Fenofibric Acid Reduces Fibronectin and Collagen Type IV Overexpression in Human Retinal Pigment Epithelial Cells Grown in Conditions Mimicking the Diabetic Milieu: Functional Implications in Retinal Permeability

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PURPOSE. To determine whether fenofibric acid (FA) reduces high glucose (HG)-induced basement membrane component overexpression and hyperpermeability in human retinal pigment epithelial (RPE) cells.

METHODS. Retinal pigment epithelial cells (ARPE-19) were cultured for 18 days in normal glucose (5 mM) or HG (25 mM) medium and studied for the effects of FA on fibronectin (FN) and collagen IV (Coll IV) expression. During last 3 days of the experiment, 100 μM FA was added to cells grown in HG medium or in HG medium plus IL-1β (HG + IL-1β) to mimic, at least in part, the inflammatory aspect of the diabetic milieu. Real-time RT-PCR was performed to determine FN and Coll IV mRNA levels, whereas protein levels were assessed by Western blot analyses. Cell monolayer morphology and barrier function were analyzed by confocal microscopy using specific antibodies against tight junction proteins, ZO-1, and claudin-1 and by measuring apical-basolateral movements of FITC-dextran, respectively.

RESULTS. FN and Coll IV expression were significantly increased in RPE cells grown in HG or HG + IL-1β medium compared with cells grown in normal medium. When cells grown in HG or HG + IL-1β medium were treated with FA, significant reductions in FN and Coll IV expression were observed. In addition, exposure to FA decreased excess permeability in a dose-dependent manner in cells grown in HG + IL-1β medium. This effect was unrelated to changes in tight junction protein content.

CONCLUSIONS. Findings from this study suggest that the downregulation of basement membrane components by FA may have a protective effect against outer blood-retinal barrier leakage associated with diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2011;52:6348–6354) DOI:10.1167/iovs.11-7282

The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial has shown beneficial effects of fenofibrate in reducing the risk for cardiovascular disease events and microvascular complications in diabetes.¹,² In particular, fenofibrate reduced total cardiovascular disease events and macular edema by 31% and proliferative diabetic retinopathy (DR) by 30% in patients with diabetes. In addition, recent data from the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial indicated that ocular complications had 40% odds of progression to DR in the group of patients receiving fenofibrate plus simvastatin compared with the group of patients treated with placebo plus simvastatin.³ However, it is unknown how fenofibrate, a hypolipemiant drug, improves retinal vascular permeability associated with DR.⁴ Fenofibrate reduces cholesterol by lowering low-density lipoprotein, very low-density lipoprotein, and triglyceride levels while increasing high-density lipoprotein levels.⁵ In addition, its beneficial effect on insulin resistance has been reported.⁶,⁷ Although the lipid-modifying effects of fenofibrate have been well documented,⁸ its mechanistic role in reducing diabetic microvascular complications, specifically diabetic macular edema formation, is unknown.

DR is a leading cause of blindness and vision loss in the working age population.⁹ Basement membrane thickening and increased vascular permeability are two major retinal vascular changes associated with the pathogenesis of this disease.¹⁰–¹² Studies have reported that HG or hyperglycemia induces the overexpression of basement membrane components, which, in turn, contributes to excess retinal vascular permeability.¹¹,¹² We have shown that normalization of basement membrane component overexpression could lead to beneficial effects in preventing excess retinal vascular permeability and to the development of acellular capillaries and pericyte loss in animal models of DR.¹¹,¹² Diabetic macular edema (DME) is a prominent clinical manifestation that frequently leads to severe loss of central vision in patients with diabetes.¹⁵ Studies indicate that tight junctions play an important role in maintenance of the inner blood-retinal barrier (BRB) and that compromised tight junctions promote the formation of DME.¹⁶,¹⁷ Similarly, the outer BRB, which is formed by RPE cells attached to one another by tight junctions, also plays an essential role in preventing the accumulation of extracellular fluid in the subretinal space of the retina.¹⁸ Compromised tight junctions in the RPE cell monolayer are known to contribute to the disruption of the outer BRB and to the impairment of neural retinal function. Studies
have shown that fibronectin (FN) and collagen IV (Coll IV) are located in the basement membrane of the RPE, and that significant thickening develops in the RPE basement membrane with aging and the formation of advanced glycation end products, two phenomena known to contribute to diabetic vascular basement membrane thickening. Because overexpression of basement membrane components and subsequent retinal capillary basement membrane thickening have been implicated in the breakdown of the inner BRB in diabetes, we examined in this study whether the overexpression of FN and Coll IV, two basement membrane components synthesized by RPE cells, may contribute to the outer BRB hyperpermeability seen in DR and whether such hyperpermeability could be prevented by FA.

