Integrin-Linked Kinase Controls Choroidal Neovascularization by Recruitment of Endothelial Progenitor Cells

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Choroidal neovascularization (CNV) complicating age-related macular degeneration (AMD) is the most common cause of severe visual loss in people older than 60 in developed countries.1 CNV is a complex process, and tissue ischemia is thought to be involved in its development.2 There is accumulating evidence that not only angiogenesis but also vasculogenesis participates in the development of CNV3–4—the peripheral blood contains a population of bone marrow-derived endothelial progenitor cells (EPC) that can differentiate into endothelial cells.

CXC chemokine receptor 4 (CXCR4) and vascular endothelial growth factor receptor 2 (VEGFR2), expressed on EPC, play crucial roles in the homing of these cells to CNV; these receptors’ counterparts molecules, stromal cell–derived factor-1 (SDF-1) and VEGF respectively, are expressed by the cell component within the CNV lesion, especially retinal pigment epithelium (RPE).3,6 However, little is known about the intracellular signaling pathways involved in orchestrating the expression of these molecules by RPE in response to hypoxia and how this is related to the recruitment of EPC to the CNV area.

Integrin-linked kinase (ILK) is a 59-kDa Ser/Thr kinase that binds to the cytoplasmic domain of βintegrin-4 and lies upstream of protein kinase B (PKB/AKT),7,8 and mitogen-activated protein (MAP) kinase,9 important molecules in inflammation and neovascularization. Recently, ILK was found to be involved in vessel formation.10,11 Moreover, ILK also participates in postnatal vasculogenesis by recruiting EPC to CNV lesions, possibly through ILK-dependent expression of SDF-1 and VEGF in RPE.

Keywords: choroidal neovascularization, endothelial progenitor cell, retinal pigment epithelium, integrin-linked kinase, hypoxia
ischemic tissues or by augmenting the angiogenic properties of EPC.

The purpose of this study was to investigate the role of ILK in CNV. We postulated that endogenous ILK in RPE would be important in regulating pathways responsible for the expression of key molecules during vasculogenesis in response to hypoxia. We showed here for the first time that ILK is a novel regulator of RPE response to hypoxia and controls the homing of EPC to CNV.

METHODS

Cell Cultures

Primary cultured human RPE cells were obtained from a mature cell line that had been preserved in our laboratory as previously described. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO₂ humidified incubator. All experiments were performed on cells from passages 3 to 6.

For EPC culture, 50 mL peripheral blood was obtained from healthy volunteers with informed consent and cultured as previously described. Briefly, the mononuclear cells were fractionated with Ficoll-Hypaque (TBD, TianJin, China), and isolated mononuclear cells were plated in human fibronectin-coated plates (Sigma-Aldrich Corp., St. Louis, MO, USA) with EGM-2MV media supplemented with 5% fetal bovine serum. The final density of plated cells was 5 × 10⁶ cells/cm². After 3 days of culture, floating cells were discarded and the media were changed. Each cluster or colony was followed daily. The EPC were used 7 days after culture. All experiments dealing with humans or human products were approved by the institutional review board of Fourth Military Medical University.

Treatment of RPE Cells

Before the experiment, RPE cells were starved with DMEM without serum overnight. Then 200 µM CoCl₂ (Sigma-Aldrich Corp.) was applied to the cells to mimic chemical hypoxia for 1, 4, 8, 12, and 24 hours. LY294002 (PI3K inhibitor, 30 µM; Sigma-Aldrich Corp.) or PD98059 (MEK inhibitor, 20 µM; Sigma-Aldrich Corp.) was used to pretreat for 1 hour, and the cells were further cultured for the indicated time.

For ILK small interfering RNA (siRNA) transfection, 3 µg ILK siRNA (sense: GACGCUCAGCAGACAUGUGGATT; antisense: UCCACAUGUCUGCUGAGCGUCTT) or scrambled siRNA (sense: UUCUCCGAACGUUCGGAGU; antisense: ACGUGACACGUCAUCGGAGU) with 6 µL transfection agent (lipofectamine 2000; Invitrogen, Carlsbad, CA, USA) was cotransfected.

