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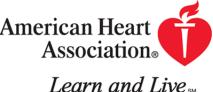# Molecular Cardiology

Integrin-Linked Kinase, a Hypoxia-Responsive Molecule, Controls Postnatal Vasculogenesis by Recruitment of Endothelial Progenitor Cells to Ischemic Tissue

Seung-Pyo Lee, MD*; Seock-Won Youn, MS*; Hyun-Jai Cho, MD; Lian Li, BA; Tae-Youn Kim, BA; Hyung-Seon Yook, BA; Jae-Woong Chung, MS; Jin Hur, MS; Chang-Hwan Yoon, MD; Kyung-Woo Park, MD; Byung-Hee Oh, MD; Young-Bae Park, MD; Hyo-Soo Kim, MD

Background—Recruitment and adhesion of endothelial progenitor cells (EPCs) to hypoxic endothelial cells (ECs) is essential for vasculogenesis in ischemic tissue; little is known, however, about the key signals or intracellular signaling pathways involved in orchestrating the expression of adhesion molecules by ECs in response to hypoxia and how this is related to the recruitment of EPCs to the ischemic tissue. Here, we report that endogenous integrin-linked kinase (ILK) is a novel molecule that responds to hypoxia in ECs that regulates the expression of stromal cell–derived factor-1 (SDF-1) and intercellular adhesion molecule-1 (ICAM-1) through nuclear factor-κB and hypoxia-inducible factor-1α and induces recruitment of EPCs to ischemic areas.

Methods and Results—Under hypoxia, both the endogenous amount and kinase activity of ILK were time-dependently upregulated in ECs, which was associated with increased ICAM-1 and SDF-1. This upregulation of ILK was mediated by stabilization of ILK by heat shock protein 90. ILK overexpression in normoxic ECs resulted in ICAM-1 and SDF-1 upregulation through dual control by nuclear factor-κB and hypoxia-inducible factor-1α. Blockade of ILK in hypoxic ECs significantly abrogated the expression of both molecules, which led to decreased EPC incorporation into ECs. A hindlimb ischemia model showed that ILK blockade significantly reduced EPC homing to ischemic limb and consequently led to poor neovascularization. Overexpression of ILK in the Matrigel plug significantly improved neovascularization in vivo, whereas the blockade of ILK resulted in the opposite effect.

Conclusions—Endogenous ILK is a novel and physiological upstream responder of numerous intracellular molecules involved in hypoxic stress in ECs and may control the recruitment of EPCs to ischemic tissue. (***Circulation***. 2006;114: 150-159.)

Key Words: angiogenesis ◼ hypoxia ◼ ischemia ◼ endothelial cell ◼ endothelial progenitor cell

$\mathbf{V}$asculogenesis, a process of new vessel formation by angiogenic progenitor cells, has been a "hot" issue since the discovery of endothelial progenitor cells (EPCs) in human peripheral blood.[1] For selective recruitment of EPCs to ischemic tissue after mobilization, "activation" of quiescent endothelial cells (ECs) and "adhesion" of EPCs to "activated" hypoxic ECs in the tissue is essential. The family of integrins is deeply involved in these processes of activation and adhesion.

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CXC chemokine receptor 4 (CXCR4) and β_2-integrin, expressed on EPCs, play crucial roles in the homing of these cells to the ischemic tissue,[2,3] whose counterpart molecules, stromal cell–derived factor-1 (SDF-1) and intercellular adhe-

sion molecule-1 (ICAM-1), respectively, are expressed by ECs.[3] However, little is known about the intracellular signaling pathways involved in orchestrating the expression of these molecules by ECs in response to hypoxia and how this is related to the recruitment of EPCs to the ischemic tissue.

Integrin-linked kinase (ILK) is a 59-kDa Ser/Thr kinase that binds to the cytoplasmic domain of β-integrin[4] and lies upstream of Akt/PKB,[5,6] mitogen-activated protein (MAP) kinase,[7] and nuclear factor (NF)-κB,[8,9] important molecules in inflammation and neovascularization. Recently, ILK was found to be involved in vessel formation during embryonic[10] or tumor[11] development. In addition, we recently showed that the level of endogenous ILK changes in EPCs and ECs after anchorage and nutrient deprivation.[6]
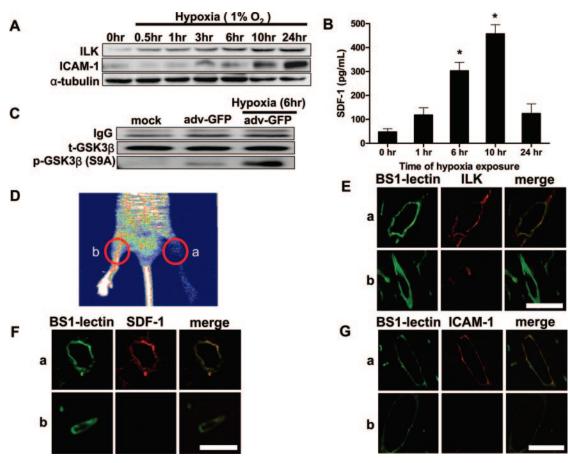
Figure 1. Changes in ILK, ICAM-1, and SDF-1 in response to hypoxia. A, Serial immunoblot analysis of the expression of ILK and ICAM-1 (n=4). B, SDF-1 excretion from hypoxic ECs measured by ELISA (*$P<0.05$ vs baseline SDF-1; n=3, mean±SEM). C, In vitro kinase assay of ILK from hypoxic or normoxic HUVECs. D, Ischemic hindlimb made by excision of unilateral femoral vessel. Laser Doppler perfusion imaging was taken just after the operation to confirm the induction of significant ischemic hindlimb (a); b denotes nonischemic control hindlimb. E–G, Costaining of both limbs with BS1-lectin and ILK (E), BS1-lectin and SDF-1 (F), and BS1-lectin and ICAM-1 (G).

