Insulin-like Growth Factor 1 (IGF-1) Stabilizes Nascent Blood Vessels*

Sarah Melissa P. Jacobo and Andrius Kazlauskas

From the Department of Ophthalmology, Harvard Medical School, The Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Boston, Massachusetts 02115

Background: Although both VEGF-A and IGF-1 promote angiogenesis, their relative contribution to neovessel stability is not completely understood.

Results: To generate stable tubes, VEGF-driven tube formation must be accompanied by IGF-1-mediated stabilization. The mechanism involves IGF-1-mediated prolonged activation of Erk, which antagonizes LPA-driven regression.

Conclusion: IGF-1 stabilizes VEGF-A-driven tube formation.

Significance: Anti-VEGF therapy can be complemented by approaches that modulate Erk or LPA pathways.

Here we report that VEGF-A and IGF-1 differ in their ability to stabilize newly formed blood vessels and endothelial cell tubes. Although VEGF-A failed to support an enduring vascular response, IGF-1 stabilized neovessels generated from primary endothelial cells derived from various vascular beds and mouse retinal explants. In these experimental systems, destabilization/regression was driven by lysophosphatidic acid (LPA). Because previous studies have established that Erk antagonizes LPA-mediated regression, we considered whether Erk was an essential component of IGF-dependent stabilization. Indeed, IGF-1 lost its ability to stabilize neovessels when the Erk pathway was inhibited pharmacologically. Furthermore, stabilization was associated with prolonged Erk activity. In the presence of IGF-1, Erk activity persisted longer than in the presence of VEGF or LPA alone. These studies reveal that VEGF and IGF-1 can have distinct inputs in the angiogenic process. In contrast to VEGF, IGF-1 stabilizes neovessels, which is dependent on Erk activity and associated with prolonged activation.

The formation of new blood vessels from the pre-existing vasculature (angiogenesis) is a deliberately orchestrated sequence of events that starts with destabilization of an existing vascular bed and ends when the newly generated vessels quiesce (1, 2). Much more is known about the formation of vessels compared with their stabilization and regression (3). Learning how to promote the regression of pathological vessels will potentiate current antiangiogenic approaches that target the formation of neovessels and, thereby, address the unmet needs of patients afflicted by diseases such as cancer or neovascular ocular disorders (4).

Extracellular cues that coordinate the angiogenic program include growth factors and bioactive lipids such as VEGF, PDGF, insulin-like growth factor 1 (IGF-1), and lysophosphatidic acid (LPA). These angiostimulators make distinct contributions to angiogenesis. For instance, VEGF organizes endothelial cells into unstable vessels, whereas PDGF recruits pericytes that promote the maturation and stabilization of the neovessels (5).

So why are newly formed vessels unstable? The composition of angiomodulators in the microenvironment of the neovessels is a determining factor. For instance, angiopoietin 2 (Ang2) induces regression, provided that the level of VEGF is low (6–9). Similarly, LPA induces regression of nascent vessels (10–12). The fact that nascent vessels persist and mature suggests that the angiogenic process also involves steps to overcome the action of regression factors.

Studies in humans and mice indicate that IGF-1 plays an essential role in developmental angiogenesis (13–16). Although many cell types respond to IGF-1, endothelial cells are the key target in the context of developmental angiogenesis. Neovascularization is compromised when IGF-1R is deleted globally or specifically in the vascular endothelium (17, 18). The finding that IGF-1 enhances VEGF-dependent signaling events (13) begins to address the mechanism by which IGF-1 mediates its proangiogenic effects.

In contrast to the vast literature documenting the ability of IGF-1 to promote angiogenesis, there are few papers that consider if this phenomenon results from stimulating the formation of new vessels versus stabilizing them and, thereby, prevents their regression. The data presented in this work indicate that IGF-1 stabilizes endothelial cell tubes and retinal neovessels that form in response to VEGF. Furthermore, IGF-1 appears to accomplish this task by antagonizing signaling pathways that are engaged by the regression factor LPA. Our findings clarify at least one way in which IGF-1 promotes angiogenesis: by stabilizing neovessels.

