Effect of Curcumin on Proliferation of Human Retinal Endothelial Cells under In Vitro Conditions

Chinnaraj Premanand,[1] *Mohan Rema,*[1] *Mahmood Z. Sameer,*[1] *Mohan Sujatha,*[2] *and Muthusamy Balasubramanyam*[1]

Purpose. To investigate the effect of high glucose on the proliferation of human retinal endothelial cells (HRECs) and to elucidate the possible mechanisms of antiangiogenic activity of curcumin, a diferuloylmethane.

Methods. Human retinal endothelial cells were isolated from the retinal tissue obtained from human donors and the culture system was established. The effect of curcumin on the proliferation of primary HRECs in the presence of low and high glucose was measured by MTT and thymidine uptake assays. Apoptosis was assessed by TUNEL assay and other adjuvant tools. Effect of curcumin on phorbol ester stimulated intracellular reactive oxygen species (ROS) generation in high glucose conditions was assessed by fluorescence assay. Finally, semiquantitative RT-PCR and Western blot analysis was performed to measure VEGF mRNA production and VEGF induced PKC-βII translocation, respectively in the presence and absence of curcumin.

Results. HREC culture was established successfully at passages 3 and 4 at 80% confluence. Curcumin effectively inhibited endothelial cell proliferation in a dose-dependent manner. At a concentration of 10 μM, curcumin significantly inhibited HREC proliferation in high-glucose–treated cells, as verified by both MTT and thymidine uptake assay. Curcumin also showed a significant ($P = 0.03$) reduction of intracellular ROS generation in HRECs. RNA expression studies showed that curcumin had an inhibitory effect on the glucose-induced VEGF mRNA expression. In addition, VEGF-mediated, membrane-associated changes in the PKC-βII translocation in HRECs was inhibited by 31% on treatment with 10 μM curcumin.

Conclusions. These data suggest an underlying mechanism whereby curcumin induces the apoptosis in HRECs by the regulation of intracellular ROS generation, VEGF expression and release, and VEGF-mediated PKC-βII translocation. (*Invest Ophthalmol Vis Sci.* 2006;47:2179–2184) DOI:10.1167/iovs.05-0580

Hyperglycemia and the consequent exposure of the intracellular milieu of the retinal capillary endothelial cells to elevated blood glucose concentrations have been implicated in the pathogenesis of vascular complications in diabetes.[1,2] The exact biochemical and molecular mechanisms of hyperglycemia-induced changes in the retina are not clear. In proliferative diabetic retinopathy (PDR), one of the sight-threatening forms of DR, there is formation of abnormal new vessels (neovascularization and angiogenesis), which are thin and fragile and tend to bleed and can result in sudden and total loss of vision.

Angiogenesis is tightly regulated by two counter-balancing systemic angiogenic stimulators, such as vascular endothelial growth factor (VEGF), and angiogenic inhibitors, such as angiostatin and pigment epithelium-derived factor (PEDF).[3–6] Vascular endothelial growth factor (VEGF), a primary factor and potent stimulator of angiogenesis, has been implicated in the pathogenesis of PDR. Of note, in a mouse model where VEGF levels were elevated, it has been demonstrated that there is a deficiency in the pericyte coverage that leads to an increase in endothelial cell proliferation.[7] This study showed that the effect of pericytes on endothelial cell proliferation varies with the level of angiogenic factors, such as VEGF.

Angiogenesis can be inhibited by antiangiogenic factors. Various antiangiogenic factors have been identified, including angiostatin and endostatin, which are all protein fragments.[8] Some small antiangiogenic molecules have also been found in natural sources such as curcumin.[9] Curcumin, which is the major yellow pigment isolated from the rhizome of the Curcuma species *Zingiberaceae* has been demonstrated in vitro to have potent antioxidant,[10] anti-inflammatory[11] and antiproliferative activities in several cell types, including human umbilical vein endothelial cells.[12]

Studies have been performed on the effect of curcumin in tumor angiogenesis. We therefore tried to investigate the effect of curcumin on retinal endothelial cell proliferation in high glucose conditions and to identify the possible mechanisms of its action on the mediators of angiogenesis.

