The four-and-a-half LIM domain (FHL) protein family is a newly identified group of proteins containing four-and-a-half LIM domains. This family consists of six members—FHL1, FHL2, FHL3, FHL4, FHL5, and ACT (Activator of CREM in Testis). The LIM domain is a zinc-binding, cysteine-rich motif consisting of two tandemly repeated zinc fingers that are thought primarily to mediate protein–protein interactions. Johannessen et al. showed that human four-and-a-half-LIM-only protein family members, including FHL2, are expressed in a cell- and tissue-specific manner and participate in various cellular processes such as regulation of gene expression, cytoarchitecture, cell adhesion, cell survival, cell mobility, transcription, and signal transduction. Our previous work has demonstrated that FHL2 is abundantly expressed in the vascular system, including blood vessels, suggesting that it might play an important physiological or pathologic role in the regulation of the circulatory system. Therefore, we have investigated the role of FHL2 protein during corneal angiogenesis induced by chemical and mechanical denudation of corneal and limbal epithelium.

Conorl transparency and avascularity are important for maintaining the proper optical performance of the cornea. Corneal neovascularization (CNV) involves the development of new vascular structures in areas that were previously avascular and is a sight-threatening condition associated with cloudy cornea. CNV may be induced by infection, inflammation, degeneration, or delayed wound-healing disorders in ocular surfaces. CNV could result from a disrupted balance between the upregulation of angiogenic factors and the downregulation of antiangiogenic factors.

The extent of CNV was quantified and analyzed in different experimental groups. Vascular endothelial growth factor (VEGF), a key mediator of vasogenic and angiogenic events, controls pathologic angiogenesis and increased vascular permeability in diseased cornea. Additionally, the prostaglandin-cyclooxygenase pathway has been reported to influence new blood vessel growth in a variety of tissues. The isoenzymes cyclooxygenase (COX)-1 and COX-2 are involved in prostaglandin biosynthesis. Generally, COX-1 is expressed in most tissues and cells, whereas COX-2 is upregulated by cytokines, inflammatory mediators, and tumor progression. Overexpression of COX-2 in colon cancer cells and endothelium increases the production of prostaglandins and angiogenic cytokines, migration of endothelial cells, and tube formation. Therefore, in the present study, the levels of VEGF and COX-2 have been measured in neovascularized cornea and compared among different groups after sodium hydroxide (NaOH) injury.

To determine the role of FHL2 in angiogenesis, we assessed the effects of corneal injury in the FHL2-null mice model that we previously generated through homologous recombination. Our results suggest that deletion of the FHL2 gene attenuates the process of corneal angiogenesis.
MATERIALS AND METHODS

Generation and Maintenance of FHL2-Deficient Mice

FHL2-null mice were generated as previously described. Briefly, the endogenous ATG start codon of FHL2 was replaced by a cDNA encoding LacZ and a pGKneo cassette. In this manner, the lacZ cDNA was brought under the control of the endogenous FHL2 promoter while it ablated the endogenous FHL2 gene. All animals used in this study (FHL2-null and wild-type littermates) were on a C57BL/6 genetic background and were genotyped by PCR. All procedures for handling mice were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital and were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

β-Gal Staining for LacZ Reporter Gene in FHL2-Null Mice

For LacZ expression analysis, wild-type and FHL2-null corneal sections were fixed and stained using reported procedures. The duration of staining was dependent on color development and ranged from 4 to 12 hours at room temperature, or 30°C. Samples were photographed and analyzed under a dissecting microscope.

Murine Models of Chemical-Induced Angiogenesis

Heterozygous interbreeding generated a cohort of 180 male FHL2-null and wild-type mice that were divided into four groups, as follows: FHL2-null control, FHL2-null injury, wild-type control, and wild-type injury. At 6 to 8 weeks of age, 50% of the wild-type and FHL2-null mice were anesthetized with tribromoethanol (Avertin; Sigma-Aldrich, St. Louis, MO). The model of angiogenesis in mouse cornea was established according to a previously established protocol. Briefly, filter paper cut by a trephine of 2-mm diameter was placed on the central cornea, and 2 μL of 0.15 M NaOH was applied to the right cornea of each mouse for 1 minute after topical application of proparacaine (Alcaine; Falcon Pharmaceuticals, Fort Worth, TX) to each eye. Corneal and limbal epithelia were removed with a corneal knife (Tooke; Katena Products, Denville, NJ) using a rotary motion applied parallel to the limbus and rinsed with normal saline. Erythromycin ophthalmic ointment was applied immediately after epithelial denudation. The most prominent neovascularization was observed on the 10th day after injury.