**TABLE 2. Primer Sequences**

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<th>Name</th>
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TPCR, melting temperature for PCR.

**FIGURE 1.** Expression of ILK in RPE cells. (A, B) Immunofluorescence staining of ILK (green fluorescence) in normoxic (A) and hypoxic (B) RPE; nucleus was counterstained by 4′,6-diamidino-2-phenylindole (DAPI). Scale bar: 50 µm. (C, D) Expression of ILK by Western blotting (C) and by RT-PCR (D).
into the cells according to the manufacturer’s instructions (Invitrogen). Twenty-four hours after transfection, cells were further cultured for another 24 hours with or without CoCl₂.

**Proliferation Assay**

To examine the effect of ILK from hypoxic RPE cells on the proliferation of EPC, RPE cells cultured in the transwell inserts were transfected with ILK siRNA, and 1 × 10⁵ EPC were cultured in 24-well plates. Twenty-four hours after transfection, EPC were cocultured with transfected RPE cells treated with or without CoCl₂ for another 48 hours. EPC were fixed with ethanol after washing with PBS three times. The total number of EPC cells was counted under a ×40 magnification field from five random fields. Each set of experiments was repeated three times.

**Migration Assay**

To examine the effect of ILK from hypoxic RPE cells on the migration of EPC, RPE cells cultured in the 24-well plate were transfected with ILK siRNA. EPC (1 × 10⁵) were added to the transwell inserts. When EPC attached to the inserts, then EPC were cocultured with transfected RPE cells for another 48 hours with or without CoCl₂. EPC were fixed with ethanol after washing with PBS three times. The total number of migrated EPC cells was counted under a ×40 magnification field from five random fields. Each set of experiments was repeated three times.

**Adhesion Assay**

To examine the effect of ILK from hypoxic RPE cells on the adhesion of EPC, RPE cells were transfected with ILK siRNA. CM-DiI–tagged EPC (1 × 10⁵) were added to the cultured RPE cells and further cultured for 2 hours. Nonadhesive EPC were removed by washing with PBS three times. The total number of EPC binding to RPE cells was counted under fluorescence using a ×40 magnification field from five random fields. Each set of experiments was repeated three times.

**ELISA Assay**

Human VEGF ELISA kits were used to measure the expression level of VEGF protein secreted by RPE cells under different conditions (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

**Induction of CNV**

A total of 36 Brown Norway rats (Vital River Laboratory, Beijing, China), weighing 180 to 220 g each, were used in the study in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the Ethics Committee of the Fourth Military Medical University, and followed the tenets of the Declaration of Helsinki.

Laser-induced CNV was performed as previously described. Each rat received bilateral treatment. The rats were anesthetized for all procedures with an intraperitoneal injection of 1% sodium pentobarbital (45 mg/kg body weight), and the ocular surface was then anesthetized with topical instillation of amethocaine hydrochloride. The pupils were dilated with tropicamide (5 mg/mL) and phenylephrine hydrochloride (5 mg/mL). Eight laser spots (130 mW, 0.1 second, 75 μm) were delivered with a diode-pumped frequency-doubled 532-nm laser (Oculight GLx; Iridex, Mountain View, CA, USA) between the retinal vessels in a peripapillary distribution in each fundus. Production of a subretinal bubble at the time of laser treatment confirmed the rupture of Bruch’s membrane.

**Treatment With ILK siRNA**

After photocoagulation, all animals were randomly divided into three groups. For the ILK siRNA group, the lasered eyes immediately received intravitreal injection as previously described with 4 μg ILK siRNA conjugated with transit-TKO reagent (Roche, Mannheim, Germany) on days 0 and 7. The control group received without intravitreal injection at all. Animals were killed after photocoagulation at days 1, 3, 7, and 14. The rats were divided into four main groups (Table 1).
Immunohistochemistry and Immunofluorescence Staining

Rats were euthanized and perfused with 4% polyoxymethylene solution. Serial 4-μm paraffin sections of rat eyes were obtained at 7 days after photocoagulation. Sections were incubated sequentially with rabbit anti-ILK antibody (1:100; Cell Signaling Technology, Beverly, MA, USA), biotinylated secondary anti-rabbit antibody (1:300; Sigma-Aldrich Corp.), and streptavidin peroxidase (Vector Laboratories, Burlingame, CA, USA), with three PBS washes in between. Specificity of staining was assessed by substitution of nonimmune serum for primary antibody. Immunoreactivity was visualized with the peroxidase substrate amino ethyl carbazole (AEC kit; TBD). Slides were counterstained with hematoxylin and observed using a bright-field microscope (Olympus, Tokyo, Japan).

