Pericyte Regulation of Vascular Remodeling Through the CXC Receptor 3

Richard J. Bodnar, Margaret E. Rodgers, William C.W. Chen, Alan Wells

Objective—To understand the role, if any, played by pericytes in the regulation of newly formed vessels during angiogenesis. In this study, we investigate whether pericytes regulate the number of nascent endothelial tubes.

Approach and Results—Using an in vitro angiogenesis assay (Matrigel assay), we demonstrate that pericytes can inhibit vessel formation and induce vessel dissociation via CXCR3-induced involution of the endothelial cells. In a coculture Matrigel assay for cord formation, pericytes prevented endothelial cord formation of human dermal microvascular endothelial cells but not umbilical vein endothelial cells. Blockade of endothelial CXCR3 function or expression inhibited the repressing effect of the pericytes. We further show that pericytes are also able to induce regression of newly formed microvascular cords through CXCR3 activation of calpain. When CXCR3 function was inhibited by a neutralizing antibody or downregulated by siRNA, cord regression mediated by pericytes was abolished.

Conclusions—We show for the first time that pericytes regulate angiogenic vessel formation, and that this is mediated through CXCR3 expressed on endothelial cells. This suggests a role for pericytes in the pruning of immature vessels overproduced during wound repair. **(*Arterioscler Thromb Vasc Biol*. 2013;33:2818-2829.)**

Key Words: angiogenesis ■ CXC chemokine receptor 3 ■ endothelial cells ■ pericytes ■ wound healing

Downloaded from http://ahajournals.org by on March 5, 2022

The replacement of lost tissue after injury requires the formation of stable vessels. During wound healing, angiogenesis is key to the regeneration of the damaged tissue.[1] The regenerative phase produces an overexuberance of vessels. However, the majority of these nascent blood vessels involute during the resolution of the wound that results in mature functional tissues. Recent studies have defined a key signaling network driving cellular and vessel involution during the resolution phase, the CXCR3 system[2–5]; however, these works have focused on major structural cellular elements, keratinocytes, fibroblasts, and endothelial cells, and have not accounted for the regulatory cells that are proposed to stabilize vessels, the pericytes. We now query whether vascular pericytes, cells that have been shown to play a significant role in wound repair and vessel maturation,[6–8] contribute to the involution of these vessels or rescue some vessels.

Pericytes are vascular mural cells that are found interacting with the abluminal surface of endothelial cell of capillaries, arterioles, and venules.[9] Although their function is not fully understood, pericytes have been found to regulate capillary diameter and blood flow,[10,11] vessel permeability, and stabilization.[8,12] However, the role of pericytes during vessel regression late in wound repair has not been probed. Herein, we provide evidence that pericytes promote in vitro vessel dissociation and perversely likely drive much of the vascular regression.

During wound resolution, the extensive newly formed vascular network regresses, and the remaining vessels attain a mature state. The mechanisms regulating regression and maturation are not well defined. Although, pericyte–endothelial interaction has been associated with mechanical stability of microvessels, their influence on vessel remodeling more likely results from the activation of signals that regulate endothelial function.[8,13] The molecular mechanisms by which pericytes regulate vessel maturation are not well understood. The majority of studies on vascular regression have investigated protection of pericyte-associated vessels. Few studies have looked at the role pericytes play in modulating remodeling and maturation of newly formed vessels before stable interactions with pericytes.

Several ligand–receptor systems have been implicated in regulating vessel stability. Of particular interest for vascular involution are the ELR-negative chemokines (CXCL4/ platelet factor 4, CXCL9/MIG, CXCL10/IP-10, and CXCL11/IP-9) and their receptor CXC receptor 3 (CXCR3), which have been shown to play an important role in regulating angiogenesis.[2,3,5,17] During the wound healing process, these ELR-negative chemokines limit the function of fibroblasts and endothelial cells during the resolving phase.[14–16] The receptor for these ligands, CXCR3, has been found to prevent angiogenesis and induce vessel dissociation of new tubes.[2,3] Coincident with this role, CXCR3 expression is significantly upregulated on endothelial cells during

Received on: November 29, 2012; final version accepted on: October 3, 2013.

From the Department of Pathology (R.J.B., M.E.B., A.W.) and Department of Bioengineering (W.C.W.C.), University of Pittsburgh, PA; and Pittsburgh Veterans Affairs Medical Center, Pittsburgh, PA (R.J.B., M.E.B., A.W.).

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.302012/-/DC1.

Correspondence to Richard J. Bodnar, Veterans Affairs Medical Center, Department of Pathology, University Drive C, Pittsburgh, PA 15240. E-mail rjb8@pitt.edu

© 2013 American Heart Association, Inc.

Nonstandard Abbreviations and Acronyms	
CXCR2	CXC chemokine receptor 2
CXCR3	CXC chemokine receptor 3
CXCR3B	CXC chemokine receptor 3 B-isoform
GFR	growth factor reduced
IP-10/CXCL10	interferon-γ–inducible protein 10 kDa
IP-9/I-TAC/CXCL11	interferon-γ–inducible protein 9 kDa, interferon-inducible T-cell alpha chemoattractant
Mig/CXCL9	monokine induced by interferon-γ
Myf-5	myogenic factor 5
Pax-7	paired box protein 7

angiogenesis,[2] whereas on mature/senescent vessels, CXCR3 expression is practically nonexistent.[17] Thus, CXCR3 activation may play a significant role in regulating vascular pruning and opens an interesting avenue for control of neovessels.

Using an in vitro Matrigel assay, we demonstrate that pericytes inhibit microvascular endothelial cord formation and that the dissociation of newly formed cords was mediated through the activation of CXCR3 on endothelial cells. We show that inhibiting CXCR3 function on microvascular endothelial cells blocked the ability of pericytes to induce cord dissociation. Furthermore, pericytes stimulated by interferon-γ (IFN-γ) secrete CXCR3 ligands. These results indicate a novel function of pericytes, namely as regulators of vascular pruning.

Materials and Methods

Material and Methods are available in the online-only Supplement.

Results

Pericytes Inhibit Endothelial Tube Formation

Pericytes have been suggested to play an important role in vessel maturation; however, the mechanism by which pericytes modulate the transition from immature, endothelial cell-only small caliber vessels to stable mature pericyte-supported capillaries is not well understood. Also, it is not known whether pericytes facilitate angiogenesis. However, during the process of wound healing, pericytes associated with small vessels in mature skin only reappear with vessels during the late regeneration/early resolution phase transition (Figure II in the online-only Data Supplement). Hence, we wanted to determine whether pericytes play a role in vessel formation.

Pericytes were isolated from human skeletal muscle biopsies[18] and purified by flow cytometry as $CD146^+/CD34^-/CD45^-/CD56^-$ population.[18,19] These cells express the pericyte marker CD146 but do not express the myogenic marker CD56, indicating they are not conventional myogenic progenitor cells (Figure IIIa in the online-only Data Supplement). In vivo cells expressing these surface antigen profile were found surrounding endothelial cells and tightly associated with microvascular structures (arterioles, venules, and capillaries), thus are designated as pericytes.[19] These pericytes remained homogenous in long-term culture and stained positive for typical pericyte markers; chondrotin sulfate proteoglycan 4, α-smooth muscle actin, and platelet derived growth factor receptor-β, verifying the consistency of their phenotype in culture (Figure IIIb in the online-only Data Supplement). Furthermore, the expression of these pericyte markers was stable up to passage 14 (Figure IIIb in the online-only Data Supplement). The expression of these markers was compared with primary retinal pericytes and primary adult human MSC. The pericytes from muscle had similar expression levels of desmin, platelet derived growth factor receptor-β, chondrotin sulfate proteoglycan 4, VEGFR1, and Tie-2 as retinal pericytes (Figure IV in the online-only Data Supplement). Human MSCs show low-to-no expression of these markers (Figure IV in the online-only Data Supplement). Although the muscle-isolated pericytes have been shown to possess some myogenic potential with the ability to generate myotubes in specific culture media,[20] these cells do not express any known myogenic cell markers (MyoD, myogenin, m-cadherin, myf-5, and Pax-7) and thus are not considered as mesenchymal progenitors.

Using an in vitro angiogenesis assay,[2,3] endothelial cells and pericytes were coincubated on growth factor reduced (GFR) Matrigel in serum-free MCDB131 media and then analyzed for the formation of endothelial cords. In our initial coculture system, the ratio of pericytes to endothelial cells was 1 to 10. We observed that coculture of HMEC-1[21] (immortalized dermal microvascular endothelial cells) and pericyte on Matrigel leads to the formation of cellular aggregates (Figure 1A; EC:pericytes) not the web-like cord structures typically formed by endothelial cells on Matrigel (Figure 1A; EC only). These cellular aggregates were not observed when pericytes were plated individually on Matrigel in the same culture media (Figure 1A; pericytes only).

