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Endothelial cell activation in a VEGF-A gradient: relevance to cell fate decisions

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Abstract

Distribution of vascular endothelial cell growth factor A (VEGF-A) as a gradient determines microvascular endothelial cell (EC) fate during organogenesis. While much is understood about mechanisms of differential distribution, less is known about how EC perceive and interpret a graded VEGF-A signal to generate positional target gene activation. Using microvascular EC, we analyzed the effect of time and graded VEGF-A input on VEGFR2 autophosphorylation, signal kinase activation and induction of immediate-early genes. The threshold and time to peak activation of VEGFR2 were dependent on signal strength over a 50-fold range in concentration with 3-fold concentration differences readily distinguished. Longer duration of exposure did not compensate for low concentration of VEGF-A, suggesting intensity and duration of signal were not interpreted equivalently. *With the same conditions* graded and time-sensitive information was transduced through the PLC γ /p44/p42MAPK signal pathway but not the parallel AKT pathway. Analysis of MAPK-induced angiogenic immediate-early genes determined EGR-1, EGR-3, and NR4A1 were dependent on graded input while NR4A2 and DSCR1 were independent with 'switch-like' induction. These data demonstrate rapid, linear integration of VEGF-A levels but independent interpretation of duration of signal and identify *potential* nodes for segregation of gradient-dependent and -independent responses. These results describe how microvascular EC fate decisions can be determined by comparatively moderate changes in VEGF signal strength, resulting in combinatorial changes in the repertoire of immediate-early genes for transcription effectors.

Keywords

morphogen; microvascular; organogenesis; cell fate

During organogenesis, important cell fate decisions are dependent upon positional information provided by graded distribution of morphogens. The impact of morphogens during development is well characterized in *Drosophila* and *Xenopus* (reviews Ashe and Briscoe, 2006; Gurdon and Bourillot, 2001). In these systems, morphogens are critical for the formation of sharp borders of distinct cell types from common progenitors during tissue and organ development. It is proposed that localized expression of vascular endothelial growth factor A (VEGF-A) provides positional information that determines endothelial cell patterning during

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organogenesis (Haigh, 2008; Ng et al., 2001; Watkins et al., 1999). Developmentally regulated alternate splicing of VEGF-A leads to expression and secretion of isoforms with variable heparin binding activity. This influences association with extracellular matrix components and creates the potential for formation of a gradient originating from the epithelial source. Much attention has focused on how the graded distribution of VEGF-A is established in developing tissues, however less is known of how endothelial cells (EC) of the primitive vascular network perceive and interpret the graded signal to generate alternate gene expression patterns.

VEGF-A is required for vasculogenesis, the de novo formation of a primitive vascular network from angioblast progenitors. Signaling through receptor VEGFR2 (aka KDR or FLK1) is essential for vascular development and blood vessel homeostasis (Carmeliet et al., 1996; Ferrara et al., 1996). Focal expression of VEGF-A, a requirement for classification as a morphogen (Gurdon and Bourillot, 2001), occurs in retina, lung and other organs (Haigh, 2008). On focal expression of VEGF-A in the retina, microvascular 'tip' EC with activated VEGFR2 migrate towards the epithelial source, while neighboring EC of the microvascular network remain at distance from the epithelium (Schwarz et al., 2009). During the early stages of lung organogenesis, before E13.5, VEGF-A is distributed evenly within the mesenchyme and functions as a competence factor for establishment of a primitive microvascular network rather than as a morphogen. Between E13.5 and E14.5 type II cells of the developing airway epithelium rapidly increase expression of heparin binding VEGF-A isoforms (Greenberg et al., 2002; Ng et al., 2001). Altering relative VEGF-A isoform expression (Galambos et al., 2002; Ruhrberg et al., 2002) or decreasing expression levels by half (Carmeliet et al., 1996; Ferrara, 1996) or increasing expression by 2-fold (Akeson et al., 2003), change VEGF-A gradient characteristics and cause significant deleterious changes in pulmonary microvascular development. The mechanism for relay of VEGF gradient related information from receptor activation through signal kinases to differential target gene induction required for lineage selection and vascular patterning is poorly characterized.

Interpretation of morphogenic gradients relies on linear interrogation of signal strength through interactions involving morphogen receptors, downstream signaling and target gene activation. Recent reviews discuss general principles common to morphogenic response (Ashe and Briscoe, 2006; Gurdon and Bourillot, 2001; Ibanes and Belmonte, 2008; Jaeger et al., 2008). First, cells sense relatively small concentration differences with often two- to three-fold changes in concentration sufficient to induce alternate gene expression patterns. Second, concentration-dependent information must be faithfully transmitted from receptor activation through signal transduction to target gene activation. For example, a five-fold increase in VEGFR2 activation should result in approximately a five-fold increase in direct target gene activation. In addition, a responding cell reads its position within a gradient without reference to neighboring cells.

The goal of this study was to determine whether the response of EC to VEGF follows these morphogenic principles and to assess how EC integrate VEGF signal with time. We utilized primary human microvascular endothelial cells (HMVEC) isolated from lung to investigate how EC convert exposure to a VEGF-A gradient field into discrete changes in gene expression. For the first time we demonstrate the ability of EC to integrate small differences in both VEGF-A concentration and duration of exposure from receptor activation through specific signal transduction pathways to induce differential patterns of immediate early gene expression.