Therefore, we postulated that endogenous ILK in ECs would be important in regulating pathways responsible for the expression of key molecules during vasculogenesis in response to hypoxia. We show here for the first time that ILK is a novel regulator of EC response to hypoxia and controls the homing of EPCs to ischemic tissue.

Methods

All materials and methods used for experimental procedures can be found in the online-only Data Supplement.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

Hypoxia Upregulates ILK and Its Downstream Molecules in ECs In Vitro and In Vivo

For analysis of the changes in ILK and the proposed downstream molecules in response to hypoxia, human umbilical vein endothelial cells (HUVECs) were exposed to 1% O_2 hypoxic stress in a time-sequential manner. ILK was upregulated after 30 minutes of hypoxic stress and up to 24 hours in a time-dependent manner (Figure 1A). Phospho-Akt (p-Akt) and phospho-Erk (p-Erk) were also upregulated in 30 minutes

and showed a biphasic increase at 6 and 24 hours of hypoxia, whereas the total amount of Akt and Erk remained unchanged (online Data Supplement Figure I). Because activation of the IκB kinase (IKK) and degradation of the inhibitor of NF-κB (IκB) would lead to the nuclear translocation of NF-κB,[8] the level of intracellular IκBβ was measured to detect the degree of nuclear translocation of NF-κB. IκBβ was downregulated, in contrast to hypoxia-inducible factor-1α (HIF-1α) and ICAM-1, which were upregulated after 1 and 3 hours of hypoxia, respectively (Figure 1A; online Data Supplement Figure I). SDF-1, which is known to be secreted and expressed on the surface of hypoxic ECs,[3] was upregulated in response to the hypoxic stress as measured by ELISA (Figure 1B). In addition to assessing the total amount of ILK in hypoxia, the kinase activity of ILK was also measured with an in vitro kinase assay. With the same amount of ILK immunoprecipitated, the degree of phosphorylation of a given substrate, glycogen synthase kinase–3β, was greater in immunoprecipitated ILK from hypoxic HUVECs than in normoxic HUVECs (Figure 1C). Thus, ILK was increased not only in its amount but also in its kinase activity under hypoxia.

To confirm that the upregulation of ILK, ICAM-1, and SDF-1 also takes place in vivo, we used an established model
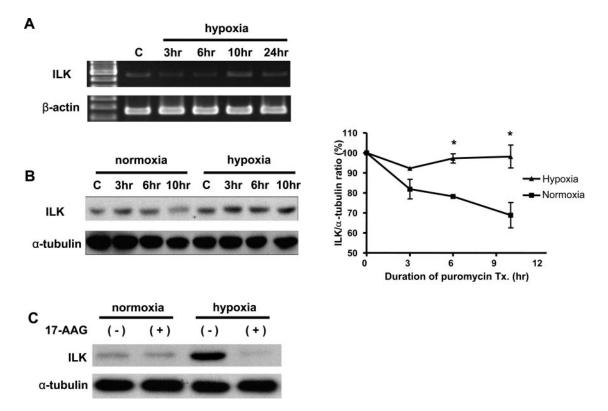
Figure 2. Mechanism of upregulation of endogenous ILK in hypoxic ECs. A, mRNA level of ILK at certain time points after hypoxia (n=3). B, Level of ILK in puromycin-treated normoxic and hypoxic HUVECs. Before puromycin treatment, HUVECs were exposed to normoxia or hypoxia for 24 hours. Data points in the plot indicate the percentage of ILK protein remaining compared with α-tubulin after puromycin treatment. *$P<0.05$ vs the same time point of normoxic ECs (n=3). C, Attenuation of the hypoxia-induced ILK increase by inhibition of Hsp90 in hypoxic HUVECs. HUVECs were treated with an Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, on initiation of hypoxia for 24 hours. Tx indicates treatment.

of ischemic hindlimb in mouse.[12] Laser Doppler imaging just after the operation confirmed the significant hypoxia induced by the procedure (Figure 1D), and the tissue specimens were harvested 6 hours after surgery. ECs in the ischemic limb showed significantly higher expression of intracellular ILK levels than nonischemic limb by immunofluorescence staining (Figure 1E). The hypoxia-induced increase in ILK was associated with increased SDF-1 (Figure 1F) and ICAM-1 (Figure 1G).