Based on the reported pericyte-endothelial ratios observed in vivo, which range from as high as 1:1 in the retina to concentrations as low as 1:100 in the peripheral vasculature,[13,22] we wanted to determine the effects of altering the pericytes to endothelial cells ratio in our model system. Pericytes were labeled with cell tracker (Red, CMTPX, Molecular Probes) before coculture on Matrigel to identify pericyte location within the cord structures. Using primary adult dermal microvascular endothelial cells (dHMECs) at endothelial cell (EC)-pericyte (peri) ratios of 10:1 and 20:1, cellular aggregates and a significant decrease in endothelial cord area were observed compared with endothelial cells incubated on Matrigel in the absence of pericytes (Figure 1B and 1D). Labeled pericytes were found in the endothelial cells aggregates (Figure 1B). When the EC:pericyte ratio reached 80:1, the formed endothelial cords were similar to the cords generated in the absence of pericytes (Figure 1B and 1D). We found similar results with the immortalized dermal endothelial cell line HMEC-1 (Figure V in the online-only Data Supplement). In addition, when dHMECs were incubated with primary human retinal pericytes, similar results were observed; however, when cocultured with hMSCs cord formation was not affected (Figure VI in the online-only Data Supplement). We also examined human umbilical vein endothelial cells (HUVECs) because these cells are the primary endothelial cells used most often when studying pericyte–endothelial interaction. HUVEC-pericyte cocultures were incubated on GFR Matrigel under the same conditions as the dHMECs and analyzed for cord formation (Figure 1C). Unlike what was

Downloaded from http://ahajournals.org by on March 5, 2022

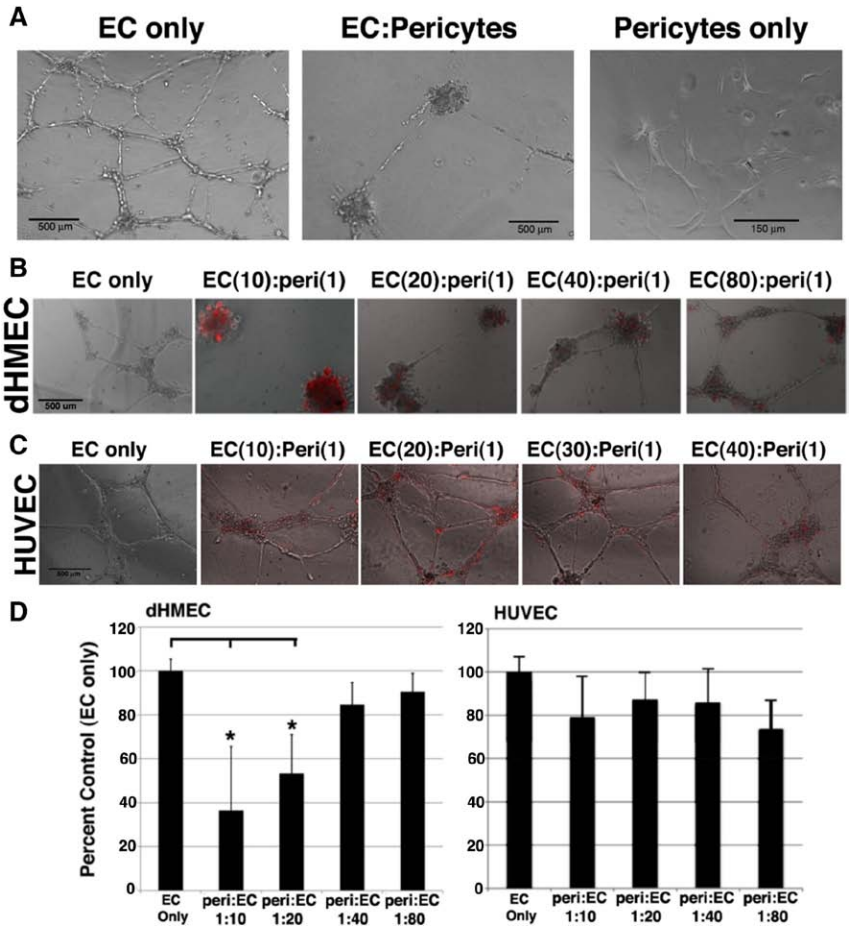

Figure 1. Pericytes inhibit microvascular endothelial cord formation. Color figure B and C can be viewed in the online-only Data Supplement. **A**, HMEC-1, pericytes, and a coculture of endothelial (10)-pericyte (1) were grown on GFR Matrigel. After 18 hours, incubation cords were formed in the wells containing only endothelial cells (EC only). In the coculture (EC-pericytes), no cords were formed but large cellular aggregates containing pericytes and endothelial are observed. The scale bar for the EC only and EC:pericytes figures represents 500 μm, and the pericytes only represents 150 μm. **B**, Primary adult dermal microvascular endothelial cells (dHMECs), and (**C**) HUVECs (unstained) were cocultured with different concentrations of pericytes (white) on GRF Matrigel for 18 hours to allow cord formation. The scale bar in the figure represents 500 μm. **D**, Quantification of the endothelial cord area was determined using MetaMorph. EC:pericytes ratios of 10:1 to 40:1 caused a significant decrease in cord formation compared with EC only. The pericyte-induced decrease in cord formation was not observed when cocultured with HUVECs. A magnification of 4× was used to image the formed tubes. Images are a representative of 5 individual wells. For the graph n=5 (average ± SEM); **P*<0.05 paired Student *t* test.

observed with dHMECs, the ability of HUVECs to form cords was unaffected by the presence of pericytes (Figure 1C). Even at a high pericyte to EC ratio (1:10), cords were still observed with HUVECs. At the different ratios examined, the number of formed cords was consistent with that observed with HUVECs alone (Figure 1D). Even at ratios of 1:1 and 1:5, we did not observe any significant difference in HUVEC cord formation when cocultured with pericytes (data not shown). To verify that inhibition of cord formation was pericyte mediated and not attributable to the addition of exogenous cells, fibroblasts were chosen because of their presence in wound healing and their ability to promote angiogenesis.[23] Endothelial cells (dHMECs and HMEC-1) were incubated with fibroblasts. Coincubation with the fibroblasts had no effect on cord formation (Figure VII in the online-only Data Supplement). Because pericytes have been shown to have multilinage differentiation capacity[19,24] and express mesenchymal marker, we analyzed whether hMSC isolated from bone marrow retained similar inhibitory properties. We found that hMSC cocultures did not inhibit endothelial cord formation (Figure VI in the online-only Data Supplement). Although the ability of pericytes to inhibit dermal endothelial cord formation was unexpected, our results showed some similarities to previous work by Orlidge and D'Amore.[25] Orlidge and D'Amore showed that cocultures of EC/retinal pericyte at ratios from 1:1 to 20:1 caused inhibition of endothelial cell proliferation. Incubation with ratios greater than EC/pericyte 20:1 showed a diminished inhibition. The inability of the pericytes to communicate with the vast number of endothelial cells was suggested as the reason for the diminished inhibition. Taken together, these data suggest that pericytes negatively regulate the ability of dermal microvascular endothelial cells (HMECs-1 and dHMECs) but not vein endothelial cells (HUVECs) to form cords in this model system. In addition, these results are highly suggestive that pericyte–endothelial cell interaction is important for pericyte regulation of endothelial function during angiogenesis.

Pericyte-Mediated Dissociation of Newly Formed Endothelial Cords

Recent work from several laboratories has shown a role for pericytes in vessel stabilization.[8,26,27] Also, pericyte association with HUVECs has been found to promote conditions facilitating tube stabilization through inhibition of MMPs, growth factor production, and matrix formation.[13,28,29] One possible explanation of the above findings could be that pericytes quiesce endothelial cells and thereby prevent cord development, but similar cell shutdown might stabilize formed vessels.

Thus, we generated endothelial cords using the Matrigel assay and subsequently exposed these formed endothelial cords to pericytes. When pericytes were incubated with HUVEC cords, no significant dissociation was observed (Figure 2), recapitulating earlier coculture findings. However, when HMECs-1 or dHMECs were allowed to form cords on incubation on GFR Matrigel and then exposed to pericytes, the cords collapsed,