Materials and Methods

Cell Culture and analysis

HMVEC-L (Lonza) used before passage 8 were cultured in EGM-2 MV (EGM bullet kit Lonza). For analysis of proliferation HMVEC were cultured overnight without VEGF and

bFGF at 2×10^4 cells/well in 96-well plates in a final volume of 100ml/well EGM media. Human recombinant VEGF-A isoform V165 (CalBiochem) was added at 0 to 2.38 nM and proliferation at 24 or 48 hr determined using WST-1 Cell Proliferation Assay Kit from Chemicon. HMVEC migration was assessed with a wound migration assay. Confluent HMVEC were cultured overnight without VEGF-A in EGM, 1% serum. A clear zone was created with a sterile swab, cell debris removed by washing and EGM with VEGF-A added to $n=5$ cultures at each concentration. Using an inverted Olympus microscope with camera, wound area was recorded at 0 time and at 24hr with 5 views at clearly identified specific locations photographed for each well. To quantify migration, in each view the area of cells within the clear zone was determined at 24 hr using MetaMorph software and defined in arbitrary units. For signal kinase analyses, cells were cultured overnight without VEGF-A and bFGF and activation media with VEGF-A165 and 1mM Na orthovanadate in EGM added. In each experiment lysates were prepared from $n=3$ independent cell cultures and each experiment was repeated 2 or 3 times. For western analysis, after activation media was rapidly removed, cells washed with ice-cold phosphate-buffered saline (PBS) and solubilized with RIPA lysis buffer containing 1% NP-40, 1mM Na orthovanadate and complete protease inhibitor cocktail (Roche Diagnostics). After sonication and centrifugation, protein in the soluble fraction was determined by BCA protein Assay (Sigma). For RNA analysis, total RNA was isolated using an RNeasy Plus Mini Kit (Qiagen,). First-strand cDNA was synthesized with Verso cDNA kit (Thermal Fisher Scientific Inc). Quantitative PCR was performed using Taqman probe and primer sets (Applied Biosystems) specific for *EGR1* (Hs00152928), *EGR3* (Hs00231780), *NR4A1* (Hs00172437), *NR4A2* (Hs00428691), and *DSCR1* (Hs00231766). A probe and primer set for human β -actin was used as the normalization standard. PCR reactions ($n=6$ per sample) were performed in a 7300 Real-Time PCR system (Applied Biosystems) using 25ng of cDNA per reaction. Real-time qRT-PCR data were calculated as relative quantification (RQ) for at least $n=3$ independent HMVEC samples at each condition. Data were plotted after normalization to cultures without VEGF-A where RQ was set equal to 1. Data are reported as mean RQ with standard error, significance determined by Student's t test.

For flow cytometry, cells were lifted with 0.02% EDTA (Gibco), washed with PBS, blocked and incubated with mouse anti-human VEGFR2 (Sigma) or isotype control for 1 h, washed and incubated with goat anti-mouse Alexa 647 secondary antibodies (Molecular Probes) for 30 min. Cells were resuspended in 2% paraformaldehyde and analyzed by FACSCalibur flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences).

For immunohistochemistry paraffin embedded embryonic tissues were sectioned at $5\mu\text{m}$ and analyzed as described previously (Greenberg et al., 2002). The primary antibodies were: for VEGFR2 Ab2479 (Cell Signaling), pMAPK Ab9101 and EGR-1 Absc-189 (Santa Cruz). Alexa-568-conjugated GSL-B4 isolectin with 10 mM CaCl_2 and 20 mM MgCl_2 was used as pan-endothelial marker (Akeson et al., 2000; Ponder et al., 1985). Secondary antibodies were biotinylated goat anti rabbit (Vector) or Alex568 conjugated goat anti rabbit (Molecular Probes). For fluorescence detection, slides were mounted with Vectashield with DAPI. Images were captured as pseudo-confocal Z-stacks with a Zeiss Axioscope Microscope.

Immunoprecipitation (IP) and immunoblotting

100 μg of soluble protein was incubated with antiVEGFR2 (Cell Signaling) for 2.5hr at 4°C , an equal volume of 20% slurry proteinA/G IgG sepharose beads (Pierce) added and incubated overnight at 4°C . Immune complexes were collected by centrifugation, washed and denatured by heating at 70°C for 10min in SDS NuPAGE sample buffer (Invitrogen). Immune complex samples were split for independent analysis of phosphorylated and total proteins due to high avidity and poor stripping. Samples were separated using NuPAGE Bis-Tris 4-12% gradient gels (Invitrogen) and transferred to PVDF membranes. For western blotting, membranes were

blocked in 1% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBS-T), incubated overnight at 4°C with primary antibodies to the phosphorylated proteins in blocking solution, washed, incubated for 1 hr at room temperature with secondary Ab conjugated with horseradish peroxidase, and activity detected with ECL-plus Chemiluminescence system (GE HealthCare) on a Fuji Imager (LAS-4000). Total cell lysates were used for signal kinase analysis. All blots were stripped and reprobed with Ab to glyceraldehyde dehydrogenase (GAPDH) for normalization. Using Fuji Imager software relative activity was determined by comparing band intensity for phosphorylated forms to band intensity for total protein compared to the intensities for each form for a constant activated control sample. Normalized values were averaged for n=3 to 5 independent cell cultures at each time point. For each point, mean background activity (without VEGF-A) was subtracted and activity reported as mean and standard error (SE). Activities are reported for VEGFR2 as pY/R2, pY159/R2, pY1175/R2, p44/p42MAP kinase as pMAPK/MAPK, AKT as pAKT/AKT and ^{PLC}γ as pPLC/PLC. Antibodies were 4G10 for phosphotyrosines (Upstate), Ab2479 to VEGFR2, Ab19A10 to pY1175, Ab2822 to PLCγ, Ab 2821 to phosphoY783 PLCγ, Ab 4685 to AKT, Ab 4058 to phosphoSer473 AKT, Ab9102 to MAP kinase and Ab 9101 to phospho-thr202/phospho-tyr204 MAP kinase were from Cell Signaling. The phosphoVEGFR2 antibody PS1013 recognizing Y1054/1059 was from CalBiochem.

Transgenic mice

To induce VEGF-A in distal lungs, time-pregnant *SPCrtTA/tetOVEGF-A* dams (Akeson et al., 2003) were administered doxycycline in food and water. At E14.5 pregnant dams were euthanized by carbon dioxide inhalation and embryos removed by caesarean section. Embryonic tissue was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 4 micron for immunohistochemistry. Non-transgenic and single transgenic littermates served as controls. All animal procedures were conducted under the protocols approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital.