ILK Is Stabilized in Hypoxia by Heat Shock Protein 90

Given the previous finding that the amount of ILK was upregulated in hypoxic ECs, the mechanism underlying this finding was further elucidated. First, ECs were exposed to hypoxia and harvested for reverse transcription–polymerase chain reaction at certain time points to look for the possibility of transcriptional activation of ILK. The mRNA level of ILK did not change in response to hypoxia (Figure 2A), which led us to hypothesize that stabilization, rather than increased transcription of ILK, would be the cause of increased ILK. To verify this hypothesis, ECs were exposed to hypoxia for 24 hours, and puromycin, a synthetic inhibitor of protein synthesis, was added for the indicated time to block new synthesis of ILK and to examine the pure effect of intrinsic stabilization, as shown in previous reports.[13] The level of ILK, shown by Western blot, significantly increased in
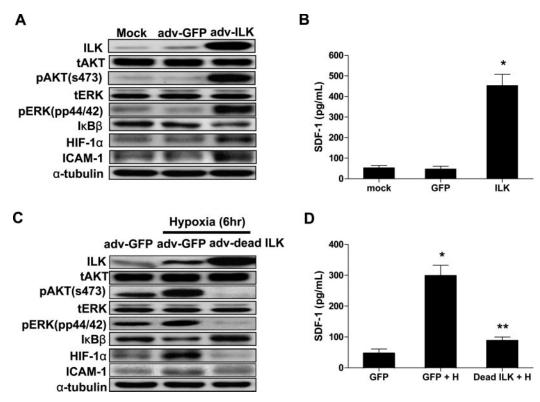
hypoxic HUVECs compared with normoxic HUVECs despite puromycin treatment (Figure 2B), which suggests that ILK was stabilized under hypoxic conditions.

Heat shock protein 90 (Hsp90), a well-known heat shock protein, is known to be a chaperone of various unstable intracellular proteins, such as endothelial NO synthase.[14] Therefore, we postulated that Hsp90 could prevent the natural degradation of ILK. Treatment of hypoxic HUVECs with a synthetic Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, abrogated the stabilization of ILK, resulting in decreased ILK despite hypoxia (Figure 2C), which suggests that Hsp90 is essential for stabilization of ILK in hypoxic ECs.

ILK Is an Important Upstream Regulator of ICAM-1 and SDF-1 Expression in ECs

On the basis of these results, we postulated that ILK could be an important upstream regulator of ICAM-1 and SDF-1. For overexpression and blockade of the kinase activity of ILK, adenovirus encoding ILK (adv-ILK) or a kinase-deficient, dominant-negative form of ILK (adv-dead ILK) was constructed with the appropriate plasmid. The kinase activity of both adenoviruses was confirmed in an in vitro kinase assay with glycogen synthase kinase–3β as described[15] previously (online Data Supplement Figures IIA and IIB).

To verify the effect of overexpressing ILK in normoxic ECs, adv-ILK–transfected HUVECs were analyzed with

Figure 3. Effect of ILK signaling on SDF-1 and ICAM-1 expression in normoxic and hypoxic ECs. A, Change in downstream molecules after transfection of adv-ILK in normoxic HUVECs (n=3). B, Upregulation of SDF-1 in normoxic HUVECs after adv-ILK transfection. *$P<0.05$ vs mock and GFP (n=3). C, Effect of blocking ILK on each downstream molecule in hypoxic HUVECs (n=3). D, Downregulation of SDF-1 in hypoxic HUVECs after blocking ILK with adv-dead ILK transfection. *$P<0.05$ vs adv-GFP transfected normoxic ECs, **$P<0.05$ vs adv-GFP transfected hypoxic ECs (n=3).

Western blot, immunofluorescence staining, and ELISA. Transfection of adv-ILK into normoxic ECs resulted in upregulation of p-Akt, p-Erk, HIF-1α, ICAM-1, and SDF-1 while downregulating IκBβ (Figures 3A and 3B). Upregulation of the final effector molecules, ICAM-1 and SDF-1, was also confirmed by immunofluorescence staining (online Data Supplement Figures IIC and IID).