EXPERIMENTAL PROCEDURES

Reagents—Lysophosphatidic acid (18:1) was purchased from Sigma-Aldrich (1-α-lysophosphatidic acid, oleoyl sodium salt, catalog no. L7260) and solubilized in methanol or chloroform:

endothelial cell; P, postnatal day; DUSP, dual-specificity phosphatase; ANOVA, analysis of variance; ATX, autotaxin; ROCK, Rho-associated coiled-coil containing protein kinase.
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Endothelial Tube Regression Assay—For endothelial tube length quantification and immunofluorescence staining, all assays were performed in 96-well plates. For regression studies, endothelial tubes were generated as described previously (19, 20) with a few modifications. 48 h prior to the tube regression assay, cells were collected in trypsin (0.5% in EDTA, 2 min at 37 °C). The suspension was neutralized with an equal volume of PBS and centrifuged at 2000 rpm for 2 min, and then the pelletted cells were resuspended in M199 medium that contained 10% bovine calf serum (no heparin or bovine brain extract). Cells were dispersed in 100-μl aliquots on 70 μl of collagen gel at a density of 45 × 10⁶ cells/well (10 × 10⁵ for HCECs) and allowed to attach overnight. 16 h after plating, the culture medium was removed, the cells were washed carefully with PBS, and 30 μl of collagen gel mix was added and allowed to solidify for 1 h at 37 °C. The collagen gel consisted of 80% PureCol collagen (Advanced Biomatrix, Inc., San Diego, CA); 0.5 μg/ml of fibronectin (catalog no. F1141) and 0.5 μg/ml laminin (catalog no. L2020), both from Sigma-Aldrich; 20 mM HEPES; 2 mg/ml NaHCO₃; and 0.2 M NaOH. Endothelial tube formation was stimulated by overlaying the collagen sandwich with endothelial basal medium (Lonza, catalog no. CC-3121) supplemented with 5% horse serum and adding 2.5 ng/ml VEGF-A. Endothelial tubes that formed in this manner persisted overnight (16 h after the initial VEGF-A addition). No significant remodeling (changes in tube length or branch points) was observed from 8–16 h within VEGF-A stimulation, and tubes within this period were considered stable. On the day of the tube regression assay (“time 0” or 16 h after VEGF-A addition), the culture medium was removed, tubes were rinsed three times with PBS, and serum-free endothelial basal medium containing treatments for regression was added. In assays where antagonists were present, the pre-existing tubes were pretreated for 15 min with the antagonist alone before the regression assay treatments were added with the antagonists. The autotaxin inhibitor HA-130 and the pan-LPA receptor antagonist 1-bromo-3-(hydroxy-4-(palmitoyl)butyl-phosphonate (BrP)-LPA were from Echelon Biosciences (Salt Lake City, UT). The pan-LPA inhibitor Ki6425 was from Cayman Chemicals. The end point of regression assays was 4 h after addition of the treatments unless indicated otherwise. Data are represented as mean ± S.E. of at least three independent experiments, and each experiment was performed in quadruplicate wells.

Collection of Tube Lysates and Immunoblot Analysis—HMREC tubes were generated in a 24-well scale as described above. We prepared HMREC tube lysates as described previously (10, 12, 21). Briefly, medium was removed, and the collagen gel sandwich was digested with two gel volumes of collagenase (type I from Clostridium histolyticum, Sigma-Aldrich) in Hank’s buffered salt solution supplemented with 100 μM Na₃VO₄. The collagen gel was digested for 1 h at 37 °C or until the gels had dissolved visibly while the tubes still remained intact. For each time point, samples from five wells were pooled. The suspensions were collected in 2-ml microspin tubes and centrifuged at 500 × g for 2 min. The supernatant containing digested collagen was removed, and the pellet containing the tubes was washed twice with ice-cold PBS. The tubes were lysed on ice for 20 min with 90 μl of 2× lysis buffer (100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 10 mM HEPES (pH 7.5), 1 mM Na₃VO₄, 10 mM NaF, 10 mM Na₃PO₄, and 1 mM PMSF) and clarified by centrifugation at 6000 × g for 2 min. Total protein concentrations were determined by BCA assay. The typical yield per sample (5 wells pooled together) was <2 mg/ml. A total of 20 μg of protein was loaded per lane on SDS-PAGE gel. For Western blot analysis of total cell lysates, pErk1/2 was probed with a rabbit monoclonal antibody against Thr-202/Tyr-204 (catalog no. 4370S, Cell Signaling Technology, Danvers, MA), pan-Erk1/2 was probed with a rabbit polyclonal antibody (catalog no. 9102, Cell Signaling Technology), and HRP-conjugated goat anti-rabbit IgG was from Santa Cruz Biotechnology (catalog no. sc-2004, Santa Cruz, CA). Films were scanned and saved in 8-bit grayscale at 300 pixels per inch resolution. Densitometry analysis was performed using the gel function of NIH ImageJ. Data are representative of n = 3 (for panels treated with VEGF-A) to 6 independent experiments.

