Abstract—Angiogenesis plays a critical role in wound repair. Endothelial cells present CXC receptor 3 (CXCR3) for chemokines expressed late in wound regeneration. To understand the physiological role CXCR3 plays in regulating endothelial function, we analyzed the ability of a CXCR3 ligand, IP-10 (CXCL10), to influence endothelial cell tube formation. Treatment of endothelial cells with IP-10 in the presence of vascular endothelial growth factor (VEGF) inhibited tube formation on growth factor–reduced Matrigel and in a subcutaneous Matrigel plug. Furthermore, IP-10 significantly inhibited VEGF-induced endothelial motility, a response critical for angiogenesis. Previous work showed that CXCR3 ligandation initiates protein kinase A (PKA) phosphorylation-dependent inhibition of m-calpain, required for induced cell motility, in fibroblasts but not epithelial cells. Here we show that CXCR3 activation in endothelial cells induces an increase in cAMP and PKA activation. Treatment of endothelial cells with Rp-8-Br-cAMP, an inhibitor of PKA, or small interference RNA to PKA was able to reverse the inhibitory effects of IP-10 on VEGF-mediated tube formation and motility. Importantly, treatment of endothelial cells with VEGF induced the activation of m-calpain, but costimulation with IP-10 significantly decreased this activity. Using Rp-8-Br-cAMP, we show blocking PKA reversed the IP-10 inhibition of VEGF-induced m-calpain activity. These data indicate that the activation of CXCR3 inhibits endothelial tube formation through a PKA mediated inhibition of m-calpain. This provides a means by which late wound repair signals limit the angiogenesis driven early in the wound response process. (Circ Res. 2006;98:617-625.)

Key Words: CXCL10 □ CXCR3 □ angiogenesis □ signal transduction □ cAMP □ receptor tyrosine kinase

Wound healing is a dynamic complex biological event.1 During the regenerative phase of wound healing angiogenesis stops, followed by involution during the remodeling phase. Key to both the initial proliferation of these vessels and the subsequent stasis and then involution of the vascular network is the response of the endothelial cells to signals from the surrounding tissues. At present it is not known whether the termination of angiogenesis occurs because of depletion of the proangiogenic or induction of antiangiogenic signals. Recently, we have found that during the regenerative phase of wound repair, chemokines are expressed to limit fibroblast immigration and motility.2 Thus, we asked whether the same signals stopped angiogenic ingrowth.

Two of the ELR (glutamic acid–leucine–arginine)-negative CXC chemokines appear late in the regenerative phase. IP-10 (interferon γ-inducible protein 10, CXCL10) is produced by endothelial cells themselves late in the regenerative phase1 and IP-9 (I-TAC, CXCL11) derives from redifferentiating keratinocytes.2 These ELR-negative chemokines are of particular interest, because they have been reported to be angiostatic.3-5 These chemokines bind to the common CXC chemokine receptor 3 (CXCR3),6 which has been found expressed on human endothelial cells.4,7 Endothelial cell chemotaxis is of significance as a rate-limiting event in neovascularization. CXCR3 ligands inhibit chemotaxis in fibroblasts8 and in endothelial cells.4 The ability of CXCR3 to induce chemotaxis in certain cells and inhibit chemotaxis in other cells, along with the recent finding of a variant CXCR3 isoform,6 suggests the existence of cell-specific signaling pathways mediated by the 2 CXCR3 isoforms. CXCR3 inhibition of fibroblast motility functions through a cAMP/protein kinase A (PKA)-mediated inhibition of m-calpain that abrogates rear retraction.9 However, the signaling pathway and cell responses mediated by CXCR3 in endothelial cells are unknown.

Herein we parse the motility promoting and inhibiting signaling pathways in human microvascular endothelial cells. We show that the ELR-negative chemokine IP-10 inhibits VEGF-induced endothelial cell tube formation concomitant with blocking motility. Furthermore, stimulation of CXCR3 by IP-10 induces an increase in cAMP production and activation of PKA. CXCR3 activation of PKA leads to the inhibition of VEGF-mediated m-calpain activation and thereby limits cell motility. We have identified a signaling pathway mediated by CXCR3 in microvascular endothelial cells that inhibits angiogenesis in vitro.
Materials and Methods

Tube Formation

Immortalized human adult dermal microvascular cells (dHMEC) (BioWhittaker) and human microvascular cell line (HMEC-1) (Centers for Disease Control and Prevention, Atlanta, Ga) were resuspended in serum-free media containing VEGF, IP-10, 8-Br-cAMP, Rp-8-Br-cAMP, Cl-1, and calpain inhibitor IV as denoted on the figure then incubated on growth factor reduced Matrigel. The formed tubes were digitally imaged and analyzed using MetaMorph (Universal Imaging Corp).

Motility Assay

Cell migration was performed as previously described. In brief, a HMEC-1 monolayer was scraped making a 1-m wide denuded area then stimulated with VEGF, IP-10, 8-Br-cAMP, Rp-8-Br-cAMP, Cl-1, and calpain inhibitor IV as denoted in the figure. The figure then incubated on growth factor reduced Matrigel. The formed tubes were digitally imaged and analyzed using MetaMorph (Universal Imaging Corp).