Next, we tested whether the hypoxia-induced increase in SDF-1 and ICAM-1 was mediated via ILK by analyzing the effects of adv-dead ILK transfection in hypoxic ECs. Indeed, reversal of ILK activation by adv-dead ILK transfection resulted in a significant attenuation of downstream molecules in response to hypoxia. Hypoxia failed to upregulate p-Akt, p-Erk, HIF-1α, ICAM-1, and SDF-1 and failed to downregulate IκBβ in ECs transfected with adv-dead ILK (Figures 3C and 3D), which was also confirmed with immunofluorescence staining (online Data Supplement Figures IIE and IIF), which suggests that ILK is an important upstream regulator of ICAM-1 and SDF-1 in hypoxic ECs.

Upregulation of ICAM-1 and SDF-1 by ILK Is Dual-Regulated by HIF-1α and NF-κB

Given the previous findings that ILK is upregulated at an early time point of ischemia in ECs and that overexpression of ILK leads to upregulation of ICAM-1 and SDF-1, the downstream pathways regulating the expression of ICAM-1 and SDF-1 by ILK were studied. We overexpressed ILK in HUVECs using adv-ILK and applied chemical blockers to

each downstream molecule. The expression of each downstream molecule was assessed with Western blot and ELISA. Despite overexpression of ILK by adenoviral transfection, which leads to the overexpression of ICAM-1 and SDF-1, the addition of LY294002, PD98059, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole, or sulfasalazine, chemical inhibitors of p-Akt, p-Erk, HIF-1α, and NF-κB, respectively, all blocked the ILK-induced expression and production of ICAM-1 (Figure 4A) and SDF-1 (Figure 4B). Interestingly, although expression of the downstream molecule ICAM-1 was decreased, HIF-1α expression was only decreased by inhibition of p-Akt and not by inhibition of p-Erk, which suggests that p-Akt can regulate HIF-1α by controlling its translation, whereas p-Erk can only regulate the activity and not the amount of HIF-1α, which is concordant with a previous report.[16]

These results, along with previous reports that SDF-1 is regulated by the transcription factor HIF-1α[3] and ICAM-1 by NF-κB,[17] suggested to us that SDF-1 and ICAM-1 expression is under dual regulation by both HIF-1α and NF-κB. To prove this hypothesis and to find out which transcription factor has the dominant effects, we performed a chromatin immunoprecipitation (ChIP) assay as described previously.[3] As expected, SDF-1 and ICAM-1 were under direct regulation of both HIF-1α and NF-κB (Figure 4C), but SDF-1 was more dominantly regulated by HIF-1α, whereas ICAM-1 was more dominantly regulated by NF-κB. In addition to the ChIP assay, the mechanism of SDF-1 and ICAM-1 upregulation by
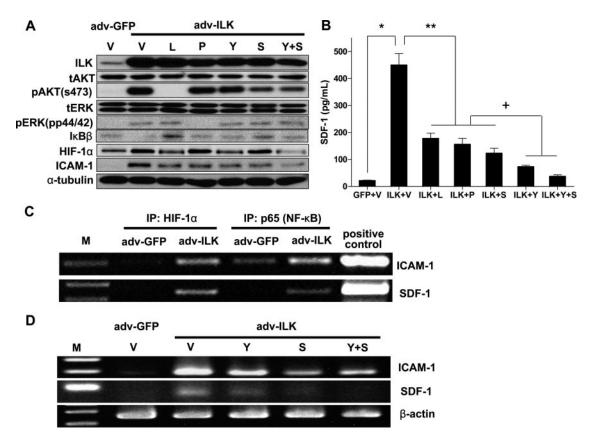
Figure 4. Mechanistic analysis of SDF-1 and ICAM-1 production by 2 transcription factors, HIF-1α and NF-κB. A, Expression of each downstream molecule after treatment of adv-ILK–transfected HUVECs with each specific chemical blocker for p-Akt, p-Erk, HIF-1α, and NF-κB. V indicates vehicle (DMSO); L, LY294002; P, PD98059; S, sulfasalazine; and Y, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl inda-zole (n=3). B, SDF-1 secretion from HUVECs cultured in the same conditions as Figure 4A. *$P<0.05$ vs adv-GFP transfected ECs, **$P<0.05$ vs adv-ILK–transfected ECs, +$P<0.05$ vs adv-ILK–transfected ECs treated with either LY294002, PD98059, or sulfasalazine only (n=4). C, ChIP analysis of SDF-1 and ICAM-1 specific genomic sequences from adv-ILK– or adv-GFP–transfected HUVECs with anti-HIF-1α and NF-κB (p65) monoclonal antibodies. D, Reverse transcription–polymerase chain reaction of ICAM-1 and SDF-1 in adv-ILK–transfected HUVECs after treatment of specific chemical inhibitors of HIF-1 and NF-κB.

adv-ILK transfection was investigated at the transcriptional level with reverse transcription–polymerase chain reaction. Transfection of adv-ILK resulted in increased mRNA level of both ICAM-1 and SDF-1. Inhibition of NF-κB by sulfasala-zine and HIF-1α by YC-1 resulted in decreased mRNA levels of SDF-1 and ICAM-1 (Figure 4D), which suggests that the upregulation of ICAM-1 and SDF-1 was controlled at the transcriptional level, under the control of HIF-1α and NF-κB.

