# Constitutive and Inflammatory Mediator-Regulated Fractalkine Expression in Human Ocular Tissues and Cultured Cells

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**PURPOSE.** Fractalkine (FKN) is a dual-adhesion molecule-chemokine that plays a role in inflammation but has not been explored in the eye. In the current study, constitutive expression of FKN was identified in human iris and retina, and its regulation by various cytokines in endothelial cells (ECs) and stromal cells from human iris, retina, and choroid was investigated.

**METHODS.** Human iris and retina explants were evaluated for FKN mRNA and protein expression using RT-PCR and immunohistochemistry, respectively. Cultured ocular ECs and stromal cells were stimulated with various inflammatory mediators (endotoxin; TNF$\alpha$; interferon-$\gamma$; interleukin (IL)-1$\alpha$, -4, -10, -13, -17, and -18; and/or CD40 ligand, or combinations thereof), with FKN mRNA being subsequently evaluated by cDNA array and/or RT-PCR and FKN protein by enzyme-linked immunoculture assay (ELICA) and/or by Western blot analysis.

**RESULTS.** Iris and retina explants constitutively expressed FKN protein in microvascular ECs and also in several stromal cell types. Iris and retina both express FKN mRNA. TNF$\alpha$ upregulated FKN in iris explants. All ocular microvascular ECs and stromal cultures expressed low FKN mRNA and/or protein levels, which were variably upregulated by endotoxin, TNF$\alpha$, interferon-$\gamma$, IL-1$\alpha$, and/or CD40 ligand, but not by IL-18. In ECs, the Th2 cytokines IL-4 and -13, but not IL-10, reduced TNF$\alpha$-induced FKN protein. IL-17, usually considered proinflammatory, reduced TNF$\alpha$-induced FKN protein in ocular ECs.

**CONCLUSIONS.** FKN is expressed in various ocular tissues and cells. Inflammatory mediator modulation of ocular FKN expression suggests that this adhesive chemokine may play important roles in regulating leukocyte efflux in inflammatory eye diseases, such as anterior uveitis and retinochoroiditis. (*Invest Ophthalmol Vis Sci.* 2003;44:1608–1615) DOI:10.1167/iovs.02-0233

During inflammation, the expression of multiple chemokines and adhesion molecules is upregulated in affected tissues.[1,2] These molecules are essential in mediating progression of inflammatory disease by directing leukocyte recruitment to the microvascular wall and subsequent trafficking into the surrounding tissues. Fractalkine (FKN) is a recently characterized transmembrane glycoprotein adhesion molecule[3] that can be proteolytically cleaved to yield a soluble chemoattractant form.[4] As a chemoattractant, FKN is unique in that it is the sole member identified so far of the $CX_3C$ (or delta) subclass of chemokines, defined by two conserved cysteines in the primary amino acid sequence with three amino acids separating them.[5] Moreover, it is the first member of the classic chemokine superfamily to be ascribed adhesive as well as chemotactic and angiogenic functions.[6] Soluble FKN is a chemoattractant and adhesion molecule for T cells, monocytes, and NK cells,[7–9] through their expression of the G-protein–coupled receptor, $CX_3CR1$.[10] FKN is expressed by several cell types, including endothelial cells (ECs), vascular smooth muscle cells, dendritic cells, and neurons.[3,11–13] In ECs, it is also strongly induced by classic proinflammatory molecules, such as tumor necrosis factor (TNF-$\alpha$) and interleukin (IL)-1,[3] and is also modulated by cytokines such as IL-4 and interferon (IFN)-$\gamma$.[14]

In the eye, inflammatory diseases share many of the molecular mechanisms involved in inflammation elsewhere in the body.[15,16] Only very recently has FKN-mediated leukocyte chemotaxis and adhesion been implicated as a major player in diverse inflammatory diseases. In these studies, FKN was shown to play important roles in mediating, for example, cardiac allograft rejection, arthritis, and psoriasis, and in modulating neuronal injury after a toxic insult.[17–20] To date, however, no investigations to our knowledge have been conducted to evaluate FKN expression in the eye, or whether such FKN expression may mediate ocular inflammation. In the present study, we demonstrate the presence of FKN in human ocular tissues and also the constitutive and inflammatory agent-regulated expression of FKN in cultured ocular microvascular ECs and stromal cells derived from human iris, choroid, and retina. These findings suggest that FKN may indeed play a regulatory role in the development and progression of ocular inflammatory disorders.

## METHODS

### Materials

Polyclonal goat (catalog no. AF365) and monoclonal mouse (catalog no. MAB3651) anti-human FKN antibodies, recombinant human TNF$\alpha$, IFN$\gamma$, CD40 ligand, and IL-1$\alpha$, -4, -10, -13, -17, and -18 were purchased from R&D Systems, Inc. (Minneapolis, MN). Additional rabbit polyclonal anti-human FKN antibodies from an alternate source were also tested in immunohistochemistry studies (catalog no. sc-7225 and sc-7226; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Fetal bovine serum (FBS) was from Hyclone Laboratories, Inc. (Logan, UT). Endothelial growth medium (EGM)-2 (Bulletkit) growth factor kits were from Clonetics, Inc. (Walkersville, MD). Culture medium MDCB-131 and all other reagents were from Sigma (St. Louis, MO). Cell culture media had no detectable endotoxin (lipopolysaccharide; LPS) contamination (E-Toxate kit; Sigma).

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## Ocular Tissues

Human eyes were obtained from anonymous donors (Lion's Eye Bank, Portland, OR) within 24 hours of death, in accordance with institution review board–approved protocol and the provisions of the Declaration of Helsinki for research involving human tissue. Irises and retinas were aseptically dissected and quartered, and explants were cultured in MCDB-131 medium supplemented with 10% FBS and growth factors (EGM-2 supplement, hydrocortisone omitted; Clonetics), and antibiotics (complete medium). After explants were acclimated for 6 hours at 37°C in a 5% $CO_2$-95% air atmosphere, some tissues were exposed to 10 ng/mL TNF$\alpha$ for up to 24 hours. At termination of the experiment, explants were washed in phosphate-buffered saline (PBS) and prepared for immunohistochemistry or were stored frozen at −80°C until RNA extraction was performed. In satellite studies to evaluate basal expression of FKN in mouse eyes, normal mice (F1 hybrid of C57BL/6J and 129/J; Jackson Laboratories, Bar Harbor, ME) received food and water ad libitum and were treated in compliance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. On death, mouse eyes were enucleated, dissected, and processed for total RNA isolation, as described for human eyes.