Immunofluorescence Staining of Endothelial Tubes—HMRECs were subjected to tube regression assays as described above. For each independent experiment (n = 3), all treatments were stimulated by overlaying the collagen sandwich with...
were done in duplicate. At the indicated time points, treatments were removed, and the collagen gel was digested with two gel volumes of type I collagenase in Hanks’ buffered salt solution with Na₂VO₄. The collagen gel was digested for 30 min at 37 °C or until the collagen gels had visibly reduced thickness while still preserving the structure of the endothelial tubes. Collagenase was removed carefully, and the intact tubes were washed 3 × 15 min with Na₂VO₄-supplemented ice-cold PBS. Tubes were fixed with 4% ice-cold paraformaldehyde for 10 min and washed with PBS 3 × 15 min. Tubes were blocked overnight at 4 °C with PBS containing 4% BSA, 10% goat serum (Sigma-Aldrich, catalog no. G9023), and 0.5% Triton X-100. pErk1/2 was probed with a rabbit monoclonal antibody (1:100 dilution, Sigma-Aldrich, catalog no. G9023), and 0.5% Triton X-100. The collagen gel sandwich was overlaid with endothelial basal lamina (endothelial tubes were costained with Alexa Fluor 594-conjugated Griffonia simplicifolia isoelectin B4 (1:100 dilution, catalog no. I21411, Invitrogen) overnight at 4 °C. For negative control, an equal molar dose of non-immune rabbit IgG (catalog no. sc-2027, Santa Cruz Biotechnology) was used in place of the pErk1/2 antibody. After primary antibody incubation, tubes were rinsed 3 × 15 min and incubated with Alexa Fluor 488-conjugated AffiniPure goat anti-rabbit IgG (1:200 dilution, H11003) overnight at 4 °C. Then tubes were rinsed 3 × 15 min. To prepare slides, the collagen gels were removed carefully from the 96-well plates by rimming the perimeter with straight Dumont 5 forceps (F.S.T. Instruments, Foster City, CA). The collagen gels were mounted on Ultra™ Frost glass slides (Denville Scientific, Inc., South Plainfield, NJ) with Vectashield mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA).

Laser-scanning Confocal Imaging—Images are representative of three independent experiments, and all conditions were done in duplicate wells. For each well, three to five randomly selected fields were imaged by sequential scanning of each channel using a Leica upright DM 6000S confocal laser-scanning microscope. Images at ×200 magnification were acquired from optical slices of ~10 μm using Leica Application Suite AF software v. 1.3.1. Confocal images for pErk1/2 are presented in monochrome for better contrast to facilitate qualitative intensity comparisons across different time points and treatments. IB4 and DAPI channels were merged using GIMP for Mac OS X.

HMREC Tube Total RNA Isolation and Real-time RT-PCR—Preformed HMREC tubes on a 24-well scale were washed three times with PBS and stimulated with the indicated treatments. After 30 min, the stimuli were removed by aspiration, the tubes were rinsed with ice-cold PBS, and total RNA was extracted using TRIzol reagent. For each treatment, six wells were pooled together to collect enough RNA. The typical yields were 250 ng/μl.