Labeling of Corneal Neovascularization

Immunohistochemical staining of vascular endothelial cells was performed on corneal flat mounts. Briefly, fresh corneas were dissected, rinsed with PBS for 30 minutes, and fixed in 100% acetone (Sigma) for 20 minutes. After the corneas were washed in PBS three times, nonspecific binding was blocked with 0.1% PBS and 2% albumin (Sigma) for 1 hour at room temperature. Corneal flat mounts were incubated with FITC-coupled monoclonal anti-mouse CD31 antibody (PharMingen, San Diego, CA) at a concentration of 1:500 in 0.1 M PBS and 2% albumin at 4°C overnight and then washed in PBS three times at room temperature. Samples were mounted with an antifading agent (Gelmount; Biomeda, San Francisco, CA) and visualized with a fluorescence microscope (Leica, Wetzlar, Germany).

Quantification Corneal Neovascularization and Statistical Analysis

For CNV quantification, image analysis software (Image-Pro Plus; Media Cybernetics, Bethesda, MD) was used to delineate CD31-stained vessels on the corneal surface. The entire mounted cornea was analyzed to minimize sampling bias. Documentation and calculations of CNV length were performed in a masked fashion. Each experiment was performed at least three times with similar results. For quantification of the differences in CNV in various groups, one-way ANOVA was used, followed by the Tukey-Kramer test for multiple comparisons. P < 0.05 was considered significant.

Western Blot Analysis

Corneal tissues from eight mice per group (FHL2-null control, FHL2-null injury, wild-type control, and wild-type injury) were collected for Western blot analysis. The cornea was dissected and homogenized with 2 mL lysis 250 (50 mM Tris-HCl [pH 7.4]), 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 25 mM NaF, and 1× protease inhibitor cocktail (Sigma). The lysis 250 soluble fraction was separated by centrifugation at 13,000 rpm for 5 minutes at 4°C. Protein concentrations from lysis 250 soluble fractions were measured by spectrophotometry at OD 280 nm. An equal volume of 2× SDS sample buffer was added to each sample. Each sample was boiled for 10 minutes and subjected to 10% SDS-PAGE, followed by Western blotting with anti-VEGF (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), COX-2 (1:1000; Novus Biologicals, Littleton, CO), or actin (1:15,000; Chemicon, Temecula, CA) antibody. The immunocomplex was visualized with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (1:1000; Elmer) and stabilized substrate (Western Blue; Promega).

RESULTS

FHL2 Gene Expression in Corneal Tissue after Chemical Injury

FHL2-null mice were generated by targeted replacement of the FHL2 gene with a LacZ reporter gene fused in-frame downstream of the FHL2 regulatory region. Therefore, cis-element-reporter constructs linking LacZ were used to trace the temporal and spatial expressions of specific gene. β-Gal staining of the FHL2-LacZ knock-in mice revealed that sporadic FHL2 expression is found in corneal epithelial cells but is upregulated in swelling epithelial cells after corneal injury (Fig. 1).

Reduction of Corneal Neovascularization in FHL2-Null Mice after Corneal Injury

One hundred twelve FHL2-null mice and 68 wild-type mice were used in this study. No significant differences in phenotype, such as eye size or corneal clarity, were found between FHL2-null mice and wild-type mice before the chemical-induced angiogenesis study. Corneal neovascularization was recorded over time after injury (1 day, 2 days, 5 days, 7 days, 10 days, and 20 days). The timing of new blood vessel appearance in the cornea was similar in wild-type and FHL2-null mice. The most prominent neovascularization was observed on the 10th day after injury in both groups. As shown in Figure 2, increased CNV after chemical injury was demonstrated by CD31 (Fig. 2A, right side) staining in wild-type and FHL2-null mice. However, the density of new blood vessel growth was significantly higher in wild-type mice than in FHL2-null mice (P < 0.001).

Western Blots of VEGF and COX-2 Expression in Wild-Type and FHL2-Null Mice after Corneal Injury

Protein levels of VEGF and COX-2 were measured by Western blot analysis using protein extracts from different groups. VEGF and COX2 expression levels were increased after chemical injury in wild-type and FHL2-null mice (Fig. 3), whereas COX-2 levels were upregulated in the FHL2-null injury group and the wild-type injury group. COX-2 levels were significantly different between wild-type control and wild-type injured cornea and between FHL2-null control and FHL2-null injured cornea (P = 0.029 and P = 0.014, respectively; Fig. 3B), but there was no significant difference in COX-2 expression levels between the FHL2-null mice injury group and the wild-type mice injury group (P = 0.167 and P > 0.05, respectively; Fig.
3B). In contrast, there was a significant difference in VEGF expression between the FHL2-null mice injury group and the wild-type mice injury group. VEGF levels were not significantly different between the FHL2-null control and the FHL2-null injured corneas (P/H11005 0.051), whereas VEGF levels were significantly different between the wild-type control and the wild-type injured corneas and between the wild-type injured and the FHL2-null injured corneas (P/H11005 0.030 and P/H11005 0.041, respectively; Fig. 3B).