## Human Ocular ECs and Stromal Cultures

Human iris, choroid, and retinal EC cultures were established as previously described.[21,22] Briefly, these tissues were aseptically dissected from donor eyes, and collagenase digested, and ECs were isolated from contaminating cells by using mouse monoclonal anti-human CD31 antibody-coated magnetic beads (Dynal Biotech, Inc., Lake Success, NY). ECs were cultured in complete MCDB-131 medium, and were used at passages 2 to 5. This process typically results in EC cultures that are more than 99.5% pure, as evaluated by morphologic criteria, their expression of CD31 and von Willebrand factor, and their uptake of acetylated LDL.[22] In some experiments, we included commercially available human retinal ECs (Applied Cell Biology Research Institute, Kirkland, WA). Iris and retina stromal cells (i.e., EC-depleted) were cultured in MCDB-131 and 10% FBS, with supplemental growth factors omitted, and were used at passages 2 to 4.

## Immunohistochemistry

Five-micrometer sections of formalin-fixed, paraffin-embedded iris and retina explants were mounted on microscope slides and deparaffinized, and sections were treated with proteinase K (20 $\mu$g/mL, 20°C, 5 minutes), to assist antigen retrieval. Sections were then washed in 0.1% Tween 20 and Tris-buffered saline (pH 7.4; TBST), and nonspecific binding sites were blocked with 2% bovine serum albumin in TBST. For FKN detection, mouse monoclonal or rabbit polyclonal anti-human FKN antibodies were used (20 $\mu$g/mL, 20°C, 4 hours to overnight), followed by the species-appropriate alkaline phosphatase–conjugated secondary antibody (1:25 dilution, 20°C, 2 hours; Sigma), all diluted in blocking solution. FKN was visualized with a fast Red substrate kit (BioGenex Laboratories, San Ramon, CA).

## RT-PCR Analysis of FKN mRNA

For mRNA studies, ocular explants, confluent ECs, or stromal cells grown in 6- or 12-well tissue culture plates were stimulated with various combinations of cytokines for up to 24 hours, and then tissues and cell monolayers were washed in PBS, and total RNA was isolated with a commercial kit (RNAPure; Genhunter Corp., Nashville, TN, or RNeasy; Qiagen, Inc., Valencia, CA). First-strand cDNA synthesis was accomplished with oligo (dT)-primed Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL Life Technologies, Rockville, MD). Gene-specific cDNA was amplified by a hot-start touchdown PCR procedure, with *Taq* polymerase (Applied Biosystems, Foster City, CA) and specific primer pairs. Twenty touchdown cycles were run, with a stepwise decrease in annealing temperature (1°C every two cycles) from 69°C to 60°C. Twelve to 20 additional cycles were then run at a constant 55°C annealing temperature, followed by a final 7-minute elongation step at 72°C. A primer pair for a constitutively expressed gene, glyceraldehyde 3′-phosphate dehydrogenase (*GAPDH*), was included in each assay as an internal control, and nuclease-free water was included as a negative control. The primer sequences used (Integrated DNA Technologies, Inc., Coralville, IA) were as follows: human FKN sense, 5′-CAGAGGAGAATGCTCCGTCTGAAG-3′, and antisense, 5′-CAGAAGAGGAGGCCAAGGAAGG-3′, 355 bp amplicon; GAPDH sense, 5′-AGCTGAACGGGAAGCTCACTGG-3′, and antisense, 5′-GGAGTGGGTGTCGCTGTTGAAGTC-3′, 209 bp amplicon.

The mRNA phenotypes were verified by restriction mapping, and the fragments generated exactly matched those predicted from knowledge of the restriction map of the cDNA and the location of the primer templates.

## cDNA Array Analysis of FKN mRNA

FKN message expression in iris and retina ECs was confirmed using cDNA arrays (Hematology/Immunology Atlas Array; Clontech, Inc., Palo Alto, CA), according to manufacturer's instructions. This kit can be used to evaluate the expression of 406 genes, including FKN, all with target sequences spotted on membranes in duplicate. Briefly, 5-$\mu$g aliquots of triple phenol-chloroform–extracted and DNase-treated (MessageClean; Genhunter, Inc.) total RNA were reverse transcribed with MMLV into $\alpha$-[$^{32}$P] adenosine triphosphate (ATP)–labeled cDNA probes, using kit-specific primer collections and provided reagents. Radiolabeled probes (2–20 $\times$ $10^6$ cpm) were hybridized overnight to cDNA arrays, which were then exposed for varying time points (2 hours to 1 week, depending on probe specific activity) to x-ray film (Biomax MS; Eastman Kodak, Rochester NY) at −80°C, using an intensifying screen (model HE; Eastman Kodak). After exposed films were developed, images were digitized and densitometrically analyzed on computer (AtlasImage 2.0 software; Clontech).

## Enzyme-Linked Immunoculture Assay

Confluent EC in 96-well culture plates were stimulated with the indicated cytokine combinations for up to 24 hours, and fluorescence ELICAs were performed as has been described in detail.[21] Briefly, after a short glutaraldehyde fixation in situ and blocking in 20% FBS-PBS, cell surface FKN was labeled with goat anti-human FKN antibody followed by an alkaline phosphatase–conjugated rabbit anti-goat IgG secondary antibody (Sigma). Detection of bound antibody used the fluorogenic alkaline phosphatase substrate, methylumbelliferyl phosphate (Sigma). Fluorescence was measured with a fluorescence microplate reader (Bio-Rad Laboratories, Hercules, CA) with 360–460 nm excitation-emission wavelengths. Background fluorescence from cells in which primary detection antibody incubation was omitted was subtracted from experimental values. All conditions were replicated in three to six wells per experiment, for the indicated number of experiments.