Serial 10-μm cryosections of rat eyes (three eyes from each group) were obtained at 3, 7, and 14 days after photocoagulation. Slides were then incubated with primary antibodies for mouse anti-hypoxia-inducible factor 1α (HIF-1α) (1:500; Chemicon, Temecula, CA, USA), goat anti-VEGF (1:100; Santa Cruz, Fremont, CA, USA), goat anti-SDF-1 (1:50; Santa Cruz), goat anti-CXCR4 (1:50; Santa Cruz), rabbit anti-ILK (1:100; Cell Signaling Technology), rat anti-CD34 (1:50; Abcam, Cambridge, MA, USA), rabbit anti-CD133 (1:50; Abcam), rabbit anti-VEGFR2 (1:50; Abcam), and mouse CK18 (1:100; Santa Cruz). This was followed by incubation with FITC-conjugated goat anti-rabbit IgG (1:100; ZhongShan Corporation, Beijing, China), AMCA-conjugated donkey anti-rat IgG (1:500; Jackson, West Grove, PA, USA), FITC-conjugated donkey anti-goat IgG (1:500; Jackson), tetramethylrhodamine (TRITC)-conjugated donkey anti-goat IgG (1:500; Jackson), TRITC-conjugated goat anti-rabbit IgG (1:100; ZhongShan Corporation), and TRITC-conjugated goat anti-mouse IgG (1:100; ZhongShan Corporation). After rinsing in PBS, slides were coverslipped with antifade medium and examined with a confocal laser scanning microscope (Fluoview 300, Olympus) using laser beams of 488 and 543 nm with appropriate emission filters for green fluorescence (503–508 nm) and red fluorescence (590–610 nm), respectively. The observation was carried out with an ultraviolet-corrected objective lens (UPLAPO40×). Digital images were captured by Fluoview application software (Olympus).

Fundus Fluorescence Angiography

The CNV lesions were studied at 14 days after laser photocoagulation by fundus fluorescence angiography (FFA) with a confocal scanning laser ophthalmoscope (Retinal Angiography; Heidelberg Engineering, Heidelberg, Germany). Fluorescein sodium (10%, 0.1 mL/kg) was injected into the tail vein of the anesthetized rats. Late-phase angiograms were obtained 5 minutes after injection. The area of CNV on FFA was measured with Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA).
Bethesda, MD, USA). The mean area of CNV was derived from measurement of all the CNV lesions (five eyes from each group) by two masked specialists.

**Choroidal Flat Mounts**

Two weeks after treatment, RPE-choroid-sclera complexes (three eyes from each group) were fixed with 4% paraformaldehyde, then incubated with HEPES-buffered saline containing 1:1000 rhodamine-conjugated *Ricinus communis* agglutinin (Vector Laboratories). CNV was visualized at 543-nm wavelength using a scanning laser confocal microscope (FV 1000; Olympus). The area of CNV-related fluorescence was measured by Image-Pro Plus software (Media Cybernetics).

**Real-Time PCR and RT-PCR Analyses**

Total RNA was prepared from either treated RPE cells or three eye cups (RPE-choroid-sclera complex) from each group using Trizol reagent (Invitrogen) and reverse-transcribed with a cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit; Fermentas, Lithuania). Real-time PCR was conducted with the ABI 7000 SDS (Applied Biosystems, Foster City, CA, USA) using SYBR green (TaKaRa Bio, Inc., Nojihigashi, Kusatsu, Shiga, Japan). Each cDNA sample was analyzed in duplicate.