In Vivo Calpain Activity (Boc-LM-CMAC) Assay

In vivo calpain activity was determined by using the membrane permeable substrate t-BOC-LM-CMAC (BOC) as described previously. In brief, cells were incubated with BAPTA/AM, 8-Br-cAMP, Rp-8-Br-cAMP, Cl-1, and calpain inhibitor IV before the addition of BOC. The cells were further incubated with VEGF and/or IP-10, as indicated on the figure. The cleavage of BOC by calpain was visualized using a fluorescence microscope.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

IP-10 Inhibits VEGF-Induced Endothelial Tube Formation

The central hypothesis posits that the ELR-negative chemokines limit vascularization late in wound healing. To examine the role the ELR-negative chemokines play in regulating the ability of endothelial cells to form vessel in vitro, both primary dHMEC and immortalized HMEC-1 cells were analyzed for their ability to form tubes on growth factor reduced Matrigel in the presence of VEGF and/or IP-10. dHMEC and HMEC-1 cells were able to form tubes, whereas addition of VEGF significantly enhanced the number of tubes formed, down to 75% of control (Figure 1A and 1B). Important for our hypothesis, IP-10 was able to override the angiogenic signals from VEGF, and limit tube formation in the presence of VEGF to an extent similar to IP-10 alone. That this inhibitory action is a general property of ELR-negative chemokines was shown as similar results were obtained when the endothelial cells were treated with IP-9 (CXCL11) and PF4 (CXCL4) (data not shown). These data strongly suggest that ELR-negative chemokines act directly

Figure 1. IP-10 inhibits endothelial cell tube formation on Matrigel. A, HMEC-1 and dHMEC cells were detached and resuspended in serum-free media with VEGF (100 ng/mL), IP-10 (200 ng/mL), or H2O (no treatment). The cells (4.5x10^4 cells/well) were added to 24-well culture plates coated with growth factor reduced Matrigel and incubated for 24 hours. A magnification of 4 was used to image the cells. B, Endothelial tube formation in A was analyzed using the MetaMorph program. The results are of N=4 (normalized average±SEM). Note, IP-10 inhibited both HMEC-1 and dHMEC tube formation in the presence of VEGF. *P=0.05. C, C57b/H6 mice were implanted subcutaneously with 750 μL of Matrigel containing VEGF (200 ng/mL) and/or IP-10 (400 ng/mL). Nine days post inoculation, the Matrigel plug was removed and stained with Masson’s trichrome for endothelial infiltration. VEGF induced the infiltration of endothelial cells, whereas IP-10 inhibited VEGF-mediated endothelial migration and vessel formation in the Matrigel plug. Arrows indicate orientation from integument (dermis) to Matrigel midline. Representative images are shown (x10) with 4-fold magnified insets.
on endothelial cells to inhibit tube formation, findings which correlate with published results.6

Examining in vivo vessel formation, Matrigel containing VEGF and/or IP-10 was implanted subcutaneously into mice. After 9 days, the Matrigel plug was removed and Masson’s trichrome staining showed that VEGF induced invasion of endothelial cells into the Matrigel compared with no treatment (Figure 1C). IP-10 inhibited this angiogenesis even in VEGF-treated Matrigel (Figure 1C). The thin tube-like structures noted in untreated and VEGF-treated plugs stained for von Willebrand factor, validating that the invasion of endothelial cells (data not shown). These data provide further evidence that IP-10 inhibits VEGF-induced vessel formation.

**IP-10 Inhibits Endothelial Cell Motility**

The above results show that IP-10 is able to inhibit endothelial cell tube formation, a process that requires endothelial cell motility. We have demonstrated that ELR-negative chemokines can inhibit fibroblast motility.2,8,9 To examine whether IP-10 inhibition of endothelial tube formation is due a similar inhibition of endothelial motility, HMEC-1 were analyzed for migration into a denuded area over 24 hours in the presence of IP-10. Treatment of HMEC-1 cells with VEGF increased cell motility compared with untreated cells, whereas IP-10 treatment significantly inhibited HMEC-1 cell migration compared with untreated cells (Figure 2A). When the cells were cotreated with VEGF and IP-10, IP-10 blocked VEGF-induced cell motility (Figure 2A). Similar migration in the presence of mitomycin C to prevent mitogenesis with or without VEGF or IP-10 stimulation (data not shown) suggests that VEGF-induced migration is not attributable to cell proliferation.

To discern whether motility per se plays an important role in angiogenesis, we found that inhibition of phospholipase C (PLC), a key regulatory protein involved in growth factor induced chemotaxis,12 inhibited HMEC-1 tube formation. HMEC-1 treated with U73122, a PLC inhibitor, was able to inhibit tube formation (Figure 2B). Inhibition of tube formation by U73122 is not a result of cell death, because U73122 did not affect HMEC-1 viability (Figure 2C). These data indicate that the ELR-negative chemokines inhibit endothelial cell migration, with the inference that loss of this process likely contributes to the noted inhibition of endothelial tube formation.