ILK Activity Controls EPC Recruitment In Vitro and In Vivo

Because SDF-1 and ICAM-1 are counterparts of CXCR4 and β_2-integrin on EPCs, respectively, and are important in EPC recruitment to ischemic tissue,[2,3] we investigated the effect of ILK modulation on the incorporation of EPCs into the normoxic EC layer in vitro. Either adv–green fluorescent protein (GFP) or adv-ILK was transfected into normoxic HUVECs, and an incorporation assay was performed as described previously.[18] Overexpression of ILK in normoxic HUVECs resulted in significantly improved EPC incorpora-tion into ECs. Coincubation with either anti–ICAM-1 or SDF-1 neutralizing antibody resulted in significantly de-creased EPC incorporation, whereas coincubation with both neutralizing antibodies had greater effects on attenuating EPC

incorporation than any single antibody (Figure 5A; online Data Supplement Figure IIIA), which confirms the significant role that both SDF-1 and ICAM-1 play in EPC incorporation.

To identify the role of ILK in the incorporation of EPCs into hypoxic ECs, incorporation assay was done under hypoxia with either adv-GFP– or adv-dead ILK–transfected HUVECs. HUVECs under hypoxia incorporated more EPCs than normoxic HUVECs, but this finding was significantly attenuated when hypoxic HUVECs were transfected with adv-dead ILK (Figure 5B; online Data Supplement Figure IIIB).

To confirm the above in vitro findings in vivo, both femoral vessels of athymic nude mice were ligated, and EPCs were injected systemically by intracardiac puncture. One ischemic hindlimb was injected intramuscularly with adv-GFP while the other was injected with adv-dead ILK to make both hindlimbs compete with each other for EPC recruitment. Immunofluorescence staining with anti-SDF-1 and anti–ICAM-1 in both limbs showed decreased expression of both molecules in the adv-dead ILK–injected limb. These 2 molecules were localized in the interstitial space, mainly around preexisting vessels, whereas there was no staining of SDF-1 and ICAM-1 in the muscle area (Figure 5C). This was associated with decreased EPC recruitment to ischemic tis-
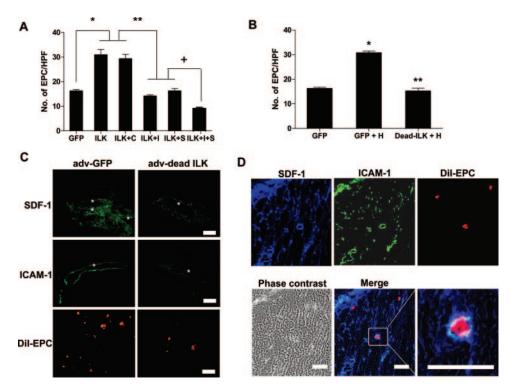
Figure 5. Effect of ILK modulation on EPC recruitment in vitro and in vivo. A, Effect of ILK overexpression on EPC incorporation in normoxic HUVECs. Ten random fields were counted from each set of experiments. HPF indicates high-power field; ILK+I+S, adv-ILK+anti–ICAM-1+anti-SDF-1; C, control antibody. *$P<0.05$ vs adv-GFP–transfected ECs; **$P<0.05$ vs adv-ILK–transfected ECs and adv-ILK–transfected ECs coincubated with control antibody; +$P<0.05$ vs adv-ILK–transfected ECs coincubated with either anti–ICAM-1 or anti-SDF-1 neutralizing antibody (n=4). B, Effect of blocking ILK activation on EPC incorporation in hypoxic HUVECs. GFP+H indicates adv-GFP–transfected hypoxic ECs; Dead-ILK+H, adv-dead ILK–transfected hypoxic ECs. *$P<0.05$ vs adv-GFP–transfected normoxic ECs; **$P<0.05$ vs adv-GFP–transfected hypoxic ECs (n=3). C, Changes in SDF-1 and ICAM-1 expression and DiI-EPC homing in hypoxic hindlimb after blocking of ILK. Bilateral hindlimb ischemia was induced with 1 limb injected with adv-GFP and the other with adv-dead ILK, and a total of 1×10^6 DiI-labeled EPCs were injected systemically via intracardiac puncture. D, Representative figure showing EPC homing to ischemic vessels coexpressing SDF-1 and ICAM-1. All scale bars are 100 μm.

sue, as measured by manual cell counting of the number of DiI-tagged EPCs in the tissue (Figure 5C; online Data Supplement Figure IIIC). DiI-tagged EPCs were colocalized to areas where SDF-1 and ICAM-1 was strongly expressed, mainly in the interstitial space of ischemic tissue (Figure 5D), when immunofluorescence stainings with anti-SDF-1 and anti–ICAM-1 were merged with the pictures for recruited EPCs. These findings demonstrate that ILK is a regulator of EPC recruitment to ischemic ECs in vitro and in vivo.