Methods

Cell Culture

To establish the HREC cultures, eyes were obtained from the eye bank after the cornea was transplanted. The eyes were transported to the laboratory in sterile medium, within 20 hours of the death of the human donors. Institutional ethics committee approval was obtained, and informed consent was obtained from the first-degree relatives of the donors separately, for the use of the retinal tissue for research. The donor eyes were obtained and managed in compliance with the Declaration of Helsinki.

Retinal tissue removed from the cadaveric eyes was digested in 0.1 mg/mL collagenase type I at 37°C for 1 hour. From the retinal tissue suspension, endothelial cells were isolated with CD 31 antibody-coated magnetic beads (Dyna beads; Dynal, Oslo, Norway).[13,14] The isolated human retinal endothelial cells were characterized by vWF fluorescence staining (Dako A/S, Glostrup, Denmark).[15] HRECs were grown in media containing endothelial complete medium (Cambrex Bio Science, Walkersville, MD), 5% fetal bovine serum (Hyclone, Logan UT), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. For experiments, the cells were transferred to serum-starved

From the [1]Madras Diabetes Research Foundation, Gopalapuram, Chennai, India; and the [2]Rajan Eye Care Hospital, T. Nagar, Chennai, India.

Supported by the Department of Science & Technology (DST) and the Fund for Infrastructure in Science & Technology (DST-FIST), Government of India.

Submitted for publication May 12, 2005; revised October 22, 2005, and January 19, 2006; accepted March 13, 2006.

Disclosure: **C. Premanand**, None; **M. Rema**, None; **M. Z. Sameer**, None; **M. Sujatha**, None; **M. Balasubramanyam**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Mohan Rema, Director and Consultant Ophthalmologist, Madras Diabetes Research Foundation, 4, Conran Smith Road, Gopalapuram, Chennai 600 086, India; drrema@vsnl.com.

medium (without growth supplement) for 18 to 24 hours before addition of the stimulatory agents. Cells at passages 3 and 4 and 80% confluence were used for the experiments.

Cell Viability and DNA Synthesis

HRECs in primary culture were detached by trypsin-EDTA and seeded in 96-well plates (approximately 1×10^5 cells/well). After 24 hours, they were challenged with high glucose concentrations. HRECs were then grown for 72 hours in either physiologic (5 mmol/L) or high-glucose (30 mmol/L) medium. HRECs without exposure to high glucose was treated with different doses of curcumin (1, 3, 10, or 30 μM). Subsequently, in another set of experiments, HRECs treated with 30 mmol/L glucose were exposed to 10 μM curcumin. After the treatment, media containing the treatment conditions were carefully removed by aspiration. The cell viability and the DNA synthesis in HRECs were studied with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, St. Louis, MO) and thymidine uptake assays, respectively.

Examination of Apoptotic Cell Death

DNA cleavage, which commonly occurs in apoptosis, was measured by TdT-mediated dUTP nick-end labeling (TUNEL) with a kit (In Situ Cell Death Detection Kit, Fluorescein; Roche Molecular Biochemicals, Indianapolis, IN). In addition, DNA fragmentation was determined. Cells ($3.0–4.0 \times 10^6$) were centrifuged, and the DNA extracted was separated in 1.5% agarose gels and visualized by staining with ethidium bromide. Because caspase-3 has been recognized as a central player in mediating apoptosis, its protein levels were also studied by Western blot analysis. Lactate dehydrogenase (LDH) activity (Diasys Diagnostic Systems, Holzheim, Germany) was measured to assess cellular necrosis.

