Developmental Endothelial Locus-1 (Del-1), a Novel Angiogenic Protein
Its Role in Ischemia

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Background—Developmentally regulated endothelial locus-1 (Del-1) is an extracellular matrix protein that is expressed by endothelial cells during embryological vascular development. We speculated that Del-1 may be reexpressed in ischemia and may be involved in endogenous angiogenesis.

Methods and Results—Del-1 protein was detected by immunohistochemistry in murine ischemic hindlimb after femoral artery excision. To determine whether exogenous Del-1 would augment angiogenesis in vivo, Del-1 or vehicle was administered for 3 weeks by intramuscular injection of murine ischemic hindlimbs. Angiogenesis was quantified by gadolinium-MRI perfusion and capillary densitometry. We used a disc angiogenesis system (DAS) to characterize the angiogenic response to vehicle (PBS), Del-1, Del-1 mutant (altered RGD domain), Del-1 minor (truncated discoidin-I–like domain), or basic fibroblast growth factor. After 14 days, the discs were extracted and sectioned to quantify vascular growth by morphometry. Endogenous Del-1 protein expression was increased in ischemic hindlimbs. Administration of Del-1 increased hindlimb vascular flow index and capillary density. In the DAS, Del-1 doubled fibrovascular growth, as did basic fibroblast growth factor. However, angiogenesis was not enhanced by the Del-1 mutant or Del-1 minor proteins.

Conclusions—Del-1 is expressed in ischemic tissue. Del-1 stimulates angiogenesis, an effect that is dependent on the RGD motif and a second signaling sequence in the discoidin-I–like domain. Exogenous intramuscular administration of Del-1 significantly enhances angiogenesis in the murine ischemic hindlimb. Del-1 may prove to be a novel therapeutic agent for patients with ischemia. (Circulation. 2004;109:1314-1319.)

Key Words: proteins ■ ischemia ■ angiogenesis

Developmentally regulated endothelial cell locus-1 (Del-1), a 52-kDa extracellular matrix protein, is expressed by endothelial cells during embryological vascular development. Del-1 is composed of 3 epidermal growth factor (EGF) repeats and 2 discoidin-I–like domains. The second EGF repeat contains an RGD motif. Ligands with the RGD sequence have been shown to interact with endothelial \( \alpha_\beta_3 \) integrin receptors. Integrin receptors are identified on endothelial cell surface and communicate with the cell through cytoplasmic signaling molecules. Activation of \( \alpha_\beta_3 \) receptors is required for vascular development and may be associated with a decrease in endothelial cell apoptosis and with downstream angiogenic signaling through protein kinases. Antagonists to \( \alpha_\beta_3 \) disrupt embryological vascular development and inhibit angiogenesis in the chick chorioallantoic membrane (CAM) assay. Del-1 has been shown to stimulate endothelial cell attachment and migration in vitro and promote angiogenic growth in the CAM assay. These Del-1–mediated events are inhibited by antibodies to \( \alpha_\beta_3 \) integrin.

Del-1 is not expressed in adult animals. However, other factors involved in embryonic vasculogenesis (eg, vascular endothelial growth factor) are reexpressed in the adult during ischemia. Furthermore, exogenous administration of these growth factors is known to enhance the angiogenic response to ischemia. Accordingly, we performed this investigation to determine (1) whether Del-1 is expressed under conditions of ischemia, (2) whether Del-1 can induce angiogenesis in adult animals, and (3) whether exogenous application of Del-1 can augment angiogenesis in response to ischemia.
Methods

Animals
Eight- to 10-week-old female wild-type C57BL/6J mice were used (Jackson Laboratories, Bar Harbor, Me, and Department of Comparative Medicine, Stanford, Calif). The mice weighed 20 to 25 g and were maintained as described previously.18

Murine Model of Ischemic Hindlimb
Mice were anesthetized with 4% chloral hydrate (intraperitoneal administration, 0.1 ml/10 g body weight). The medial surface of both hindlimbs were shaved and then cleaned with Betadine solution. A 1.5-cm longitudinal incision was performed, which extended from the knee to the inguinal ligament. Through this incision, the superficial femoral artery was dissected free along its length. After the distal ends of both the external iliac and superficial femoral arteries were ligated with 7.0 silk suture (Ethicon), complete excision of the femoral artery was performed. An additional set of mice underwent sham operation. The incisions were then closed with discontinuous stitches of 5.0 silk suture (Ethicon). Ampicillin (1 mg/10 g body weight IP) was administered after surgical procedure.