Downloaded from http://ahajournals.org by on March 5, 2022

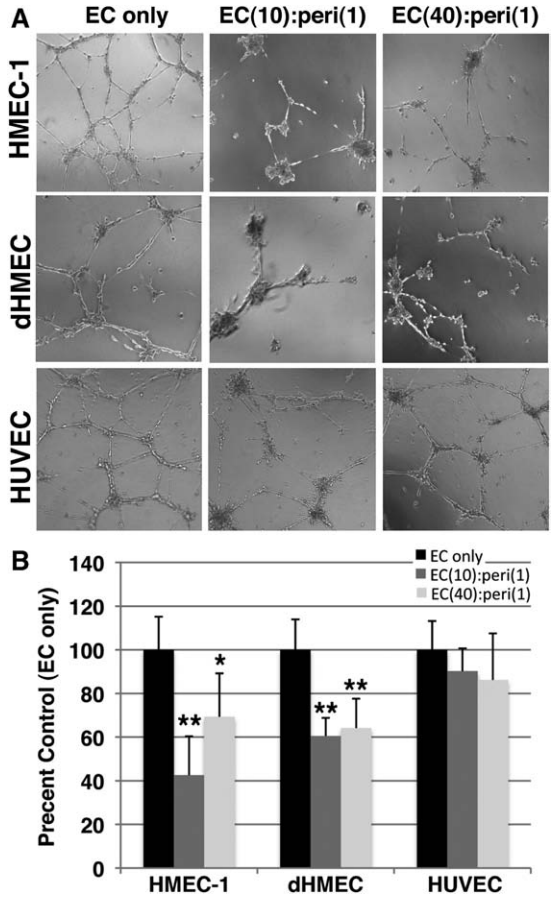

Figure 2. Pericytes induce microvascular endothelial cord dissociation. **A**, HMEC-1, primary adult dermal microvascular endothelial cells (dHMECs) and HUVECs (unstained) were incubated on GFR Matrigel and allowed to form cords. The formed cords were incubated with different concentrations of pericytes (red; [EC{10}:peri{1} or EC{40}:peri{1}]) or without pericytes (EC only) for an additional 15 hours. Pericyte incubation with dermal endothelial cords caused a significant dissociation of cord structures compared with endothelial cords without pericytes. Pericyte-mediated dissociation of cord structures was not observed with HUVECs. Images are a representative of 5 individual wells. **B**, Quantification of the endothelial cord area was determined using MetaMorph. Incubation of dermal endothelial cords with pericytes at ratios of 10:1 and 40:1 (endothelial:pericytes) caused a significant increase in cord dissociation compared with endothelial cells alone (EC only). The pericyte-induced cord dissociation was not observed when cocultured with HUVECs. n=5 (average±SEM); $^{**}P<0.01$ and $^{*}P<0.05$ paired Student *t* test.

and the endothelial cells formed endothelial-pericyte aggregates (Figure 2) similar to the coculture aggregates in Figure 1A and 1B. The cord collapse/dissociation was not attributable to an extended incubation on the Matrigel because the wells only containing endothelial cells maintained a web-like cord structures (Figure 2; EC only). In addition, incubation of retinal pericytes with dHMEC cords promoted cord dissociation similar to the pericytes isolated from muscle; however, dissociation was not observed when dHMEC cords were incubated with hMSC (Figure VIII in the online-only Data Supplement). Additionally, we show under our experimental conditions that endothelial cords incubated in MCDB131 media without serum or supplements are stable up to 48 hours.[2,3] The addition of 5% serum caused the cords to dissociate after 8 hours (Figure I in the online-only Data Supplement). These data suggest that pericytes isolated from different tissue can induce the dissociation of newly formed dermal endothelial cords but hMSC do not promote cord destabilization. Furthermore, the data indicate that pericyte regulation of vessel formation may be dependent on endothelial linage because inhibition was observed with dermal microvascular (HMEC-1, dHMECs) but not large vein (HUVECs) endothelial cells, and it is well recognized that endothelial cells differ according to type, function, and location.[30] In aggregate, these data provide new evidence that pericytes can be inhibitory to the development and stabilization of newly formed microvessels.

CXCR3 Activation Is Responsible for Pericyte Inhibition of Endothelial Function

CXCR3 is expressed as 2 isoforms (CXC chemokine receptor 3 A-isoform and CXC chemokine receptor 3 B-isoform [CXCR3B]), but endothelial cells have only been found to express CXCR3B.[17] We have previously demonstrated that activation of CXCR3B inhibits endothelial migration[3] and causes vessel dissociation.[2] Also, it has been shown by us and others that HUVECs express few if any CXCR3.[2,31,32] Because endothelial cells only express CXCR3B,[17] we investigated whether CXCR3 expression is responsible for the pericyte-mediated effects on HMEC-1 and dHMEC cord formation and dissociation (Figures 1 and 2). The treatment of dermal microvascular ECs (HMEC-1 and dHMECs) with CXCR3 neutralizing antibody (recognizes both CXCR3 isoforms) enhanced the ability of the endothelial cells to form cords when cocultured with pericytes compared with IgG treatment, whereas treatment of HUVECs with CXCR3 neutralizing antibody had no significant effects on cord formation (Figure IX in the online-only Data Supplement). As an additional control, endothelial cells were treated with an anti-CXC chemokine receptor 2 neutralizing antibody; this antibody did not alter the pericyte-mediated cord inhibition (Figure X in the online-only Data Supplement). To further test whether CXCR3 is responsible for inhibition of cord assembly, CXCR3 expression was downregulated with CXCR3 siRNA (that targets both CXCR3 mRNA species). CXCR3 expression was significantly downregulated in the dermal endothelial cells up to 4 days after transfection (Figure 3A). CXCR3 expression is virtually nonexistent in HUVECs and completely absent with CXCR3 siRNA treatment (Figure 3A); this is similar to the negligible expression we have previously shown by flow cytometry.[2] The siRNA-treated endothelial cells were coincubated with pericytes (EC[20]:peri[1]) and plated on GFR Matrigel 2 to 4 days after siRNA treatment. Downregulation of CXCR3 enhanced the ability of dHMECs to form cords in the presence of a high concentration of pericytes but had no effect on HUVECs (Figure 3B and 3C). dHMECs treated with control siRNA had a significant decrease in cord formation compared with the CXCR3 siRNA–treated dHMECs (Figure 3C).

To extend this putative mechanism and demonstrate that endothelial cord dissociation (Figure 2) was attributable to CXCR3 expression on endothelial cells, CXCR3 siRNA–transfected microvascular cells (HMEC-1 and dHMECs) were allowed to form cords then incubated with varying concentrations of pericytes. Incubation with pericytes did not disrupt formed cords

Downloaded from http://ahajournals.org by on March 5, 2022

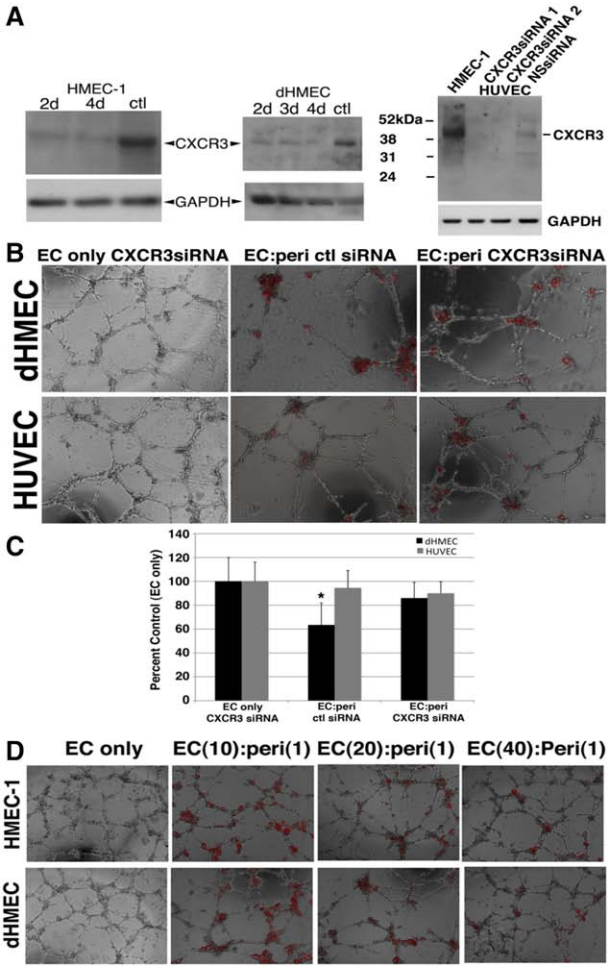

Figure 3. Knockdown of CXCR3 reversed the inhibitory effects of pericytes on cord formation. Color figure B and D can be viewed in the online-only Data Supplement. **A**, Western blot of HMEC-1 and primary adult dermal microvascular endothelial cells (dHMECs) 2 to 4 days and HUVEC 4 days after CXCR3 siRNA transfection. Control was 4 days after siRNA transfection with a nonspecific oligo (ctl). The CXCR3 antibody (ProteinTech) specifically recognizes CXCR3B. **B**, dHMECs and HUVECs were transfected with nonspecific siRNA (ctl) or CXCR3 siRNA. Between 2 to 4 days after transfection, the endothelial cells were cocultured with pericytes at a ratio of 20:1 (EC:peri) and incubated on GFR Matrigel for 18 hours. **C**, Quantification of the endothelial cord area was determined using MetaMorph software. **D**, Dermal endothelial cells (HMECs-1 and dHMECs) were transfected with CXCR3 siRNA, then cocultured with pericytes at various ratios on GFR Matrigel; 4× objective was used to image the formed tubes. Treatment of HMEC-1 and dHMEC cords with CXCR3 siRNA enhanced endothelial cord formation in the presence of pericytes when compared with control siRNA treated dermal ECs. n=3 (average±SEM); **P*<0.05 paired Student *t* test.

(Figure 3D). These data provide new evidence that pericytes can negatively regulate dermal microvascular formation and mediate destabilization of nascent vessels through activation of CXCR3.

