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Objective—New vessel formation is a dynamic process of attachment, detachment, and reattachment of endothelial cells (ECs) and endothelial progenitor cells (EPCs) with each other and with the extracellular matrix (ECM). Integrin-linked kinase (ILK) plays a pivotal role in ECM-mediated signaling. Therefore, we investigated the role of ILK in ECs and EPCs during neovascularization.

Methods and Results—In human umbilical cord vein ECs and EPCs, endogenous ILK expression, along with subsequent cell survival signals phospho-Akt and phospho–glycogen synthase kinase 3β, was reduced after anchorage or nutrient deprivation. Even brief anchorage deprivation resulted in retarded capillary tube formation by ECs. Adenoviral ILK gene transfer in ECs and EPCs reversed the decrease in cell survival signals after anchorage or nutrient deprivation, leading to enhanced survival, reduced apoptosis, and significantly accelerated the functional recovery after reattachment. And ILK overexpressing EPCs significantly improved blood flow recovery and prevented limb loss in nude mice hindlimb ischemia model. Furthermore, the efficacy of systemic delivery was equivalent to local injection of ILK-EPCs.

Conclusions—ILK overexpression protects ECs and EPCs from anchorage- or nutrient-deprived stress and enhances neovascularization, suggesting that ILK is an optimal target gene for genetically modified cell-based therapy. Neovascularization is a dynamic process of detachment and reattachment of ECs and EPCs. Endogenous ILK expression was decreased in various stress conditions, and the gene transfer of ILK protected ECs and EPCs from temporary anchorage or nutrient deprivation. Furthermore, ILK gene transfer in EPCs significantly enhanced neovascularization in vivo. (Arterioscler Thromb Vasc Biol. 2005;25:1154-1160.)

Key Words: angiogenesis ■ endothelium ■ ischemia ■ Integrin-linked kinase

New vessel formation or angiogenesis is a complex process1,2 that may be simplified as a sequential process of endothelial cell (EC) detachment from the extracellular matrix (ECM), reattachment onto new ECM, and final incorporation into new vasculature. The ability of ECs to form capillary-like structures is essential for angiogenesis in vivo. However, in ischemic tissue, because of the disruption of the microenvironment, which is necessary for sprouting and recruiting of pre-existing ECs, the survival and incorporation of ECs are not facilitated.3

There is accumulating evidence that endothelial progenitor cells (EPCs) contribute to vessel growth in ischemic and inflamed tissue in adults.4-6 Despite its promising application for tissue regeneration, the limited endogenous pool and the possible functional impairment associated with a variety of physiological and pathological conditions limit its use on clinical patients.7 Various types of genetic modifications of EPCs are under investigation to overcome such limitations.8,9 Most of the studies focused on the survival and proliferation of EPCs, and few studies, if any, focused on the overall vasculogenic sequence of EPCs, which are usually mobilized or injected at a remote site from target tissue in the anchorage-deprived detached status, and finally reattach and incorporate into the ischemic target area.

The dynamic interactions between cell–ECM and cell–cell play critical roles during angiogenesis.10 Cell adhesion to the ECM results in differentiation, proliferation, and suppression of apoptosis.11 Integrin-linked kinase (ILK) is a novel, ankyrin repeat–containing serine threonine kinase that exhibits cell type-dependent activation and inhibition,12 the activity of which is rapidly and transiently stimulated by cell–ECM interaction.13 It is an important regulator of cell–ECM inter-
action that leads to cell survival and death.\textsuperscript{14} However, the function of this gene on ECs and EPCs during the angiogenic process is largely unknown.

Therefore, we investigated the basal expression pattern of ILK in ECs and EPCs in various stress conditions, such as detachment (anchorage deprivation), reattachment, and nutrient deprivation, and examined the effects of ILK gene transfer on survival, apoptosis, and capillary formation in vitro, along with the downstream signaling pathway of ILK that controls the survival and apoptosis of EPCs. Moreover, the effects of ILK-modified EPCs on neovascularization in vivo, was also studied in a hindlimb ischemia model.