RT-PCR was performed with Taq polymerase (Promega, Madison, WI, USA). Then 1% ethidium bromide-stained agarose gels were scanned using a Fluor-Multimager (BioRad, West Berkeley, CA, USA). Band intensities for PCR products were quantitated by Gelpro 4.5 software (Media Cybernetics). See Table 2 for primer sequences.

**Western Blot Analysis**

At each time point the cells were collected, lysed, and then assessed for protein concentration (Bradford assay; Bio-Rad Laboratories, Munich, Germany). Electrophoresis of proteins was performed with 12% or 15% SDS-polyacrylamide gels; 15 μg proteins was loaded on each lane. After the protein was electrotransferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA), the membrane was blocked in a solution of 5% (wt/vol) skim milk powder in PBS (pH 7.5) for 1 hour at room temperature and then probed overnight at 4°C with AKT/pAKT (Ser473; 1:500; Cell Signaling Technology), ERK/pERK (Thr202/Tyr204; 1:200, Santa Cruz), HIF-1α (1:500, Chemicon), SDF-1 (1:200, Santa Cruz), or ILK (1:500, Cell Signaling Technology) antibodies. After washing with PBS, horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) were incubated for 1 hour at room temperature. Immunoreactivity was visualized by enhanced chemiluminescence reagent (ECL; Amersham Biosciences, Marlborough, MA, USA). For sequential blotting with additional antibodies, the membranes were stripped using a Restore Western blot stripping buffer and reprobed with the indicated antibodies. Protein levels were quantitated by densitometry and normalized to the β-actin levels. Band intensities were quantitated by Gelpro 4.5 software (Media Cybernetics).

**Statistical Analysis**

All results were expressed as the mean ± SD. Statistical analyses were made using 1-way ANOVA followed by least significant difference (L) and Student-Newman-Keuls (S) tests for multiple comparisons by SPSS 19.0 software (Chicago, IL, USA). A P value <0.05 was considered statistically significant.

**RESULTS**

**Hypoxia Upregulates ILK and Its Downstream Molecules in RPE**

We first determined the expression of ILK in RPE. ILK was mainly expressed in the cytoplasm and membrane of RPE (Fig. 1A). Hypoxia strongly increased its expression at both the protein level (Figs. 1B, 1C) and the mRNA level (Fig. 1D) in a time-dependent manner. According to our previous results, HIF-1α and VEGF were upregulated in cultured RPE cells under...
FIGURE 5. Effect of ILK siRNA on EPC proliferation, migration, and adhesion induced by hypoxic RPE cells. *Compared to normoxic sc siRNA-treated group $P < 0.05$; • compared to hypoxic sc siRNA-treated group $P < 0.05$ ($n = 5$).

FIGURE 6. Expression of ILK, HIF-1α, SDF-1, and VEGF by RPE cells in rat CNV by immunofluorescence staining. HIF-1α, SDF-1, and VEGF expression (red fluorescence) by RPE cells that were identified by CK-18 (blue fluorescence) in CNV area 7 days after photocoagulation. Scale bars: 50 μm.
In this experiment, we further confirmed that hypoxia also greatly induced SDF-1 expression in hypoxic RPE in a time-dependent manner (Fig. 2). The highest protein level for SDF-1 was at 24 hours after CoCl₂ treatment (Fig. 2B). Real-time PCR results indicated hypoxia also increased SDF-1 mRNA with greatest expression at 4 hours after hypoxia (Fig. 2A).

**ILK Is an Important Upstream Regulator of SDF-1 and VEGF in Hypoxic RPE**

In order to check whether ILK could be an important upstream regulator of SDF-1 and VEGF, we silenced the expression of ILK in RPE by transfecting the cells with one
ILK siRNA. Twelve hours after transfection, cells were further treated with CoCl₂ for another 12 hours. As expected, ILK siRNA significantly attenuated the expression of ILK, HIF-1α, VEGF, and SDF-1 induced by CoCl₂ at both mRNA and protein levels (Figs. 3A–C), indicating that HIF-1α, SDF-1, and VEGF were at least partly under the regulation of ILK in RPE cells under hypoxia.