CXCR3B Expression Is Responsible for Pericyte-Mediated Inhibition of Cord Formation

To investigate the role of CXCR3B in pericyte-mediated inhibition of dermal microvascular endothelial cord formation, HUVECs were transfected to express CXCR3B. Immunoblot analysis demonstrated that the HUVEC-CXCR3B transfectants (HUVEC-3B) had a 5-fold increase in CXCR3, a level that was comparable with what was observed in HMEC-1 cells (Figure 4A). We have previously shown that HMEC-1 cells have a 6- to 7-fold increase in CXCR3 surface expression compared with HUVECs, thus our Western blot data are in line with our flow cytometry data.[2] The cells were cocultured with pericytes and then incubated on GFR Matrigel for 18 hours to allow cord formation. The presence of pericytes with the HUVEC-CXCR3B inhibited cord formation similarly to their effect on HMEC-1 cells (Figure 4B and 4C). Pericytes did not impede HUVEC cord formation transfected with a control empty vector (Figure 4B and 4C). Thus, increasing CXCR3 expression levels in HUVECs to that observed in dermal endothelial cells enables pericytes to inhibit cord formation, further supporting the role of CXCR3 in mediating inhibition of microvessel endothelial cord formation by pericytes.

Pericyte Secretion of CXCR3 Ligands Inhibits Cord Formation and Mediates Cord Dissociation

Our model of pericyte–endothelial cell communication posits that the pericytes secrete CXCR3 ligands that would act on the endothelial cells inhibiting their function. It has recently been shown that pericytes secrete IP-9/CXCL11 when treated with IFN-γ.[33] We analyzed IFN-γ–treated pericytes to verify that our pericytes produce CXCR3 ligands. IFN-γ treatment induced IP-9 and IP-10 production and secretion (Figure XI in the online-only Data Supplement). Although these data demonstrate that pericytes can secrete CXCR3 ligands and have the potential to negatively regulate endothelial function via interaction with CXCR3, we wanted to verify that these CXCR3 ligands are capable of mediating the effect of pericytes on endothelial cord formation and dissociation.

To identify whether the factors secreted from activated pericytes act in a paracrine manner and are responsible for the negative regulation of endothelial cell cord formation, dHMECs and HUVEC were resuspended in IFN-γ–stimulated pericyte conditioned media. Incubation of the dHMECs in pericyte conditioned media not exposed to IFN-γ showed similar cord formation as dHMECs incubated in MCDB131 media (control). However, when dHMECs were incubated in IFN-γ–stimulated pericyte conditioned media, a significant decrease in cord formation was observed compared with control (MCDB131; Figure 5A and 5B). Incubation of HUVECs in either of the pericyte conditioned media had no significant effects on cord formation (Figure 5A and 5B). To verify whether the observed inhibition of cord formation by pericyte conditioned media was mediated by CXCR3 ligands, dHMECs were incubated with CXCR3 neutralizing antibody. The dHMEC monolayer was incubated with CXCR3 neutralizing antibody (1 μg/mL) or equal concentration of species matched IgG for 30 minutes before detaching. The cells were resuspended in pericyte conditioned media with additional CXCR3 neutralizing antibody (1 μg/mL) or IgG and then incubated on GFR Matrigel for 18 hours. Incubation of dHMECs with CXCR3 neutralizing antibody blocked the inhibitory effects of pericyte conditioned media on cord formation (Figure 5C and 5D), suggesting that

Downloaded from http://ahajournals.org by on March 5, 2022

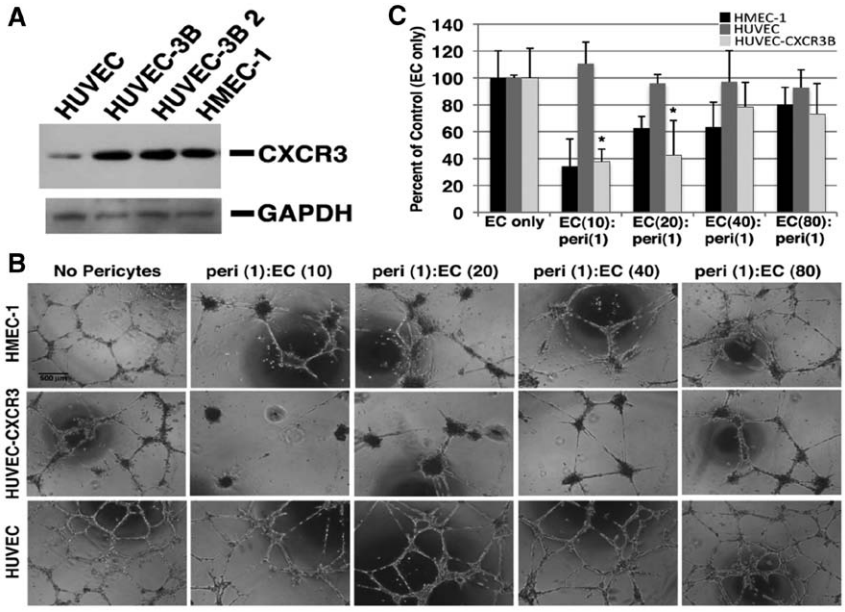

Figure 4. Overexpression of CXCR3B on HUVECs inhibits cord formation in pericyte coculture. **A**, HUVEC p7 overexpressing CXCR3B (HUVEC-3B), HUVEC p7 vector transfected (HUVECs), and HMEC-1 were analyzed by Western blot for CXCR3 expression. The HUVEC-3B cells show CXCR3B expression levels equivalent to HMEC-1. GAPDH was used as a loading control for equal protein loading between samples. **B**, HUVECs, HUVEC-CXCR3, and HMEC-1 were cocultured with pericytes at a ratio from 1:10 to 1:80 (pericyte:endothelial) then incubated on GFR Matrigel. A magnification of 4× was used to image the formed tubes. The scale bar in the figure represents 500 μm. Images are a representative of 3 individual wells. **C**, Quantification of the endothelial cord area was determined using MetaMorph. Incubation of pericytes with HUVEC-CXCR3 at ratios of 1:10 and 1:20 caused a significant decrease in cord formation when compared with vector transfected HUVECs. The observed cord formation of pericytes with HUVEC-CXCR3 at ratios of 10:1 and 20:1 was similar to HMEC-1 at the same ratio. The ability of pericytes to inhibit the CXCR3 expressing HUVECs to form cords further suggests that CXCR3 plays a role in pericyte-mediated inhibition of cord formation. n=3 (average±SEM); *P<0.01 paired Student t test.

Downloaded from http://ahajournals.org by on March 5, 2022

pericyte-secreted CXCR3 ligands are the major factors responsible for pericyte-mediated inhibition of cord formation. In addition, treatment of endothelial cells with CXC chemokine receptor 2 neutralizing antibody did not reverse the inhibitory effects of pericyte conditioned media on cord formation, with the results being similar to those obtained in the presence of IgG (Figure XII in the online-only Data Supplement). We had previously shown that IP-10 treatment of HMEC-1 inhibited cell migration through a PKC-mediated pathway, and inhibition of EC migration was responsible for the inability of dermal ECs to from cords.[3] dHMEC migration was found to be inhibited when treated with IFN-γ–stimulated pericyte conditioned media, and this inhibition could be reversed when the cells were incubated with CXCR3 neutralizing antibody (Figure XIII in the online-only Data Supplement). PKC activation has also been shown to be activated in TNF-α–induced apoptosis.[34] In addition, pericytes were shown to inhibit endothelial proliferation, in a cell concentration–dependent manner.[25] Thus, we examined whether pericytes interaction with endothelial cells promoted endothelial cell apoptosis. Coculture of pericytes and endothelial cell on plastic for a 72-hour period did not enhance endothelial cell death (Figure XIV in the online-only Data Supplement). Together these data suggest that pericyte-mediated inhibition of cord formation is attributable to inhibition of endothelial cell function and not through induction of an apoptotic pathway under these experimental conditions.

To determine whether IFN-γ stimulation of pericytes has a greater inhibitory effect on endothelial cord formation, pericytes were stimulated with INF-γ for 4 hours. The pericytes were detached and coincubated with dHMECs at various concentrations. The cells were incubated for 18 hours on GFR Matrigel to allow for cord formation. The IFN-γ–stimulated pericytes showed a greater inhibition of cord formation at the 40:1 and 80:1 EC-pericyte ratio compared with nonstimulated pericytes (Figure 6A and 6B). To verify that the inhibition of tube formation by the IFN-γ–treated pericytes was attributable to the secretion of CXCR3 ligands, dHMECs were pretreated with CXCR3 neutralizing antibody (1 μg/mL) or nonspecific IgG and subsequently cocultured with IFN-γ–stimulated pericytes at varying EC-pericyte ratios in the presence or absence of CXCR3 neutralizing antibody. Incubation of the dHMECs with CXCR3 neutralizing antibody reversed the inhibitory effects of the INF-γ–stimulated pericytes (Figure 6C and 6D).

We have previously shown IP-10 treatment of dermal microvascular endothelial cord–induced dissociation through a μ-calpain–mediated cleavage of integrins.[2] To determine whether the observed pericyte-mediated cord dissociation (Figure 2) was attributable to activation of calpain, we pretreated HMEC-1 cords with a cell permeable calpain inhibitor (CI-1, ALLN) before the addition of pericytes. Treatment of the HMEC-1 cords with CI-1 significantly reduced pericyte-mediated cord dissociation compared with diluent at EC:pericyte ratios of 20:1 and 40:1 (Figure XV in the online-only Data Supplement). Taken together, these data suggest that IFN-γ–stimulated pericytes induce cord regression through CXCR3-mediated EC detachment from the basement membrane. These data provide supporting evidence that pericytes are able to cause regression of nascent microvessels through activation of CXCR3.