ILK Activity Controls Neovascularization In Vivo
To verify the role of ILK activity in controlling new vessel formation in hypoxic tissue, we used 2 models of ischemia, an ischemic hindlimb model and an in vivo Matrigel model. Because bilateral hindlimb ischemia showed an exceptionally high mortality rate in athymic nude mouse, the unilateral hindlimb ischemia model was used for the follow up of serial laser Doppler perfusion imaging analyses. Blocking ILK activity in the ischemic limb with adv-dead ILK transfection resulted in limb amputation (autoamputation/foot necrosis/limb salvage: 6/0/0), whereas the natural augmentation of ILK activity in control, adv-GFP limbs resulted in a significant increase in limb perfusion time sequentially (Figures 6A and 6B; online Data Supplement Figure IVA) and thus less amputation (autoamputation/foot necrosis/limb salvage:

1/4/1; online Data Supplement Figure IVB). Capillary staining of the ischemic limb with BS1-lectin showed poor neovascularization in adv-dead ILK–transfected mice (Figure 6C) and BS1-lectin–positive cells (Figure 6D).

The in vivo Matrigel assay showed similar results. Plugs mixed with adv-ILK were associated with significantly higher infiltration of cells than plugs mixed with adv-GFP or adv-dead ILK, as seen by hematoxylin-and-eosin staining (Figure 7A). The infiltration of CD31- and alkaline phosphatase–positive ECs was greatest in the adv-ILK plugs and poorest in the adv-dead ILK plugs. Moreover, there were significantly more vascular networks that penetrated the plug from the muscle layer and more vascular branches in the Matrigel in the adv-ILK plugs, as seen by the BS1-lectin and phase-contrast merged image (Figure 7A). In particular, the adv-ILK Matrigel plugs showed morphologically mature vessels in contrast to adv-GFP or adv-dead ILK Matrigel plugs, which only showed dispersed ECs (Figure 7A). These findings were confirmed by counting the CD31- and alkaline phosphatase–positive cells (Figure 7B). Although there was an insignificant decrease of alkaline phosphatase–positive cells in adv-dead ILK Matrigel, there was a significant increase and decrease in CD31-positive ECs infiltrating both adv-ILK and adv-dead ILK Matrigel, respectively. These in vivo findings suggest that ILK activity is important in controlling neovascularization.
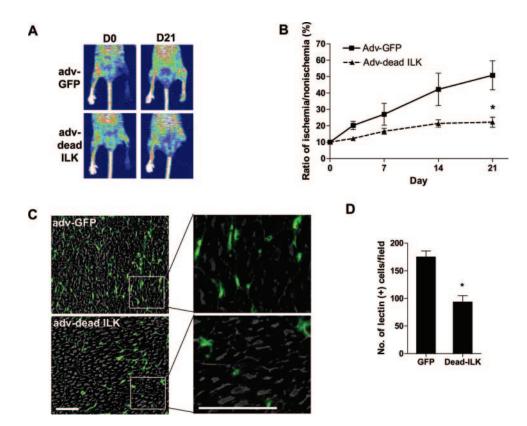
Figure 6. Effect of blocking of ILK on neovascularization in hindlimb ischemia model. A and B, Improvement of ischemic hindlimb perfusion in adv-GFP–injected groups compared with adv-dead ILK–injected groups after induction of hindlimb ischemia and EPC injection. Laser Doppler perfusion imaging was checked at day 3, 7, 14, and 21 after operation, and recovery of perfusion was assessed. *P<0.05 vs adv-GFP–transfected limb (n=5 or 6 for each group). C, Capillary density of adv-GFP–injected control ischemic limb vs adv-dead ILK–injected ischemic limb 21 days after operation. All scale bars are 100 μm. D, Degree of neovascularization in each ischemic hindlimb was corroborated with cell counting of BS1-lectin–positive ECs (*P<0.05 vs adv-GFP–transfected limb).

Discussion

The most important findings of the present study are that in ECs, endogenous ILK is a novel intracellular responder to hypoxic stress and that it lies upstream of important regulators of hypoxia, HIF-1 and NF-κB, controlling the expression of SDF-1 and ICAM-1, key molecules previously shown to be involved in selective recruitment of EPCs during vasculogenesis. The present study has several novel findings. First, we found that ILK is an endogenous, intracellular responder to hypoxia in ECs. Second, we showed that ILK could be stabilized by Hsp90 under hypoxic conditions in ECs. Third, we showed that HIF-1 is regulated by ILK via Akt/PKB and MAP kinase in an O_2-dependent manner. Fourth, not only were SDF-1 and ICAM-1 regulated by HIF-1[3] and NF-κB,[17] respectively, but there was also cross regulation of SDF-1 by NF-κB and of ICAM-1 by HIF-1. Finally, genetic modulation of ILK in ECs was shown to regulate postnatal neovascularization in vivo. This study not only shows that ILK is a key signaling molecule in hypoxic ECs but also extends our knowledge on the role of ILK in neovascularization.