## Western Blot Analysis

For Western blot studies, confluent EC monolayers were washed in PBS and then collected in immunolysis buffer (20 mM Tris base, 137 mM NaCl, 10% glycerol, 1% Triton X-100, and protease inhibitors). Lysates from approximately 5 $\times$ $10^4$ ECs per lane were electrophoresed on 4% to 20% linear gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After gels were blocked with 1% bovine serum albumin in PBS, the same detection antibody pair used in the ELICAs was used to detect FKN on Western blots. A chromogenic alkaline phosphatase substrate kit (5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium [BCIP/NBT]; Vector Laboratories, Burlingame, CA) was used to visualize FKN bands.

## Statistical Analysis

ELICA data represent mean fluorescence per well $\pm$ SD, in some cases normalized to unstimulated control values, for the indicated number of replicates. Asterisks in figures indicate a significant difference ($P <$

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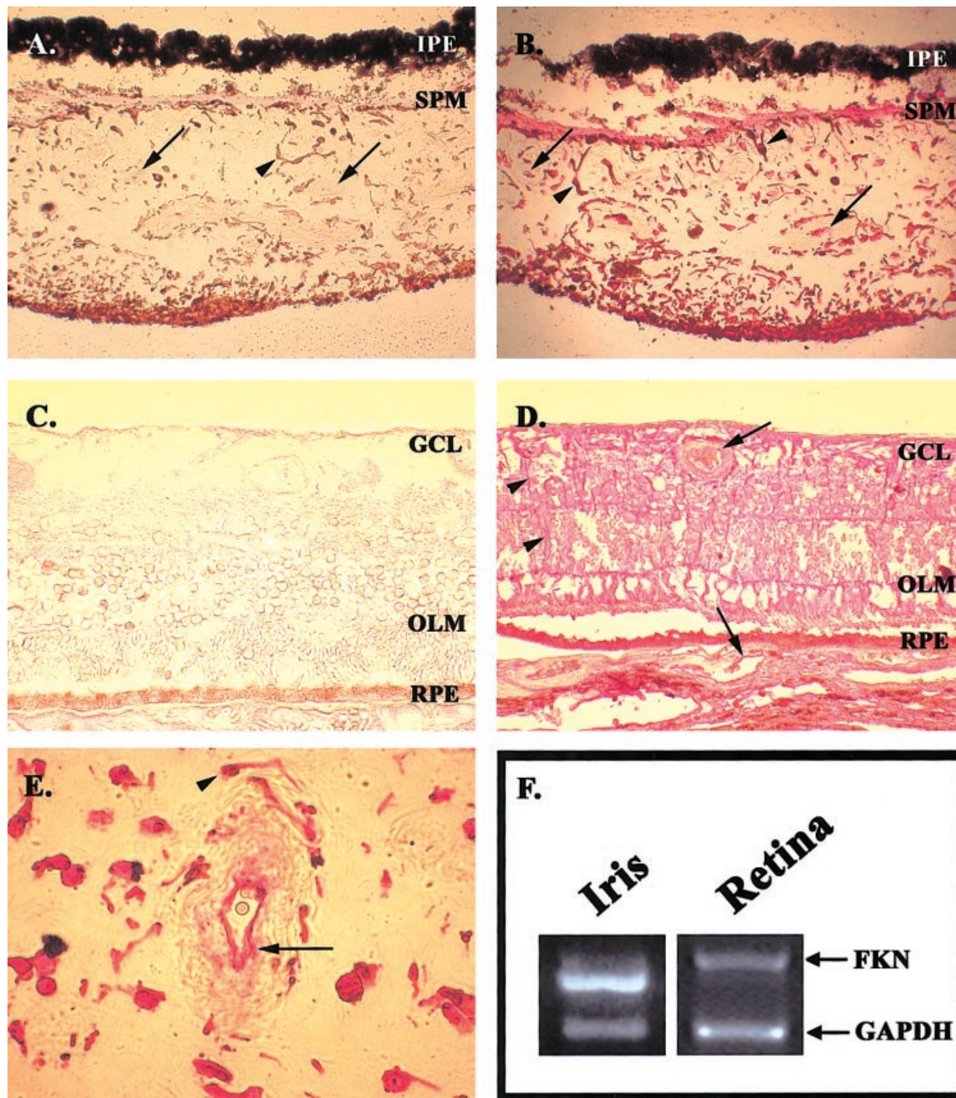


**Figure 1.** Immunohistochemical analysis of FKN protein expression in human iris and retinal tissues. Negligible background staining existed in iris (**A**) and retina (**C**) control sections, in which an isotype-matched nonimmune primary antibody was used in place of the FKN-detection antibody. Widespread constitutive expression of FKN protein was detected in iris tissue (**B**, **E**), with the highest expression localized in microvascular ECs (*arrows*), in the sphincter pupillae muscle layer (SPM), and in the anterior pigmented border (**B**, *bottom*). At higher magnification (**E**), significant FKN expression was localized to iris microvascular ECs (*arrow*) and perivascular cells, presumably pericytes (*arrowhead*), and to other stromal cell types. The iris pigment epithelium (**B**, IPE) may express FKN, in that all other pigmented cells in the iris (i.e., dispersed melanocytes [*arrowheads*, **B**] and the highly pigmented anterior border of the iris), all showed positive FKN signals, but heavy pigmentation of the IPE would obscure any specific signal identification. (**D**) Most vascular ECs of the retina and choroid stained positively for FKN (*arrows*). In addition, expression of FKN was notable in Müller cells (*arrowhead*), in cells of the GCL and RPE, and in the OLM. Weaker diffuse FKN-specific staining, observed in stromal regions of iris and retina, probably represents detection of secreted FKN protein. All tissues were sectioned at 5 $\mu$m. Identical widespread FKN staining patterns were observed in both iris and retina tissues when FKN was labeled with a variety of different detection antibodies, validating these observations. (**F**) RT-PCR analysis detected constitutive expression of FKN mRNA in iris and retinal tissues. Shown are data from one representative experiment of two conducted in tissues of eyes of different donors. FKN and GAPDH amplicons were the same size in iris and retinal explants. The apparent size disparity is because gels of PCR products were run for different lengths of time. Original magnification: (**A**–**D**) $\times$200; (**E**) $\times$1000.

0.05) between the means of experimental and respective control groups, by ANOVA (SigmaStat, ver. 2.0; SPSS Science, Chicago, IL).