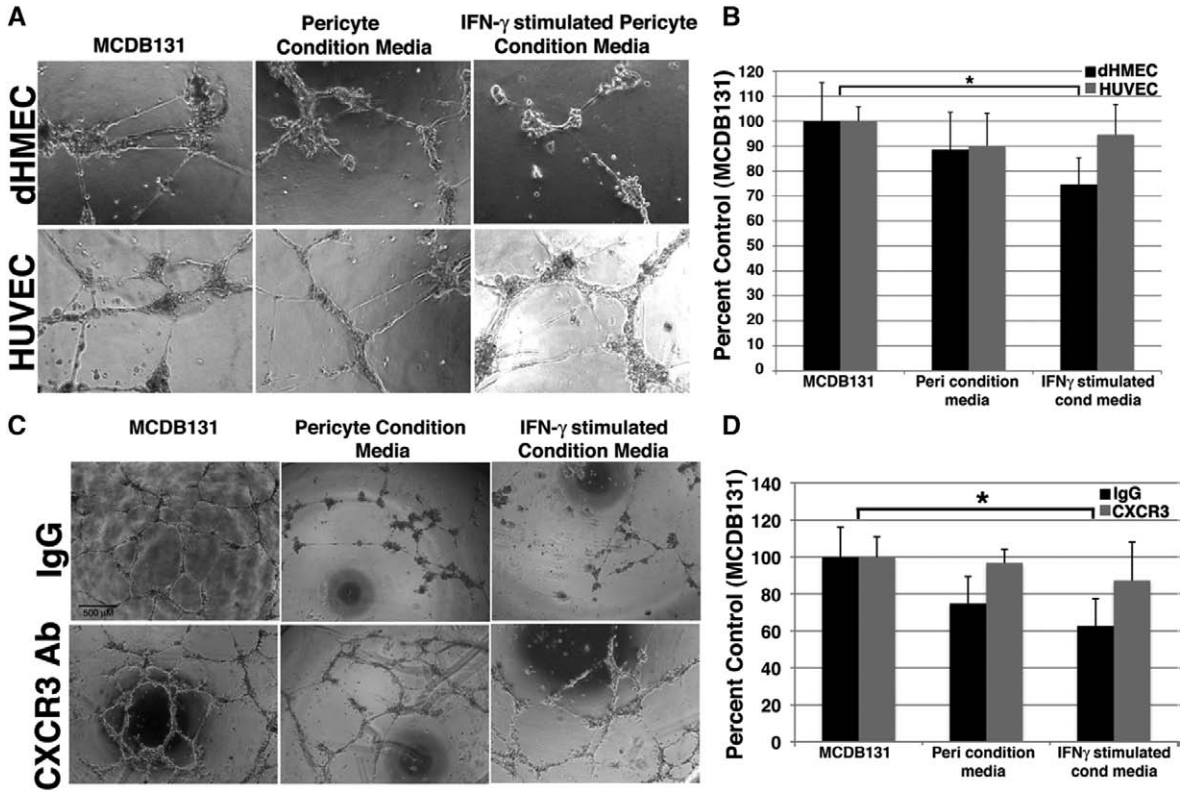

Figure 5. Pericyte condition media inhibit microvascular endothelial cord formation. **A**, HUVECs and primary adult dermal microvascular endothelial cells (dHMECs) were resuspended in MCDB131 or pericyte condition media then incubated on GFR Matrigel for 18 hours to allow cord formation. IFN-γ–stimulated pericyte condition media inhibited dHMEC cord formation but did not affect HUVECs. **B**, Quantification of the endothelial cord area was determined using MetaMorph. Incubation of dHMECs with IFN-γ–stimulated pericytes condition media caused a significant decrease in cord formation when compared with MCDB131 media. **C**, dHMECs were preincubated with CXCR3 neutralizing antibody or species matched IgG then resuspended in pericyte condition media containing antibody. The cells were incubated on GFR Matrigel to form cords. CXCR3 antibody treatment reversed the inhibitory effect of pericyte condition media on cord formation compared with IgG. **D**, Quantification of the endothelial cord area was determined using MetaMorph. Incubation of dHMECs with CXCR3 neutralizing antibody reversed the inhibitory effects of the IFN-γ–stimulated pericyte condition media compared with IgG. The results are of n=3 (average±SEM). *P<0.05 paired Student t test.

Discussion

The formation and persistence of new blood vessels is regulated by a complex system of signaling events. The basic steps in vessel formation include endothelial activation, migration, and proliferation, which are followed by the development of a vascular cord, lumen formation, stabilization, and finally vessel maturation. The pruning and stabilization steps in this process are necessary for the long-term stability of the newly formed vessel. Although extremely important for normal tissue homeostasis, little is known about the signaling pathways involved in this process. New approaches targeting angiogenesis as a therapeutic for various diseases with excessive angiogenesis (cancer, macular degeneration, rheumatoid arthritis, etc) have led to increased investigation of the signaling pathways regulating vessel maturation and stabilization. One of the hallmarks associated with vessel stabilization and maturation is the association of the mural cell, pericyte, or smooth muscle cell, with the newly formed vessel. It is generally agreed that mural cell interaction with endothelial cells promotes the maturation of the vessel but how these mural cells, in particular pericytes, regulate vessel stabilization is still unclear. Here, we provide new data indicating that pericytes have the ability to inhibit the formation of new vessels and to prune excessive immature vessels, presumably as a means to ultimately achieve vessel stabilization and maturation. We show that pericyte inhibition of dermal microvascular vessel formation is at least in part through activation of the chemokine receptor CXCR3.

Pericytes are mesenchymal derived cells that function as support cells and play a role in vascular homeostasis. Pericytes are most commonly found within the intimal layer of the basement membrane of capillaries and venules. Their long, highly branched dendrite-like cytoplasmic processes envelop the vessel establishing focal contacts allowing for the transmission of various signaling cues assisting in maintaining vascular structure and function.[12,13] Pericytes have been found to possess a variety of physiological functions, which include stabilization of vessels, regulation of vascular tone, and promotion of vessel maturation in addition to mesenchymal potentiality.[10,35] Recently, purified human muscle pericytes ($CD146^+/CD34^-/CD45^-/CD56^-$) have been shown to facilitate the repair of ischemic mouse myocardium through direct interaction and paracrine cross-talk with endothelial cells.[36] Moreover, adventitial pericyte progenitors derived from human saphenous veins strongly enhanced cord formation in Matrigel culture when cocultured with saphenous vein–derived endothelial cells and significantly augmented

