$) and the changes between the cells of the SS and the consomic SS-13^{BN} rats were also

significantly different ($p = 0.02$). In contrast, the binding changes between the cells of the BN and the consomic SS-13^{BN} rats were not significantly different ($p = 0.15$). Thus, VEGF binding levels in endothelial cells from the SS rat strain, which is impaired in its angiogenic response, did not change in response to hypoxic conditions, while the VEGF binding levels in endothelial cells from the BN rat as well as the consomic SS-13^{BN} rat did change significantly.

Discussion

It was hypothesized that vascular endothelial cells derived from the BN and the consomic SS-13^{BN} rats would exhibit different VEGF receptor-mediated binding patterns compared with those isolated from the SS rat under the effect of hypoxia, a known stimulus for angiogenesis, because the BN and the consomic SS-13^{BN} rats undergo normal angiogenesis while the Dahl salt sensitive (SS) rats do not. The results shown in Fig. 3 and 4 demonstrates that there was little difference in VEGF binding to endothelial cells from the SS rat strains under normoxic or hypoxic conditions, whereas increased binding was observed for the cells from the BN and consomic SS-13^{BN} rats under hypoxic conditions. Because the cell capture occurs through a specific interaction between VEGF receptors on the cell surface and the immobilized VEGF on the substrate, the observed changes in cell-binding likely reflect changes in VEGF receptor expression level on the endothelial cells under hypoxic conditions. Alternatively, it is possible that the accessibility or affinity of the receptor to VEGF binding is altered, rather than the level at which it is expressed. In any event, as the cell-binding assay employed here directly evaluates VEGF binding to the cell surface, the observed changes in binding are likely to be relevant to the activation of VEGF receptor-mediated signaling. In contrast, antibody-mediated approaches such as western blots, fluorescence-activated cell sorting (FACS), and immunohistochemical staining do not provide comparable information on cell surface localization and ligand-binding activity. Such antibody-based assays do not distinguish between internalized and cell-surface expressed forms of the receptor, nor do they distinguish between active and inactive forms of the receptor. Such assays also rely heavily upon antibody specificity, which in many cases can be problematic. The cell-binding assay employed in the present study has the advantage of not relying upon the use of such complex reagents to provide information on the activity of cell-surface receptors, and instead provides direct information upon the ligand-binding activity of interest.

In summary, VEGF, an important regulator of angiogenesis, exerts its biological effects through binding to VEGF receptors present on the endothelial cell surface. The use of consomic rat strains has shown that critical genetic factors affecting angiogenesis are located on chromosome 13, but the nature of those factors has not been clear. In the present study a novel cell-binding assay was employed to evaluate changes in the functional expression of VEGF receptors on endothelial cells from rat strains differing in their angiogenic responses. It was found that functional VEGF receptor expression on endothelial cells from the BN and the consomic SS-13^{BN} rats increased under the effect of hypoxia, while the cells from the SS rat did not exhibit a change in functional VEGF receptor expression. This result provides strong evidence that the impaired angiogenic response in the SS rat is due to a defect in regulation of the expression or accessibility of VEGF receptor on endothelial cell surfaces.

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Figure 1. Immobilization of proteins on aldehyde-terminated self-assembled monolayers (SAMs) on gold. An aldehyde-terminated gold substrate is reacted with primary amine groups present on proteins. The imine formed in Fig. 1B is reduced to a stable secondary amine using NaBH_3CN .

Figure 2.
Optical images of endothelial cells from the Sprague-Dawley (SD) rat strain captured on a protein-modified substrate. VEGF (2A) and BSA (2B) were spotted on aldehyde-terminated SAMs.

Figure 3. Optical images of endothelial cells treated under normoxic and hypoxic conditions and captured on the VEGF-modified substrates. Endothelial cells isolated from the SS (3A), BN (3B), and SS-13^{BN} (3C) rat strains were captured.

Figure 4. Percentage changes of densities of the bound endothelial cells from SS, BN, and the consomic SS-13^{BN} rats under the effect of hypoxia. Error bars indicate mean $\pm$ s.d. of five separate experiments.