**IP-10 Stimulates cAMP Production and PKA Activation in Endothelial Cells**

In fibroblasts, IP-9–induced activation of PKA is required for inhibition of motility,9 whereas little, if any, cAMP accumulates in response to IP-9 in keratinocytes in which IP-9 is motogenic.13 To determine whether stimulation of endothelial cells with IP-10 can induce an increase in cAMP, HMEC-1
cells were incubated with VEGF, and/or IP-10, and then assayed for total cAMP production. Treatment of HMEC-1 cells with IP-10 showed a 3-fold increase in cAMP production compared with untreated and VEGF-treated cells (Figure 3A), as previously reported.6 When the cells were stimulated with a combination of VEGF and IP-10, there was still a significant increase in cAMP production compared with VEGF alone (Figure 3A).

PKA is an inhibitor of endothelial cell migration and tube formation.14,15 We sought to determine whether the increase in cAMP by IP-10 stimulation of CXCR3 mediates the activation of PKA. Incubation of HMEC-1 cells with IP-10 alone showed a significant increase in substrate phosphorylation by PKA compared with control and VEGF-treated cells (Figure 3B), as previously reported. The PKA activity was shown by an increase in the amount of the synthetic substrate phosphorylated by PKA when HMEC-1 cells were treated with IP-10 in the presence of VEGF, compared with VEGF alone (Figure 3B). These results indicate that incubation of endothelial cells with IP-10 activates PKA. Thus, the signaling pathway activated by CXCR3 involves PKA activation.

**PKA Regulates Endothelial Cell Tube Formation and Motility**

If PKA plays a significant role in endothelial cell tube formation and motility, then the cell permeable cAMP analogs 8-Br-cAMP, a PKA activator, and Rp-8-Br-cAMP, a PKA inhibitor, should regulate endothelial cell tube formation and motility. Incubation of HMEC-1 cells with 8-Br-cAMP inhibited the ability of the cells to form tubes compared with the untreated group (Figure 4A and 4B). 8-Br-cAMP also significantly inhibited VEGF-induced tube formation (Figure 4A and 4B). When the HMEC-1 cells were incubated with Rp-8-Br-cAMP in the presence of IP-10, Rp-8-Br-cAMP was able to reverse the inhibitory effects of IP-10 on tube formation (Figure 4C and 4D). To further show that IP-10 induced PKA activation plays a role in inhibiting endothelial tube formation on Matrigel, HMEC-1 cells were preincubated with Rp-8-Br-cAMP, then stimulated with VEGF, IP-10 and/or 8-Br-cAMP. As predicted, Rp-8-Br-cAMP diminished the inhibitory effects of IP-10 and 8-Br-cAMP on VEGF-induced HMEC-1 tube formation (Figure 4E). Next, RNA interference was used to ablate the catalytic subunit of PKA (PKA Cβ small interference RNA [siRNA]) in HMEC-1 cells. Ablation of PKA reversed the inhibitory effects of IP-10 and 8-Br-cAMP on tube formation; scrambled constructs had no effect on IP-10 and 8-Br-cAMP inhibition of VEGF-induced tube formation (Figure 4F). These results suggest that IP-10–mediated activation of PKA inhibits endothelial cell tube formation.

HMEC-1 cells were analyzed for migration in the presence of 8-Br-cAMP and Rp-8-Br-cAMP. Incubation of HMEC-1 cells with 8-Br-cAMP significantly inhibited cell motility compared with untreated cells (Figure 5A). Also, 8-Br-cAMP significantly inhibited VEGF–induced cell motility to a level comparable to 8-Br-cAMP only (Figure 5A). When the cells were treated with Rp-8-Br-cAMP in the presence of IP-10, the Rp-8-Br-cAMP abrogated the inhibitory effects of IP-10 (Figure 5B). These results further indicate that PKA activation by CXCR3 plays a major role in inhibiting endothelial cell motility.
In endothelial cells, PKA activation has been found to induce apoptosis.\textsuperscript{15} To verify that IP-10–mediated inhibition of tube formation was not caused by apoptosis, we analyzed the viability of cells treated with IP-10 by TUNEL assay. The treatment of HMEC-1 cells with IP-10 alone showed a 8% increase in the number of cells undergoing apoptosis, but no difference in the number of apoptotic cells was observed when IP-10 was incubated with VEGF compared with VEGF or nontreated cells (data not shown).

### m-Calpain Activation Is Inhibited by IP-10 in Endothelial Cells

In fibroblasts, CXCR3-activation of PKA inhibits motility secondary to inhibitory phosphorylation of m-calpain.\textsuperscript{8,9} Using a membrane permeable synthetic calpain substrate, Boc-LM-CMAC (BOC), that can be cleaved by both m- and \(\mu\)-calpain, we find that VEGF induces calpain activity in endothelial cells (Figure 6A). The increase in fluorescence attributable to BOC cleavage in VEGF-stimulated dHMEC cells was prevented by the pan-calpain inhibitor CI-1.