Results

EC sense and respond to small changes in VEGF-A

The full range of cellular response to a morphogen can be elicited with a 20- to 50-fold change of concentration (Ashe and Briscoe 2006). For instance, VEGF-A induces proliferation (Fig 1A) and migration (Fig 1B) over a 20-fold range of concentration. To determine the dynamic concentration range for response of EC to VEGF-A, the kinetics of VEGFR2 autophosphorylation were analyzed in vitro with time series analysis. HMVEC were stimulated with hVEGF-A165 (20ng/ml or 0.48nM) through 10 min (Fig 1C). VEGFR2 was isolated from total cell lysates by IP pulldown and relative receptor phosphorylation (pY/R2) determined from Western blot analysis with phosphotyrosine-specific antibody. Receptor phosphorylation was rapid, Fig 1C, detectable within 1 min and reaching steady state at 2 min, as previously reported for HUVEC (Pan et al., 2007). The dose-response of VEGFR2 phosphorylation was determined at 2 min, Fig 1D. Autophosphorylation was induced by as little as 2 ng/ml (0.048nM) VEGF-A. The EC50 of 650pM (Fig 1E) was in the range of the Kd for binding of VEGF-A165 to VEGFR2 reported for microvascular EC of 600 to 700pM (Wang et al., 2002). These results demonstrate that the full response to VEGF-A is elicited in HMVEC over a relatively steep 50-fold concentration range. Within this range, 3-fold changes in concentration are readily distinguished as differences in VEGFR2 phosphorylation. These results confirm the relationship between the kinetics of VEGF-A binding to VEGFR2 and receptor autophosphorylation. For analyses of downstream activation events with time, concentrations at just above the threshold of response (0.12nM or 5 ng/ml), near the EC50 (0.48nM or 20ng/ml) and just below saturated response (1.9nM or 80ng/ml) were used to represent effective concentration zones within a VEGF-A gradient field.

Concentration and temporal integration of VEGF-A signal

VEGF-A activates a complex network of signal transduction initiated primarily by the autophosphorylation of VEGFR2 (Olsson et al., 2006). To determine possible interrogation points for differential response to VEGF-A concentration and duration of exposure, we analyzed the kinetics of VEGFR2 tyrosine phosphorylation and activation of downstream signal kinases PLC γ , p44/42MAPK and AKT.

Within the intracellular domains of VEGFR2, six tyrosines are autophosphorylated following ligand binding (Matsumoto et al., 2005). Autophosphorylation of tyrosine (Y) 1059 in the kinase-regulating site is required for optimal EC response to VEGF-A (Zeng et al., 2001). Tyrosine 1175 plays an essential and direct role in linking receptor phosphorylation to signal transduction. On ligand binding and receptor phosphorylation, PLC γ directly binds pY1175 in the intracellular domain of VEGFR2, leading to activation of the MAPK signal pathway (Takahashi et al., 2001). Binding of adapter protein Shb to pY1175 mediates activation of PI3 kinase and AKT (Dayanir et al., 2001). Mutation of the comparable amino acid Y1173 in mice is embryonic lethal due to severe vascular defects (Sakurai et al., 2005).

HMVEC were cultured with increasing VEGF-A, lysates collected at times up to 10 min and analyzed following IP pulldown of VEGFR2 for quantification of relative pY1059/R2 and pY1175/R2. Activation of Y1059 and Y1175 occurred rapidly with maximal phosphorylation reached between 2 and 4 min, Fig 2A, B, C. At 4 min, maximal pY1059/R2 at high VEGF-A (1.9nM) was 2-fold higher than with mid dose VEGF-A (0.48nM) and 5-fold higher than with low dose VEGF-A (0.12nM). The differential for Y1175 at 4 min was 2-fold between mid and high dose and 10-fold between low and high dose. Duration of phosphorylation signal varied with VEGF-A level. At high concentration, pY1059/R2 and pY1175/R2 declined rapidly with significant decrease between peak at 4 min and 6 min. At mid dose both relative pY1059/R2 and pY1175/R2 remained at or near peak levels from 2 to 10 min. Longer culture at either 0.12 or 0.48nM did not induce peak activation observed at high concentration (1.9nM) for short duration. These results demonstrate that gradient generated changes in VEGF-A concentration are rapidly and faithfully transmitted through the differential kinetics of VEGFR2 autophosphorylation at Y1059 and Y1175. Moreover, VEGF-A concentration and duration of signal were not sensed by HMVEC in an equivalent manner.

Autophosphorylation of VEGFR2 leads to recruitment of signal kinase proteins and other accessory proteins critical for regulation of multiple downstream signal pathways (Olsson et al., 2006). We examined the transmission of graded VEGFR2 activation through the parallel PLC γ / p44/42MAPK and AKT pathways. In the first 10 minutes, activation kinetics of PLC γ and p44/42MAPK (Fig 2D, E) were similar to the activation of VEGFR2. Peak PLC γ activity was reached with high dose VEGF-A between 4 and 6 min. Moreover, at low dose VEGF-A, activity reached 60% of peak and was constant from 2 to 10 min. Downstream of PLC γ , the activation p44/42MAPK (ERK1/2) was slightly delayed, reaching peak after 6 min at high dose VEGF-A (Fig 2E). Further, relative pMAPK/MAPK at mid and low dose VEGF-A reached dose-dependent maxima at 4 min with activity sustained at that level through 10 min. As with VEGFR2 autophosphorylation, there was an approximately 3-fold difference in relative peak response between low and high dose. Further, longer duration input at low concentration was not comparable to short duration input at high concentration for activation of both PLC γ and p44/p42MAPK.