ILK Regulates the Expression of SDF-1 and VEGF in Hypoxic RPE Through PI3K/AKT and MEK/ERK Pathways

We have illustrated that PI3K/AKT and MEK/ERK are two signaling pathways that at least partly control the expression of HIF-1α and VEGF in hypoxic RPE cells. We next studied whether these two pathways were also involved in the regulation of SDF-1 in cultured RPE cells. When PI3K/AKT inhibitor (LY294002) and MEK/ERK inhibitor (PD98059) were applied to the RPE cells separately, 1 hour before CoCl₂ treatment, both the phosphorylation of AKT and ERK and the expression of SDF-1 were dramatically attenuated (Figs. 2B, 2C). Therefore, we concluded that the upregulation of HIF-1α, SDF-1, and VEGF in hypoxic RPE was mediated by PI3K/AKT and MEK/ERK pathways.

Since ILK governed the expression of HIF-1α, SDF-1, and VEGF, we then investigated whether ILK regulated its downstream molecules through these two pathways. As shown in Figure 3D, the phosphorylation of both AKT and ERK was significantly decreased by ILK siRNA in hypoxic RPE, whereas their total protein levels were not affected.

The above results show that PI3K/AKT and MEK/ERK pathways were under the regulation of ILK. ILK could also govern the expression of HIF-1α, SDF-1, and VEGF in hypoxic RPE through PI3K/AKT and MEK/ERK pathways.

ILK-siRNA–Transfected RPE Cells Attenuate the Proliferation, Migration, and Adhesion of EPC

We then further studied whether RPE-derived ILK played a role in the recruitment of EPC in vitro. Primary cultured human peripheral blood EPC were positive for CD31, CD34, CD133, CXCR4, and VEGF receptor VEGFR2 (Fig. 4). We next cocultured RPE-EPC cells to investigate the effect of ILK expressed by RPE cells on EPC ex vivo recruitment. The results indicated that RPE cells under hypoxia greatly induced the proliferation, migration, and adhesion of EPC cells. When ILK expression was abolished by transfecting the RPE cells with one ILK siRNA, the proliferation, migration, and adhesion of EPC cells were also significantly attenuated (Fig. 5). These results mean that hypoxic RPE cells had a positive effect on EPC recruitment through production of more ILK.

ILK and Its Downstream Molecules Are Upregulated in Laser-Induced Rat CNV

Previously we reported that HIF-1α and VEGF were stimulated in CNV. In this paper, we first confirmed that ILK and its downstream molecules were expressed in the RPE cells within CNV (Fig. 6). We next checked the expression pattern of ILK and SDF-1 in CNV. We found that in the normal rat retina ILK was mainly distributed in the ganglion cell layer, the inner nuclear layer, and the blood vessels of both retina and choroid (Fig. 7A, green arrows). Moreover, ILK was strongly induced in the CNV area and the retina above the lasered lesion (Fig. 7B, green arrows). ILK protein level was greatly upregulated 1 day after photocoagulation and peaked at day 3 (Fig. 7C). The mRNA level had a similar expression trend (Fig. 7D). For SDF-1, in the normal retina SDF-1 was mainly expressed in the ganglion cell layer, the inner and outer plexiform layers, and the RPE layer. SDF-1 was greatly elevated within the lasered lesions by photocoagulation with a peak expression at day 7 at both mRNA and protein levels (Fig. 8).
Intravitreous Injection of ILK siRNA Prohibits the Recruitment of EPC to Laser-Induced Rat CNV

Our results showed that EPC indeed participated in the development of rat CNV. We found that in CNV lesions there were CD34/CD133-positive cells, which indicated that EPC really existed in rat CNV lesions (Fig. 9). We further found that EPC within the CNV area also expressed CXCR4 and VEGFR2 (Fig. 9). In our previous work we found that EPC were recruited to the CNV area 3 days after photocoagulation, with the number increasing to its greatest level at 7 days, and then decreasing after 14 days. EPC not only constituted CNV; it also played a role in the development of CNV through secreting growth factors such as bFGF, VEGF, MMP9, and IL-10. Based on the above in vitro results we further studied the effect of ILK in vivo. Intravitreous injection of ILK siRNA could severely decrease the number of EPC within the CNV area (Fig. 10).