In cells, m-calpain is activated, at least in part, by ERK phosphorylation on serine 5016 and \(\mu\)-calpain is activated secondary to a calcium flux.\textsuperscript{13} Therefore, we used BAPTA/AM, a membrane permeable calcium chelator, to distinguish between m and \(\mu\)-calpain activation in cells as this blocks activation of \(\mu\)- but not m-calpain.\textsuperscript{13} Preincubation of dHMEC cells with BAPTA/AM did not prevent VEGF-induced BOC cleavage (Figure 6A). Similar results were also observed in HMEC-1 (data not shown). Thus, VEGF activates m-calpain, as BAPTA/AM did not quench this cleavage. Finding that IP-10 itself led to BOC fluorescence complicated whether IP-10 blocked VEGF-induced calpain activity (Figure 6B). This was not surprising as CXCR3 ligandation can induce PLC\(\beta\)-mediated calcium flux activation of \(\mu\)-calpain.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** IP-10–mediated PKA activation inhibits endothelial tube formation on Matrigel. A, HMEC-1 cells were treated with 8Br-cAMP (250 \(\mu\)mol/L) or Rp8Br-cAMP (50 \(\mu\)mol/L) in the absence (no treatment) or presence of VEGF (100 ng/mL). The cells were incubated on growth factor–reduced Matrigel for 24 hours and then imaged. Note, the PKA activator 8-Br-cAMP inhibited VEGF-mediated tube formation. B, Endothelial tube formation in A was analyzed using MetaMorph. The bar graph is the MetaMorph data expressed as a percentage of the control (no treatment). The results are of \(N=3\) (average±SEM). C, HMEC-1 were treated as described in A but incubated with IP-10 (200 ng/mL) instead of VEGF. The PKA inhibitor Rp-8-Br-cAMP reversed the inhibitory effects of IP-10 on tube formation. D, Endothelial tube formation in C was analyzed using MetaMorph as described in B. As seen, Rp8Br-cAMP was able to reverse the inhibitory effects IP-10 had on endothelial cell tube formation. E, HMEC-1 were treated as described in A but incubated with Rp-8-Br-cAMP, 8-Br-cAMP, VEGF, and IP-10 together. Again, Rp-8-Br-cAMP is able to reverse the inhibitory effects that IP-10 and 8-Br-cAMP have on VEGF-mediated tube formation. N=3; 1 representative experiment shown. F, HMEC-1 cells were transfected with siRNA for the catalytic \(\beta\) subunit of PKA (AAGAGTTTCTAGCCAAAGCCA) or a scrambled (AACCGTCGATTTCACCGGG) oligo.\textsuperscript{23} The siRNA ablated the protein expression of PKA \(\beta\) as seen in the Western blot. HMEC-1 transfected with PKA \(\beta\) siRNA were resistant to the inhibitory effects of IP-10 and 8-Br-cAMP comparable to VEGF. Note that IP-10 and 8-Br-cAMP were able to inhibit VEGF-induced tube formation in HMEC-1 cells transfected with the scrambled siRNA.
in keratinocytes.\textsuperscript{13} In accord with these earlier findings, IP-10 induced \( \mu \)-calpain activity as it was inhibited by BAPTA/AM (Figure 6B). When the cells were treated with both VEGF and IP-10, cleavage of BOC was observed (Figure 6C), but when the cells were preincubated with BAPTA/AM and then treated with VEGF and IP-10 there was almost no discernable BOC fluorescence (Figure 6C), indicating that IP-10 inhibits VEGF-mediated \( \mu \)-calpain activity.

Importantly for the hypothesis being tested, growth factor activation of m-calpain is prevented by PKA-mediated phosphorylation of a specific sequence in domain III.\textsuperscript{9} Thus, we assessed the ability of 8-Br-cAMP and forskolin to inhibit VEGF-induced m-calpain activity. Cleavage of BOC was observed when the cells were incubated with VEGF alone or in the presence of BAPTA/AM (Figure 7A). When the cells were pretreated with either 8-Br-cAMP or forskolin before VEGF treatment BOC cleavage was significantly reduced (Figure 7A). These data suggest that the activation of PKA plays a role in inhibiting m-calpain activity. Next we wanted to determine whether Rp-8-Br-cAMP could reverse the inhibitory effects of IP-10 on VEGF-mediated m-calpain activation. HMEC-1 cells were pretreated with BAPTA/AM, so that only m-calpain activity would be noted, and then incubated with Rp-8-Br-cAMP before stimulating the cells with VEGF and IP-10. Treatment of HMEC-1 cells with BAPTA/AM, VEGF and IP-10 showed almost no cleavage of BOC (Figure 7B). When the cells were treated with BAPTA/AM, Rp-8-Br-cAMP, VEGF, and IP-10 cleavage of BOC was observed (Figure 7B). These data show that the PKA inhibitor Rp-8-Br-cAMP is able to reverse the inhibitory effect IP-10 has on VEGF-mediated m-calpain activation. Thus, these results provide sufficient evidence to conclude that CXC receptor 3 activation of PKA inhibits m-calpain activity in endothelial cells.

m-Calpain Activation Is Required for Endothelial Tube Formation and Motility

PKA is a signaling molecule in a variety of inhibitory pathways and has been shown in other endothelial cell lines to inhibit Raf activation.\textsuperscript{14} Thus, to determine whether m-calpain inhibition is critical to IP-10 blockade of angiogenesis, rather than a distracting epiphenomenon, HMEC-1 cells were pretreated with the nonspecific calpain inhibitor, CI-1, or a m-calpain selective inhibitor, calpain inhibitor IV, and analyzed for tube formation or migration. Treatment of HMEC-1 cells with calpain inhibitor IV or CI-1 almost completely abolished the ability of HMEC-1 cells to form tubes or to locomote into a denuded area (Figure 8A and 8B). We then verified that these calpain inhibitors did indeed inhibit m-calpain. Analysis of the cleavage of BOC verifies that calpain inhibitor IV does inhibit VEGF-mediated m-calpain activation (Figure 8C). In addition, this pathway appears similar to those from other receptors with tyrosine kinase activity, as the MEK inhibitor, PD98059, was also able to inhibit m-calpain activation by VEGF (Figure 8C). These results provide sufficient evidence that m-calpain activation is required for VEGF-mediated endothelial motility and, thereby, tube formation.