In the present study we demonstrated that FA, the active metabolite of fenofibrate, prevents the breakdown of the RPE barrier under conditions that mimic the diabetic milieu. This effect is related to the protective role of FA in reducing FN and Coll IV overexpression produced by RPE cells. Results from this study suggest that FA may impart beneficial effects in preventing or arresting the development of DME in diabetic patients by ameliorating abnormal basement membrane component synthesis in the outer BRB.

**Materials and Methods**

**Cell Culture**

ARPE-19 cells representing a spontaneously immortalized human RPE cell line were obtained from American Type Culture Collection (Manassas, VA). Cells from passage 18 were cultured for 18 days at 37°C under 5% (vol/vol) CO₂ in medium (DMEM/F12) supplemented with 10% (vol/vol) fetal bovine serum (HyClone; Thermo Fisher Scientific, Logan, UT) and 1% (vol/vol) penicillin/streptomycin (HyClone; 10% (vol/vol) fetal bovine serum (HyClone; Thermo Fisher Scientific, Logan, UT) and 1% (vol/vol) penicillin/streptomycin (HyClone; Thermo Fisher Scientific) in N condition (5.5 mM glucose) and HG conditions (25 mM glucose). To study the potential protective effect of FA on the barrier function of RPE cells, FA (100 µM) was added to the standard culture medium daily for the last 3 days of the experiment (days 15–17). For studies examining the effect of different doses, cells were exposed to 25 or 100 µM FA after the conditions described for 100 µM FA. Cells were also treated with IL-1β (10 ng/mL) for the last 2 days of the experiment (days 16, 17) and were subjected to serum starvation (1% FBS) during the treatments. To rule out a potential bias by an osmotic effect, the experiment was also performed using mannitol (5.5 mM glucose + 19.5 mM mannitol vs. 25 mM glucose) as an osmotic control agent.

**In Vitro Permeability**

For permeability studies, ARPE-19 cells were seeded at 400,000 cells/mL (80,000 RPE cells/well) in 0.35 cm² polyester filters (HTS-Transwells; Costar, Corning, NY). For real-time PCR and Western blot analyses, cells were seeded directly on plastic at 20,000 cells/mL for immunofluorescence and polarization studies, cells were seeded on glass cover slips at 20,000 cells/mL. The permeability of RPE cells was determined at 18 days in culture by measuring the apical-to-basolateral movements of fluorescein isothiocyanate (FITC) dextran (40 kDa) (Sigma, St. Louis, MO). The test molecule was added to the apical compartment of the cells in a concentration of 100 µg/mL. Samples (200 µL) were collected from the basolateral side at baseline and 75 minutes after the addition of the molecules. The medium in the basolateral compartment was replaced by fresh medium after the collection of every sample. A minimum of four wells were used for each time measurement. Absorbance was measured at 485 nm of excitation and 528 nm of emission with a microplate reader (SpectraMax Gemini; Molecular Devices, Sunnyvale, CA).

**Real-Time RT-PCR**

To study the mRNA level of FN and Coll IV, first-strand cDNA was synthesized using a cDNA synthesis kit (Superscript; Invitrogen, Carlsbad, CA). Primer sets for performing real-time quantitative qPCR for CollIV (accession no. NM_001135009) and FN (accession no. X15906) and housekeeping gene hypoxanthine phosphoribosyl transferase 1 (HPRT; accession no. NM_012583) were designed using a Web-based primer design program (www.roche.com). All real-time qPCR measurements were performed on a PCR system (7500; Applied Biosystems, Foster City, CA) using the standard temperature cycling protocol for the relative quantification assay. Each measurement was run in triplicate for each sample. Selected samples were run after sequential dilution to confirm that the detected signals were within the linear amplification range. Results were first normalized to the expression level of the endogenous housekeeping gene HPRT. Selected samples were tested against two additional housekeeping genes, 18S and glyceraldehyde-3-phosphate dehydrogenase, and the results were no different from the results obtained using HPRT. Further information is presented in Table 1.