Downloaded from http://ahajournals.org by on March 5, 2022

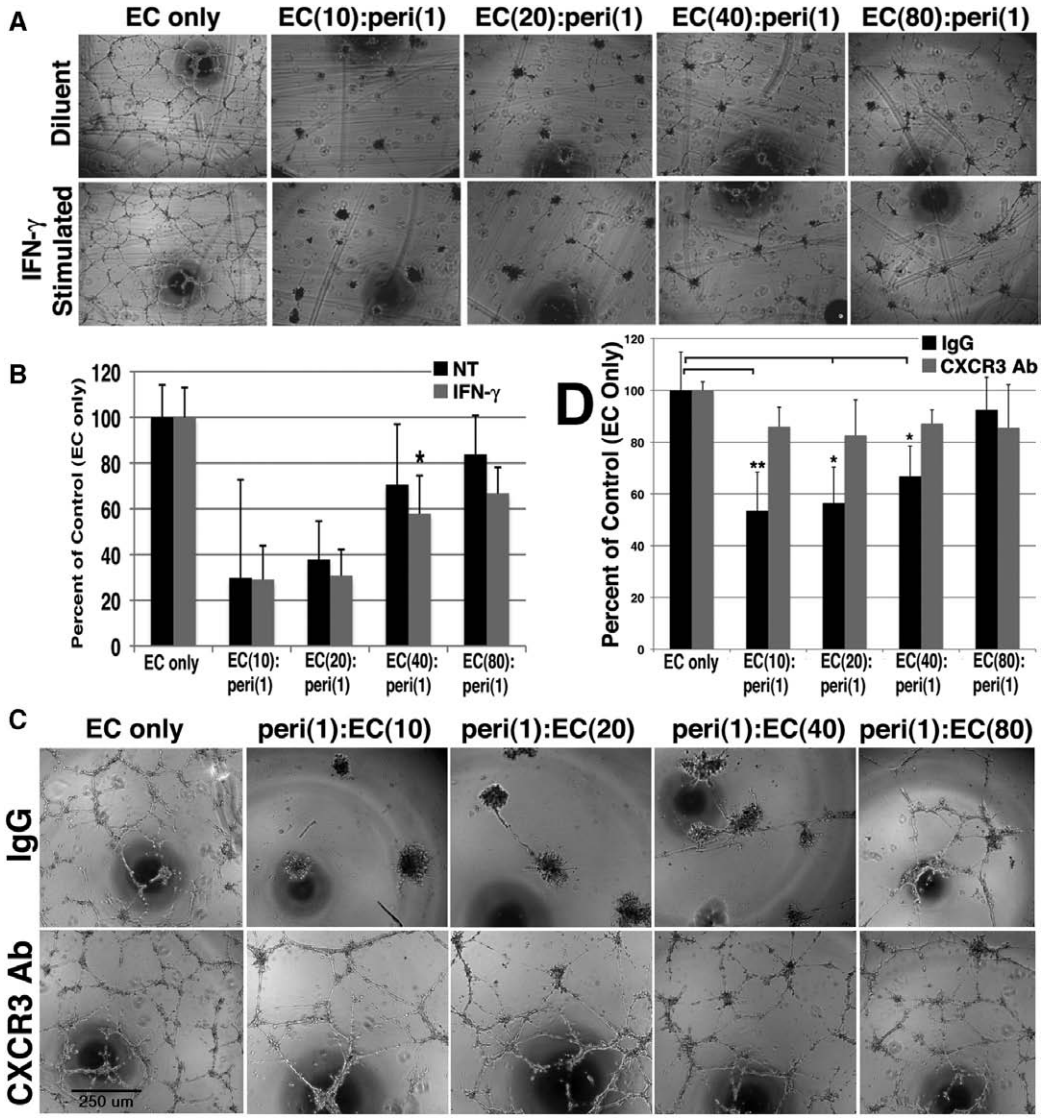

Figure 6. Endothelial cord formation is inhibited by IFN-γ–stimulated pericytes. Color figure A and C can be viewed in the online-only Data Supplement. **A**, Pericytes were treated with 25 nm IFN-γ (IFN-γ stimulated) or diluent for 4 hours. The pericytes were then coincubated with primary adult dermal microvascular endothelial cells (dHMECs) at indicated concentrations. The cells were incubated on GFR Matrigel for 18 hours to form cords. Incubation with IFN-γ–stimulated pericytes showed a decrease in dHMEC cord formation at the 40:1 and 80:1 dilution compared with control (diluent). **B**, Quantification of the endothelial cord area was determined using MetaMorph. IFN-γ stimulation of pericytes before coincubation with dHMEC showed a significant enhanced ability to inhibit dHMEC cord formation at the 40:1 dilution. Although, significance was not obtained at the 80:1 dilution, there was still a decrease in cord formation compared with diluent. The results are of n=3 (average±SEM). **P*<0.05 paired Student *t* test. **C**, dHMECs were pretreated with CXCR3 neutralizing antibody (1 μg/mL) or IgG for 1 hour. The cells were then coincubated with IFN-γ stimulated pericytes (25 nmol/L for 4 hours) at indicated concentrations. The cells were incubated on GFR Matrigel for 18 hours to form cords. Incubation of the cell with CXCR3 neutralizing antibody enhanced cord formation compared with IgG. **D**, Quantification of the endothelial cord area was determined using MetaMorph. Incubation of dermal endothelial cells with CXCR3 neutralizing antibody reversed the inhibitory effects of the IFN-γ–stimulated pericytes on cord formation when compared with nonspecific IgG. n=5 (average±SEM); **P*<0.05 paired Student *t* test.

angiogenesis when transplanted intramuscularly into ischemic hindlimbs and intramyocardially into infarcted myocardium in mouse models.[24,37]

For the most part, pericytes are thought to be a key regulator of vascular homeostasis, regulating vessel permeability and tone. Although there is much evidence for this, the evidence on the role of pericytes mediating vessel stabilization is conflicting. Although studies have shown that vessel regression was prevented through pericyte association,[28] other studies have shown that pericyte association did not prevent vascular involution.[38,39] Furthermore, the loss of pericyte association with the vasculature does not promote vessel destabilization inasmuch as their role in regulating microvessel structure.[40] Recent studies showed the ability of mesenchymal stem cells and pericyte to inhibit vessel formation.[41,42] Thus, pericytes may regulate both destabilization of nascent vessels and stabilization of mature vasculature.

In this study, we provide in vitro data indicating that pericytes can inhibit vessel formation and promote vessel dissociation. We found that human pericytes, isolated from muscle[18]

Downloaded from http://ahajournals.org by on March 5, 2022

and retina, are able to inhibit dermal microvascular cord formation but did not observe inhibition by hMSCs (Figure 1; Figure VI in the online-only Data Supplement). Here, we show that when pericytes and dermal endothelial cells are cocultured on matrigel at endothelial:pericyte ratios from 10:1 to 40:1, there is a significant decrease in the number of cords formed compared with endothelial cells alone, where as the addition of fibroblasts or hMSCs isolated from bone marrow did not affect cord formation (Figures VII and VIII in the online-only Data Supplement). It has been found that the physiological distribution of pericytes can be as high as 1 to 1 in the retina and as low as 1 to 100 in skeletal muscle, thus these results are not attributable to nonphysiological concentrations of endothelial cells and pericytes.

Because this assay is more vasculogenesis than angiogenesis, and current data indicating pericytes play a more significant role in angiogenesis, we changed the assay conditions to be more similar to in vivo conditions. Using the Matrigel assay, dermal endothelial cells were incubated on GFR Matrigel to allow cord formation. After cords were formed, pericytes were added and incubated further. This assay looks at the interaction of pericyte with newly formed cords. Our findings show that addition of pericytes, even at a ratio of 1:40, caused dissociation of dermal endothelial cords (Figure 2; Figure VIII in the online-only Data Supplement). These results do not fully accord with what others have previously observed in vitro.[24,36,43] We identified 2 major differences, which could be responsible for the contradictory results. In many studies, bovine pericytes[29,43] are used to investigate pericyte–endothelial interaction where our study uses human pericytes. Second, in a majority of in vitro studies investigating pericyte–endothelial interaction, the endothelial cells used are vein endothelial cells (HUVECs or SVECs),[24,36,43] HUVECs express low to no CXCR3.[31,32] To verify whether our results were a result of cell specificity, we performed the vasculogenesis Matrigel assay with HUVECs. Our results show that coincubation of our human pericytes isolated from muscle did not effect the ability of HUVECs to form cords (Figure 1). These results correlate with the in vitro findings from other studies that used HUVECs.[36,43] Furthermore, when HUVECs were transfected to express CXCR3B, the ability of these cells to form cords when cocultured with pericytes was inhibited similar to what is observed in microvascular endothelial-pericyte cocultures (Figure 4B and 4C; Figure VI in the online-only Data Supplement). In addition, we show that bone marrow–isolated hMSCs did not affect dermal endothelial cord formation (Figure VI in the online-only Data Supplement). These findings indicate that pericytes may differentially regulate specific subtypes of endothelial cells. When HUVECs were used in the cord dissociation assay, again we observed no significant dissociation of HUVEC cords (Figure 2). This provided further evidence to suggest that pericyte regulation of endothelial function may be dependent on cell linage.

We have indicated that a major difference between our studies and others is the origin of the pericytes (nonhuman) or endothelial type (vein). This caveat could possibly be observed in our studies. The pericytes used are from nondermal origin (muscle and retina), cells are obtained from different donors, possible phenotypic changes of the cells attributable to in vitro culturing of primary cells and the in vitro assay system. At this time, our in vitro findings are only suggestive with what may be occurring in vivo. The novelty of this study is it provides a physiological mechanism for neovascular dissociation that can occur in vivo during wound healing. Second, it provides a physiological function for CXCR3 in regulating vessel stability and a novel mechanism for pericyte function.

CXCR3 expression on dermal endothelial cells was first described by Salcedo et al[32] providing a mechanism by which the CXCR3 ligands IP-10 and platelet factor 4 can inhibit endothelial function but its physiological function is still not understood. Our previous studies[2] showed that the ELR-negative chemokines (platelet factor 4 [CXCL4] and IP-10 [CXCL10]) and their receptor CXCR3 were responsible and played a significant role in mediating vessel dissociation, but whether physiological vascular regression occurred through activation of CXCR3 was not established. When CXCR3 was blocked, the inhibitory effects of the pericytes on cord formation were reversed (Figure IX in the online-only Data Supplement). This suggested that activation of CXCR3 was responsible for the pericyte-induced inhibition. It is unknown whether pericytes are able to secrete IP-9 (CXCL11), IP-10 (CXCL10), or Mig (CXCL9); therefore, we looked at whether stimulation of pericytes with interferon-γ (IFN-γ), a known stimulator of IP-9, IP-10, and Mig secretion from inflammatory cells. Mesangial cells, mural cells of the kidney, have been shown to express the CXCR3 ligand, IP-10.[44] Here, we are the first to show that pericytes express IP-9 and IP-10 (Figure XI in the online-only Data Supplement). This suggests that pericytes possess the ability to negatively regulate vessel formation and induce vessel regression and may be the mechanism involved in vessel regression during wound healing. There are studies that have indicated CXCR3 ligands can inhibit endothelial cell function in a CXCR3-independent mechanism. Although we have not ruled out this possibility, our data are highly suggestive that inhibition is through CXCR3 because the use of a CXCR3 neutralization antibody (Figure IX in the online-only Data Supplement) and CXCR3 knockdown by siRNA (Figure 3) reversed the pericyte-mediated inhibition of cord formation. Also, pericytes did not inhibit cord formation when cocultured with HUVEC (Figures 2 and 3), which lack CXCR3 expression but over expression of CXCR3B induce inhibition of cord formation when cocultured with pericytes (Figure 4).