ROS Assay

To investigate the effect of curcumin, its impact on the generation of reactive oxygen species (ROS) was studied. Approximately 2.0×10^6 cells were exposed to 30 mmol/L D-glucose for 72 hours in 1% serum-starved medium. They were then washed, trypsinized, and resuspended in HEPES buffer (Ca^{2+}- and Mg^{2+}-free [pH 7.4]). A 10-μM solution of dihydrorhodamine (Invitrogen, Carlsbad, CA) was added and kept for incubation at room temperature (RT) for 30 minutes. After the removal of extracellular dye, HRECs were challenged with 50 nM phorbol 12-myristate-13-acetate (PMA) in the presence or absence of 10 μM curcumin. Fluorescence intensity representing the intracellular generation of ROS was monitored at an emission wavelength of 536 nm, after excitation at 505 nm, as DHR 123 forms the fluorescent product rhodamine 123 on oxidation by ROS.[16] Rhodamine 123 was used for calibration. ROS levels were calculated based on the standard curve and the values were represented as nanomolar per 10^6 cells.

Semiquantitative RT-PCR

To study the inhibitory effect of curcumin on VEGF induced RNA expression, HRECs were processed for total RNA extraction (TRIzol Reagent; Invitrogen). cDNA was prepared from 4 μg total RNA by reverse transcription in a volume of 20 μL. PCR was performed, electrophoresis was run, and RNA samples were tested for genomic DNA contamination. The primer sequence, annealing temperature, and number of cycles were as follows: VEGF: forward primer 5′-ACCATGAACTTTCTGCTGTC-3′ and reverse primer 5′-TCACCGCCTCGGCTTGTC-3′, 65°C, 30 cycles. GAPDH (positive control): 5′-GGAGTCAACGGATTTGGT-3′ and reverse primer 5′-GTGATGGGATTTCCATTGAT-3′, 57°C, 30 cycles. Each RT-PCR experiment was conducted in duplicate. Finally, the ratios of the concentration of GAPDH to those of the VEGF isoform ($VEGF_{164}$) were calculated. All results represent the average density of positive bands obtained from at least three separate experiments.

Western Blot Analysis

The effect of curcumin on VEGF-induced PKC βII activation (translocation of PKC βII from the cytosol to the plasma membrane) was assessed by measuring the protein expression levels both in the cytoplasm and plasma membrane. The cells were treated with an effective concentration of 10 μM curcumin for 30 minutes at 37°C before stimulation with 10 ng/mL VEGF for 15 minutes. HRECs exposed to VEGF but not to curcumin served as the control.

HRECs were subjected to subcellular fractionation using a 0.25-M sucrose lysis buffer followed by 100,000*g* ultracentrifugation for 1 hour at 4°C. Protein concentrations were quantified using Bradford's reagent with bovine serum albumin as the standard. Approximately, 20 μg of cytosolic and membrane proteins were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane using a semi-dry transferring unit (GE Healthcare). The membrane was blocked for 1 hour at room temperature with TBS containing 5% BSA. After three washes with TBS/Tween-20 (0.1%), the membrane was incubated in TBS/Tween at 4°C (overnight) with polyclonal antibodies, anti-PKC βII (1:500) for translocation studies and anti-caspase 3 (1:500) for the detection of apoptosis. The membrane was next washed with TBS/Tween and incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000). Antigen detection was performed with an enhanced chemiluminescence detection system (GE Healthcare).

Enzyme-Linked Immunosorbent Assay

The conditioned media from HRECs treated with or without curcumin (3 and 10 μM) and cultured in high glucose concentrations were subjected to mouse VEGF ELISA in triplicate (R&D Systems, Wiesbaden-Nordenstadt, Germany). The absorbance at 450 nm (correction wavelength set at 510 nm) was measured in a microplate reader, (model 680; Bio-Rad, Munich, Germany). The standard graph was generated with the microplate reader software (Microplate Manager, ver. 4, with four-parameter logistic fit; Bio-Rad).