## Results

### FKN Expression in Human Ocular Tissues

By immunohistochemistry, FKN protein expression was observed in iris and retina-choroid tissues (Figs. 1A–E). In all ocular tissues examined, arteriolar, venular, and capillary ECs tended to stain prominently, although occasional FKN-negative vessels were observed. A variety of stromal cell types also displayed strong constitutive expression of FKN, including perivascular cells (presumed to be of pericyte-smooth muscle cell lineage; Fig. 1E), and several other stromal cell types, as well. We attribute such widespread staining to the fact that FKN exists in both a soluble and cell surface–bound molecule with a diverse expression profile. Similar staining patterns were observed in normal skin by others using the same methodology and one of the same detection antibodies that we used.[14] We validated our findings in the same ocular tissues, using a variety of different anti-FKN detection antibodies (see the Methods section), with results identical with those shown in Figure 1.

In the iris, the smooth muscle cells of the sphincter pupillae muscle were strongly positive (Fig. 1B), whereas the ciliary muscles stained much more faintly (not shown). We were unable to discern the extent of staining in the myoepithelial cells of the iris dilator muscle, because of obscuration by the large amounts of pigment in these cells and in the abutting pigment epithelial layer. Nearly all the iris melanocytes scattered throughout the stroma and more concentrated at the anterior iris border, however, were strongly positive for FKN.

Extensive cellular FKN staining was observed in a variety of cell types throughout the retina (Figs. 1C, 1D). Staining was most pronounced, however, in the vasculature, the inner nerve fiber layer, and abutting the ganglion cell layer (GCL), the outer plexiform layer, the pigment epithelium (RPE), and within the outer limiting membrane (OLM), compared with the fainter staining of the nuclear and photoreceptor layers. Müller cells, discernible by their long transretinal processes, also strongly

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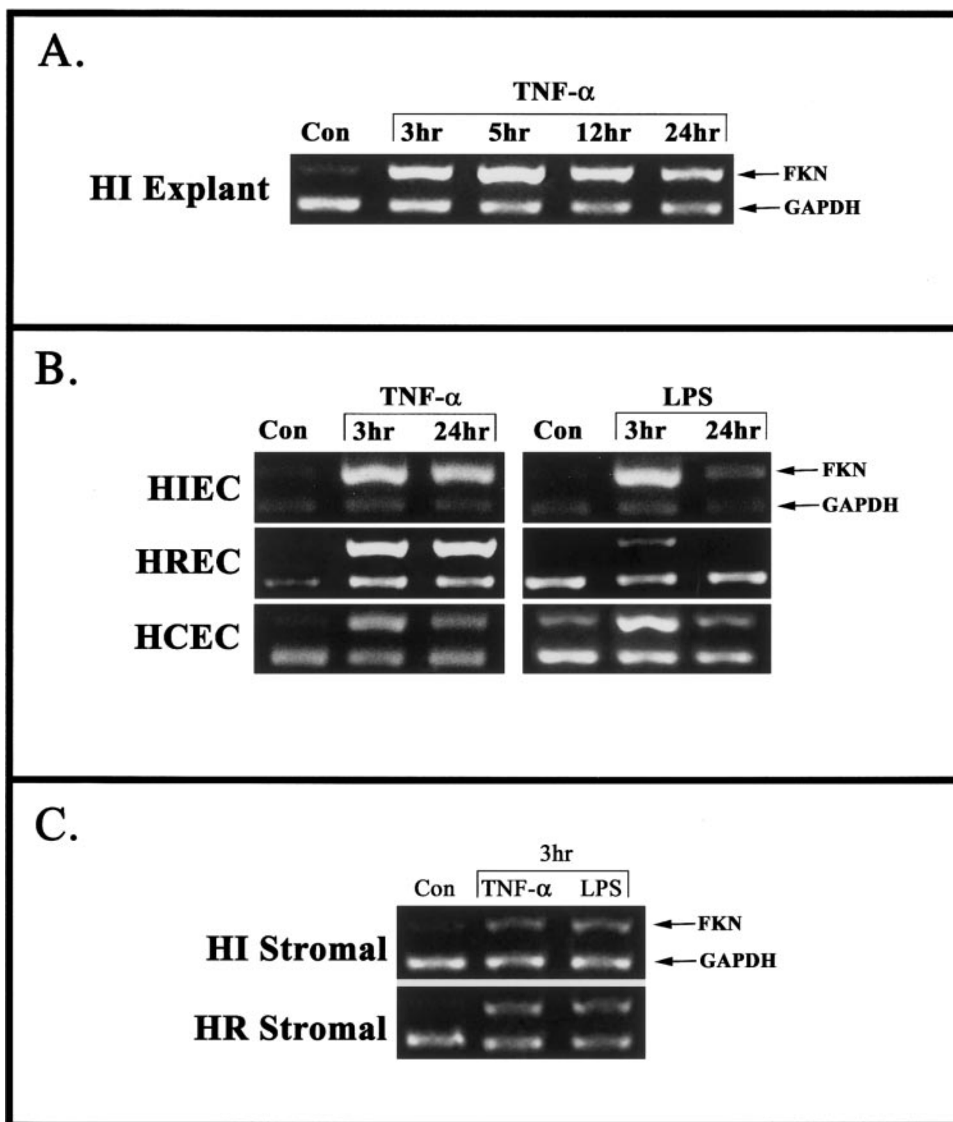


**Figure 2.** RT-PCR evaluation of FKN mRNA in human iris explants and in cultured ocular ECs and stromal cell cultures. (**A**) Low but detectable constitutive expression of FKN mRNA in unstimulated iris explants (Con) was time-dependently upregulated by stimulation with 10 ng/mL TNF$\alpha$. Shown is a representative of three experiments. (**B**) Cultured human ECs from iris (HIECs), choroid (HCECs), and retina (HRECs) expressed low but detectable basal FKN mRNA that was upregulated by TNF$\alpha$ (10 ng/mL) and LPS (10 $\mu$g/mL). TNF$\alpha$ caused a more persistent upregulation than did LPS, in all three EC types. Shown are data from one experiment representative of four experiments for HIECs, three for HRECs, and three for HCECs. (**C**) Stromal cells (EC-depleted) cultured from collagenase-digested irises (HI Stromal) and retinas (HR Stromal) showed low basal FKN signal that was upregulated by 3 hours' stimulation with TNF$\alpha$ (10 ng/mL). Typically, RT-PCR product bands intensities were weaker in TNF$\alpha$- and LPS-stimulated stromal cell samples than in stimulated EC samples. Shown are data from one experiment representative of two conducted with tissues from different donors. In all RT-PCR reactions, GAPDH was coamplified as an internal control.

expressed FKN. The adjacent choroid showed strong FKN expression in ECs and other stromal cells (Fig. 1D).