At a superficial level, these data may seem contradictory to the current dogma of the requirement or necessity of pericyte association with the neovasculature to promote vessel stability. It is well established that mature vessels are associated with pericytes and there is a synergism between the 2 cells in maintaining a functional vasculature. Numerous studies have shown that endothelial cells secrete PDGF to promote pericyte migration and attachment and pericytes secrete several factors including Ang-1 to promote endothelial cell survival through the activation of survivin, promote cell–cell adhesion through Rho activation, and inhibit proliferation through Grb-2 activation.[45] Pericytes also form adherens and

Downloaded from http://ahajournals.org by on March 5, 2022

gap junction with endothelial cells for direct communication between the 2 cells.[22,46] Whereas these literature reports, which are not refuted by the data herein, relate to mature vessels, the findings herein only indicate that pericytes have the ability to promote regression of immature vessels.

This function of promoting regression of immature or nascent vessels is a critical distinction. In the context of wound healing, there is an overexuberant vascularization that requires pruning as the tissue matures. The initiation of angiogenesis from the severed vascular stump includes the dissociation of pericytes from these cells, followed by migration and proliferation of the endothelial cells to form new vessels. In the retina, pericyte were found to associate with endothelial cells after the neovasculature is formed,[28] whereas pericytes have been found at the tips of angiogenic sprouts in the corpus luteum.[47] The circumstance under which pericytes interact with the neovasculature may dictate their regulation of vessel stability. In both situations, an inflammatory response is not observed but an inflammatory response is a key regulator of wound healing. Thus, specific environmental conditions (inflammatory response) may dictate the ability of pericyte to promote vessel involution. These questions still need to be addressed and are currently being investigated by our laboratory.

Our findings are consistent with pericytes both inducing involution of immature vessels,[2,3] while stabilizing mature vasculature. CXCR3 expression is nearly absent on mature vessels but expression is regained during wound-induced angiogenesis[2,48] We show during wound healing, initial angiogenesis occurs in the absence of pericytes (days 3–7) and by day 12 a majority of pericytes are associated with vessels that are absent of CXCR3 expression (Figure XVI in the online-only Data Supplement). During the initial phases of angiogenesis endothelial cells express CXCR3 and are absent of pericyte association (days 3 and 4). At days 7 and 9 after wounding, CXCR3 expression on endothelial cells is diminishing and pericytes are now observed in proximity of vessels. At day 9, there is a large population of pericytes surrounding vessels lacking CXCR3 expression. By day 12, the pericytes were predominantly found only associated with vessels absent in CXCR3 expression. Although these in vivo data do not provide direct evidence that pericytes mediate vessel dissociation, it is highly suggestive that pericyte association with the neovasculature occurs in the late stages of the granulation phase. Furthermore, our data show the majority of pericytes are associated with vessels absent in CXCR3 expression. Using a CXCR3−/− mouse, we showed that CXCR3 is necessary for regulating vessel growth and plays a significant role in vessel regression.[2,5] This study demonstrated that CXCR3 signaling is necessary for physiological vessel regression but does not provide an exact mechanism for CXCR3 activation. Herein, we provide a possible mechanism in which pericytes initiate vessel regression through activation of CXCR3, with inflammatory cytokines such as IFN-γ stimulating upregulation of the CXCR3 ligands IP-10 (CXCL10) and IP-9 (CXCL11). Our data show that pericytes inhibit EC migration (Figure XIII in the online-only Data Supplement) and promote cord dissociation through calpain activation (Figure XV in the online-only Data Supplement). To put this all in the context of wound healing, angiogenesis begins to occur around day 3 to 4 after wounding and continues reaching maximum density around day 14. In the wound bed, it is estimated that the newly formed vasculature can be in excess of 10-fold. Around days 11 to 14, natural killer cells and T-helper cells become activated.[49] These cells are a major source of IFN-γ. At this time, vessel regression is occurring. This scenario is observed during wound healing in our mouse model (Figure XVI in the online-only Data Supplement). Although we do not know the mechanism regulating the downregulation of CXCR3 expression on maturing vessels, we have shown that the vessels that remain after day 14 are absent of CXCR3 expression.[2] These events suggest that IFN-γ released from activated T-cells may initiate vessel regression of immature or nonfunctional vessels through the secretion of CXCR3 ligands, IP-10 and IP-9, from pericytes and fibroblasts. Even in the absence of IFN-γ induced secretion of CXCR3 ligands, pericytes may possess an alternative mechanism for promoting the activation of CXCR3 on endothelial cells. Endothelial cells have been found to synthesize and store IP-10 in small vesicles throughout the cytoplasm, but the mechanism responsible for IP-10 secretion is not known.[50] Thus, paracrine signaling or cell–cell communication from stromal cell (myofibroblasts, macrophages, lymphocytes, pericytes) could mediate the release of stored IP-10 from endothelial cells leading to an autocrine activation of CXCR3 promoting vessel regression. Although these studies do not provide direct in vivo evidence for pericyte activation of CXCR3 on endothelial cells, they do provide a plausible physiological mechanism.

In a recently published study, Simonavicius et al[42] showed that activation of the pericyte receptor endosialin/CD248, type I transmembrane glycoprotein, and accessory receptor for the PDGF receptor[51] promotes selective vessel regression during development. In this study, during eye development pericyte expression of endosialin was shown to promote endothelial cell apoptosis. The authors provide 2 possible mechanisms: (1) endosialin could disrupt endothelial adhesion to the matrix or (2) impairs cross-talk between integrins and VEGFR2. In addition, the authors suggest that endosialin activity could be restricted to select vessels. Because little is known about endosialin expression and signaling, it is conceivable that endosialin is not expressed by pericytes during wound regeneration. Conversely, we have shown that vessel regression through CXCR3 occurs through anoikis.[2] Thus, it is plausible that endosialin-mediated regression could also be required for the CXCR3-mediated regression (and conversely, the nonexamined CXCR3 signaling axis also could be necessary during endosialin-mediated vascular involution). Whether endosialin plays a direct or indirect role in vessel regression during wound healing remains to be elucidated in studies beyond the scope of the present communication.

In summary, these data provide first evidence of a physiological function for CXCR3 in mediating vessel regression. Here, we show that pericytes in addition to their role in promoting vessel stabilization and regulating vascular tone can promote vessel regression through activation of the CXCR3 signaling pathways in endothelial cells. The results of this

Downloaded from http://ahajournals.org by on March 5, 2022

study indicate a new mechanism for the regulation of vessel stability. The identification of CXCR3 as a possible key regulator of vessel regression puts forth an attractive approach to target CXCR3 for the treatment of neoangiogenesis.

Acknowledgments

We thank the members of the Wells laboratory for helpful discussions and suggestions. We thank Drs Bruno Peaúlt and Mihaela Crisan for providing the pericyte cell line and Dr Darwin Prockop for the hMSC. We also thank Dr Paula Romagnani for providing the CXCR3B construct.

Sources of Funding

These studies were supported by a grant from the National Institutes of Health/National Institute of General Medical Sciences to A. Wells and a Department of Veterans Affairs MREP Award to R.J. Bodnar.

Disclosures

None.