Statistical Analysis

All experiments were repeated at least thrice to confirm the results. Data on proliferation studies are expressed as optical density (mean ± SD) of the results obtained with positive control conditions (cells with no treatment) with each experiment. Statistical comparison among groups was calculated with the two-tailed *t*-test. $P \leq 0.05$ was considered statistically significant.

Results

HREC culture was established. Cells at passages 3 and 4 were used in all experiments.

Figure 1 shows the effects of different concentrations of curcumin on human retinal endothelial cell viability in culture. Treatment with various concentrations of curcumin (1, 3, 10, or 30 μM) for 72 hours markedly attenuated the basal proliferation of HRECs. The survival of HRECs was inversely correlated with curcumin concentration, indicating its dose-dependent inhibitory effect. The median inhibitory concentration (IC_{50}) was calculated as 8.2 ± 0.05 μM, and hence 10 μM of curcumin was adjudged as an optimal concentration and was used throughout the study.

The HRECs treated with medium containing 30 mmol/L D-glucose showed no significant increase in the cell viability (0.62 ± 0.08) over basal medium (0.54 ± 0.04; Fig. 2A). However this was inhibited significantly by 10 μM curcumin to the extent of 42% (0.36 ± 0.09; $P = 0.003$).

The effect of curcumin on DNA synthesis in the 30 mmol/L D-glucose treated HRECs were also examined by the thymidine incorporation method. HRECs (30 mmol/L glucose treated) were exposed to 10 μM curcumin for 72 hours and during the last 20 hours were pulsed with thymidine. Although HRECs treated with 30 mmol/L glucose showed no significant increase ($P = 0.167$) in proliferation when compared with cells treated

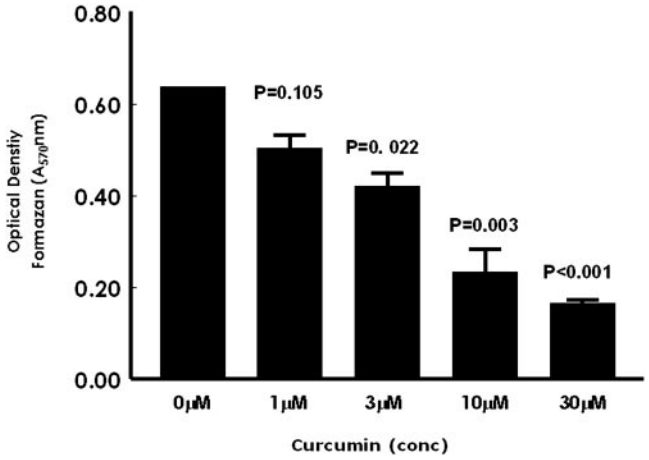

FIGURE 1. Dose–response effect of curcumin on human retinal endothelial cell viability. Curcumin (1–30 μM) was tested on HREC viability at 72 hours of incubation. Data are shown as optical density measured at 570 nm, spectrophotometrically, compared with control cells without curcumin (0 μM) and are the mean ± SD of results in four separate experiments. $P < 0.05$.

with 5 mmol/L glucose, there was an increasing trend in high-glucose condition (Fig. 2B). Nevertheless, it is important to note that curcumin significantly ($P = 0.002$) inhibited the high-glucose–mediated changes in HRECs.

To confirm the mechanism of antiproliferative effect of curcumin on HRECs, a TUNEL assay was performed. Figure 3A shows that the number of apoptotic bodies was significantly higher in HRECs treated with 10 μM curcumin, when exposed to 30 mmol/L D-glucose, compared with HRECs treated with 30 mmol/L glucose alone. The endothelial cell population detected by the TUNEL technique represented almost exclusively apoptotic rather than necrotic cells.

DNA fragmentation experiments demonstrated that DNA from HRECs treated with various concentrations of curcumin for 72 hours showed a typical smudged ladder pattern of internucleosomal fragmentation (Fig. 3B), whereas no DNA fragmentation was observed in cells with no treatment. To confirm that curcumin-induced cell death was not caused by necrosis, LDH activity was measured spectrophotometrically. HRECs treated with different concentrations of curcumin (10 and 30 μM) showed no significant increase in the amount of LDH activity ($P = 0.09$ and $P = 0.260$, respectively).