Unlike PLC γ and p44/p42MAPK, activation of AKT increased through 10 min, independent of the VEGF-A concentration range examined (Fig 2 F). AKT activation occurred at a low threshold of VEGF-A with time of exposure the primary kinetic determinant. Ashe and Briscoe (Ashe and Briscoe, 2006) describe this type of off or on response as 'switch-like'. These data suggest that dose-dependent VEGFR2 activation is propagated downstream through the

PLC γ /MAPK signal pathway but not through the AKT pathway in the range of VEGF-A concentrations tested. The AKT pathway may respond in a dose-dependent manner under other conditions, for instance at very low concentrations or after prolonged exposure. The PLC γ /MAPK pathway may transduce initial concentration dependent information while the AKT pathway mediates more prolonged differential response to concentration. Further analysis is needed to determine the mechanism for segregation of gradient-dependent and independent responses to VEGF-A at the transition between VEGFR2 phosphorylation and signal kinase activation. Of interest is determining the role of adapter proteins like Shb associated with pY1175 in determining differential activation kinetics of signal pathways.

Target gene activation relative to VEGF-A concentration—To investigate the forward interrogation of dose and duration of signal, we analyzed the expression of several genes rapidly activated by PLC γ /MAPK and implicated in EC activation and angiogenesis including early growth response protein -1 and -3 (EGR-1, EGR-3), nuclear receptors NR4A1 and NR4A2 and Down syndrome critical region 1 (DSCR1) (Liu et al., 2008; Liu et al., 2000) (Minami et al., 2004). Although transiently expressed, transcription factors EGR-1, EGR-3, NR4A1 and NR4A2 are essential mediators of long-term angiogenic response (Liu et al., 2008; Liu et al., 2000). For instance, interference RNA to EGR3 and NR4A1 inhibits EC proliferation and migration (Liu et al., 2008). DSCR is a cytoplasmic protein that negatively regulates activity of NFAT transcription factors, a critical activation path for endothelial cells. The NFAT path is primarily induced through modulation of Ca⁺² and indirectly associated with receptor kinase activation (Armesilla et al., 1999; Schweighofer et al., 2007). In these experiments RNA was isolated from HMVEC stimulated for 1, 2, 3 or 24 hr with increasing VEGF-A and relative mRNA transcript levels quantified by using the linear dynamic range of real-time QPCR.

To confirm these genes were direct targets of VEGF-A, cells were preincubated for 30 min with cyclohexamide, simulated with 0.48nM VEGF-A and RNA isolated after 1 hr. Transcript levels for each gene were insensitive to cyclohexamide, Fig 3, demonstrating that these genes do not require protein synthesis for transcriptional activation and confirming their status as immediate-early genes.

Real-time analysis demonstrated several patterns of expression for these genes relative to VEGF-A concentration and duration (Fig 4). Maximal transcripts for EGR3, NR4A1, NR4A2 and DSCR were detected at 1 hr (Fig 4 B-E) and at 2 hr for EGR1 (Fig 4A). Two concentration-dependent profiles were discernable at these peak times. Graded VEGF-A induced proportionate and quantitative changes in gene targets EGR-1, EGR-3 and NR4A1. However, for NR4A2 (Fig 4D) and DSCR (Fig 4E) at 1hr, transcripts were maximal at both mid and high dose and at low dose 10% of maximal for NR4A2 and 40% of maximal for DSCR, Fig 4E. These profiles demonstrate that NR4A2 and DSCR require a relatively high threshold of VEGF-A for induction and suggested these target genes may not be differentially regulated in a VEGF-A gradient field.

The pattern of target gene transcripts was also variable with time of analysis. While maximal and still proportionate EGR-1 transcripts were detected at 3 hr (Fig 4A), EGR3 and NR4A1 and NR4A2 transcripts were greatly diminished after 2 h (Fig. 4 B, C, D). In the latter, short transcript half-life likely determines the time-limited profile of these transcripts. Of note, at low dose VEGF-A, both EGR-1 and EGR-3 were relatively constant through 3 hr, suggesting sustained response was maintained at low but not high levels of VEGF-A. By 24 hr, only transcripts for EGR-1 and EGR-3 were significantly above baseline and only at high dose VEGF-A (for EGR1 2.4-fold, $p=0.0028$; for EGR3 3.6-fold, $p<0.003$, Fig 4 A, B). These data predict that EC fate decisions are biased by combinatorial changes in the repertoire of

transcription effectors dependent on both the position of an EC within a VEGF-A gradient and the time after first encounter with ligand.

VEGFR2 autophosphorylation and signal kinase activation occurs rapidly, reaching peak signal within 10 min (Fig 2). Pulse chase analysis was used to determine whether receptor activation in this rapid phase is sufficient to induce immediate early gene transcripts. EC were stimulated with 0.48nM VEGF-A and after 10 min, the media replaced with VEGF-free media. At 1 hr RNA was isolated and EGR-1 transcripts determined, Fig 5. This pulse-chase analysis determined that a 10 min exposure was sufficient to induce EGR-1 transcript levels equivalent to levels with 1 hr continuous exposure to VEGF-A.

Graded VEGF-A induced direct, proportionate and quantitative changes in the gene targets EGR-1, EGR-3 and NR4A1. However targets NR4A2 and DSCR1, also downstream of the MAPK pathway, required a higher threshold of VEGF-A for activation. These data demonstrate that as with other well-characterized pathways (Ashe and Briscoe, 2006), combinatorial inputs from both concentration-dependent and -independent mechanisms regulate immediate early gene response to VEGF-A. These data predict that within a VEGF-A gradient field, positional information is transduced as changes in the repertoire of immediate early genes for early response transcription effectors. Activation of this class of transcriptional effectors appears to serve as another node for bifurcation of dose-dependent versus dose-independent information.

Interpretation of sustained VEGF-A exposure

During organogenesis focal expression of VEGF-A can be sustained through several stages of development. For instance, from E14.5 until birth endothelial cells within the microvascular network of the developing mouse lung are continuously exposed to a VEGF-A gradient field. To model receptor activation and signal kinase dynamics in long-term steady state yet sub-saturating conditions, HMVEC were cultured overnight at each VEGF-A dose. Analysis of pY/R2 (Fig 6A) demonstrated that HMVEC continue to respond to VEGF-A although VEGFR2 activation required higher ligand concentrations than at early times (Fig 1), reaching significant pY/R2 at mid and high dose but not low dose. At 24 hr relative activation of PLC γ (Fig 6B) and MAPK (Fig 6C) but not AKT (Fig 6D) were dose-dependent, although activation required higher concentrations of VEGF-A than at early times, Fig 2. Significant activation of AKT was not detected (Fig 6D).