Discussion

The ELR-negative CXC chemokines, including IP-10 and IP-9, have antiangiogenic properties,\textsuperscript{3,5} but the mechanism by which these chemokines inhibit angiogenesis has not been determined. Only recently has the CXC receptor 3, the only defined receptor for all ELR-negative CXC chemokines, been shown to be expressed on endothelial cells.\textsuperscript{4,7} In this study, we provide evidence that the ELR-negative chemokines inhibit angiogenesis through a PKA-mediated pathway via its effects on calpain. The evidence shows that IP-10 stimulation of microvascular endothelial cells increases cAMP levels, promoting the activation of PKA and thereby inhibiting endothelial cell migration. Herein we show that VEGF-mediated m-calpain activation is necessary for in vitro endothelial motility and tube formation and IP-10 inhibits VEGF-mediated m-calpain activation. To verify that this is PKA mediated, we show that Rp-8-Br-cAMP, a PKA inhibitor, and
the ablation of PKA using siRNA reversed the IP-10 inhibition of VEGF-mediated m-calpain activation. These results demonstrate that IP-10 inhibits endothelial cell migration through a PKA-mediated inhibition of m-calpain.

The data provide herein suggest a novel pathway for the regulation of angiogenesis. In endothelial cells, CXCR3 activation by its ligands mediates an increase in cAMP, thus activating PKA. This then inhibits m-calpain activity; m-calpain has numerous cellular functions, with a key function being to control locomotion by enabling rear detachment during haptokinetic motility. Thus, these results suggest that the inhibitory mechanism of the ELR-negative chemokines is by limiting the ability of endothelial cell migration through inhibition of rear-cell detachment.

An issue arises during wound healing of how these ELR-negative CXC chemokines regulate angiogenic drive. One such chemokine, PF4 (CXCL4) is released at high levels by the platelet clot. This early PF4 release and angiostasis would act to limit vascularization of the nascent clot and aid in hemostasis and prevention of fluid loss. During the inflammatory phase, in which the provisional matrix starts to take shape, the levels of PF4 drop and vascularization is noted. Only later during the resolution and remodeling phase do CXCR3 ligands reappear. This timing is consistent with our finding that the angiostatic effects of the ELR-negative CXC chemokines are concentration dependent and thus reversible (data not shown). Thus, there appears to be a proangiogenic state of low CXCR3 activation during the later...
inflammatory and earlier regenerative phases of wound healing wherein vascularization is noted.

The implications for CXCR3-mediated angiogenesis likely extend beyond wound repair. The regulation of angiogenesis is thought to be a coordinate regulation of angiogenic and angiostatic factors, thus a dysregulation of either of these factors can lead to pathological conditions such as idiopathic pulmonary fibrosis, endometriosis, and diabetic retinopathy. The downregulation of the angiostatic chemokine IP-10 has been found to be a factor in the development of idiopathic

Figure 8. Inhibition of m-calpain inhibits endothelial tube formation and migration. A, HMEC-1 cells were resuspended in serum-free media. The cells were treated with the m-calpain–specific inhibitor calpain inhibitor IV or CI-1 (5 μmol/L) in the absence (no treatment) or presence of VEGF (100 ng/mL). The cells were incubated on Matrigel for 12 hours then imaged. The images are representative of 4 experiments. Calpain inhibitor IV at the 100 μmol/L concentration almost completely abolished VEGF-mediated tube formation. B, HMEC-1 cells were analyzed for the ability to migrate into a denuded area using a 2D scratch assay. A 1-mm “scratch” was made in the HMEC-1 monolayer. The cells were incubated in serum-depleted media alone (no treatment) or containing VEGF (100 ng/mL) and/or calpain inhibitor IV (50 or 100 μmol/L) or CI-1 (5 μmol/L) for 24 hours. The area unoccupied by the cells was determined and shown as a percentage of the control (no treatment) and results are of N=3 (average ± SEM). *P<0.01. Inhibition of m-calpain significantly reduced VEGF-mediated cell migration. C, HMEC-1 cells were grown on gelatin coated glass chamber slides. The cells were preincubated with either CI-1 (10 μmol/L), calpain inhibitor IV (50 μmol/L), PD98059 (2.0 μmol/L), or BAPTA/AM (50 μmol/L) before incubation with BOC (27 μmol/L); concentrations were determined empirically. The cells were stimulated with either VEGF (100 ng/mL) or IP-10 (200 ng/mL) for 1 hour. Calpain activation was analyzed by fluorescence microscopy. Fluorescence within the cell indicates calpain activity. Note that both CI-1 and calpain inhibitor IV inhibited VEGF-mediated m-calpain activity. Also, the inhibition of calpain activity by the MEK inhibitor (PD98059) suggests that m-calpain is activated by the MAPK pathway, a known pathway activated by VEGF. Images are representative of N=3.
pulmonary fibrosis\textsuperscript{17} and endometriosis.\textsuperscript{18} These studies provide further evidence that understanding the signaling pathways mediated by the ELR-negative chemokines may provide new therapies in the treatment of such pathological conditions. Tumor growth, on the other hand, is not necessarily attributable to a dysregulation of the angiogenic/angiostatic signaling pathways but a recruitment of new vessels by the tumor cells themselves. IP-10 or Mig have been presented to successfully inhibit non–small cell lung cancer\textsuperscript{20} and Burkitt’s lymphoma.\textsuperscript{21} These studies demonstrate the importance of the endogenous expression of angiostatic factors in the regulation of tumor growth. IP-10, in tumor studies, has been found to cause tumor necrosis and vascular damage at the tumor site while not eliciting an appreciable inflammatory response.\textsuperscript{20}