To study the possible therapeutic effect of Del-1 to induce angiogenesis in a murine ischemic hindlimb, daily intramuscular Del-1 major protein injections (2.5 μg per leg) were performed at 3 different sites in the hindlimb for a period of 3 weeks (n=6). The control group received daily intramuscular injections of vehicle (PBS, n=6) for the 3-week period. We have used a similar mode of administration (ie, intramuscular) and time course (daily for 3 weeks) in this murine model of hindlimb ischemia.19

Immunohistochemistry

Tissue Preparation
At 3 weeks after surgery, mice were killed with an overdose of 4% chloral hydrate IP and cervical dislocation, and the adductor and semimembranous muscles were collected for capillary density assessment. Briefly, a longitudinal incision in the medial thigh was made to expose the entire hindlimb muscle. The adductor and semimembranous muscles were removed and immediately frozen in OCT. Subsequently, 5-μm sections were taken from the mid region of each muscle in a transverse orientation. The sections were air-dried and fixed in acetone.

Del-1 Immunostaining
To determine whether Del-1 protein expression is upregulated in the setting of ischemia, we used anti-Del-1 antibody on ischemic hindlimb 3 weeks after surgery. The tissues were removed from paraffin with xylene and rehydrated with graded alcohol washes from 100% through 70% alcohol. Tissue slides were digested with proteinase K (DAKO) for 15 minutes at room temperature. Endogenous peroxidase was blocked with 3% H2O2 in methanol for 10 minutes. Tissues were then blocked with normal goat serum, and then primary polyclonal rabbit anti-human Del-1 (1:6000) was added and incubated for 60 minutes at room temperature in a humidified chamber. Tissue slides were then washed and incubated with biotin-labeled goat anti-rabbit polyclonal antiserum at room temperature for 30 minutes. Streptavidin–horseradish peroxidase (1:100) was added and incubated for 30 minutes at room temperature. Development of the peroxidase color reaction was performed as described above. Hematoxylin was used to counterstain the tissues. Mice were identified in either an ischemia-induced group (n=6) or a control non–ischemia-induced group (n=6).

Capillary Densitometry
Immunohistochemistry was performed with anti-mouse CD31 antibodies, and a commercial kit (ABC Vector) was used to identify the endothelial cells. An eosin counterstain was used to differentiate myocytes. Capillaries and myocytes were identified and counted by light microscopy (20×). For each section, 4 different fields were selected and the total number of capillaries and myocytes per field was determined. These values were averaged to provide a determination of capillary density for each experimental limb. To ensure that the value for capillary density was not underestimated owing to muscle atrophy or underestimated owing to interstitial edema, capillary density was expressed as a ratio of capillaries to myocytes present in the same field.

Murine Hindlimb Blood Flow Measurements

Magnetic Resonance Imaging
First-pass perfusion imaging was performed with a multisection, fast imaging technique as we have described previously.19 Magnetic resonance (MR) images were acquired with a Signa 1.5-T scanner (GE Medical Systems). A fast-spoiled GRASS (SPGR) sequence (repetition time 11.7 ms, time to echo 3.7 ms, flip angle 90°, 256×128 matrix, field of view 4×2 cm) was used. Gadopentetate dimeglumine (Magnevist; Berlex Laboratories), at a dose of 0.02 mmol/kg, was injected through a catheter inserted into the left carotid artery of a mouse, which was advanced into the thoracic aorta. Perfusion imaging was performed at the level of the hindlimb. Simultaneously, images were acquired at the level of the common iliac artery so as to obtain signals for the arterial input function. Signal intensity–versus-time curves were plotted by spatially averaging the signal intensities over the regions of interest. This approach takes advantage of the MR contrast provided by gadolinium. Gadolinium is injected into the aorta, and MR images of the region of interest (hindlimb in this case) are taken in rapid succession. Signal intensity increases as gadolinium-containing blood flows into the limb. If there is an obstruction, and flow is arriving through collaterals, the rate of increase in signal intensity is delayed. If collateralization is poor or insufficient, the rate is delayed to a greater degree.