**Western Blot Analysis**

Western blot analysis was performed to determine the relative levels ofZO-1, claudin-1, FN, and Coll IV protein in the RPE cells from each group. RPE cells were homogenized, and protein was isolated as previously described. Bacitracin acid assay (Pierce Chemical, Rockford, IL) was used to determine total protein concentrations. Western blot analysis were performed with 25 µg protein/lane; after electrophoresis, the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) using a semidry apparatus according to Towbin’s procedure. The membranes were blocked with 5% nonfat dry milk for 2 hours and then exposed to rabbit FN (Millipore, Billerica, MA; 1:1000) and rabbit Coll IV (Fitzgerald Industries, Acton, MA; 1:2500) antibody solution overnight at 4°C. Blots were washed with Tris-buffered saline containing 0.1% Tween-20 and then incubated with goat anti-rabbit IgG secondary antibody (Cell Signaling, Billerica, MA) solution (1:3000) for 1 hour and goat anti-rabbit (1:20,000) or goat anti-mouse (1:10,000) for 1 hour (Pierce; Thermo Scientific). The membranes were again washed as described and then were exposed to a chemiluminescent protein detection system (Immun-Star; Bio-Rad) film (Fujifilm, Tokyo, Japan). Protein loading in the gels was confirmed by Ponceau’s staining and tubulin antibody (Cell Signaling; 1:1000), and the densitometric values were used for adjustment of any differences in loading. Densitometric analysis of the Western blot signals was performed at nonsaturating exposures and analyzed using the ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

**Immunohistochemistry**

For immunohistochemistry and polarization studies, cells were grown for 18 days at confluence in 24-well plates containing one circle...

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coverslip of glass (12-mm diameter) (Thermo Scientific, Menzel-Gläser; Braunschweig, GE) inside each well. Cells were washed with PBS and fixed with methanol (ZO-1 and claudin-1) or paraformaldehyde (FN and Coll IV) for 10 minutes, washed again with PBS twice, and blocked with 2% BSA and 0.05% Tween in PBS overnight at 4°C. Mouse anti-ZO-1, rabbit anti-claudin-1 (Zymed Laboratory Gibco, Invitrogen, San Diego, CA), rabbit anti-FN, rabbit anti-Coll IV (Abcam, Cambridge, MA), and mouse anti-Na+/K+ ATPase (Millipore), all diluted to 1:200, were incubated for 1 hour at room temperature (RT). After washing with PBS, cells were further incubated with Alexa 488 goat anti-rabbit and Alexa 594 donkey anti-mouse secondary antibodies (Invitrogen) for 1 hour at RT. After washing with PBS, the slides were mounted with mounting medium containing DAPI for fluorescence (Vectorshield; Vector Laboratories, Burlingame, CA). Images were acquired with a confocal laser scanning microscope (FV1000; Olympus, Hamburg, Germany).

Statistical Analysis

Data are presented as mean ± SD. The values of the control groups were normalized to 100%, and values from all other groups were expressed as percentages of control; statistical analysis was performed using the normalized values. Comparisons between groups were performed using ANOVA followed by the Student’s t-test, and P < 0.05 was considered statistically significant.

RESULTS

Effect of FA on High Glucose- and IL-1β-Induced Fibronectin Overexpression in RPE Cells

Western blot analysis showed significantly increased FN protein expression in RPE cells grown in HG or HG + IL-1β medium compared with those grown in normal medium (179% ± 14% of normal, P < 0.05; 195% ± 10% of normal, P < 0.05, respectively). When RPE cells grown in HG medium were treated with FA, a significant reduction in FN protein level was observed compared with RPE cells grown in HG medium (121% ± 9% of normal vs. 179% ± 14% of normal, P < 0.05). Similarly, when RPE cells grown in HG medium supplemented with IL-1β were treated with FA, FN expression was significantly reduced compared with RPE cells grown in HG medium supplemented with IL-1β (87% ± 10% of normal vs. 194% ± 14% of normal, P < 0.05) (Figs. 1A, 1B).