For real-time RT-PCR analysis of DUSP transcripts, the following PCR primers were obtained from the Massachusetts General Hospital PrimerBank and Oligo Synthesis Core Facility (Boston, MA): DUSP1 (NM_004419), ACCACACCGTGTCCACTTC (forward) and TGGGAGAGGTCGATAT-GGG (reverse); DUSP2 (NM_004418), TGCCCCAACAC- TTTGAGG (forward) and AGTCAAATGAAGCTATGGCCT (reverse); DUSP4 (NM_001394), GGCCGCTAGAGGT- TTTCC (forward) and TGGTGTGTAATGGGTGTCC (reverse); DUSP5 (NM_004419), GCCAGCTTATGACCAAGGTG (forward) and GTCCGTGGAGACATTCCAG (reverse); DUSP6 (NM_001946), GAAATGGCAGATAGCAGAGCG (forward) and CGACCAGCTGTATAGCTCCTG (reverse); DUSP7 (NM_001947), GACCTGTCCGAGAGATATG (forward) and GAAAATCTGTTGTAGTGAACCT (reverse); DUSP8 (NM_004420), TCAGTCTTCGACACTCTC (forward) and CGCTGTCTGCTGTCTAG (reverse); and GAPDH (NM_002046), GAGCCAGATGCCCTC-CACAAAT (forward) and GGCTGTTCGATTACTTCCTCTATGG (reverse). The design of the one-step PCR using Power RNA-to-Ct SYBR Green Mix (Applied Biosystems) with 100 ng of total RNA as template was first tested using a 40-cycle reaction in a benchtop thermocycler. The expected amplicons were validated by running PCR products on a 2% agarose gel. Quantitative real-time RT-PCR was performed on an Eppendorf Replex2. Relative expression of DUSP transcripts was calculated using the 2⁻ΔΔCt method relative to GAPDH and normalizing with vehicle control. Data represent mean ± S.E. of three experiments. Each experiment was performed in duplicate.

Animals—The 8-week-old male C57Bl/6j mice (Jackson Laboratories, Bar Harbor, ME) used in this study were acclimatized for at least 48 in the institute’s animal facility in 12-h light-dark cycles with ad libitum access to food and water. The animals were euthanized by CO₂ narcosis. Animal handling procedures adhered to protocols approved by the Institutional Animal Care and Use Committee and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Marine Retinal Explants—We prepared retinal explants as described previously (10, 12, 22). Immediately after euthanasia, the eyes were enucleated and kept in PBS. The retinas were dissected under a Zeiss Stemi 2000-c stereomicroscope equipped with a KL 1500 LCD cold light source. When the retinas were dissected, four relaxing cuts were made to get four evenly sized quadrants. Each quadrant was further cut into four 1 × 1 mm pieces, yielding 16 pieces per eye for explants. Each piece was placed within a collagen gel (described above), with the ganglion cell layer facing up, in a 48-well tissue culture plate. The collagen gel sandwich was overlaid with endothelial basal medium containing 10% horse serum, 12 μg/ml bovine brain extract, 100 μg/ml heparin, and 25 ng/ml VEGF-A. Culture medium was replaced every 48 h after photographing the explants for phase-contrast image acquisition. Neovessel sprouting was typically observed starting on day 7, with significant and robust outgrowth starting on days 10–14. Thereafter, retinal explants with neovessel outgrowths that showed no significant or robust increase in length or caliber within a 48-h window were considered to be at their peak and stable (typically days 18–21). Retinal explants that reached this period were then treated with the indicated agents for regression assays.

For the experiments reported here (n = 3, 2 mice/experiment), the success rate of neovessel sprouting from retinal explants was 78–87%. Retinal explants of comparable robustness (number of neovessel outgrowths, length of sprouts) were selected for paired or grouped comparisons before adding the indicated treatments to minimize variability between explants from the same eye. In regression assays, culture medium that
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A

Normalized Tube Length (length at 4h/length at 0h)

LPA, 10 μM

Vehicle IGF-1 VEGF-A

B

0h, peak 4h post-peak

LPA

Vehicle

BrP-LPA Ki6425 HA-130

C

Normalized Tube Length (length at 4h/length at 0h)

Vehicle BrP-LPA Ki6425 HA-130

D

Normalized Tube Length (length at 4h/length at 0h)

VEGF-A Vehicle BrP-LPA HA-130 IGF-1

E

Normalized Tube Length (length at 4h/length at 0h)

LPA Vehicle IGF-1 insulin

n = 3 ns

***
contained the indicated treatments were replaced every 12 h for 3 days. Data represent three independent experiments, and each experiment was done in duplicate. The explants were derived from the left and right mouse eye.