The results of this study provide a signaling mechanism for the inhibition of angiogenesis by the ELR-negative chemokines and, thus, provides a possible mechanism by which to regulate physiological and pathological angiogenesis. Further characterization of the signaling mechanism mediated by CXCR3 may provide new insights into the development of new therapeutic strategies to promote or inhibit blood vessel formation. Furthermore, understanding the regulation of CXCR3 expression may be another method by which the regulation of angiogenesis maybe controlled.

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**References**

IP-10 Blocks Vascular Endothelial Growth Factor-Induced Endothelial Cell Motility and Tube Formation via Inhibition of Calpain
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Materials and Methods

Materials:

The tissue culture plates were purchased from Falcon (Franklin Lakes, NJ). The chamber slides were purchased from Nalgene (Naperville, IL). Growth factor reduced Matrigel was purchased from BD Bioscience (Bedford, MA). VEGF, IP-10 and Calpain Inhibitor I (ALLN) were purchased from BIOMOL (Plymouth Meeting, PA). Forskolin, leupeptin, 3-Isobutyl-1-Methylxanthine, EDTA, and PMSF were purchased from Sigma (St. Louis, MO). Adenosine 3’,5’-cyclic monophosphate, 8-bromo-, sodium salt (8-Br-cAMP), adenosine 3’,5’-cyclic monophosphorothioate, 8-bromo-, Rp-isomer, sodium salt (Rp-8-Br-cAMP), calpain inhibitor IV, and BAPTA/AM were purchased from Calbiochem (San Diego, CA). The calpain substrate, 7-amino-4-chloromethylcoumarin, t-BOC-L-leucyl-L-methionine amide (Boc-LM-CMAC) was purchased from Molecular Probes (Eugene, OR). The monoclonal antibody against GAPDH was purchased from Abcam (Cambridge, MA). The cAMP assay kit was purchased from Amersham (Piscataway, NJ). The PKA assay kit was purchased from Promega (Madison, WI). The TUNEL assay kit was purchased from Promega (Madison, WI) The siRNA oligos were purchased from IDT (Coralville, IA). All concentrations of materials used were determined empirically either herein or in prior studies.

Cell Culture:

Human adult dermal microvascular cells (dHMEC) and the immortalized human microvascular cell line (HMEC-1) were used. The dHMEC were purchased from BioWhittaker (Walkersville, MD) and grown in 10% FBS-EBM-2MV media (Clonetics, Walkersville, MD). The cells used for experiments were between passages 5 and 8. The HMEC-1 line was obtained at passage 14, and used before passage 20, from the Center for Disease Control (Atlanta, GA) and grown in 10% FBS-MDCB 131 media (Gibco, Gaithersburg, MD) supplemented with 10 mM L-Glutamate (Gibco), 1 ng/mL EGF (BD Biosciences, Bedford, MA), and 1 µg/mL hydrocortisone (Sigma, St. Louis, MO).

Tube Formation:

HMEC-1 were grown to 75-80% confluency then incubated in 0.5% dialyzed MDCB-131 media for 24 hrs. The cells were then resuspended in serum-free MDCB 131 media supplemented with VEGF (100 ng/mL), IP-10 (200 ng/mL), 8-Bromo-cAMP (250 µmol/L), Rp-8-Bromo-cAMP (50 µmol/L), individually and in combination as denoted on the figures, for 24 hrs at 37°C in 5% CO₂. For the dHMEC, the cells were grown to 60% confluency then incubated
in 1% dialyzed FBS-EBM-2 media for 6 hours at 37°C. The cells were detached and resuspended in serum-free EBM-2 media and treated as indicated in the figure. The cells (5.0 x 10^4) were incubated in 24 well plates coated with 300 µL of Matrigel (1:2 dilution with EBM media) for 24 hrs at 37°C in 5% CO₂. Both cell types were then imaged for tube formation using an Olympus CK2 microscope (4X objective) equipped with a Sony CCD-IRIS camera employing Xclaim™ video player software (ATI Technology, Marlborough, MA). Analysis of tube formation was performed using MetaMorph (Universal Imaging Corporation, Downington, PA). Tube formation was evaluated by computer-guided imaging to ascertain extended structures on the matrix through analysis of cell structure density as quantified by threshold area. The results are shown as a percent of the “no treatment” (diluent only) control.