Analysis by flow cytometry demonstrated a dose-dependent decrease in the peak channel for detection of VEGFR2 indicating the number of cell surface receptors diminished as cells were cultured long-term with VEGF-A (Fig 6E). Western analysis (Fig. 6F), determined that total cellular VEGFR2 protein was unaffected by ligand concentrations up to 0.48nM VEGF-A. Only at 1.9nM VEGF-A was total cellular VEGFR2 decreased by 30%, $p=0.046$. These data suggest that during sustained exposure to high dose VEGF-A, both decreasing cell surface and total cellular VEGFR2 protein act to down modulate receptor activation leading downstream to reduced amplitude and a possible generalized shift in dose-dependence to higher concentrations.

Increased VEGF-A in vivo alters gradient and response of microvascular EC

Gurdon and Bourillot suggest that interaction between neighboring cells is not required for interpretation of position within a gradient (Gurdon and Bourillot, 2001). The in vitro experiments described above were conducted at subconfluence and the results support the concept of cell autonomous response. To address whether this applies in vivo, we analyzed the activation state of EC in E14.5 lung using immunofluorescence for pMAPK and expression of EGR-1. We found heterogeneity of response within the primitive vascular bed as not all EC had activated p44/42MAPK, Fig 7A. Other cell types were also positive for pMAPK including

alveolar epithelial cells and an occasional mesenchymal cell (arrowheads, Fig 7A). Further, some but not all microvascular EC expressed EGR-1 (Fig. 7B). These observations suggest that although the cells are part of a primitive vascular network, EC respond as individual cells within a VEGF-A gradient field.

If VEGF-A acts as a morphogen during development, in vivo overexpression should shift gene expression in the direction predicted by in vitro experiments. We previously described a conditional, lung-specific transgenic model (*SP-CrtTA/tetOVEGF-A* bitransgenics) with 2- to 4-fold increase in VEGF-A in developing mouse lung (Akeson et al., 2003). In this model, the VEGF-A165 transgene is exclusively expressed by type II epithelial cells, the normal cellular site of focal VEGF-A production in the developing lung. At E14.5, conditional lung-specific induction of VEGF-A alters the patterning of the microvascular network without affecting either EC proliferation or apoptosis (Akeson et al., 2003). While experimentally difficult to visualize, we predict that overexpression of VEGF-A disrupts the typical gradient, creating a broad high concentration zone of VEGF-A. The three dimensional structure of the lung makes quantification difficult, however immunofluorescence results for thin tissue sections suggested the majority of EC in *SP-CrtTA/tetOVEGF-A* lungs patterned in the zone adjacent to the epithelium and a high number of these cells were positive for pMAPK and EGR-1, Fig. 7 C, D. The few EC further from the epithelium appeared to have lower or undetected pMAPK, Fig. 7C. The frequency of MAPK activation in nonendothelial cells was similar in wild type and *SP-CrtTA/tetOVEGF-A* lungs (arrowheads Fig 7C). These data suggest that in vivo, as in vitro, changes in VEGF-A concentration are transduced through signal kinase pathways to alter downstream target gene expression and ultimately to change in cell fate. This includes the changes in patterning illustrated in Figure 7 and changes in endothelial lineage selection. For instance, we have previously shown with this model that modest VEGF-A overexpression shifts the balance between formation of blood vasculature and lymphatic vasculature in embryonic lung (Mallory et al., 2006).

Discussion

This study determines that microvascular EC response to VEGF-A meets established criteria for a morphogenic response (Ashe and Briscoe, 2006; Gurdon and Bourillot, 2001; Ibanes and Belmonte, 2008; Jaeger et al., 2008) and provides insight into how concentration-dependent patterns of target gene expression are established. In vitro the full range of response is achieved over a 50-fold difference in VEGF-A with linear VEGFR2 autophosphorylation interrogated across PLC γ /p44/p42MAPK phosphorylation and activation of target genes EGR-1, EGR3 and NR4A1. Within the same VEGF-A range, concentration-dependent information was not transduced through the AKT signal pathway. It is possible that the AKT pathway may transmit concentration-dependent information at for instance very low VEGF or at later times. However these data suggest that within the dynamic VEGF-A range for VEGFR2 phosphorylation, there is bifurcation of concentration-dependent and concentration-independent information at the level of signal kinase activation. Further analysis is required to understand how crosstalk between signal pathways influences concentration-dependent output. Induction of transcriptional effectors also acts a node for differential transmittance of dose-dependent information, as some but not all of VEGF-A and MAPK activated immediate early genes analyzed had induction profiles proportional to VEGF-A concentration.

Both in vitro and in vivo analyses shown here support the cell autonomous response of microvascular EC, another criteria for morphogenic response. Within the microvascular network of the developing mouse lung, individual cells were activated as indicated by phosphorylated MAPK or EGR-1 induction but neighboring cells, presumably experiencing similar VEGF-A concentration, were not activated. Unequal response of EC receiving seemingly similar positional information is also seen in the influence of VEGF-A on

specification of tip and stalk phenotypes by adjacent EC (Gerhardt et al., 2003). The differential activation of jagged and DII4 in the Notch signaling pathway specifies migration versus proliferation and determination of tip versus stalk phenotype (Benedito et al., 2009; Gerhardt et al., 2003). Ruhrberg, Gerhardt et al. (2002) report that VEGF-A gradient position determines branch point location as the microvascular network is refined. Arterial differentiation is induced through the PLC γ /MAPK and Notch signaling pathways (Siekman et al., 2008) and our model predicts that induction of arterial differentiation via PLC γ /MAPK is dependent on position of EC within the gradient.