Disc Angiogenesis System
To study whether Del-1 induces angiogenesis, we used the disc angiogenesis system (DAS).19,20 In addition, using this system, we assessed whether the RGD sequence and the discoidin-I–like domains were necessary for the functionality of Del-1. Accordingly, some discs were treated (as described below) with Del-1, a Del-1 mutant (RGD mutated to RAD), or Del-1 minor (with a truncation of the discoidin-I–like domain).

Preparation of the Disc
The DAS consisted of a disc (11 mm in diameter and 1 mm thick) made of a polyvinyl alcohol sponge (Kanebo PVA, Rippey Co). Both sides were covered with nitrocellulose cell-impermeable filters (Millipore filters, 0.45 μm in pore diameter) of the same diameter as the sponge disc, fixed to the sponge with Millipore glue #1 (xx70000.00, Millipore). As a result, cells (and thus vessels) could penetrate or exit only through the rim of the disc.21,22 To study the direct effect of an agent on angiogenesis, such agents were added directly to the disc. Briefly, a 1.5-mm core (pellet) was cut from the disc center. Both the pellets and discs were sterilized before assembly in a laminar flow hood. The pellet was loaded with up to 20 μL of the agent in solution and subsequently air-dried. We placed pellets in the disc: vehicle (PBS; Sigma Chemical Co; n=5); Del-1 (0.2 mol; n=5); Del-1 mutant (0.2 mol; n=5); Del-1 minor (0.2 mol; n=5; Progenitor); or basic fibroblast growth factor (bFGF; 20 μg; Scios; n=5). The pellet was then coated with ethylene-vinyl acetate copolymer (Elvax, Dupont, Chemcentral Corp) for slow release of the test substance from the pellet into the disc. The pellet was then reinserted into the disc before the disc was sealed with the Millipore filters.

Implantation of the Disc
The mice were anesthetized with 4% chloral hydrate (0.1 mL/10 g body weight IP). The flanks and posterior surface of the thorax were shaved and cleaned with saturated 70% isopropyl alcohol. A 2-cm incision was made in the skin of the flank contralateral to the implantation site. Blunt dissection through the subcutaneous tissue
produced a channel into which the PBS-moistened disc was inserted. The skin was closed with 5.0 silk suture.19,20

Disc Removal and Preparation
Two weeks after disc implantation, the mice were killed with an intraperitoneal overdose of 4% chloral hydrate and cervical dislocation. A careful incision was made next to the skin overlying the implanted disc, and the disc was gently removed from the implantation site. Attached tissue was carefully detached from the disc. After the disc was removed, 1 filter was separated from the disc. Discs were then fixed in 10% formalin and embedded flat in paraffin. Subsequently, 5-μm sections were made in a plane through the center of the disc and parallel to the disc surface. With the size of the disc used in the present study, our experience indicates that 2 weeks is an optimal time to examine fibrovascular growth. With later time points, fibrovascular growth could completely invest the disk, particularly in growth factor–stimulated animals, which would make analysis difficult (ie, after the disc is fully invested with fibrovascular growth, further increases cannot be seen over time in the growth factor–treated group, whereas fibrovascular growth in the vehicle-treated group may continue at a slower rate, artificially diminishing the true difference between the groups).