It is also inferred from the Western blot experiment that caspase-3 (a marker for apoptosis) was increased in HRECs treated with curcumin. There was no significant change in the caspase 3 expression levels in cells treated with either 5 or 30 mmol/L glucose. However, HRECs treated with 10 and 30 μM curcumin, cultured in high glucose conditions exhibited significant increase in the expression of caspase 3 in the lysate, as shown in Figure 3C. Taken together, these data indicate that curcumin induces apoptosis in HRECs.

In the experiments in which intracellular ROS generation was measured in high-glucose–treated HRECs (Fig. 4), addition of PMA induced an increase in the generation of ROS from 0.82 ± 0.1 to 1.5 ± 0.08 nM/10^6 cells ($P = 0.01$); this increase was significantly ($P = 0.03$) reduced by curcumin.

RT-PCR of human HRECs using VEGF primers revealed two alternative splicing variants that comprised 571- and 441-bp DNA fragments corresponding to $VEGF_{164}$ and $VEGF_{188}$ amplifications. When expression of VEGF and GAPDH mRNA under normal and high-glucose concentrations was tested, HRECs in the normal glucose group expressed low levels of VEGF and the ratio of GAPDH to $VEGF_{164}$ was 0.30 ± 0.04. The ratio in the high glucose group was markedly increased to 0.48 ± 0.1 at 24 hours ($P = 0.016$), as shown in Figure 5. In the high-glucose group curcumin significantly decreased VEGF mRNA expression at 24 hours. The ratio of $VEGF_{164}$ to GAPDH was decreased to 0.44 ± 0.08, 0.23 ± 0.06, and 0.16 ± 0.05 in the high-glucose group treated with 1, 10, and 30 μM curcumin, respectively.

The levels of VEGF released in the media from the HRECs treated with curcumin were significantly decreased, when compared with HRECs exposed to 30 mmol/L glucose alone, as estimated with ELISA. HRECs treated with 30 mmol/L glucose showed a 1.7-fold increase in the VEGF levels in the conditioned media when compared with basal conditions, whereas there was a significant decrease in VEGF release, when 3 and 10 μM curcumin were added to HRECs cultured in a high-glucose condition (~25% [$P = 0.008$] and 65% [$P < 0.001$]), respectively, as shown in Figure 6.

Because angiogenic processes involve both VEGF and PKC signals, the effects of VEGF on PKC expression profiles in the presence and absence of curcumin were tested. The protein expression studies revealed that there was a 25% increase in the membrane PKC βII expression in cells treated with VEGF 10 ng/mL, when compared with the basal condition. This increase in the PKC βII expression was significantly inhibited to 31% ($P = 0.02$), when the VEGF-induced retinal cells were pretreated with 10 μM curcumin, as shown in Figure 7.

DISCUSSION

In the present study, we observed that curcumin inhibits the proliferation of human retinal endothelial cells, in a dose-dependent manner by inducing cell death in HRECs cultured in high glucose conditions. As revealed by three different exper-