Intravitreous Injection of ILK siRNA Inhibits the Development of Laser-Induced Rat CNV

We finally examined the effect of ILK on the development of rat CNV. We showed that intravitreous injection of ILK siRNA significantly decreased the size of CNV by flat-mount staining and the extent of fluorescence leakage by FFA (Fig. 11).

DISCUSSION

Several studies of CNV now provide evidence that EPC may be the major contributor to the formation and growth of CNV.17
The contribution of EPC to the CNV lesion was thought to be 20% to 45%. In our study, we confirmed that EPC is involved in the development of rat CNV not only through differentiating into endothelial cells but also through releasing several proangiogenic factors. The mechanisms involved in homing, recruitment, and migration of EPC to areas of NV remain to be fully elucidated. Several studies have indicated the importance of ILK in vasculogenesis, such as hindlimb ischemia and myocardial infarction. In our experiment, we also found that the expression of ILK was dramatically increased in the CNV area, especially during the early stage of CNV. Intravitreal injection of ILK siRNA significantly impaired the growth of CNV, and ILK presence was required for VEGF-stimulated endothelial cell migration, tube formation, and MAP kinase pathways. SDF-1 and VEGF expressed by RPE mediate the chemotaxis and binding of EPC to ischemic vessels.

We have already confirmed that RPE is an important cell component in CNV and involved in the growth of CNV through releasing several proangiogenic factors. It was the main contributor for ILK, HIF-1α, VEGF, and SDF-1 in the CNV area. Our previous work estimated that hypoxia played a major role in triggering ocular neovascularization by inducing several angiogenic factors. Thus, we hypothesized that ILK may function as a regulator of the intracellular signaling in hypoxic RPE and, ultimately, a regulator of VEGF and SDF-1. Both the protein and the mRNA levels of VEGF and SDF-1 were increased in hypoxic RPE and this upregulation was abrogated by ILK siRNA, which means that VEGF and SDF-1 were under the regulation of ILK in RPE cells under hypoxia. PKB/AKT and ERK/MAP kinase pathways were involved in hypoxic cell signaling in RPE. ILK presence was required for VEGF-induced phosphorylation of AKT in retinal endothelial cells. Phosphorylation of AKT and ERK resulted in stimulation of HIF-1α, which contributes to the upregulation of VEGF and SDF-1. The present study further confirmed that these two pathways were also under the regulation of ILK. Inhibiting the expression of ILK caused a decrease in AKT and ERK phosphorylation, and inhibition of AKT and ERK phosphorylation resulted in the reduction of SDF-1 and VEGF protein and mRNA levels. These data suggest that ILK has a crucial role in SDF-1 and VEGF expression via activation of AKT and ERK pathways in RPE cells. ILK regulates HIF-1α-mediated VEGF expression in glioblastoma and prostate cancer cells, and is essential for VEGF-stimulated endothelial cell migration, tube formation, and tumor angiogenesis. Here we found that ILK also promoted EPC angiogenic effect in vitro. RPE transfection with ILK siRNA severely abrogated the proliferation, migration, and adhesion of EPC to RPE.

In conclusion, we found that in RPE cells, endogenous ILK is a novel intracellular responder to hypoxic stress and that it lies upstream of the important regulator of hypoxia, HIF-1, controlling the expression of SDF-1 and VEGF via PKB/AKT and MAP kinase pathways. SDF-1 and VEGF expressed by RPE played crucial roles in the angiogenic effect of EPC through their counterpart molecules CXCR4 and VEGFR2 expressed on EPC in vitro. The present study has several novel findings. First, we found that ILK is an endogenous, intracellular responder to hypoxia in RPE. Second, HIF-1α is regulated by ILK via PKB/AKT and MAP kinase under hypoxia. Third, SDF-1 and VEGF expressed by RPE and under the regulation of ILK played key roles in EPC vasculogenesis in vitro. Finally, inhibiting the expression of ILK severely prohibited the growth of CNV.

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