Image Analysis and Quantification—For tube assays and retinal explants, image acquisition and data quantification were performed as described previously (10, 12). Briefly, phase-contrast images at ×100 magnification were acquired with a Nikon Eclipse TE2000-S inverted microscope (Melville, NY) equipped with Diagnostic Instruments RT Slider charge-coupled device camera. 8-bit grayscale images at 1600 × 1400 ppi resolution were acquired using Spot Advanced for Mac OS X (Diagnostic Instruments, Sterling Heights, MI). Images were imported into National Institutes of Health ImageJ. For quantification of tube assays in a 96-well plate, a collected image was quantified by tracing the tubes using the brush tool (5-pixel brush width). The final values shown, “normalized neovessel length,” represent the total pixel units at the end point (4 h) divided by the pixel units at the start (0 h, which is the peak of tube formation) of the regression assay. Each treatment was done in quadruplicate wells per independent experiment (n = 3 or more). For the visual representation of tubes in Fig. 1B, images were digitally zoomed ×8, and 1400 × 1400 ppi areas were selected randomly.

In retinal explant assays, phase contrast images at day 0 (start) and day 3 (end point) of regression assays were imported into National Institutes of Health ImageJ. Neovessels were traced using the paintbrush function, and total neovessel length was expressed as arbitrary pixel units. Normalized neovessel length represents the total pixel units at the end point (day 3) divided by the pixel units at the start (day 0, which is the peak of neovessel formation) of the regression assay. All representative images from each assay were prepared for figures using GIMP 2.8 for Mac OS X.

Data Analysis—Data analyses and graphing were performed with Prism 6.0 (GraphPad Software, San Diego, CA). Two-tailed Student’s t test was used for paired comparisons. One-way analysis of variance followed by Tukey’s post-hoc test was used for comparison of more than two groups. Comparisons in which p < 0.05 (*) at α ≥ 0.05 were considered statistically significant. ** denotes p < 0.01, and *** denotes p < 0.001.

RESULTS

VEGF-A Promoted Regression of Endothelial Tubes, whereas IGF-1 Stabilized Them—To study remodeling and destabilization of nascent capillaries in vitro, tubes were generated uniformly from primary HMRECs treated with VEGF-A in a modified version of the collagen gel sandwich assay described previously (19, 20). When such retinal endothelial tubes formed, they were stable and did not significantly remodel in terms of total length or branch points for at least 4 h in serum- and growth factor-free medium (Fig. 1, A and B) (12, 19). Adding purified LPA to such tubes induced regression (Fig. 1, A and B). As expected, LPA receptor antagonists (BrP-LPA or Ki6425) prevented regression, whereas an ATX inhibitor (HA-130) had no effect on this response because it was driven by exogenous LPA (Fig. 1C). Regression in this model system is not initiated by apoptosis, which is what happens to nascent tumor vessels when VEGF-A is acutely neutralized (24–26). Rather, LPA triggers regression by stimulating the migration and subsequent disorganization of tubes organized from primary endothelial cells (10–12).

In light of the existing observations that VEGF increases the level of ATX (10, 27, 28), we anticipated that VEGF would induce regression and that this response would be driven by LPA. Indeed, VEGF triggered regression of newly formed tubes (Fig. 1, A, B, and D). This response was blocked by antagonists of either LPA receptors or ATX (Fig. 1D), indicating that VEGF was acting via endogenously produced LPA. In contrast to VEGF, IGF-1 failed to promote regression (Fig. 1, A, B, and D). This observation is consistent with the fact that IGF-1R does not engage PLC, which is activated by VEGFR2 and is required to increase the level of ATX (29, 30). Furthermore, IGF-1 prevented both VEGF- and LPA-stimulated regression (Fig. 1, A, B, and D). In contrast to IGF-1, insulin was unable to limit LPA-driven regression (Fig. 1E). Moreover, an IGF-1R-specific tyrosine kinase inhibitor (NVP-AEW541), but not an insulin receptor-specific antagonist (S961), abolished the IGF-1-mediated block of regression in the presence of LPA (data not shown).

The data in Fig. 1 reveal that VEGF promotes the regression of newly formed tubes and that the underlying mechanism is likely to involve the ATX-mediated production of LPA. Furthermore, IGF-1 not only failed to promote regression but antagonized this response.

IGF-1 Stabilized Tubes Organized from Four Types of Primary Endothelial Cells—In Fig. 1, regression was monitored in serum-free medium, and, under these conditions, this response was dependent on addition of LPA or agents that stimulated LPA production (such as VEGF-A). We sought to extend these findings to additional experimental conditions and types of endothelial cells.