In our hands, as shown by immunohistochemistry in Figure 1, FKN was diffusely detectable throughout the iris and retina, with more concentrated cellular localization, whereas some extracellular regions also stained positively, although less strongly. This widespread detection of FKN protein is conceivably a result of a staining artifact. We believe, however, that this diffuse staining accurately reflects the distribution of FKN, because both a monoclonal antibody and three polyclonal antibodies independently resulted in similar staining; other investigators, using the same antibodies, have clearly shown similar diffuse FKN expression in other tissues such as skin[14]; and our recent observations based on Western blot analysis of EC-conditioned medium (Rookhuizen D, unpublished data, 2002) show that FKN is constitutively secreted by ECs and is markedly and time-dependently upregulated by activation of TNF$\alpha$ (10 ng/mL, 0–24 hours). Thus, secreted FKN probably is the source for the observed diffuse extracellular matrix immunolocalization of this protein.

In concordance with our immunohistologic findings, we observed clear constitutive expression of FKN mRNA in both human iris and retina explants, using RT-PCR (Fig. 1F). Similar constitutive expression was also observed in murine iris and retina explants (not shown). With human iris explants, ex vivo TNF$\alpha$ (10 ng/mL) stimulation markedly increased expression of FKN mRNA, with levels greatest between approximately 3 and 5 hours and beginning to decline only after 24 hours of stimulation (Fig. 2A).

## Upregulation of FKN mRNA by TNF$\alpha$ and LPS

To better elucidate potential regulation of FKN in specific ocular cell types, we evaluated FKN message levels in ECs cultured from human iris (HIECs), choroid (HCECs), and retina (HRECs; Fig. 2B), as well as in cultured EC-depleted stromal cells from human irises and retinas (Fig. 2C). By RT-PCR, we observed low but detectable FKN expression in all three ocular EC types, which was markedly and time-dependently upregulated by stimulation with either TNF$\alpha$ (10 ng/mL) or LPS (10 $\mu$g/mL), with TNF$\alpha$ causing a more persistent upregulation (Fig. 2B). IL-1$\alpha$ similarly upregulated FKN in these ocular ECs (not shown). These findings were supported by parallel studies of cDNA arrays (Fig. 3). By gene array, we similarly detected weak constitutive FKN signals in HIECs and HRECs that were markedly increased after 3-hour stimulations with either LPS or TNF$\alpha$. In human iris and retinal stromal cell cultures, we observed low but detectable basal expression of FKN mRNA that was, as with ECs, upregulated by LPS and TNF$\alpha$ (Fig. 2C).

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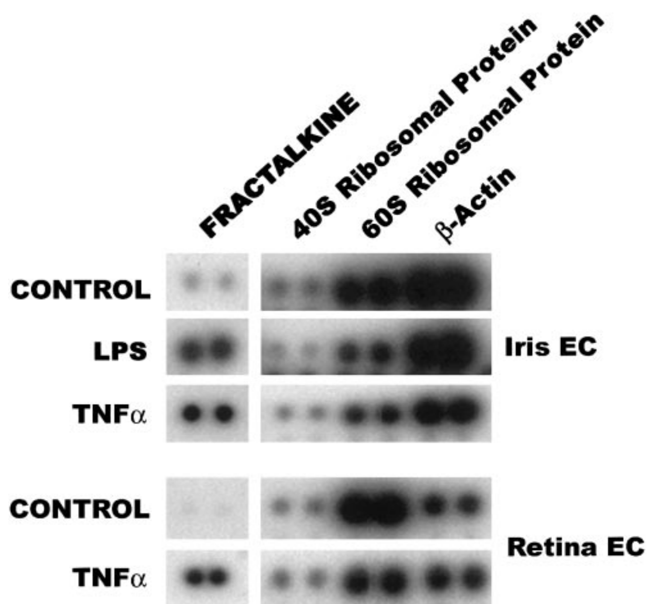


**FIGURE 3.** Gene array analysis of FKN mRNA in cultured human ocular ECs. Low constitutive expression of FKN was markedly upregulated in iris and retinal ECs after 3 hours' stimulation with LPS (10 μg/mL) or TNFα (10 ng/mL). Signals for ribosomal proteins 40S and 60S and for β-actin (three of the eight housekeeping genes on the gene array) are included as internal controls. Data are from one experiment representative of at least three independent experiments per condition. Similar results were also obtained in one experiment with human choroid ECs (data not shown).

## Constitutive and TNFα- and LPS-Induced FKN Protein Expression in Ocular ECs

Using ELICA, we saw FKN protein expression profiles in ocular ECs that correlated well with their observed mRNA profiles. In HIECs, HCECs, and HRECs alike, we detected measurable, albeit low, constitutive FKN protein on cell surfaces (Fig. 4). After activation with TNFα or LPS, FKN protein was significantly upregulated in a dose- and time-dependent fashion in all three EC types, with TNFα being the more efficacious stimulus. For example, in HIECs, although both LPS (10 μg/mL) and TNFα (10 ng/mL) caused maximum induction after 6 hours of stimulation, FKN levels were significantly higher when stimulated with TNFα (Fig. 4A). In addition, TNFα caused a more persistent elevation in cell surface expression of FKN protein in these three EC types. In HIECs, whereas FKN levels began declining toward baseline after only 12 hours of exposure to LPS, they remained at maximum level through 24 hours of TNFα exposure (Fig. 4A). Similar results were also obtained in several experiments using HCECs and HRECs (not shown). We confirmed FKN protein upregulation by TNFα in HIECs by Western blot analysis (Fig. 4B). In two experiments comparing HIECs with HRECs in concentration-dependent induction of FKN by TNFα, both EC types displayed a similar $EC_{50}$ of approximately 100 pg/mL (i.e., 5.7 pM; Fig. 4C).