Quantification of Results
The disc sections were stained with hematoxylin and eosin for light microscopy and histomorphometric measurement of radial growth and stained with toluidine blue for quantitative determination of total area of fibrovascular growth. A video microscope and a computer-assisted digital image analysis system (NIH Image 1.59b9) were used to calculate the entire area of fibrovascular growth in the toluidine blue–stained disc, expressed in millimeters squared. As described in a previous study, total fibrovascular growth area is directly proportional to the total area of the disc occupied by blood vessels.19,20 Therefore, the measurement of such total area is used as an index of angiogenesis.19–24

Vascular Continuity Assessment
To visualize the microvessels in the disc sections and to establish continuity between the systemic and disc vasculatures, luconyl blue dye was injected into the left carotid artery before the mice were killed. Animals were anesthetized with 4% chloral hydrate (0.1 mL/10 g body weight IP). An incision was made in the ventral midline of the neck. After the carotid sheath was exposed, the left carotid artery was separated from the neurovascular bundle and secured by two 4.0 silk sutures. An incision was made in the carotid, and a 1.5-cm length of PE10 tubing (Becton Dickinson) was introduced into the carotid artery and advanced to the ascending aorta just distal to the aortic valve. Approximately 1.0 mL of luconyl blue dye was then slowly injected from a 1-mL syringe through the tubing into the thoracic aorta. The presence of blue dye in the fibrovascular network in the disc was detected by light microscopy. Microscopy revealed microvessels lined by a single layer of endothelium with erythrocytes contained within their lumen. Luconyl blue dye was observed throughout the total area of the disc (Figures 1a and 1b).

Statistical Analysis
All data are given as mean±SEM. Statistical significance was tested with an unpaired 2-tailed t test for comparisons between groups or with ANOVA when appropriate. Statistical significance was accepted for P<0.05.

Results
Upregulated Expression of Del-1 in Response to Ischemia
Immunohistochemistry of ischemic hindlimb tissues indicated that Del-1 expression is reactivated in the setting of muscle tissue ischemia. Ischemic tissue harvested from the ischemic hindlimb (Figure 2A) was compared with the normal control hindlimb (Figure 2B) with immunohistochemistry studies. By comparison with tissue sections from the normal murine hindlimb, tissue sections from ischemic hindlimbs manifested a marked increase in Del-1 immunostaining, primarily in the interstitial space. Little intracellular staining was seen in the skeletal muscle cells. Competition with blocking peptide that was used to generate the antibody confirmed the specificity of Del-1 immunostaining (Figure 2C).

Angiogenic Effects of Del-1: Structure-Function Studies
Del-1 protein significantly increased angiogenesis as reflected by an increase in fibrovascular growth in the DAS (22.7±1.7 versus 12.2±3.6 mm², Del-1 versus vehicle, P<0.05). The angiogenic factor bFGF also significantly stimulated angiogenesis (23.3±4.0 versus 12.2±3.6 mm², bFGF versus vehicle, P<0.05). Del-1–induced angiogenesis was abrogated when the RGD domain of Del-1 was mutated to RAD (Del-1 mutant, 11.8±0.9 mm², P<0.05 by comparison with Del-1). Similarly, Del-1–induced angiogenesis was abrogated by a deletion in the amino terminal discoidin-I–like domain (Del-1 minor, 10.8±1.7 mm², P<0.05 by comparison with Del-1; Figure 3).
Capillary density was calculated as the ratio of capillaries to myocytes in the murine ischemic hindlimbs. After 3 weeks of daily Del-1 major protein intramuscular injection, angiogenesis was significantly increased on the basis of the elevation in the capillary-to-myocyte ratio (3.67±0.20 versus 2.28±0.17, Del-1 versus vehicle, P<0.05; Figure 4). Signal intensity–versus-time curves, plotted by spatially averaging the signal intensities over the ischemic hindlimb, were consistent with greater limb blood flow in the Del-1–treated animals (data not shown).

**Discussion**

This is the first report that Del-1 is expressed in adult animals in response to ischemia and that it plays an important role in adult angiogenesis. The protein encoded by Del-1 plays a critical role in embryonic vascular development. However, at the time of birth, the gene becomes quiescent, and Del-1 is no longer expressed in normal adult tissues.