Methods

Cell Culture

Human umbilical vein ECs (HUVECs; Cambrex) were plated on 2\% gelatin-coated (Sigma) dishes and cultured in EC basal medium (EBM) or EC growth medium (EGM; Clonetics) supplemented with 5\% FBS.

For EPC cultures, 50 cc of peripheral blood was obtained from healthy volunteers with informed consent. The mononuclear cells were fractionated as described previously.\textsuperscript{15,16} Isolated mononuclear cells were resuspended by EGM–2MV media with 5\% FBS. Cells were plated on gelatin-coated plates with $2 \times 10^5$ cells/cm\textsuperscript{2} density. Three-week cultured cobblestone-shaped EPCs were used as we reported previously.\textsuperscript{16,17} All experiments dealing with humans or human products were approved by the institutional review board of Seoul National University Hospital.

To examine the kinetics of ILK expression on suspension culture, HUVECs and EPCs were trypsinized and seeded on the polyhydroxyethylmethacrylate (poly-HEMA)–coated dishes. Poly-HEMA solution (10 mg/mL; Sigma), an adhesion inhibitor, was dispensed to cover the entire surface of each well or dish as described previously.\textsuperscript{19,20}

Recombinant ILK Adenovirus

Construction of adenovirus ILK (Ad-ILK), human wild-type ILK plasmid (Upstate), was cloned into a shuttle vector (pAdTrack-CMV). The recombinant shuttle vector was cotransfected with adenoviral genome (pAdEasy–1)–containing green fluorescence protein (GFP) gene also into Escherichia coli (BJ5183), where homologous recombination occurred. The construct of Ad-ILK was confirmed by DNA sequencing, and the expression was confirmed by immunoblot analysis. Adenovirus–encoding GFP was used as control viral transduction.

Western Blotting, Cell Viability Assays, and Apoptosis Analysis

Immunoblot assays were performed by modification of the procedures described previously.\textsuperscript{19} The membrane was incubated with primary antibody, ILK (Cell Signaling), phospho-AKT (S473; Cell Signaling), and phospho–glycogen synthase kinase-3β (GSK3β; S9; Cell Signaling), overnight at 4°C. Enhanced chemiluminescence (ECL) or ECL–PLUS (Amersham) was used for detection.

Cell viability was assessed by cell proliferation reagent WST-1 assay, as instructed by the manufacturer (Roche), and apoptosis was measured by fluorescence-activated cell sorting (fluorescence-activated cell sorter [FACS]) of hypodiploid DNA, and observations of pyknotic nuclei and phosphatidylserine staining by modification of procedures described previously.\textsuperscript{19,20}

In Vitro Capillary Formation

Matrigel (Becton Dickinson Labware) basement membrane matrix was thawed and mixed 1:1 with EBM. Diluted Matrigel was added to chamber slide. After 1 hour incubation at room temperature, $2 \times 10^5$ cells were added to the chamber slide with 300 μL EGM. The slide was observed under an inverted microscopy 12 hours later. Five representative fields were taken, and the average of the total area of tubes completely formed by cells or the total length of the tubes was compared.

In Vitro Incorporation

Twenty-four hours after gene transfer, Ad-ILK EPCs and Ad-GFP EPCs were incubated on a monolayer of HUVECs. Five hours after incubation, nonadherent cells were removed by washing with PBS. The total number of adhesive EPCs in each well was counted under a ×100 magnification field of a fluorescent microscope.\textsuperscript{8}