**In Vivo Angiogenesis Assay:**

The angiogenesis assay was performed as previously described¹. C57B1/6 female mice age 8-9 months were inoculated with Matrigel that was supplemented with VEGF (200 ng/mL), IP-10 (400 ng/mL) or in combination. Matrigel (750 µL) was injected subcutaneously into the ventral side of the mouse in the groin area near the dorsal midline. Nine days post inoculation the Matrigel was removed. The Matrigel plug was placed in paraffin and sections were stained with Masson’s trichrome by a clinical histology laboratory (VA Medical Center, Pittsburgh, PA).

**Motility Assay:**

Cell migration was performed as previously described². In brief, HMEC-1 cells were plated at 2.5 x 10^5 cells/well in 12 well culture plates in complete growth media and incubated for 24 hrs at 37°C in 5% CO₂. The cells were washed one time with PBS and then incubated in 0.5% dialyzed MDCB 131 media for 24 hrs at 37°C in 5% CO₂. The monolayer was scraped making a 1 mm wide denuded area. The cells were then stimulated with VEGF (100 ng/mL), IP-10 (200 ng/mL), 8-Bromo-cAMP (250 µM), Rp-8-Bromo-cAMP (50 µM), alone and in combination for 24 hrs at 37°C in 5% CO₂. Images were taken at zero and 24 hrs, and the relative distance traveled by the cells into the acellular area was determined using MetaMorph. Motility was determined by the decrease in the area of the denuded region. The change in area is represented as a percent of control.

**cAMP Assay:**

HMEC-1 cells were plated in 10 mm culture dishes and grown to near confluency in complete growth media. The cells were then incubated in serum-reduced media (0.5% dialyzed...
FBS for HMEC-1) for 24 hrs at 37°C in 5% CO₂. The cells were washed once with PBS before incubating in serum-free media containing 0.75 mM 3-Isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor. The cells were stimulated with forskolin (25 µM), VEGF (100 ng/mL) or IP-10 (200 ng/mL), alone and in combination. The media was removed and ice cold 80% ethanol was added and the cells incubated on ice for 15 min. The extracts were then lyophilized and resuspended in 100 µl of water. Total cAMP was quantified using a cAMP assay kit (Amersham), performed according to manufacturer’s protocol.

PKA Assay:
HMEC-1 were grown in complete growth media to semiconfluency then, further incubated in 0.5% dialyzed FBS MCDB 131 media for 24 hrs. The cells were stimulated with VEGF (100 ng/mL) and/or IP-10 (200 ng/mL) for 15, 30 and 60 min. The cells were lysed with an ice-cold hypotonic solution (50 mmol/L Tris pH 7.4, 1 mmol/L EDTA, 10 µg/mL aprotinin and 1 mmol/L PMSF). The cells were incubated on ice for 30 min prior to centrifugation to remove the cell membranes. PKA activity was measured using a cAMP dependent protein kinase kit (Promega), performed according to manufacturer’s protocol.

RNA Interference:
The use of synthetic small interfering RNA (siRNA) to down-regulate PKA expression was performed as previously described. The following sequences used were purchased from Integrated DNA Technologies (Coralville, IA); PKA Cβ, AAGAGTTTCTAGCCAAAGCCA; scrambled, AACCGTGCAGTTCACCGGG. HMEC-1 cells were plated at 4.0 X10⁵ cells/60 mm dish incubated for 18 hrs in complete media. The cells were transfected with 40 pmol of either the PKA Cβ or scrambled siRNA using DarmaFECT 1 transfection reagent. The cells were incubated for 48 hrs at 37°C in 5% CO₂. The media were removed and replaced with complete media and incubated for 24 hrs at 37°C in 5% CO₂. The cells were detached and resuspended in 0.5% dialyzed FBS MDCB 131 media, centrifuged at 1,200 rpm for 10 min at 25°C. The cells were resuspended in 0.5% dialyzed FBS MDCB 131 media containing VEGF (100 ng/mL), IP-10 (200 ng/mL), 8-Br-cAMP (200 µmol/L), alone or in combination. The cells (5.0 x 10⁴) were incubated in 24 well plates coated with 300 µL of Matrigel (1:2 dilution with EBM media) for 24 hrs at 37°C in 5% CO₂. The cells were imaged with an Olympus CK2 microscope (4X objective) equipped with at Sony CCD-IRIS camera employing Xclaim™ video player software.

To verify that PKA was ablated by the siRNA, a fraction of the cells were centrifuged and the cell pellets were lysed. The lysates were separated by SDS-PAGE and immunoblotted.
for PKA Cβ using the polyclonal antibody against the C-terminus of human PKAβ catalytic
domain (Santa Cruz Biotechnology, Santa Cruz, CA) and a secondary anti-rabbit antibody. PKA
Cβ was visualized using ECL kit (Amersham).