**Angiogenic Effects of Del-1 in the Ischemic Hindlimb**

**Capillary Density**

Capillary density was calculated as the ratio of capillaries to myocytes in the murine ischemic hindlimbs. After 3 weeks of
In the embryo, Del-1 is expressed by endothelial progenitor cells and is secreted into the extracellular matrix. Del-1 supports adherence and migration of endothelial cells, mediated largely through the αβ3 integrin receptor. Accumulating evidence indicates that angiogenesis requires signaling through both growth factor and integrin signaling pathways. Endothelial cells deprived of either influence will undergo programmed cell death. Indeed, in the chick CAM assay, antibodies directed against αβ3 suppress Del-1–induced angiogenesis. A mutant form of Del-1, when the RGD motif has been altered (RGD→RAD), also disrupts angiogenesis in this model. These studies indicate that Del-1 activates angiogenesis in part by an integrin signaling pathway. Consistent with this hypothesis is the observation that cultured endothelial cells adhere to and spread on Del-1 in much the same way that they interact with other known integrin ligands such as vitronectin. Furthermore, integrin signaling pathways are activated in these cells, as manifested by the recruitment of talin and vinculin into highly phosphorylated focal complexes. Moreover, adherence to Del-1 triggers the phosphorylation of p125FAK, Shc, and MAPK with a magnitude and time course similar to that observed with the known integrin ligands vitronectin and fibronectin. Taken together, these data indicate that Del-1 supports endothelial cell adherence and triggers an integrin-mediated signaling cascade that participates in the angiogenic response.

In addition to promoting adherence and migration, Del-1 likely acts as an endothelial cell survival factor via its interaction with αβ3. By inducing expression of the bcl-2 gene, a known antiapoptotic gene, αβ3 opposes endothelial cell apoptosis. In cultured dermal microvascular endothelial cells, vascular endothelial factor potentiates endothelial tube formation. This effect is associated with an increased expression of integrin αβ3, as well as the bcl-2 gene. By contrast, antagonists to αβ3 induce endothelial expression of the p53 gene and cause apoptosis of vascular endothelial cells.

Del-1 protein comprises 3 epidermal growth factor repeats and 2 discoidin-I-like domains. An RGD motif of Del-1 is critical for its angiogenic potency. Mutation of the RGD to RAD sequence reduces its angiogenic effect in the CAM assay to one third of that of the native protein. In the present study, we observed that the angiogenic effect of Del-1 was abrogated by the RGD to RAD mutation. A minor form of the Del-1 protein is elaborated by endothelial cells, in which the discoidin-I-like domain is truncated. We observed that this minor form of Del-1 lacked angiogenic effects.

Although Del-1 is not ordinarily expressed after birth, we now provide immunohistochemical evidence that ischemia reactivates the expression of Del-1. In our model, Del-1 was heavily expressed in the ischemic hindlimb. However, we did not definitively identify the source of Del-1 in the ischemic hindlimb. Although Del-1 has an endothelial cell–restricted pattern of Del1 expression in the embryo, in the adult, tumor cells and endothelial cells are capable of expressing this protein. It is possible that infiltrating endothelial progenitor or inflammatory cells could contribute to the accumulation of Del-1 in the interstitial space. Nevertheless, we have clearly shown that Del-1 has angiogenic properties in the setting of ischemia in the adult animal. Intramuscular administration of Del-1 into the ischemic hindlimb augmented angiogenesis, as demonstrated by capillary densitometry. Furthermore, the increase in capillary densitometry was functionally significant, as demonstrated by increases in MR perfusion, an indicator of limb blood flow.

To conclude, this investigation revealed that Del-1, a protein expressed during embryonic vasculature development, is reexpressed in the setting of ischemia. Exogenous Del-1 enhances the angiogenic response to ischemia. The angiogenic effect of Del-1 is mediated in part by activation of integrin-mediated signaling and requires an RGD motif and a discoidin-I-like domain. Additional studies are warranted to determine its potential for therapeutic angiogenesis. Indeed, a trial of Del-1 gene therapy for treatment of peripheral arterial disease is now imminent?

**Note Added in Proof**

Two recent articles have suggested that gene transfer of plasmid or adenoviral constructs encoding Del-1 may improve blood flow and/or tissue function in murine, rabbit, and porcine models of ischemia.

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