In Vivo Transplantation of Genetically Modified EPCs in Hindlimb Ischemia Model

Female athymic nude mice (n=41; Daehan Biolink; Korea) 7 to 8 weeks old weighing 17 to 20 g were anesthetized, and an ipsilateral femoral artery was surgically removed as described previously.\textsuperscript{21} EPCs transduced with Ad-ILK or Ad-GFP were washed gently $5 \times$ with PBS, harvested by brief trypsinization, and resuspended with growth factor–free EBM. The suspension volume of injected cells was $1 \times 10^6$ μL. On the basis of our previously reported laboratory data, the dose of injected cells was determined to be from $1 \times 10^4$ to $2 \times 10^5$ per mouse because too high of a dose of EPCs may salvage most limbs and conceal the effect of Ad-ILK on angiogenic potential of EPCs. As an administrative route for EPCs, we chose direct cell infusion into systemic arterial circulation through left ventricular cavity to determine the tolerance of EPC anchorage-deprived stress during angiogenesis or direct local muscular injection into 4 separate sites of thigh muscle to minimize cellular stress. All animal experiments were performed after receiving approval of the Institutional Animal Care and Use Committee of Clinical Research Institute in Seoul National University Hospital and complied with National Research Council Guidelines for the Care and Use of Laboratory Animals (revised 1996).

Physiological and Histological Evaluation of In Vivo Angiogenesis Effect

Each mouse was followed by serial recording of surface blood flow of hindlimb on days 0, 3, 7, 14, and 21 by laser Doppler perfusion imager (Moor Instrument). Photos of limb were also recorded and visually analyzed as “limb salvage” (completely normal status without a sign of ischemia), “foot necrosis” (necrosis of toe or below knee), or “auto amputation” (necrosis or loss of tissue above knee).

Tissue vascular density was determined at the microvascular level as a histological evaluation of neovascularization. At day 21, mice were euthanized, and the lower thigh muscles of ischemic limb were harvested and snap-frozen in liquid nitrogen–chilled isopentane. Tissues were embedded in OCT compound (Sakura Finetek), and multiple 10-μm-thick slices were prepared. Tissue sections were stained with tetramethylrhodamine B isothiocyanate (TRITC)–jugulated murine EC–specific Bandeiraea simplicifolia lectin 1 (BS-1; Sigma). Five random fields were selected from each slice, and fluorescent vascular ECs were counted.

Statistical Analysis

All data are presented as the mean±SEM. Statistical significance was evaluated by means of Student’s $t$ test or ANOVA with Bonferroni’s correction using SPSS version 11.0. The incidence of limb salvage was evaluated by $\chi^2$ test. A $P$ value of $<0.05$ was considered to denote statistical significance.

Results

Expression of Endogenous ILK in ECs and EPCs Decreased in Various Stresses

Western blot analysis was used to characterize the endogenous expression of ILK in HUVECs under various stressful conditions for each cell type. The expression of endogenous ILK in HUVECs under various stressful conditions, such as detachment (anchorage deprivation), reattachment, and nutrient deprivation, and examined the effects of ILK gene transfer on survival, apoptosis, and capillary formation in vitro, along with the downstream signaling pathway of ILK that controls the survival and apoptosis of EPCs. Moreover, the effects of ILK-modified EPCs on neovascularization in vivo, was also studied in a hindlimb ischemia model.
conditions such as detachment (anchorage deprivation), reattachment, and nutrient deprivation (Figure 1). Endogenous ILK expression in ECs was transiently upregulated during 6 hours after detachment, and then it was decreased rapidly. However, on reattachment on ECM after detachment, the expression of ILK recovered. Next, we compared ILK expression patterns between HUVECs and EPCs by serial immunoblot analysis (Figure 1) and reconfirmed by paired immunoblot (Figure 1A, available online at http://atvb.ahajournals.org). Overall, the serial ILK expression pattern of EPCs was similar to HUVECs. However, in EPCs, the basal level of endogenous ILK was upregulated, peaking at 6 hours of anchorage deprivation as cultured on poly-HEMA (adhesion inhibitor)–coated dish, and then decreased much more slowly than HUVECs (Figure 1B). After 48 hours of detached stress, EPCs were reattached on gelatin-coated dish, which led to increased expression of ILK and its downstream molecules phosphorylated forms of Akt and GSK3β, lasting for 24 hours. In the nutrient deprivation (serum-free and growth factor–free media), ILK expression rapidly decreased in HUVECs, whereas it was maintained and decreased slowly in EPCs.