**DISCUSSION**

In the present study we demonstrate that the pathologic progression of corneal injury-induced neovascular lesions is reduced in FHL2-deficient mice. Chemical and mechanical denudation of the corneal and limbal epithelia induce angiogenesis by tilting the balance between angiogenic and antiangiogenic factors toward an angiogenic response. Previous studies have shown that FHL2, a member of the four-and-a-half LIM domain-only protein family, is expressed in heart, blood vessels, and skeletal muscle during development. Additionally, it has been reported that some four-and-a-half LIM domain-only proteins participate in cell lineage determination and pattern formation during development. To investigate the expression and function of the FHL2 gene, we used homologous recombination to target a LacZ cDNA into the endogenous FHL2 locus. β-Galactosidase staining of FHL2-LacZ knock-in corneal sections shows that LacZ expression increases significantly in the corneal epithelium layer after chemical injury. Except for corneal epithelial cells, there is no LacZ expression in the corneal stroma or endothelium layer of FHL2-LacZ knock-in mice. Our previous studies have shown a significant increase in FHL2 expression in the developing vasculature, indicating that FHL2 plays an important role in the development of the circulatory system. In this study, we demonstrate that deletion of the FHL2 gene attenuates the neovascularization response to corneal injury. These results clearly point toward an important pathophysiologic role for FHL2 during angiogenesis.

VEGF, a potent angiogenic stimulator, has been reported to promote proliferation, migration, proteolytic activity, and capillary tube formation in endothelial cells. During CNV, VEGF is highly expressed in the vascular endothelial cells of limbal vessels and in newly formed vessels in the stroma but is

**Figure 1.** Localization of FHL2 gene expression in corneal tissue. β-Gal staining of FHL2-LacZ knock-in mice reveals FHL2 expression in the different layers of epithelium (A, 200×; C, 600×, arrow head, blue spot); FHL2-LacZ overexpression in swelling epithelial cells after corneal injury (B, 200×; D, 600×, arrow head, blue spot).

**Figure 2.** Comparison of CNV in FHL2-null mice and wild-type mice after alkali injury. (A) CNV after 10th day of injury was observed in wild-type (+/+) injury and FHL2-null mice (−/−) injury. Wild-type mice (+/+) show more CNV after corneal injury (+/+) injury, whereas FHL2-null mice show less CNV (−/−) injury. CD31 immunostaining was shown on the right side. (B) The extent of neovascularization was much lower in FHL2-null mice (−/−) compared with wild-type mice (+/+) demonstrated by CD-31 stains and semiquantitative analytical methods.
induced angiogenesis. Furthermore, our data show that dele-
that VEGF might play a more important role during chemical-
tween wild-type and wild-type mice after chemical injury (Fig. 3). We found no
lower in the P/H11021 compared with wild-type controls (bar 3 vs. 4; left). (*P < 0.05) Moreover, the protein level of VEGF was significantly
attenuation of CNV after alkali injury. Further work is needed
to the nucleus. CREM and ACT.
Transcriptional cascades during spermatogenesis: pivotal role of
inn/TCF-mediated transcription and ERK-2–mediated induction
of transcription.53–55 Future work will focus on elucidating the molecular mechanisms that mediate FHL2-regulated inhibition of CNV.
In conclusion, FHL2 is expressed in the cornea, and its
eexpression is significantly increased after corneal injury. Dele-
tion of FHL2 is associated with relative resistance to angio-
genesis, including a decrease in VEGF expression and inflamma-
tion of FHL2 protein in the ocular surface may be partly
responsible for CNV after chemical-induced inflammatory
angiogenesis. These results suggest that FHL2 is intimately asso-
ciated with the regulation of inflammation function and the
process of angiogenesis. To our knowledge, this is the first
report documenting the potential involvement of FHL2 in the
attenuation of CNV after alkali injury. Further work is needed
to delineate the complex interactions between angiogenic fac-
tors and antiangiogenic factors and will increase our under-
standing of the molecular mechanism of regulation of angio-
genesis in the corneas of FHL2-null mice.

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References
1. Kadrmas JL, Beckerle MC. The LIM domain: from the cytoskeleton
2. Chu PH, Ruiz-Lozano P, Zhou Q, Cai C, Chen J. Expression patterns of
FHL/SLIM family members suggest important functional roles in
skeletal muscle and cardiovascular system. Mech Dev. 2000;95:
259–265.
3. Fimia GM, Morlon A, Macho B, De Cesare D, Sassone-Corsi P.
Transcriptional cascades during spermatogenesis: pivotal role of
protein, is highly expressed in ced gill pillar cells and responds to
wall tension. Am J Physiol Regul Integr Comp Physiol. 2004;287:
R1141–R1154.
multifunctional roles of the four-and-a-half-LIM only protein FHL2.


34. Purcell NH, Darwin D, Bueno OF, Muller JM, Schule R, Molkentin JD. Extracellular signal regulated kinase 2 interacts with and is negatively regulated by the LIM-only protein FHL2 in cardiomyocytes. Mol Cell Biol. 2004;24:1081–1095.