Several factors are likely to contribute to dissimilar response of microvascular EC at the same position within a VEGF-A gradient field. Variable expression of VEGFR2 binding partners including VEGFR1 and neuropilin-1 (NRP-1) or of the soluble, ligand-sequestering form of VEGFR1 (sFLT-1) by EC within the microvascular network could contribute to the response of individual cells. Kappas et al (Kappas et al., 2008) propose that local discontinuous expression of sFLT-1 regulates VEGF-A signal amplitude and creates a mosaic of expression. NRP-1 as a coreceptor enhances VEGF-A165 binding to VEGFR2 (Soker et al., 1998). Variable expression of NRP-1 could account for differential activation of EC within a vascular bed. Also, the microvasculature is actively expanding throughout lung organogenesis (Schachtner, 2000). Differential response to VEGF-A by EC within the population may be cell cycle dependent, with the response of new daughter cells dependent on the VEGF-A concentration zone into which they replicate. Finally, receptor desensitization due to decreasing cell surface receptor and diminished VEGFR2 synthesis could account for differences in cellular response with time.

Cellular interpretation of morphogens is specified by both spatial and temporal input (Ashe and Briscoe, 2006; Kholodenko, 2006). The temporal activation profile of VEGFR2 autophosphorylation and PLC γ /MAPK activation at low, mid and high VEGF-A showed that EC sense duration of signal and concentration differently. This is unlike systems where duration is sensed in a manner similar to signal strength, for example digit specification by Shh (Harfe et al., 2004). VEGFR2 autophosphorylation and signal kinase activation were very rapid, detected in 30 sec, but extending the time of exposure at low concentration did not generate a response equivalent to short exposure at high concentration. Further, induction of immediate early target gene EGR-1 required only a short pulse of VEGFR2 activation suggesting that initial and not sustained receptor occupancy determines response. Moreover, analysis of very long-term exposure demonstrated down regulation of cell surface VEGFR2, suggesting a mechanism for desensitization. This was confirmed by diminished receptor autophosphorylation and increased threshold for activation of PLC γ and MAPK. This data predicts that first exposure and not long-term exposure sets the critical parameters for response.

While the essential role of VEGFs and their receptors is well appreciated, many important questions about transcriptional mechanisms downstream remain unanswered. In this study we report that VEGF gradient derived positional information is transduced through specific signal kinase pathways. Analysis of a limited set of immediate early genes (Fig 4) suggested how positional and temporal information within a VEGF-A gradient field is transduced through combinatorial changes in transcription effectors that could influence microvascular EC fate decisions. Recent theoretical approaches have modeled the distribution and availability of VEGF-A in tissues accounting for low affinity binding of heparin-binding VEGF-A isoforms including VEGF-A165 to extracellular matrix (Ibanes and Belmonte, 2008; Mac Gabhann and Popel, 2004; Mac Gabhann and Popel, 2007), making approximations of positional concentrations possible. Receptor activation analysis (Fig 1) demonstrate the dynamic range for EC response to be between 0.10 and 2.0nM VEGF-A. Using a gradient field of 55 μ m (Ruhrberg et al., 2002) and the theoretical approach of Mac Gabhann and Popel (Mac Gabhann and Popel, 2007), we calculated that to achieve a gradient from 2.0nM to 0.10nM would require

a decrease in VEGF-A of 25% with each 5µm, Fig. 8. The result is an uneven slope of VEGF-A distribution, another characteristic of morphogen fields (Ashe and Briscoe, 2006; Gurdon and Bourillot, 2001). This approach predicts cells less than 15µm from the VEGF-A source would be in a high concentration zone (> 1.0nM) while cells further than 40µm from the source would be in a zone of low concentration (<0.25nM). Our data (Fig 4) predicts that within the zone closest to the epithelial source of VEGF-A (<15µm), EC response would be initially determined by expression of high levels of EGR-3, NR4A1, NR4A2 and DSCR1. In the zone 40 µm or more from the source, DSCR1 transcripts would be predominant. As exposure continues EGR1 transcripts predominate in high and mid concentration zones (<40µm). By 24hr, only EGR1 and EGR3 would have significant influence on cellular response. While these results suggest how EC transduce positional information, system-wide computational analyses are needed to fully elucidate cell-context dependent effects of signal strength and duration of signal on EC response to a VEGF-A gradient field.

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References

- Akeson AL, et al. Temporal and spatial regulation of VEGF-A controls vascular patterning in the embryonic lung. *Dev Biol* 2003;264:443–55. [PubMed: 14651929]
- Akeson AL, et al. Embryonic vasculogenesis by endothelial precursor cells derived from lung mesenchyme. *Dev Dyn* 2000;217:11–23. [PubMed: 10679926]
- Armesilla AL, et al. Vascular endothelial growth factor activates nuclear factor of activated T cells in human endothelial cells: a role for tissue factor gene expression. *Mol Cell Biol* 1999;19:2032–43. [PubMed: 10022890]
- Ashe HL, Briscoe J. The interpretation of morphogen gradients. *Development* 2006;133:385–94. [PubMed: 16410409]
- Benedito R, et al. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* 2009;137:1124–35. [PubMed: 19524514]
- Carmeliet P, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;380:435–9. [PubMed: 8602241]
- Dayanir V, et al. Identification of tyrosine residues in vascular endothelial growth factor receptor-2/FLK-1 involved in activation of phosphatidylinositol 3-kinase and cell proliferation. *Journal of Biological Chemistry* 2001;276:17686–92. [PubMed: 11278468]
- Ferrara N. Vascular endothelial growth factor. *European Journal of Cancer* 1996;32A:2413–22. [PubMed: 9059329]
- Ferrara N, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996;380:439–42. [PubMed: 8602242]
- Galambois C, et al. Defective pulmonary development in the absence of heparin-binding vascular endothelial growth factor isoforms. *Am J Respir Cell Mol Biol* 2002;27:194–203. [PubMed: 12151311]
- Gerhardt H, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 2003;161:1163–77. [PubMed: 12810700]
- Greenberg JM, et al. Mesenchymal expression of vascular endothelial growth factors D and A defines vascular patterning in developing lung. *Dev Dyn* 2002;224:144–53. [PubMed: 12112468]
- Gurdon JB, Bourillot PY. Morphogen gradient interpretation. *Nature* 2001;413:797–803. [PubMed: 11677596]
- Haigh JJ. Role of VEGF in organogenesis. *Organogenesis* 2008;4:247–56. [PubMed: 19337405]
- Harfe BD, et al. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* 2004;118:517–28. [PubMed: 15315763]