ILK was reported to be an important regulator of angiogenesis.[10,11] There have not been any reports, however, about the intracellular signals involved in this pathway, nor has the role of ILK been investigated in the response of ECs to hypoxia. Previously, it was shown that the Akt/PKB,[19] Erk/MAP kinase,[20] and NF-κB[21] pathways are involved in

hypoxic cell signaling in ECs, which in turn have all been proved to be downstream molecules of ILK in various epithelial cells.[6,7,9] Thus, we hypothesized that ILK may function as a regulator of the intracellular signaling in hypoxic ECs and, ultimately, a regulator of ICAM-1 and SDF-1. From our results, we propose a scheme for the intracellular signaling pathways involved in EPC recruitment in Figure 8. Although this scheme is shown as a 1-way pathway, the possibility of an autocrine circuit that involves SDF-1→p-Akt→CXCR4→SDF-1,[22] shown as a dotted arrow in Figure 8, cannot be ignored. In fact, ILK has been shown to be involved in this autocrine circuit, and inhibition of ILK in hypoxic ECs leads not only to decreased SDF-1 expression but also to CXCR4 expression (data not shown).

The present results also suggest that upregulation of ILK in hypoxia is due to increased protein stabilization rather than transcription, because ILK mRNA was not changed after hypoxia, and ILK decreased after the addition of an Hsp90 inhibitor. Hsp90 was previously reported to be a stabilizer of ILK in 293T cell lines, and enhanced expression of Hsp90β resulted in increased intracellular levels of ILK,[23] but the present finding of ILK and Hsp90 interaction in ECs is novel. Although protein stabilization of ILK accounted for the upregulation of ILK in hypoxic ECs, the mechanism of persistent ILK production was not elucidated in this work. There are few previous works on this subject, and ILK has
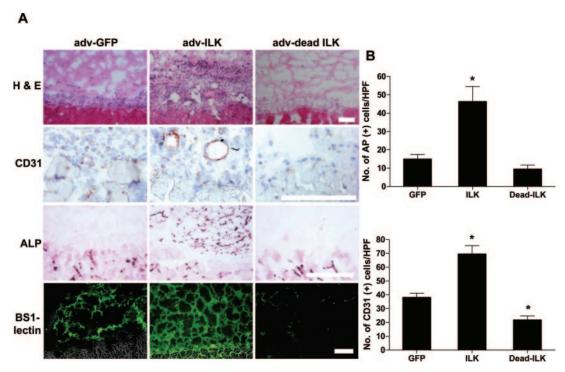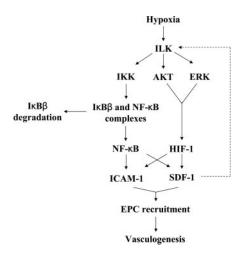
Figure 7. Effect of ILK modulation on in vivo Matrigel implantation model. A, Representative figures of each Matrigel stained for specific markers of ECs, CD31, alkaline phosphatase (ALP), and BS1-lectin. All scale bars are 100 μm (n=4 for each group). B, Manual counting of cells positive for CD31 and alkaline phosphatase. Five random fields were chosen and counted for each section of each Matrigel (*P<0.05 vs adv-GFP Matrigel). H&E indicates hematoxylin and eosin.

previously been described to be upregulated by transforming growth factor (TGF)-β in renal tubular epithelial cells.[24] However, neither blockade of TGF-β with neutralizing antibody nor blockade of intracellular TGF-β signaling with Smad7 transfection could block the upregulation of ILK in

Figure 8. Schematic model of SDF-1 and ICAM-1 expression under the control of ILK in hypoxic ECs. Hypoxia increases the amount and activity of ILK in ECs, where Hsp90 is involved in its stabilization. ILK in turn phosphorylates and activates Akt/PKB and MAP kinase. These are associated with stabilization of HIF-1 and its nuclear translocation. Another downstream molecule of ILK is IKK, which degrades IκBβ, leading to NF-κB nuclear translocation. HIF-1 and NF-κB work together to express various molecules involved in EPC recruitment, such as SDF-1 and ICAM-1, which in turn cooperate to selectively home EPCs to ischemic tissue.

hypoxic ECs (data not shown). In a previous report by Melchior et al,[25,26] there were 2 isoforms of ILK, namely, ILK1 and ILK2, that could only be discriminated by *Bam*HI cDNA restriction. ILK1, which has no TGF-β responsive element in its promoter site and is not restricted by *Bam*HI, was found to have typical characteristics of a housekeeping gene. In concordance with these characteristics, the upregulation of ILK in the present set of experiments was found to have the same characteristics of ILK1, the cDNA of which was not restricted by *Bam*HI restriction and the production of which was not blocked by blocking TGF-β (data not shown). This may explain in part why ILK is persistently produced in ECs and why the ILK level decreases after puromycin treatment.