References

1. Babu M, Wells A. Dermal-epidermal communication in wound healing. *Wounds*. 2001;13:183–189.
2. Bodnar RJ, Yates CC, Rodgers ME, Du X, Wells A. IP-10 induces dissociation of newly formed blood vessels. *J Cell Sci*. 2009;122(Pt 12):2064–2077.
3. Bodnar RJ, Yates CC, Wells A. IP-10 blocks vascular endothelial growth factor-induced endothelial cell motility and tube formation via inhibition of calpain. *Circ Res*. 2006;98:617–625.
4. Yates CC, Krishna P, Whaley D, Bodnar R, Turner T, Wells A. Lack of CXC chemokine receptor 3 signaling leads to hypertrophic and hypercellular scarring. *Am J Pathol*. 2010;176:1743–1755.
5. Yates CC, Whaley D, Kulasekeran P, Hancock WW, Lu B, Bodnar R, Newsome J, Hebda PA, Wells A. Delayed and deficient dermal maturation in mice lacking the CXCR3 ELR-negative CXC chemokine receptor. *Am J Pathol*. 2007;171:484–495.
6. Davis GE, Saunders WB. Molecular balance of capillary tube formation versus regression in wound repair: role of matrix metalloproteinases and their inhibitors. *J Investig Dermatol Symp Proc*. 2006;11:44–56.
7. Edelman DA, Jiang Y, Tyburski J, Wilson RF, Steffes C. Pericytes and their role in microvasculature homeostasis. *J Surg Res*. 2006;135:305–311.
8. von Tell D, Armulik A, Betsholtz C. Pericytes and vascular stability. *Exp Cell Res*. 2006;312:623–629.
9. Allt G, Lawrenson JG. Pericytes: cell biology and pathology. *Cells Tissues Organs*. 2001;169:1–11.
10. Kutcher ME, Herman IM. The pericyte: cellular regulator of microvascular blood flow. *Microvasc Res*. 2009;77:235–246.
11. Peppiatt CM, Howarth C, Mobbs P, Attwell D. Bidirectional control of CNS capillary diameter by pericytes. *Nature*. 2006;443:700–704.
12. Gaengel K, Genové G, Armulik A, Betsholtz C. Endothelial-mural cell signaling in vascular development and angiogenesis. *Arterioscler Thromb Vasc Biol*. 2009;29:630–638.
13. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. *Circ Res*. 2005;97:512–523.
14. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen*. 2008;16:585–601.
15. Yates CC, Whaley D, Y-Chen A, Kulesekaran P, Hebda PA, Wells A. ELR-negative CXC chemokine CXCL11 (IP-9/I-TAC) facilitates dermal and epidermal maturation during wound repair. *Am J Pathol*. 2008;173:643–652.
16. Zaja-Milatovic S, Richmond A. CXC chemokines and their receptors: a case for a significant biological role in cutaneous wound healing. *Histol Histopathol*. 2008;23:1399–1407.
17. Lasagni L, Francalanci M, Annunziato F, Lazzeri E, Giannini S, Cosmi L, Sagrinati C, Mazzinghi B, Orlando C, Maggi E, Marra F, Romagnani S, Serio M, Romagnani P. An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *J Exp Med*. 2003;197:1537–1549.
18. Crisan M, Deasy B, Gavina M, Zheng B, Huard J, Lazzari L, Péault B. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. *Methods Cell Biol*. 2008;86:295–309.
19. Crisan M, Yap S, Casteilla L, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell*. 2008;3:301–313.
20. Chen CW, Corselli M, Péault B, Huard J. Human blood-vessel-derived stem cells for tissue repair and regeneration. *J Biomed Biotechnol*. 2012;2012:597439.
21. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol*. 1992;99:683–690.
22. Gerhardt H, Betsholtz C. Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res*. 2003;314:15–23.
23. Newman AC, Nakatsu MN, Chou W, Gershon PD, Hughes CC. The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Mol Biol Cell*. 2011;22:3791–3800.
24. Campagnolo P, Cesselli D, Al Haj Zen A, Beltrami AP, Kränkel N, Katare R, Angelini G, Emanueli C, Madeddu P. Human adult vena saphena contains perivascular progenitor cells endowed with clonogenic and proangiogenic potential. *Circulation*. 2010;121:1735–1745.
25. Orlidge A, D'Amore PA. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol*. 1987;105:1455–1462.
26. Betsholtz C, Lindblom P, Gerhardt H. Role of pericytes in vascular morphogenesis. *EXS*. 2005:115–125.
27. Hughes CC. Endothelial-stromal interactions in angiogenesis. *Curr Opin Hematol*. 2008;15:204–209.
28. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development*. 1998;125:1591–1598.
29. Stratman AN, Malotte KM, Mahan RD, Davis MJ, Davis GE. Pericyte recruitment during vasculogenic tube assembly stimulates endothelial basement membrane matrix formation. *Blood*. 2009;114:5091–5101.
30. Fajardo LF. The complexity of endothelial cells. A review. *Am J Clin Pathol*. 1989;92:241–250.
31. Murdoch C, Monk PN, Finn A. Cxc chemokine receptor expression on human endothelial cells. *Cytokine*. 1999;11:704–712.
32. Salcedo R, Resau JH, Halverson D, Hudson EA, Dambach M, Powell D, Wasserman K, Oppenheim JJ. Differential expression and responsiveness of chemokine receptors (CXCR1-3) by human microvascular endothelial cells and umbilical vein endothelial cells. *FASEB J*. 2000;14:2055–2064.
33. Suyama T, Furuya M, Nishiyama M, Kasuya Y, Kimura S, Ichikawa T, Ueda T, Nikaido T, Ito H, Ishikura H. Up-regulation of the interferon gamma (IFN-gamma)-inducible chemokines IFN-inducible T-cell alpha chemoattractant and monokine induced by IFN-gamma and of their receptor CXC receptor 3 in human renal cell carcinoma. *Cancer*. 2005;103:258–267.
34. Wang F, Liu HM, Irwin MG, Xia ZY, Huang Z, Ouyang J, Xia Z. Role of protein kinase C beta2 activation in TNF-alpha-induced human vascular endothelial cell apoptosis. *Can J Physiol Pharmacol*. 2009;87:221–229.
35. Díaz-Flores L, Gutiérrez R, Madrid JF, Varela H, Valladares F, Acosta E, Martín-Vasallo P, Díaz-Flores L Jr. Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. *Histol Histopathol*. 2009;24:909–969.
36. Chen CW, Okada M, Proto JD, Gao X, Sekiya N, Beckman SA, Corselli M, Crisan M, Saparov A, Tobita K, Péault B, Huard J. Human pericytes for ischemic heart repair. *Stem Cells*. 2013;31:305–316.
37. Katare R, Riu F, Mitchell K, Gubernator M, Campagnolo P, Cui Y, Fortunato O, Avolio E, Cesselli D, Beltrami AP, Angelini G, Emanueli C, Madeddu P. Transplantation of human pericyte progenitor cells improves the repair of infarcted heart through activation of an angiogenic program involving micro-RNA-132. *Circ Res*. 2011;109:894–906.
38. Baluk P, Lee CG, Link H, Ator E, Haskell A, Elias JA, McDonald DM. Regulated angiogenesis and vascular regression in mice overexpressing vascular endothelial growth factor in airways. *Am J Pathol*. 2004;165:1071–1085.
39. Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, McDonald DM. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. *Am J Pathol*. 2004;165:35–52.

Downloaded from http://ahajournals.org by on March 5, 2022

40. Lindahl P, Johansson BR, Levéen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science*. 1997;277:242–245.
41. Otsu K, Das S, Houser SD, Quadri SK, Bhattacharya S, Bhattacharya J. Concentration-dependent inhibition of angiogenesis by mesenchymal stem cells. *Blood*. 2009;113:4197–4205.
42. Simonavicius N, Ashenden M, van Weverwijk A, Lax S, Huso DL, Buckley CD, Huijbers IJ, Huijber IJ, Yarwood H, Isacke CM. Pericytes promote selective vessel regression to regulate vascular patterning. *Blood*. 2012;120:1516–1527.
43. Stratman AN, Schwindt AE, Malotte KM, Davis GE. Endothelial-derived PDGF-BB and HB-EGF coordinately regulate pericyte recruitment during vasculogenic tube assembly and stabilization. *Blood*. 2010;116:4720–4730.
44. Merkle M, Ribeiro A, Wörnle M. TLR3-dependent regulation of cytokines in human mesangial cells: a novel role for IP-10 and TNF-alpha in hepatitis C-associated glomerulonephritis. *Am J Physiol Renal Physiol*. 2011;301:F57–F69.
45. Thomas M, Augustin HG. The role of the Angiopoietins in vascular morphogenesis. *Angiogenesis*. 2009;12:125–137.
46. Hirschi KK, Burt JM, Hirschi KD, Dai C. Gap junction communication mediates transforming growth factor-beta activation and endothelial-induced mural cell differentiation. *Circ Res*. 2003;93:429–437.
47. Amselgruber WM, Schäfer M, Sinowatz F. Angiogenesis in the bovine corpus luteum: an immunocytochemical and ultrastructural study. *Anat Histol Embryol*. 1999;28:157–166.
48. Romagnani P, Annunziato F, Lasagni L, Lazzeri E, Beltrame C, Francalanci M, Uguccioni M, Galli G, Cosmi L, Maurenzig L, Baggiolini M, Maggi E, Romagnani S, Serio M. Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. *J Clin Invest*. 2001;107:53–63.
49. Fishel RS, Barbul A, Beschorner WE, Wasserkrug HL, Efron G. Lymphocyte participation in wound healing. Morphologic assessment using monoclonal antibodies. *Ann Surg*. 1987;206:25–29.
50. Øynebråten I, Bakke O, Brandtzaeg P, Johansen FE, Haraldsen G. Rapid chemokine secretion from endothelial cells originates from 2 distinct compartments. *Blood*. 2004;104:314–320.
51. Tomkowicz B, Rybinski K, Sebeck D, Sass P, Nicolaides NC, Grasso L, Zhou Y. Endosialin/TEM-1/CD248 regulates pericyte proliferation through PDGF receptor signaling. *Cancer Biol Ther*. 2010;9:908–915.

Significance

CXC chemokine receptor 3 (CXCR3) signaling pathway inhibits endothelial migration and promotes apoptosis, but its physiological role in regulating endothelial cell function during angiogenesis is not known. In this study, we have identified a physiological function for CXCR3 in promoting the regression of the nascent vasculature and a novel mechanism for pericytes to promote vessel regression through CXCR3 activation on endothelial cells. Identification of an endogenous antiangiogenic pathway that could be targeted to not only stop the progression of angiopathies but even reverse the hypervascularity would provide for novel molecular targets to alleviate angiopathies. This could then treat such diseases as retinopathy or ineffective wound healing. This study could lead to the development of preclinical and clinical treatments for diseases in which pathological angiogenesis is an underlying or principal cause of disease progression.

Downloaded from http://ahajournals.org by on March 5, 2022