**In Vivo Calpain Activity (Boc-LM-CMAC) Assay:**

In vivo calpain activity was determined by using the membrane permeable substrate t-
BOC-Leu-Met-chloromethylaminocoumarin (Boc-LM-CMAC) as described previously. In
brief, HMEC-1 and dHMEC cells were plated at 1.25 x 10^4 cells/chamber in an eight well
chamber slide (Nalge) and grown in complete media for 24 hrs. The cells were then incubated in
serum-reduced media (0.5% dialyzed FBS for HMEC-1 or 1% dialyzed FBS for dHMEC) and
incubated for 24 or 6 hrs respectively. The cells were incubated with BAPTA/AM (5 µM) or CI-
1 (5 µM) for 15 min prior to the addition of Boc-LM-CMAC (27 µmol/L) as specified by the
figures and legends. The cells were further incubated for 20 min then, incubated with VEGF
(100 ng/mL), IP-10 (200 ng/mL), 8-Bromo-cAMP (250 µmol/L), Rp-8-Bromo-cAMP (50
µmol/L), and/or forskolin (25 µmol/L) for 30 min as indicated by the figure. The wells were
removed and the slide washed with PBS before covering with a glass cover slide. The cleavage
of Boc-LM-CMAC by calpain was visualized using a fluorescence microscope (Olympus BX40)
with a UV blue filter (Olympus MNUA) and images were digitally captured using a SPOT®
camera and SPOT® software (Diagnostic Instruments, Sterling Heights, MI).

**References:**

2. Chen P, Gupta K, Wells A. Cell movement elicited by epidermal growth factor receptor
requires kinase and autophosphorylation but is separable from mitogenesis. *Journal of Cell
3. Dumaz N, Marais R. Protein kinase A blocks Raf-1 activity by stimulating 14-3-3 binding
and blocking Raf-1 interaction with Ras. *Journal of Biological Chemistry*. 2003;278:29819-
29823.
activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase
Supplemental Figure 1: IP-10 treatment does not affect cell number
A) HMEC-1 cells were detached and resuspended in serum free media with VEGF (100 ng/mL), IP-10 (200 ng/mL) or H2O (no treatment). The cells ($4.5 \times 10^4$ cells/well) were added to 24 well culture plates coated with growth factor reduced Matrigel and incubate for 24 hrs. The cells were then fixed, permeabilized then stained with propidium iodine to identify cell nuclei. A magnification of 4X was used to image the cells.

B) Cell nuclei were counted and graphed as a percentage of no treatment (control). The results are of $N = 4$ (normalized average ± SD). Note, although IP-10 treatment shows a slight decrease in cell number compared to VEGF treatment this decrease is not statistically significant.
Supplemental Figure 2: IP-10 Inhibits Endothelial Cell migration into Matrigel
C57b/H6 mice implanted subcutaneously with 750 µl of Matrigel containing VEGF (200 ng/ml), and/or IP-10 (400 ng/ml). Nine days post inoculation the Matrigel plug was removed, paraffin embedded then stained for von Willebrand factor (Abcam) to verify invading cells are endothelial cells. The cells observe infiltrating into Matrigel alone or supplemented with VEGF stained for von Willebrand factor indicating the cells are endothelial cells. Outside arrow indicates orientation from integument (dermis) to Matrigel midline; on picture arrows highlight some vWF-staining tubes. Representative images are shown (40X).
Supplemental Figure 3

Supplemental Figure 3: Inhibitory concentration of IP-10
To determine the concentration of IP-10 that produced maximal inhibition of tube formation on Matrigel, HMEC-1 cells were incubated for 24 hrs on Matrigel with increasing concentrations of IP-10 from 25-1000 ng/mL in the absence or presence of VEGF (100 ng/mL). Tube formation was analyzed using MetaMorph as area occupied by the cells. The IC50 was determined to be 170 ng/mL. The results are of 3 individual experiments performed in duplicate (average ± SEM).
Supplemental Figure 4: Viability of IP-10 treated cells
To determine whether IP-10 causes endothelial cells to undergo apoptosis, we treated HMEC-1 cells with IP-10 and analyzed them by TUNEL assay. HMEC-1 cells were grown on gelatin covered chamber slides. The cells were incubated for 12hrs in 0.5% dialyzed FBS media then, treated with VEGF (100 ng/mL), IP-10 (200 ng/mL) and actinomycin D (0.3 μmol/L) for 24 hrs. The cells were then analyzed for apoptosis by nuclear staining by TUNEL assay. Although there is a significant difference in cell viability between no treatment and IP-10 (p = 0.02), viability is at 87% thus, should not be a significant factor in the inhibition of initial tube formation. N=3 with 400 cells counted each (average + SEM). For actinomycin D treated cells N=1.
Supplemental Figure 5: Cell proliferation
To determine if VEGF or IP-10 affect cell proliferation, we analyzed cell number of HMEC-1 cells after 24 hrs incubation with VEGF or IP-10. Cells were plated at 50,000 cells/well in 12 well plates. The cells were incubated for 12 hrs then, incubated in 0.5% dialyzed FBS media for 24 hrs. The cells were then treated with VEGF (100 ng/mL), IP-10 (200 ng/mL) in the presence or absence of mitomycin C (0.4 µmol/L) for 24 hrs. The cells were counted using a coulter counter. N=6 (average + SEM). Note, there is no significant difference between the mitomycin C treated or untreated cells.
Supplemental Figure 6

Supplemental Figure 6: Inhibitory concentration of 8-Br-cAMP
To determine the concentration of 8-Br-cAMP that produced maximal inhibition of tube formation on Matrigel, HMEC-1 cells were incubated for 24 hrs on Matrigel with increasing concentrations of 8-Br-cAMP from 25-800 ng/mL in the presence of VEGF (100 ng/mL). Tube formation was analyzed using MetaMorph as area occupied by the cells. The IC50 was determined to be 100 ng/mL. The results are of 3 individual experiments (average + SEM).