Real-time RT-PCR results showed significantly increased FN mRNA levels in RPE cells grown in HG or HG + IL-1β medium compared with RPE cells grown in normal medium (349% ± 41% of normal, P < 0.05; 423 ± 53% of normal, P < 0.05, respectively). FA significantly reduced FN mRNA overexpression in RPE cells grown in HG or HG + IL-1β medium compared with untreated RPE cells grown in HG or HG + IL-1β medium, respectively (247% ± 34% vs. 349% ± 41% of normal, P < 0.05; 282% ± 15% of normal vs. 423% ± 53% of normal, P < 0.05, respectively; Fig. 1C).

Effect of FA on High Glucose- and IL-1β-Induced Collagen Type IV Overexpression in RPE Cells

Western blot analysis showed significantly increased Coll IV protein expression in RPE cells grown in HG or HG + IL-1β medium compared with those grown in normal medium (232% ± 25% of normal, P < 0.05; 276% ± 21% of normal, P < 0.05, respectively; Fig. 2). When RPE cells grown in HG medium or HG medium supplemented with IL-1β were treated with FA, a significant reduction in Coll IV expression compared with RPE cells grown in HG medium or HG medium supplemented with IL-1β, respectively, was observed (113% ± 17% of normal vs. 232% ± 25% of normal, P < 0.05; 168% ± 22% of normal vs. 276% ± 21% of normal, P < 0.05, respectively; Figs. 2A, 2B).

Real-time RT-PCR results showed significantly increased Coll IV mRNA levels in RPE cells grown in HG or HG + IL-1β medium compared with RPE cells grown in normal medium (221% ± 28% of normal, P < 0.05; 301% ± 23% of normal, P < 0.05, respectively). FA significantly reduced Coll IV mRNA overexpression in RPE cells grown in HG or HG + IL-1β medium compared with untreated RPE cells grown in HG or HG + IL-1β medium, respectively (127% ± 39% of normal vs. 221% ± 28% of normal, P < 0.05; 206% ± 19% of normal vs. 301% ± 23% of normal, P < 0.05, respectively; Figs. 2A, 2B).

Effect of FA on High Glucose- and IL-1β-Induced Increased Barrier Permeability in RPE Cells

The effect of different conditions tested on the permeability of ARPE-19 monolayers is displayed in Figure 3. HG alone
mildly increased excess permeability, whereas IL-1β alone significantly increased permeability. Interestingly, both (HG + IL-1β) dramatically increased permeability in what appeared to be a synergistic effect. Data related to osmotic control experiments using mannitol indicated that the excess permeability and the effects of HG + IL-1β are independent of hyperosmotic effects. When cells grown in HG medium supplemented with IL-1β were treated with 25 μM FA, a significant reduction in permeability was observed (164.6 ± 38.3 vs. 224.9 ± 26.4; P = 0.05). This protective effect on monolayer permeability was more evident in cultures treated with 100 μM FA (149.9 ± 15.5 vs. 224.9 ± 26.4; P = 0.005).

**FIGURE 2.** Effect of FA on Coll IV protein and mRNA levels in RPE cells. (A) Representative Western blot image shows FA reduces HG- and HG + IL-1β-induced Coll IV overexpression. (B) Graphical representation of Western blot data. Coll IV protein level is significantly increased in RPE cells grown in HG or HG + IL-1β. When treated with FA, RPE cells grown in HG medium showed a significant reduction in Coll IV expression compared with untreated HG cells (*P < 0.05). Similarly, FA treatment reduced Coll IV overexpression in cells grown in HG + IL-1β medium compared with untreated cells grown in HG + IL-1β medium (**P < 0.05). (C) Real-time RT-PCR result indicates increased Coll IV mRNA expression in cells grown in HG or HG + IL-1β medium. FA significantly reduces Coll IV overexpression in both groups (*HG vs. FA, P < 0.05; **HG + IL-1β vs. HG + IL-1β + FA, P < 0.05).

**DISCUSSION**

Findings from the present study indicate that FA treatment prevents increased RPE permeability induced by HG + IL-1β and that this beneficial effect of FA is associated with decreases in HG- and HG + IL-1β-induced FN and Coll IV overexpression.