In the presence of serum, tubes regress without addition of purified LPA because serum contains LPA, ATX, and the ATX...
FIGURE 2. IGF-1 stabilized tubes generated from primary endothelial cells derived from various vascular beds. A, IGF-1 prevented serum-stimulated regression. These experiments were done as in Fig. 1A, except that, during the 4-h regression period, the collagen sandwich was overlaid with medium containing 5% (instead of 0%) serum. B, serum-stimulated regression was prevented by antagonists of LPA receptors or ATX. These experiments were done as in Fig. 1C, except that, during the 4-h regression period, the collagen sandwich was overlaid with medium containing 5% (instead of 0%) serum. C–F, IGF-1 antagonized serum-induced regression of tubes organized from four different types of primary endothelial cells. The experiments were done as in A, except that, during the regression period, the collagen sandwich was overlaid with medium containing 1% (instead of 5%) serum. Regression of tubes beyond the 4-h time point is also shown. HUVEC, human umbilical vein endothelial cell; BREC, bovine retinal endothelial cell. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
substrate lysophosphatidyl choline (30–32). As expected, tubes regressed when 5% serum was present, and this response was suppressed by LPA receptor antagonists (Fig. 2, A and B). Furthermore, inhibiting ATX prevented serum-driven regression (Fig. 2B). These observations indicate a requirement for ATX, which is not only present in serum but also produced by cells.
We observed previously that blocking ATX reduced the level of LPA (35). Importantly, IGF-1 prevented regression under these conditions, whereas VEGF did not and, instead, even augmented regression slightly (Fig. 2A), as observed when purified LPA was driving this response (Fig. 1A). Even 1% serum was sufficient for regression, and, under these conditions, IGF-1 also stabilized tubes whereas VEGF did not (Fig. 2C). We conclude that IGF-1 stabilized tubes organized from a variety of endothelial types derived from distinct vascular beds and under various conditions.

The data in Fig. 2, C and D, also indicate that the rate of regression followed comparable but not identical time courses for all four types of endothelial cells. Possible reasons include differences in expression of LPA receptors and/or responsiveness to LPA or relative expression and basal activation of IGF-1R. The concept that the response to LPA may not be uniform is supported by our recent observation that prolonged exposure of endothelial cells to high glucose blunts their ability to respond to LPA (10, 12, 24), which indicates that LPA responsiveness is not a fixed parameter even within a single cell type.

**IGF-1 Mediated Stabilization Was Dependent on Erk Activity**

What is the mechanism by which IGF-1 antagonizes LPA-mediated regression? In light of previous publications that IGF-1 activates Erk (29, 30), which antagonizes LPA-stimulated regression (36–38), we considered whether Erk activation was associated with stabilization. In this set of experiments, the regression assay conditions were the same as in Fig. 1 (serum-free medium), and time points that precede overt regression were chosen to compare tubes of similar lengths. Fig. 3, A and B, shows that LPA, VEGF, and IGF-1 all activated Erk and that, when IGF-1 was present, Erk stayed active longer.

Monitoring Erk activity in tube lysates, as was done in Fig. 3, A and B, required extraction of the tubes from the collagen gel (31–34). We observed previously that blocking ATX reduced the level of LPA (35). Importantly, IGF-1 prevented regression under these conditions, whereas VEGF did not and, instead, even augmented regression slightly (Fig. 2A), as observed when purified LPA was driving this response (Fig. 1A). Even 1% serum was sufficient for regression, and, under these conditions, IGF-1 also stabilized tubes whereas VEGF did not (Fig. 2C).
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A

B

C

D

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(32). Using this approach, we previously reported differences in the phosphorylation state of myosin light chain kinase (10, 12). Although it is difficult to assess to what extent this procedure itself influenced the activation state of Erk, we did observe the predicted differences between stimulated and unstimulated samples.

To further address this caveat, we evaluated Erk activity by immunofluorescence, which did not involve extraction of the tubes from the collagen gel. The same correlation between enduring Erk activity and tube stability was observed using this approach. Erk stayed active longer in tubes exposed to IGF-1 alone or in combination (IGF-1 + VEGF or IGF-1 + LPA) compared with tubes exposed to VEGF or LPA alone (Fig. 3C). The pErk1/2 signal is shown in monochrome. Fig. 3C, inset, shows the Isolectin B4-positive (IB4+) endothelial tubes in red, and the nuclei are blue. The pErk1/2 signal was sensitive to a Mek inhibitor, PD98059, and was not observed when a non-immune isotype control was used (Fig. 3D). Therefore, two different approaches indicated that IGF-1-mediated stabilization of tubes was associated with enduring activation of Erk1/2.