## Regulation of FKN Expression by Other Inflammatory Mediators

IFNγ and IL-4 are prototypical T helper cell (Th) type 1 and Th2 cytokines, respectively, with major roles in regulating inflammation.[23] Thus, we investigated potential effects of these agents in mediating both constitutive and TNFα-induced FKN in ocular ECs and stromal cells. Stimulating cultured HIECs with 10 ng/mL IFNγ modestly upregulated constitutive expression of FKN, at both the protein level (Fig. 5A) and the

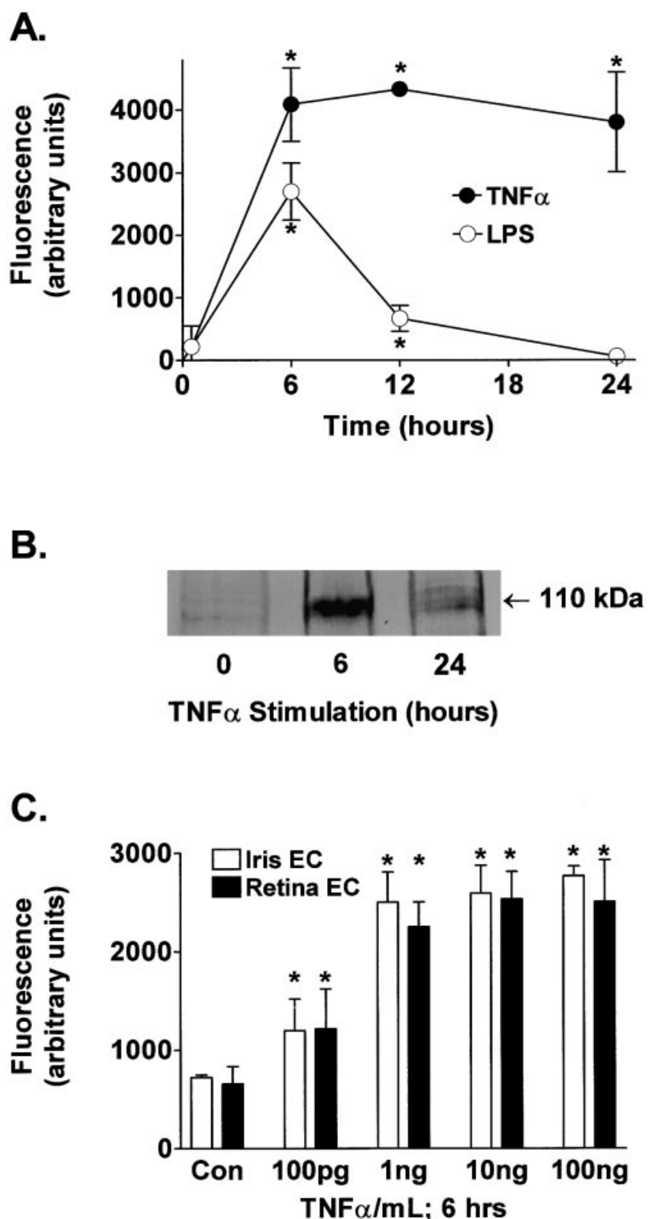


**FIGURE 4.** TNFα and LPS upregulated expression of FKN protein in cultured human ocular ECs. (**A**) Time-dependent induction of cell surface FKN was measured in TNFα- (10 ng/mL) and LPS-stimulated (10 μg/mL) HIEC monolayers using a fluorescence ELICA. Constitutive FKN was upregulated as early as 4 hours after TNFα stimulation, with maximum protein levels attained after 6 hours. With LPS stimulation, FKN levels declined by 12 hours' stimulation and had returned to control levels by 24 hours. Upregulation of FKN was more persistent with stimulation by TNFα, with which maximum induced levels were maintained for at least 24 hours. Data are representative of one of four experiments conducted with HIECs, each condition having been conducted in quadruplicate wells. Similar results were obtained in three experiments with both HRECs and HCECs (data not shown). (**B**) Western blot analysis of iris EC lysates ($5 \times 10^4$ cells/lane) that had been stimulated with 10 ng/mL TNFα for up to 24 hours confirmed upregulation of FKN protein. Data are from one experiment representative of two. (**C**) Dose-dependent upregulation of FKN by TNFα in ocular ECs. Iris and retinal ECs were stimulated with the indicated cytokine concentration for 6 hours, and cell surface FKN protein was measured by fluorescence ELICA. Data are from one experiment representative of four, with experiments for each condition having been conducted in three to four wells per experiment. All ELICA data represent mean fluorescence ± SD. *$P < 0.05$ versus the unstimulated control, by ANOVA. Con, unstimulated controls.

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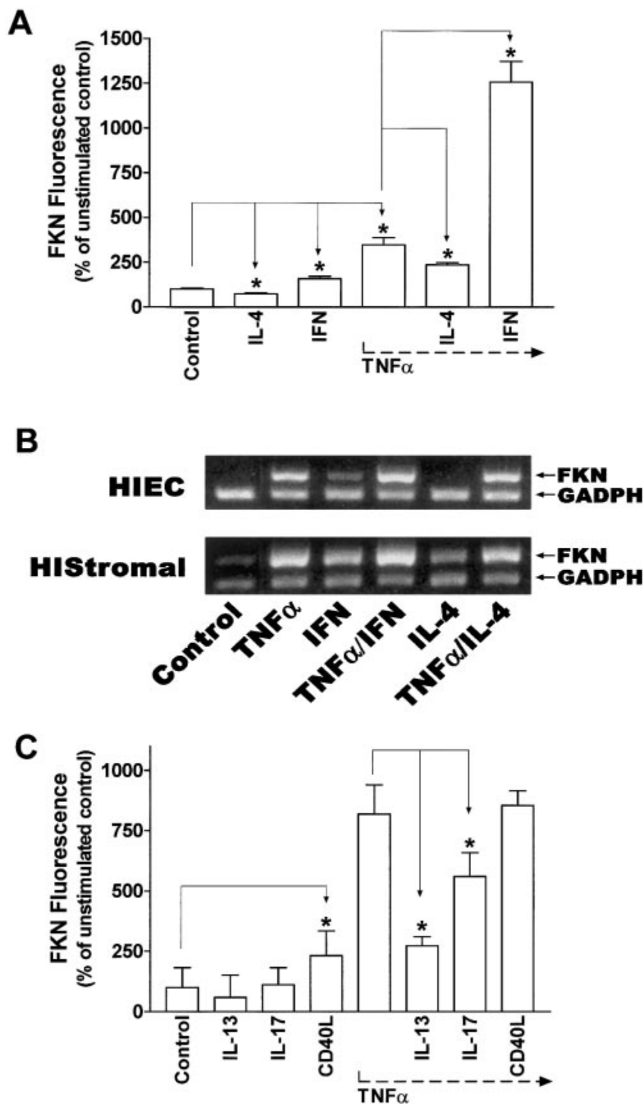