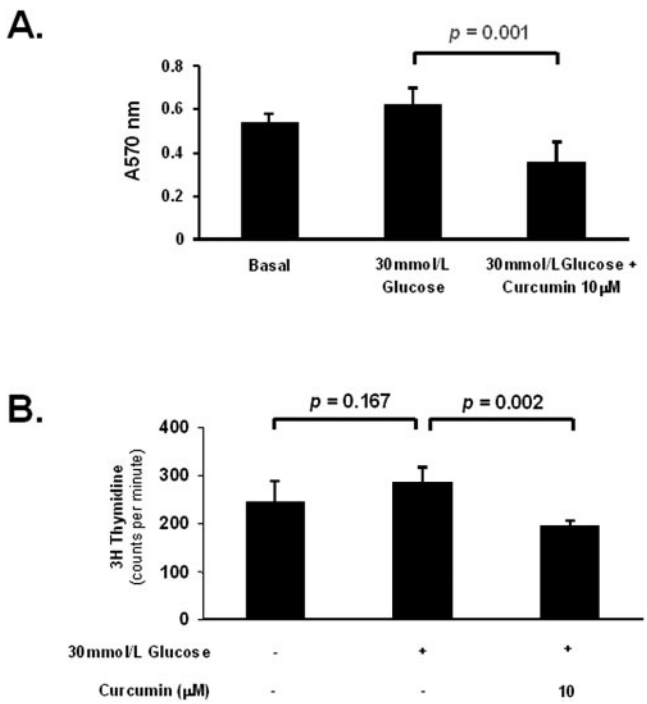

FIGURE 2. Human retinal endothelial cell viability and DNA synthesis in high-glucose and curcumin treatment conditions. HRECs were treated with 30 mmol/L glucose and 10 μM curcumin for 72 hours. (**A**) Cell viability was measured through the formation of formazan products by MTT and (**B**) DNA synthesis measured as thymidine uptake. Data points show the mean ± SD of five separate experiments. $P < 0.05$.