In light of the correlation between prolonged Erk activation and stabilization (10, 12, 36, 38), we considered whether Erk was required for IGF-1 to stabilize endothelial tubes. Indeed, two different Erk pathway inhibitors, PD98059 and U0126 (data not shown), prevented IGF-1 from stabilizing tubes. LPA- and VEGF-driven regression proceeded in the face of IGF-1 when Erk activity was blocked (Fig. 4, A and B, and data not shown). We concluded that IGF-1-mediated stabilization required Erk activity. The results in Figs. 3 and 4 support the conclusion that prolonged Erk activation is required for IGF-1-dependent stabilization. A plausible reason for why short-lived Erk activation is not sufficient to stabilize nascent tubes is that persistent antagonism of LPA-dependent signaling events such as Rho/ROCK is required to prevent regression (Fig. 4C) (10, 12, 38–41, 42).

An intriguing question that arises from this set of results is why the dynamics of Erk activity were not the same for distinct agents that are capable of engaging this signaling pathway. Part of the answer may involve distinct mechanisms by which LPA, VEGF, and IGF-1 engage the Erk pathway (43, 44). Furthermore, these agents may not equivalently trigger signaling events that limit the duration of Erk activation, such as the induction of DUSPs, also called MAP kinase phosphatases), which dephosphorylate and thereby inactivate MAP kinase family members, including Erk (45). Indeed, we observed that LPA and VEGF, which activated Erk transiently, induced the expression of DUSP1 and DUSP6 (Fig. 4D and data not shown).

IGF-1, which stimulated prolonged Erk activity, did not increase the expressions of these DUSPs (Fig. 4D and data not shown). Furthermore, IGF-1 suppressed the ability of LPA to induce DUSP1, DUSP2, and DUSP5 (Fig. 4D and data not shown). Therefore, the differential expression of DUSPs was associated with the distinct kinetics of Erk activation by the various agents used in this study.

**DISCUSSION**

The data presented here suggest that there is a division of labor among proangiogenic agents. IGF-1 stabilizes nascent vessels whose formation is driven by VEGF. The underlying mechanism of IGF-1-mediated stabilization involves prolonged activation of Erk, which antagonizes LPA-driven regression.

Although our results indicate that Erk activity and, thereby, Rho/ROCK antagonism (36), was required for IGF-1 to stabilize nascent tubes, there may be additional signaling events that...
play a role during this phase of neovessel remodeling. For instance, in mesenchymal stem cells, IGF-1 induces phosphorylation of p190 RhOAP and, thereby, results in its translocation and increased ability to inactivate Rho (46). This suggests that, in addition to activating Erk, IGF-1R signaling may induce Rho inactivation via alternate routes. It would be interesting to see whether this particular alternate route to Rho inactivation also operates in vascular endothelial cells.

To begin to understand why IGF-1 activated Erk longer than either VEGF or LPA, we focused on DUSPs. Our results indicate that VEGF and LPA stimulated an increase in DUSP1, whereas as IGF-1 did not (Fig. 4D). Consequently, the faster decline in Erk activity in tubes exposed to LPA or VEGF alone may be at least in part, due to an inducible increase in the expression of certain DUSPs. We also observed that IGF-1 promoted translocation of activated Erk to the nucleus (Fig. 3C) (47, 48). It would be interesting to know whether nuclear translocation protects Erk from dephosphorylation and/or results in transcriptional modifications that contribute to the persistence of nascent capillaries.

Given that Erk contributes to the migration of cells, it was somewhat of a surprise that antagonizing the Erk pathway suppressed LPA-dependent regression, which involves cell migration. A likely explanation emerges when one considers cell types in which the Erk pathway is required for migration. In vascular smooth muscle cells, blocking the Erk pathway reduces PDGF-driven migration, whereas this intervention has no effect on fibroblasts (49–51). Similarly, LPA or VEGF-stimulated migration of endothelial cells is unaffected by Erk pathway antagonists (52, 53). In this context (Fig. 4C and Refs. 10, 12, 36), Erk antagonized LPA-driven regression because it suppressed Rho/ROCK, which was essential for this response.

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