**Figure 5.** Polarized effects of Th1 versus Th2 cytokines on FKN expression by cultured iris ECs. The classic Th1 cytokine IFNγ upregulated both constitutive FKN protein (**A**) and mRNA (**B**) levels in HIECs. IFNγ also acted synergistically with TNFα to upregulate FKN protein in these cells. The Th2 cytokine IL-4 downregulated both constitutive FKN protein and FKN protein induced by TNFα- and/or IFNγ in iris ECs, although no effect was detected at the mRNA level by RT-PCR. In RT-PCR experiments, GAPDH was coamplified and served as an internal control. In iris stromal cells, both IFNγ and TNFα upregulated FKN mRNA. In two experiments, IL-4 also appeared to induce modest upregulation of FKN in these cells (**B**). In the protein experiments (**A**), all stimulations were for 6 hours with 10 ng/mL of each agonist, except for TNFα which was used at 1 ng/mL, and data presented are from two experiments conducted in quadruplicate wells. Similar results were obtained in two experiments using HRECs. For RT-PCR (**B**), all stimulations were conducted for 3 hours using 10 ng/mL of each agonist, and data are from one of two experiments. (**C**) Results of ELICA showed that neither IL-13 nor -17 had any effect on basal expression of FKN protein in HIECs, whereas CD40 ligand (CD40L) caused a significant (twofold) increase in constitutive FKN (all used at 10 ng/mL for 8 hours). When superimposed on stimulation with TNFα (10 ng/mL, 8 hours), both IL-13 and -17 significantly decreased TNFα-induced FKN protein, whereas CD40L had no effect on TNFα-induced FKN. Data are representative of two experiments in HIECs, with experiments for each condition conducted in six wells per experiment. ELICA data represent mean fluorescence ± SD, after normalization to the unstimulated control. $^*P < 0.05$ versus indicated control, by ANOVA.

mRNA level (Fig. 5B). Costimulation of HIECs with IFNγ and low dose (i.e., 1 ng/mL) TNFα resulted in a synergistic elevation in cell surface expression of FKN (four- to ninefold over summed effects of both cytokines acting alone) that was obvious at 6 hours (Fig. 5A) and sustained through 24 hours (not shown). Similar effects on expression of FKN protein were obtained using HRECs and HCECs (not shown). Iris stromal cultures responded similarly to stimulation by IFNγ and TNFα, as was observed with ECs (Fig. 5B).

In contrast to the inducing effect of IFNγ on endothelial FKN, the Th2 cytokine IL-4 (10 ng/mL), significantly decreased both constitutive and agonist-induced expression of FKN protein during the same time frame (Fig. 5A). Similar effects of IFNγ and IL-4 on expression of FKN protein were also obtained with a large-vessel endothelium representative, human aortic ECs (not shown). Addition of IL-4 inhibited both TNFα-induced FKN and the synergistically elevated TNFα-IFNγ–induced expression of FKN in HIECs by approximately 50%, after 6 hours of simultaneous stimulation with all three agents. This inhibitory effect was not discernible, however, at the mRNA level by standard semiquantitative RT-PCR (Fig. 5B).

We also tested the ability of other inflammatory reagents to modulate FKN expression in ECs (Fig. 5C; all used at 10 ng/mL, 8 hours). The Th2 cytokine IL-13, although it had no significant effect on basal FKN protein levels in HIECs, caused a marked (~65%) decrease in TNFα-induced FKN after 8 hours of concomitant stimulation. IL-10, another Th2 cytokine, however, was without effect on either constitutive or agonist-induced FKN in HIECs and HRECs (not shown). IL-17, generally considered to be proinflammatory,[24] had no effect on basal FKN expression in HIECs, but unexpectedly caused a significant (~40%) decrease in TNFα-induced FKN protein levels. Similar results were obtained using IL-17 on TNFα-stimulated human dermal microvascular ECs (data not shown). IL-18, another proinflammatory cytokine related to the IL-1 family, had no effect on either basal or TNFα-induced FKN protein in HIECs (data not shown), consistent with previous work showing no involvement of IL-18 in experimental autoimmune uveitis.[25] CD40 ligand caused a significant (twofold) elevation in basal FKN protein levels in HIECs, but caused no observable additional increase when superimposed on stimulation with TNFα.

## Discussion

In various studies of uveitis and retinitis, aberrant expression of chemokines and adhesion molecules appears to play a critical role in disease initiation and progression.[15,16,26] FKN is a recently characterized multifunctional adhesion and chemoattractant molecule,[3] that is upregulated in the vasculature during a variety of inflammatory diseases,[17–19] in which it plays a key role in regulating leukocyte influx into affected tissues. In vitro, FKN expression is modulated by inflammatory cytokines in ECs and in smooth muscle cells.[3,11,14] Given the mechanistic parallels between various ocular inflammatory diseases and inflammation elsewhere in the body, we sought to investigate whether FKN is expressed in tissues of the eye and to better understand how such expression might be regulated by the local cytokine milieu. To this end, we tested ocular tissue explants, as well as cultured microvascular ECs and stromal cells derived from iris, retina, and/or choroid.