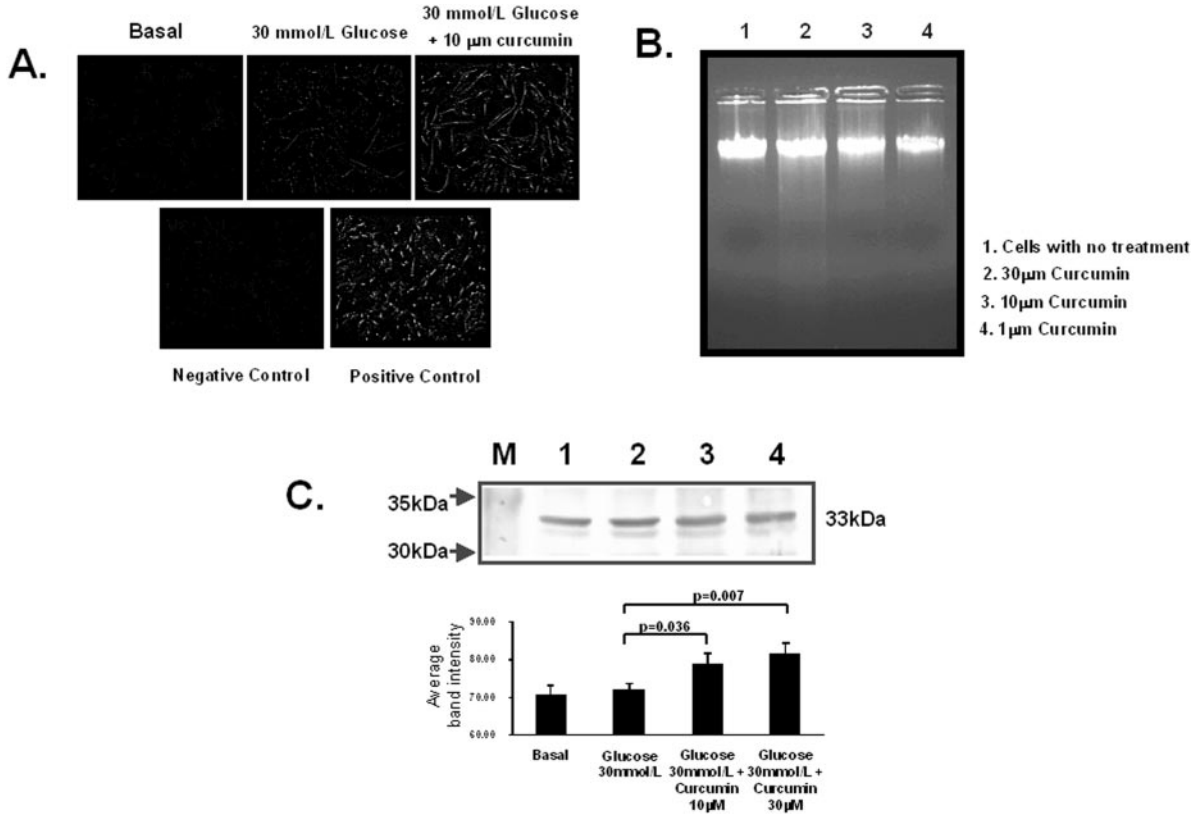

FIGURE 3. Examination of cell death. (**A**) TUNEL staining showing cells undergoing apoptosis as indicated by intense (*green*) staining in condensed nuclei. (**B**) HRECs treated for 72 hours with curcumin caused an increase in large DNA fragments. *Lane 1*: control (untreated), *lanes 2*, *3*, and *4*: treated with 30, 10 and 1 μM curcumin, respectively. Data are representative of results in one of three independent experiments with similar results. (**C**) Expression of caspase 3 shown by Western blot analysis in HRECs. A representative blot from three separate experiments is presented, along with average band intensity quantifications.

iments—TUNEL, DNA fragmentation and caspase-3 expression—it appears that the antiproliferative effect of curcumin in HRECs is mediated by induction of apoptosis.

Curcuma longa has been used from antiquity as a condiment, medically as an anti-inflammatory agent, and as a dye. It is also known as an angiogenesis inhibitor and inhibits multistep progression of angiogenesis in vitro and in vivo.[17,18] It inhibits proliferation of human umbilical vein endothelial cells (HUVECs) though induction of G_0/G_1 cell cycle arrest.[12] It also inhibits basic fibroblast growth factor (bFGF)–induced corneal neovascularization in vivo and activation of MMP9 by FGF during angiogenesis.[17] However, the control mechanisms of endothelial cell proliferation by curcumin have not yet been determined, especially in the microvasculature. In this study, to the best of our knowledge, a mechanistic antiproliferative effect of curcumin on HREC is being reported for the first time.

Previous studies showed that high glucose produces an increase in intracellular ROS generation and an upregulation of PKC activity.[19,20] In agreement with previous reports,[20–24] it was found in this study that high glucose produced an increase in ROS. According to Nishikawa et al.,[19] the activation of PKC seems to be dependent on free radical generation, because inhibiting oxidative stress significantly reduces PKC activation. The PKC inhibitor BIM1 and the selective PKC β inhibitor LY379196 were equally effective in diminishing the production of free radicals and reducing the NAD(P)H activation.[20] However the precise mechanisms underlying this phenomenon may require further clarification. In our study, treatment of HRECs with curcumin resulted in marked reduction of intracellular ROS generation and thereby appeared to interfere with the downstream molecular mechanisms of angiogenesis.

Our study also clearly demonstrates that high concentrations of glucose in HRECs upregulated VEGF mRNA expression. This finding is very similar to a previous report that high glucose concentrations markedly increase VEGF mRNA expression in human vascular smooth muscle cells and that

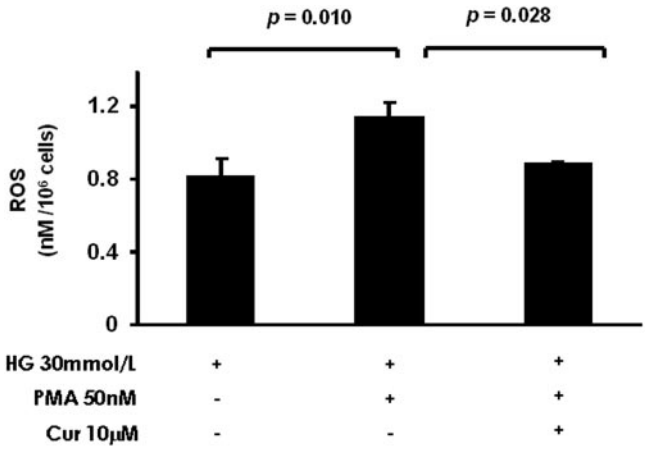

FIGURE 4. Effect of curcumin on high glucose induced intracellular ROS generation. The bar diagram depicts the results of HRECs treated with 50 nM PMA in high-glucose conditions. The intracellular generation of ROS was monitored with a spectrofluorometer. Results are presented as the mean ± SD of results in six independent experiments.