**ILK Protects HUVECs From Apoptosis by Anchorage Deprivation and Promotes Cell Proliferation and Tube Formation After Reattachment**

Adenovirus encoding the human ILK gene followed by GFP gene under the control of a CMV promoter (Ad-ILK) was constructed to investigate the effects of ILK overexpression. The survival, apoptosis of HUVECs, and the expression of downstream molecules of ILK were examined 48 hours after anchorage deprivation in cells transfected with Ad-ILK. Survival of HUVECs transfected with Ad-ILK increased significantly by 2-fold (P value <0.01) compared with Ad-GFP HUVECs (Figure 2A) as measured by WST-1 assay. In addition, ILK gene transfer protected HUVECs from detachment-induced apoptosis (apoptotic fraction 41% versus 63% for Ad-ILK versus Ad-GFP HUVECs) as measured by FACS analysis. Accordingly, the expression of downstream
molecules, active phospho-Akt, a well-known survival signal, and inactive phospho-GSK3β, a well known antiapoptotic signal, were considerably increased in Ad-ILK HUVECs compared with Ad-GFP HUVECs (Figure 2B). After 24 hours of anchorage deprivation, HUVECs were allowed to reattach on gelatin-coated dishes. After reattachment, cell proliferation significantly increased in Ad-ILK HUVECs compared with Ad-GFP HUVECs (Figure 2C and 2D), which led to functional improvement as measured by in vitro Matrigel tube formation assay (Figure 2E), suggesting that overexpression of ILK mediates recovery of cell proliferation and function after reattachment.

**ILK Protects HUVECs From Nutrient Deprivation**

To demonstrate the effect of ILK gene transfer under nutrient deprivation, HUVECs were starved serum and growth factor in EBM for 24 hours. The viability of Ad-ILK HUVECs, measured by WST-1 assay, was twice that of Ad-GFP HUVECs (Figure 2F; P value <0.01), and the apoptotic fraction was significantly reduced in the Ad-ILK HUVECs (50% versus 65% for Ad-ILK versus Ad-GFP HUVECs). Along with increased survival and resistance to apoptosis, Ad-ILK HUVECs again showed significantly greater tubulogenic capacity compared with the Ad-GFP HUVECs in the nutrient-deprived condition. Not only the network length of the tubes, but also the tube area of capillaries completely encircled with HUVECs was significantly greater in Ad-ILK HUVECs (Figure 2G and 2H).

**Impact of ILK Gene Transfer in EPCs**

We then investigated the possibility of genetic modification of EPCs with wild-type ILK. After full transduction with either wild-type ILK or GFP, downstream molecule expression was similar to HUVECs overexpressing ILK or GFP (Figure 3A). To confirm the functional improvement of ILK-modified EPCs, in vitro incorporation of EPCs on HUVEC monolayer was tested. Significantly more Ad-ILK EPCs incorporated on the HUVEC monolayer compared with Ad-GFP EPCs (Figure 3B and 3C). Under the anchorage- or nutrient-deprived condition, EPCs overexpressing ILK showed improved cell viability and reduced apoptosis compared with Ad-GFP EPCs, similar to what was observed in HUVECs (Figure 3D through 3G).