- Ibanes M, Belmonte JC. Theoretical and experimental approaches to understand morphogen gradients. *Mol Syst Biol* 2008;4:176. [PubMed: 18364710]
- Jaeger J, et al. Regulatory feedback in pattern formation: towards a general relativistic theory of positional information. *Development* 2008;135:3175–83. [PubMed: 18776142]
- Kappas NC, et al. The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching. *J Cell Biol* 2008;181:847–58. [PubMed: 18504303]
- Kholodenko BN. Cell-signalling dynamics in time and space. *Nat Rev Mol Cell Biol* 2006;7:165–76. [PubMed: 16482094]
- Liu D, et al. The zinc-finger transcription factor, early growth response 3, mediates VEGF-induced angiogenesis. *Oncogene* 2008;27:2989–98. [PubMed: 18059339]
- Liu L, et al. Egr-1 gene is induced by the systemic administration of the vascular endothelial growth factor and the epidermal growth factor. *Blood* 2000;96:1772–81. [PubMed: 10961876]
- Mac Gabhann F, Popel AS. Model of competitive binding of vascular endothelial growth factor and placental growth factor to VEGF receptors on endothelial cells. *Am J Physiol Heart Circ Physiol* 2004;286:H153–64. [PubMed: 12714333]
- Mac Gabhann F, Popel AS. Interactions of VEGF isoforms with VEGFR-1, VEGFR-2, and neuropilin in vivo: a computational model of human skeletal muscle. *Am J Physiol Heart Circ Physiol* 2007;292:H459–74. [PubMed: 16980341]
- Mallory BP, et al. Lymphangiogenesis in the developing lung promoted by VEGF-A. *Microvasc Res* 2006;72:62–73. [PubMed: 16806288]
- Matsumoto T, et al. VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. *Embo J* 2005;24:2342–53. [PubMed: 15962004]
- Minami T, et al. Vascular endothelial growth factor- and thrombin-induced termination factor, Down syndrome critical region-1, attenuates endothelial cell proliferation and angiogenesis. *J Biol Chem* 2004;279:50537–54. [PubMed: 15448146]
- Ng YS, et al. Differential expression of VEGF isoforms in mouse during development and in the adult. *Dev Dyn* 2001;220:112–21. [PubMed: 11169844]
- Olsson AK, et al. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* 2006;7:359–71. [PubMed: 16633388]
- Pan Q, et al. Neuropilin-1 binds to VEGF121 and regulates endothelial cell migration and sprouting. *J Biol Chem* 2007;282:24049–56. [PubMed: 17575273]
- Ponder BA, et al. An allelic difference determines reciprocal patterns of expression of binding sites for Dolichos biflorus lectin in inbred strains of mice. *Journal of Embryology & Experimental Morphology* 1985;87:229–39. [PubMed: 4031755]
- Ruhrberg C, et al. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev* 2002;16:2684–98. [PubMed: 12381667]
- Sakurai Y, et al. Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. *Proc Natl Acad Sci U S A* 2005;102:1076–81. [PubMed: 15644447]
- Schachtner S, Wang YQ, Baldwin HS. Qualitative and quantitative analysis of embryonic pulmonary vessel formation. *Am. J. Respir. Cell Mol. Biol* 2000;22:157–165. [PubMed: 10657936]
- Schwarz MA, et al. Emerging pulmonary vasculature lacks fate specification. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L71–81. [PubMed: 18952755]
- Schweighofer B, et al. Signals and genes induced by angiogenic growth factors in comparison to inflammatory cytokines in endothelial cells. *Clin Hemorheol Microcirc* 2007;37:57–62. [PubMed: 17641395]
- Siekmann AF, et al. Modulation of VEGF signalling output by the Notch pathway. *Bioessays* 2008;30:303–13. [PubMed: 18348190]
- Soker S, et al. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998;92:735–45. [PubMed: 9529250]
- Takahashi T, et al. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. *EMBO Journal* 2001;20:2768–78. [PubMed: 11387210]

- Wang D, et al. Expression and endocytosis of VEGF and its receptors in human colonic vascular endothelial cells. *Am J Physiol Gastrointest Liver Physiol* 2002;282:G1088–96. [PubMed: 12016135]
- Watkins RH, et al. Differential expression of VEGF mRNA splice variants in newborn and adult hyperoxic lung injury. *Am J Physiol* 1999;276:L858–67. [PubMed: 10330042]
- Zeng H, et al. Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (KDR) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. *Journal of Biological Chemistry* 2001;276:32714–9. [PubMed: 11435426]

Figure 1. HMVEC respond to a narrow range of VEGF-A

HMVEC proliferate (A) and migrate (B) in response to VEGF-A. Cells were factor-starved overnight then cultured with 0 to 2.38nM hVEGF-A and proliferation (A) assessed at 24 hr (•) or 48 hr (▲). Migration (B) was assessed by creating a clear zone or wound in cultures of confluent HMVEC and determining cell migration into the zone in the presence of 0 to 2.38nM VEGF-A for n=5 wells at each concentration at 24hr. VEGFR2 phosphorylation (C, D, E) was assessed by culturing HMVEC with VEGF-A at 0.48nM (20ng/ml) for the indicated times (C) or with VEGF-A for 2 min at the indicated concentrations (D). Representative Western analyses comparing phosphoVEGFR2 (pY) and VEGFR2 (R2) at each time (C) or concentration (D). Band intensities were quantified using MultiGage software (Fuji) as described in Methods. (E) Relative VEGFR2 tyrosine phosphorylation (pY/R2) was determined for lysates from 3 or 4 independent HMVEC cultures at each VEGF-A concentration and half maximal pY/R2 response (EC50) of 650pM was determined using dose-response analysis of Prism4 (GraphPad). Concentrations used in subsequent analyses denoted with*.