**FIGURE 3.** Effect of FA on ARPE-19 cell monolayer permeability. Data from permeability assays indicate that FA has a protective effect on HG + IL-1β-induced increased barrier permeability in a dose-dependent manner. Monolayer permeability of cells grown in 5.5 mM D-glucose (white bar), 5.5 mM D-glucose + 19.5 mM mannitol (dark gray bar), 25 mM D-glucose (HG; black bar), N + IL-1β (dotted black bar), HG + IL-1β (light gray bar), HG + IL-1β + FA (25 mM; striped bar), and HG + IL-1β + FA (100 mM; dotted white bar). Results are expressed as the mean ± SD (n = 4). *P < 0.05 compared with N. **P < 0.01 in comparison with N.
This suggests that FA can prevent the breakdown of BRB permeability at least in part by normalizing ECM protein overproduction. In addition, we confirmed previous reports showing that the altered amount of tight junction proteins was not necessarily the only factor regulating tight junction functionality and that the distribution of the tight junction proteins plays an important role in barrier permeability.\textsuperscript{23,24} In fact, the protective effect of FA on RPE disruption induced by HG + IL-1β is in part mediated by its ability to prevent the aberrant distribution of tight junction proteins. The capacity of FA in maintaining the tight junction distribution and its suppressive effect on ECM overproduction could be involved in the beneficial effect.

\textbf{FIGURE 4.} Evidence for tight junction and polarity in ARPE-19 monolayer. Confocal image showing the expression of occludin (green) and the apical marker enzyme Na⁺/K⁺ ATPase (red). Nuclei were stained with DAPI (blue). (A) Confocal vertical (X-Z) sections showing predominant apical Na⁺/K⁺ ATPase localization and apical staining pattern for the tight junction protein occludin in cells grown in NG medium. (B) ARPE-19 cells cultured under HG supplemented with IL-1β showing disruption of the cell monolayer and partial loss of polarization, which is prevented after treatment with FA 100 μM (C).

\textbf{FIGURE 5.} Effect of FA on localization and distribution of tight junction and ECM proteins in ARPE-19 cells. (A) Immunohistochemistry of ARPE-19 cells showing disruption of the monolayer induced by HG + IL-1β and the beneficial effects of FA in preventing the disorganization of tight junction proteins in the cell monolayer. Merged images show colocalization of claudin-1 and ZO-1 (yellow). Note that claudin-1 immunostaining appears green and ZO-1 immunostaining appears red. (B) Immunohistochemistry of ARPE-19 showing downregulation effect of 100 μM FA on FN (green). (C) Immunohistochemistry of ARPE-19 showing the downregulation effect of 100 μM FA on Coll IV expression (green). Nuclei were stained with DAPI (blue). Scale bar, 20 μm.
effects of fenofibrate on DME. However, further investigation to
determine the mechanisms by which FA affects ECM protein
expression and tight junction protein distribution are needed.

Importantly, our findings from this study implicate a down-
regulation effect of FA on extracellular matrix protein levels,
which could play a role in preventing vascular permeability and
in underscoring the importance of FN and Coll IV in forming a
selective permeable outer BRB. In this regard we have
previously shown that reducing basement membrane
thickening by downregulating extracellular matrix compo-
nents including FN and Coll IV is effective in preventing the
apoptosis and increased permeability associated with DR.\textsuperscript{11,25}
Additionally, studies on RPE monolayers cultured on laminin-
coated filters indicated that extracellular matrix components
promote RPE morphology and the formation of a selective
permeability barrier to various tracers.\textsuperscript{26}

Increased levels of proinflammatory cytokines play a key
role in the pathogenesis of DME.\textsuperscript{17,27,28} Treatment of RPE cells
with either serum, interferon-\gamma, tumor necrosis factor-\alpha, hepa-
tocytew growth factor (HGF), interleukin (IL)-1 \beta or placental
growth factor-1 increases permeability and alters the expres-
son or content of tight junction molecules.\textsuperscript{23,29 –31} Because
IL-1 \beta plays an important role in the development of DR\textsuperscript{32-34}
we decided to use the cytokine together with HG conditions to
mimic the diabetic milieu. A significant overexpression of FN
and Coll IV was observed after treating ARPE-19 cells with
IL-1 \beta in the presence of HG, and this overexpression was
associated with an increase in permeability. Overall, these
findings indicate that a higher content of basement membrane
components may contribute to the impairment of barrier
function, leading to excess permeability. In addition, the over-
expression of basement membrane components known to be
induced by inflammatory cytokines such as IL-1 \beta\textsuperscript{35-36} may be
involved in hyperpermeability, which occurs in DR.