**Genetically Modified EPCs Enhance Angiogenesis In Vivo**

In an athymic mouse hindlimb ischemia model, we tested the systemic and local injection methods to assess the impact of ILK transfection in vivo. One day after unilateral femoral artery excision, athymic mice received either systemic (via intracardiac injection) or local injections (via direct muscular injection) of Ad-ILK EPCs or Ad-GFP EPCs (as control). To determine the tolerance of anchorage deprivation and reattachment stress, we performed systemic arterial delivery and, to minimized anchorage-deprivation, direct local delivery was performed. We observed capillary density, serial tissue perfusion, and fate of ischemic limb. In the histological examination of skeletal muscle sections at day 21, capillary density observed in the mice transplanted with ILK-EPCs was significantly higher than in the mice with GFP-EPCs (Figure 4); a similar result was observed in serial tissue perfusion by laser Doppler images (Figure 5). More than half of the mice that received systemic injection of $2 \times 10^5$ GFP EPCs (n=6 of 11) developed extensive necrosis of the ischemic hindlimb, and eventually, the limbs were auto amputated during serial examination (Figure 5C). This finding meant that systemic delivery of $2 \times 10^5$ native EPCs was insufficient for neovascularization in the athymic nude mouse, which has inherent impaired angiogenesis. However, when EPCs genetically modified by ILK transfection were systemically injected, even at the $20 \times$ less dose (ILK4; $1 \times 10^4$ ILK-EPCs), it resulted in a significantly better outcome than the systemic delivery of GFP-EPCs (ILK4 versus GFP, P <0.01, Figure 4, capillary density; ILK4 versus GFP, P <0.01; n=6). ILK overexpression also reduced EPCs apoptosis by 28% as measured by FACS analysis.

**Figure 3.** ILK overexpression in EPCs in vitro. ILK gene transfer improved the in vitro survival and function of EPCs. A, Adenoviral transduction was performed and confirmed with inverted fluorescence microscope in ex vivo expanded human EPCs. The upregulated expressions of ILK and its downstream molecules were confirmed by Western blots. B, In vitro incorporation of ILK overexpressing EPCs on HUVEC monolayer. More ILK-EPCs incorporated into the HUVEC monolayer compared with GFP-EPCs. Green dots represent the incorporated EPCs. C, Quantitative analysis of EPC incorporation observed at 4 hours of incubation (P<0.01). D and E, At 48 hours after anchorage deprivation, ILK-EPCs were about twice as viable as GFP-EPCs in WST-1 assay (P<0.05; n=6). The effect of ILK gene transfer to reduce apoptosis by anchorage deprivation was less dramatic in EPCs in FACS analysis, which may be associated with the high basal resistance of EPCs.
In the comparison between systemic and local delivery of ILK-modified EPCs in the ischemic limbs, capillary density in the local delivery group was greater than in the systemic injection group (Figure 4; ILK5L versus ILK5; \( P < 0.02 \)), but recovery of tissue perfusion and final outcome was quite comparable between 2 groups (Figure 5C). Between 2 different cell dose (2\( \times 10^5 \) and 1\( \times 10^5 \)), systemically delivered ILK-EPCs groups, there was no significant difference in capillary density, tissue perfusion, and final outcome.

**Discussion**

This study reveals that ILK is important in EC and EPC biology, playing a pivotal role to control the cell survival, proliferation, and apoptosis leading to modulation of capillary tube formation in stressful conditions unavoidable during the process of angiogenesis, such as anchorage or nutrient deprivation. Furthermore, we show that EPCs genetically modified by ILK get the satisfactory outcome to salvage the ischemic limb. Our data show for the first time the potential of ILK as a target to improve the efficacy of cell-based therapeutic angiogenesis.

Cell attachment is mediated mainly through the engagement of ECM with integrin molecules.22 It has been shown recently that the protein kinase signaling pathways control anchorage-dependent cell survival positively and negatively.23 ILK was first identified as a protein that binds to the \( \beta_1 \)-integrin subunit, and ILK expression has been documented in the cardiac muscle, skeletal muscle, kidney, and pancreas.12 Integrin-mediated cell anchorage is essential for cell survival. It has been demonstrated in epithelial cells and tumor cells that overexpression of ILK results in the stimulation of anchorage-independent cell growth, cell cycle progression, and suppresses apoptosis.10,24,25 In ECs, it has been shown that...
oxidized low-density lipoprotein (LDL) induces ILK expression, and that overexpression of ILK can prevent oxidized LDL–induced apoptosis.26