Figure 2. Dose-dependent activation propagated through VEGFR2 autophosphorylation and some but not all associated signal kinases

Relative phosphorylation of pY1059/R2(A, C), pY1175/R2(B, C), pPLC/PLC(D), pMAPK/MAPK (E) and pAKT/AKT (F) was determined by quantitative Western analysis from lysates prepared at 0, 2, 4, 6 and 10 min of activation without VEGF-A or with either 0.12 (■), 0.48 (▲) or 1.9 nM (▼) VEGF-A. For analysis of pY1059/R2 and pY1175/R2 (A, B, C) total VEGFR2 was isolated by immunoprecipitation followed by Western analysis. (C) A representative blot from cells stimulated for 4 min with VEGF-A at concentrations indicated. Analysis of pPLC/PLC (D), pMAPK/MAPK (E) and pAKT/AKT (F) was completed on total cell lysates. Data are presented as the mean fold activation with standard deviation for 3 to 5 independent HMVEC cultures at each condition. (G) Representative blots for phosphorylated and total PLCγ, AKT and MAPK at 2, 4, 6 and 10 min at the indicated concentrations of VEGF-A.

Figure 3. Immediate early genes direct VEGF-A targets

To determine whether immediate early genes EGR-1, EGR-3, NR4A1, NR4A2 and DSCR1 were direct targets of VEGF-A, protein synthesis was inhibited by pretreating HMVEC 30 min without (-) or with (+) 50µg/ml cyclohexamide (CH). The HMVEC were then cultured without (-) or with (+) 0.48nM VEGF-A for 1 hr. RNA was isolated and quantified by real-time QPCR with normalization to human β actin. All data represent the mean relative quantification (RQ) with error bars indicating standard deviation for n=3 independent HMVEC cultures. Data were plotted after normalization with mean RQ=1 for each transcript in cultures without CH and VEGF-A.

Figure 4. VEGF-A-activated immediate-early genes respond in either dose-dependent or 'switch-like' modes

HMVEC were cultured for 1, 2, 3 or 24 hr without VEGF-A (0) or with low dose (L, 0.12nM), mid dose (M, 0.48nM) or high dose (H, 1.9nM) VEGF-A, RNA isolated and transcripts for (A) EGR-1, (B) EGR-3, (C) NR4A1, (D) NR4A2 and (E) DSCR-1 determined by real-time QPCR with normalization to human β actin. Data represent the mean relative quantification (RQ) with error bars indicating standard deviation for $n=3$ to 6 independent HMVEC cultures at each condition. Data were plotted after normalization with RQ=1 for transcripts from cultures without VEGF-A.

Figure 5. Pulse chase analysis of EGR-1 induction

HMVEC were cultured for 1 hr with 0.48nM VEGF-A (open bars) or for a 10 minutes (m) pulse without or with 0.48nM VEGF-A, the cells washed and cultured a further 50 minute chase in VEGF depleted media (gray bars) for a total culture of 1 hr. RNA was isolated, quantified by real-time QPCR as described above.

Figure 6. EC response with long-term exposure to VEGF-A

HMVEC were stimulated for 18hr with 0, 0.12, 0.48 or 1.9nM VEGF-A. Quantitative western analysis determined the effect of VEGF-A concentration on (A) pY/R2, (B) pPLC/PLC, (C) pMAPK/MAPK, (D) pAKT/AKT. Data are the mean with standard deviation for n=3 assays. (A) Compared to activity without VEGF-A, pY/R2 was 2.3-fold higher at 0.48nM VEGF-A (#p=0.036) and 9-fold higher at 1.9nM VEGF-A (*p=0.0013) with significance determined by Student's t test. (B) PLC γ activity was significant at mid and high dose, 1.7-fold increase, (*p=0.0010) and 3-fold increase (*p<0.0001), respectively. (C) p44/42MAPK activation was significantly increased at 0.48nM (1.5-fold, *p=0.0013) and 1.9nM VEGF-A (1.8-fold, *p<0.0001). (D) Significant AKT activation was not detected at any VEGF-A concentration.

Cell surface VEGFR2 (E) as determined by flow cytometry decreased after 24 hr culture with increasing VEGF-A. Cells without VEGF-A (green line), with 0.12nM (pink), 0.48nM (blue) or 1.9nM (orange) compared to isotype control (black). (F) Total cellular VEGFR2 was determined by quantitative Western analysis for n=3 cultures of HMVEC at each concentration and shown as relative band intensity.

Figure 7. Activation of pulmonary microvascular EC in wild type (A, B) and SP-CrtTA/tetOVEGF-A transgenic mice (C, D) at E14.5

Wild type and *SP-CrtTA/tetOVEGF-A* littermates were treated in utero with doxycycline from E10.5 to E14.5 to induce lung-specific VEGF-A transgene expression. Immunostaining for pMAPK by EC (magenta, arrows in A and C) and nonEC (red, arrowheads A and C) and EGR-1 by EC (red, arrows in B and D) is indicated. EC stained with isolectin GSL B4 (blue) and all nuclei detected with DAPI (green). Autofluorescence of red blood cells is bright pink (*).

Figure 8. Model of VEGF-A gradient to estimate positional concentration

Model of VEGF-A concentration gradient from a focal source using the approach of MacGabbhan (Mac Gabhann and Popel, 2007). Total distance was set at 60 μm , just beyond the VEGF gradient distance reported by Ruhrberg et al (Ruhrberg et al., 2002). It was assumed that near saturating VEGF-A of 1.9nM would occur within 5 μm of the VEGF-A source and that 0.12nM VEGF-A, just above the threshold of response, would occur at 55 μm . Considering increments of 5 μm , it was mathematically determined that there would be a 25% decrease in VEGF-A in each increment to achieve this differential of concentration across 55 μm .