In addition, Akt/PKB and Erk/MAP kinase activity has been known to be upregulated at 30 minutes and downregulated in 2 hours in response to hypoxia.[27] In the present study, Akt/PKB and Erk phosphorylation showed a biphasic response, increasing up to 6 hours. The difference in the duration of enhanced expression may be due to the type of ECs and culture medium used for the experiment. In the present study, the upregulation of ILK occurred at 30 minutes of exposure to hypoxia in vitro, which suggests that ILK is a very early, if not a primary, responder to hypoxia.

The regulation of SDF-1 and ICAM-1 by both HIF-1 and NF-κB implies that the expression or production of end products in hypoxia is not regulated by a single pathway but rather by a complex network of several transcription factors. However, the binding of HIF-1 to the SDF-1 promoter site was stronger than that to ICAM-1, and the opposite was true for NF-κB, as shown in the ChIP assay, which suggests that

although effector molecules are controlled by several transcription factors, there may be certain signals that are more predominant. Maintaining a complex web of signaling may be a means of maintaining the ability to respond to various exogenous stresses in an organism, even if a single pathway may be blocked.

Selective recruitment and binding of leukocytes to inflamed endothelium is a critical step in inflammation. Likewise, we speculated that the same process would be important in vasculogenesis, because EPCs share many common features, such as surface molecules, with peripheral blood monocytes.[28] CXCR4 and β_2-integrin on EPCs have been shown to be important molecules for chemotaxis and binding of EPCs to ischemic vessels.[2,3] On the basis of these previous results, we hypothesized and demonstrated that the presence of SDF-1 and ICAM-1 on hypoxic ECs is important for adhesion of EPCs to hypoxic ECs and thus for selective recruitment of EPCs to ischemic tissue. Of course, the possibility that EPCs may reside in the pericellular area in the incorporation assay done in vitro and that EPCs also may be recruited to the perivascular area and not actually incorporated into the neovessels in vivo cannot be overlooked. Nonetheless, the main thesis of the present study was to show the importance of ILK in recruiting these EPCs to hypoxic ECs rather than the role of EPC itself in new vessel formation. Although there may be several molecules responsible for EPC recruitment, and EPC-associated neovascularization may not be entirely responsible for ischemic tissue salvage, homing of the EPCs by SDF-1 and ICAM-1 in hypoxic vessels may be an important component of ischemic tissue salvage, as shown in Figure 8.

Finding strategies to control vasculogenesis is an area of intense interest in the field of cardiovascular research. Options to maximize the efficiency of vasculogenesis include mobilization of EPCs, functional modification of mobilized EPCs, and selective recruitment of EPCs to ischemic areas. Considering the scarcity of EPCs even in mobilized peripheral blood, optimizing the selective recruitment of EPCs to ischemic areas would be an efficacious method of enhancing vasculogenesis, and it would be very helpful to find the key molecule in this process. In association with our previous findings,[6] ILK may be a good candidate molecule, either for enhancing the function of EPCs or for enhancing the recruitment of EPCs to ischemic tissue.

In conclusion, ILK is an important intracellular molecule that regulates the response of ECs to hypoxic stress, and modulating the activity of ILK would be a good target to control the process of vasculogenesis. Unraveling the natural response of ECs to exogenous stimuli may provide good novel targets to enhance or suppress EPC recruitment and neovascularization.

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CLINICAL PERSPECTIVE

For clinicians facing the difficult task of saving organs or tissues at risk of ischemic death every day, enhancing the growth of blood vessels can be an attractive method of saving these organs. Since the discovery of endothelial progenitor cells (EPCs) in adult human peripheral blood, clinicians are now faced with the problem of augmenting the recruitment of these cells to the most appropriate tissue. Although understanding the mechanism of this homing process can be a crucial step in bringing this procedure into clinical practice, there have been few reports on this problem. What is the key molecule that orchestrates the expression of adhesion molecules in hypoxic endothelium, and how does it do so? How is this related to the recruitment of EPCs to the appropriate, ischemic tissue? Does control of this molecule ultimately lead to control of neovascularization? These are the key questions that we tried to answer. In this work, integrin-linked kinase (ILK) has been shown to be an important mediator of neovascularization, possibly by controlling the homing of EPCs to ischemic tissue. Furthermore, this process was mediated by stromal cell–derived factor-1 and intercellular adhesion molecule-1, the key molecules known to be important in EPC recruitment in ischemic tissue; ILK has also been found to be upstream of various molecules known to be responsive to ischemia, such as hypoxia-inducible factor-1 and nuclear factor-κB. In conclusion, this work provides new insight into the mechanism of neovascularization by EPC recruitment and emphasizes the possible importance of manipulating ILK in ischemic tissue in the future.