By immunohistochemical staining of ocular tissues, we observed clear constitutive expression of FKN in the ECs and in other stromal cells of the iris, retinal, and choroidal microvasculature, consistent with a role in mediating leukocyte extravasation. We also observed in these ocular tissues weaker but diffuse specific FKN staining throughout the stromal regions. This is probably attributable to the existence of both cell-

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bound and secreted FKN within the stromal tissues, both of which were detectable by the panorama of anti-FKN antibodies that we used in immunolocalization studies. This observation is consistent with the findings of others who used similar methods to localize FKN in other tissue types.[14] Such widespread constitutive expression is likely to mediate normal leukocyte recirculation during routine immune surveillance. Its upregulation in cultured ocular ECs by known inflammatory agents supports a further role for FKN in mediating ocular inflammation. That a variety of extravascular cell types throughout the eye strongly express FKN (e.g., Müller cells in the retina and the sphincter pupillae muscle in the iris), implies that additional functional roles may exist for FKN beyond its likely role in mediating the initial leukocyte efflux from ocular microcirculation. In the brain, for example, neuron-derived soluble FKN binds its cognate receptor on nearby microglial cells, and subsequent paracrine interactions are neuroprotective.[20] It is possible that a similar relationship exists in the retina, wherein Müller cells, a glial cell type that strongly expresses FKN, may use FKN-mediated cellular communication as a mechanism to protect and/or support juxtaposed retinal neurons.

Cultured ocular ECs expressed low amounts of FKN mRNA and protein, and these levels were potently upregulated by stimulation with TNF$\alpha$, in partial concordance with previous observations in other EC types.[14] One notable difference is that in ocular microvascular ECs we saw the maximum induction of FKN mRNA after 3 hours of stimulation, whereas the aforementioned study in HUVECs showed that achievement of maximum TNF$\alpha$-induced FKN mRNA levels required 18 to 24 hours, using a similar agonist concentration.[14] This difference is possibly a reflection of site-derived endothelial heterogeneity[21,27] and if so, probably does not signify a gross difference between micro- and macrovascular ECs, because in our hands, human aortic ECs also showed a rapid upregulation of FKN in response to identical stimulation (data not shown). Proinflammatory cytokines such as TNF$\alpha$ are significantly elevated in the local milieu in various ocular inflammatory scenarios.[15,16] Thus, there may be a functional link between upregulated FKN and the increased leukocyte efflux that is characteristic of ocular diseases such as anterior uveitis or retinochoroiditis. Our observation of both constitutive and inducible expression of FKN in stromal cells suggests that FKN may be important, not only in initial extravasation, but also in guiding leukocyte migration further into perivascular tissues, once these cells have left the circulation, or in mediating their physical interactions with resident stromal cells after migration.

Our observations of a polarized FKN response in iris and retinal ECs when stimulated with Th1 versus Th2 cytokines confirm and extend previous findings.[14] In both our report and in previous studies, the Th1 cytokine IFN$\gamma$ acted both alone and in synergy with TNF$\alpha$ to upregulate FKN, whereas the Th2 cytokines IL-4 and -13 decreased both TNF$\alpha$- and TNF$\alpha$-/IFN$\gamma$–induced FKN expression in these cells.[14] In our study, IL-4, but not IL-13, significantly decreased the low basal expression of FKN protein in ocular ECs as well. The inductive effect of IFN$\gamma$ versus the suppressive effect of IL-4 and -13 on FKN expression in ocular ECs suggests that FKN may play a role in mediating ocular diseases such as iritis and uveoretinitis, which are considered to be primarily Th1-driven conditions.[28–30] In support of this, both IL-4 and -13 have been shown to decrease inflammatory parameters in various experimental models of ocular inflammation[30,31] and to downregulate FKN in HUVECs.[14] In addition, we observed no effect of IL-10, another Th2 cytokine known to be anti-inflammatory in some models of uveitis,[32] on either basal or TNF$\alpha$-induced expression of FKN in ocular ECs. Local IL-10 levels increase in some ocular inflammatory states, and its levels correlate with the downregulation of Th2 responses and with disease resolution.[32–34] In some models of experimental uveoretinitis, IL-10 activity appears to require the concerted action of IL-4.[32,35] The requirement of the coordinated activity of multiple Th2 cytokines in mitigating inflammatory processes may be reflected in the observed inability of IL-10, by itself, to modulate FKN expression in iris and retinal ECs.

IL-17 is the prototype member of an emerging cytokine family.[24] Identified expression of IL-17, to date, has been limited to a subset of activated memory T lymphocytes that appear to be distinct from the classic Th1–Th2 categorization.[36] All the yet reported biological effects of IL-17 are of a proinflammatory nature, and IL-17 is elevated in diverse inflammatory diseases including rheumatoid arthritis, asthma, psoriasis, transplant rejection, and systemic sclerosis.[37–41] At the cellular level, IL-17 stimulates chemokine and/or cytokine expression in ECs, fibroblasts, and epithelial cells[38,42,43] and induces adhesion molecules on ECs and keratinocytes.[39,41] To the best of our knowledge, ours is the first investigation of the action of IL-17 in tissues or cells derived from the eye or on expression of FKN. We hypothesized that IL-17 would act in a proinflammatory capacity and upregulate the expression of FKN in ocular ECs. To our surprise, IL-17 alone did not alter basal FKN expression in HIECs, but in fact significantly antagonized TNF$\alpha$-induced FKN expression in these cells. This novel observation using ECs derived from the eye exemplifies the complex interplay of cytokine signals that govern microvascular involvement in inflammation and raises the likelihood that IL-17 plays novel regulatory roles in ocular immune disease.

The CD40/CD40 ligand dyad is an often-studied proinflammatory signaling system.[44] In the eye, CD40 ligation has been linked to rejection of corneal transplants, keratoconjunctivitis sicca, and Sjögren's syndrome and causes release of IL-6 and -8 release by RPE.[45–47] ECs express both CD40 and its ligand, and ligation upregulates the expression of adhesion molecules,[48,49] although controversy exists concerning the specific molecular responses. Although one group has reported the upregulation of FKN mRNA in CD40 ligand-stimulated HUVECs,[14] we extend these findings to the protein level and also to the realm of the ocular microvasculature.

In summary, we describe for the first time the constitutive and cytokine-modulated expression of FKN in intact human iris and retinal explants and in microvascular ECs and stromal cell cultures derived from various ocular tissues. Considering the common expression of FKN in the eye and in other tissues and its proven role in mediating leukocyte extravasation in inflammatory diseases elsewhere in the body, these observations implicate FKN as a potential key regulator of ocular inflammation.

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