The endogenous ILK expression pattern, in response to various stresses observed in our study, revealed that it is only transiently upregulated and then decreased rapidly in ECs and EPCs, which is insufficient to maintain cell survival and proliferation in these stressful conditions. Although overall ILK expression pattern in EPCs was similar to ECs, the expression of ILK in EPCs was higher at baseline and decreased more slowly than ECs after exposure to stressful conditions, suggesting that the higher basal expression and maintenance of such expression levels of ILK may play an important role in opposing stresses. Taking this into account, we examined the effect of ILK gene transfer in ECs and EPCs under various stresses. The effects of ILK overexpression on viability and apoptosis of EPCs in anchorage or nutrient deprivation condition were somewhat different from those of HUVECs. Under either anchorage-deprived or nutrient-deprived stress, ILK gene transfer significantly increased the viable cell numbers of EPCs as well as HUVECs. Regarding the apoptosis, ILK gene transfer significantly reduced apoptosis of EPCs and HUVECs in nutrient-deprived stress. But in anchorage-deprived condition, antiapoptotic effect of ILK gene transfer was less dramatic in EPCs than HUVECs; ILK gene transfer reduced apoptosis of EPCs from 48% to 42% and that of HUVECs from 63% to 41%. This may be explained by the higher basal resistance of EPCs against anchorage deprivation than HUVECs. Thus, the beneficial effect of ILK gene transfer would be more dramatic in HUVECs than in EPCs.

The results of our tests provide potential insights into the mechanisms responsible for neovascularization, which require a process of sequential detachment from ECM and reattachment onto new ECM. The interaction of ECs with the ECM inhibits default apoptotic pathways, which become activated when cell–ECM interactions are disrupted. However, IL- overexpressing ECs and EPCs were able to overcome anoikis,27 suspension-induced apoptosis, in vitro. In our vivo experiment, the dose of native EPCs (2×103), delivered systemically, was subtherapeutic to salvage the ischemic hindlimb. In contrast, genetic modification with ILK resulted in a significantly improved outcome even at a dose, which was only one twentieth (1×103) of the control experiment. Furthermore, the efficacy of systemic delivery of EPCs with ILK overexpression was comparable to that of local injection of EPCs. These findings suggest that the efficacy of systemic administration of EPCs to salvage ischemic limb is significantly dependent on the inevitable stresses, such as anchorage deprivation during systemic administration and nutrient deprivation in ischemic tissue. Genetic modification with ILK may be a very effective strategy to overcome such inevitable stresses during cell therapy for ischemic tissue.

As for which cell signal pathway mediates these changes, we found that the ILK/Akt/GSK3β axis is activated in ECs and EPCs. ILK is believed to function as the effector of phosphatidylinositol 3-kinase signaling, which positively regulates protein kinase B/Akt activity and negatively regulates GSK3 activity.28,29 In addition to regulating the Akt/GSK axis, ILK mediates the transduction of signals from ECM components to intracellular protein.30 Therefore, strategies targeting ILK overexpression may be highly effective in cell-based therapeutic vasculogenesis in ischemic area.

In conclusion, we showed for the first time that ILK enhanced survival, reduced apoptosis, and significantly improved function of ECs and EPCs to augment angiogenesis. This kind of genetic modification to strengthen the EPCs against the anchorage or nutrient deprivation could improve the efficacy of therapeutic vasculogenesis by enabling the systemic administration of the reduced number of cells required for optimal neovascularization.

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References


Figure I. ILK expression pattern in endothelial cells and endothelial progenitor cells. A, Paired immunoblot analysis was used to reconfirmed the endogenous expression level of the ILK in HUVECs and EPCs exposed to various stresses. B, Four blots were used for quantification of serial ILK expression pattern between two cell types. The expression of ILK greater at baseline in EPCs compared with HUVECs, and decreased to a lesser degree after exposure to anchorage-deprivation. Tubulin was used for loading control. * $P<0.05$, ILK in EPC versus HUVEC.