Microvascular basement membrane is an important compo-
nent of the blood barrier system, which participates in the
regulation of vascular permeability. Thus, any changes to the
basement membrane structure or its composition may ad-
versely affect its function. Previous studies demonstrated the
ability of fenofibrate to decrease extracellular matrix accumu-
lation in renal cortex of streptozotocin-induced diabetic rats\textsuperscript{37}
and in kidneys of spontaneously hypertensive rats.\textsuperscript{38} In addi-
tion, fenofibrate treatment was shown to affect extracellular
matrix changes associated with systolic failure seen in ascend-
ing aortic constriction in chronic pressure overload mice.\textsuperscript{39}
Our results from this study parallel these findings and dem-
strate fenofibrate treatment’s beneficial effects on pathologic
changes associated with the overexpression of extracellular
matrix proteins.

The exact cellular mechanisms by which FA influences
extracellular matrix component levels is unclear. Recent stud-
ies have focused on the ability of FA to activate peroxsome
proliferator-activated receptor alpha (PPAR\alpha), a transcription
factor that regulates the genes involved in cellular lipid cata-
bolism. The activation of PPAR\alpha increases lipolysis and
the elimination of triglyceride-rich particles from plasma and also
increases the synthesis of apoproteins, which leads to a reduc-
tion in very-low-density and low-density fractions and an increase
in the high-density lipoprotein fraction containing apoprotein.
PPAR\alpha may regulate extracellular matrix turnover through con-
sequently inhibiting matrix metalloproteinases\textsuperscript{38,39} or decreasing
plasminogen activator inhibitor-1.\textsuperscript{57} However, the exact pathway
involving PPAR\alpha and its downstream effectors has not been com-
pletely defined.

Other studies have investigated how fenofibrate may sup-
press oxidative stress and MAPK activation, thus decreasing
TGF-\beta levels and ultimately affecting extracellular matrix accu-
mulation.\textsuperscript{58} Finally, one cannot rule out other mechanisms
whereby fenofibrate may affect vascular permeability. One
report demonstrated that fenofibrate is able to reduce apopto-
sis in human retinal endothelial cells, which is associated with
DR.\textsuperscript{40} The mechanism by which fenofibrate exerted its anti-
apoptotic effect was found to be AMP-activated protein kinase
(AMPK)-dependent and PPAR\alpha-independent. Preventing un-
wanted apoptosis in the retinal vasculature may help maintain
vessel integrity and prevent leakage associated with DR.

In addition, we have recently shown that RPE disruption induced
by IL-1 \beta is prevented by FA because of its ability to suppress
AMPK activation.\textsuperscript{44} This finding indicates that suppression
rather than activation of AMPK is the mechanism by which FA
prevents the hyperpermeability induced by HG + IL-1 \beta. In the
same paper, we reported that AMPK activation in human RPE
from diabetic donors was significantly higher than from non-
diabetic donors and very similar to that obtained in ARPE-19
cells cultured under high (25 mM) glucose + IL-1 \beta. Taken
together, our results suggest that the suppression of AMPK
activation is a mechanism by which fenofibrate may prevent or
arrest diabetic macular edema.

A limitation of the present study is that it focuses on the
effects of FA only on the outer BRB. As such, further studies are
needed to investigate the effect of FA on the inner BRB and the
contribution of FA on overall BRB breakdown. However, find-
ings from this study documented an important proof of con-
cept that HG-induced excess accumulation of basement mem-
brane components of the outer BRB is involved in increased
retinal permeability and that the protective effect of FA against
leakage of the outer BRB is at least in part linked to the
inhibitory effect of FA on specific basement membrane com-
ponent expression in the RPE cells. The ability of FA to prevent
basement membrane component overexpression may have
significance for other diabetic microangiopathies beyond DME.

Acknowledgments

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