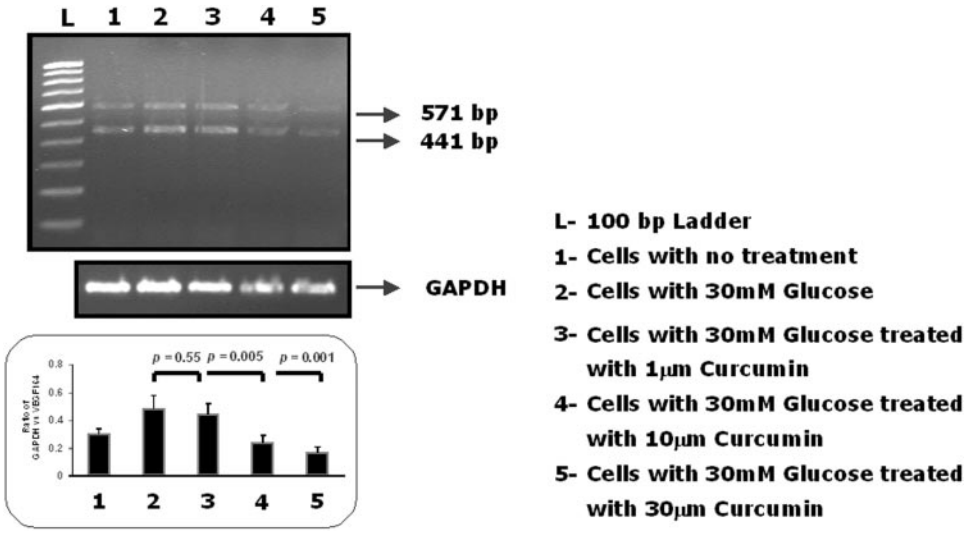

FIGURE 5. Expression of VEGF mRNA in response to high glucose and curcumin. Confluent HRECs were cultured for 72 hours in medium with 5 mM and 30 mmol/L glucose, or in medium containing the indicated concentrations of curcumin. Changes in mRNA levels are reported as the ratios of the concentration of GAPDH to $VEGF_{164}$. The densitometric analysis was performed with molecular analysis software, and the levels of expression were plotted ($n = 4$).

hyperglycemia could contribute directly to the development of endothelial dysfunction and neovascularization in diabetic retinopathy.[25] Increased ROS production has been shown to mediate transcriptional changes in hormones and growth factors, including induction and upregulation of VEGF.[26] Moreover, direct exposure of cells to ROS was shown to induce VEGF expression.[27] Thus, our work also suggests that VEGF induction in high-glucose–treated HRECs could be a downstream effect of increased ROS generation.

It is interesting to observe that curcumin also inhibits PKC βII translocation induced by VEGF in HRECs. In the angiogenesis cascade, VEGF appears to mediate its mitogenic effects predominantly through the activation of PKC[28] and PKC translocation to membrane in several cell types including the primary bovine retinal endothelial cell (BREC) cultures.[29,30] Because PKC activation could also upregulate VEGF expression,[25] there may be a vicious cycle of upregulation of both VEGF and PKC signals culminating in angiogenesis processes.

In conclusion, our study has delineated molecular effects of curcumin on HRECs exposed to high glucose, with some plausible mechanisms. The antiproliferative effect of curcumin may be partly related to its antioxidant property and partly to its biological activities that interfere with VEGF production and VEGF-induced PKC βII translocation. Further research is necessary to translate this knowledge into therapeutic applications.

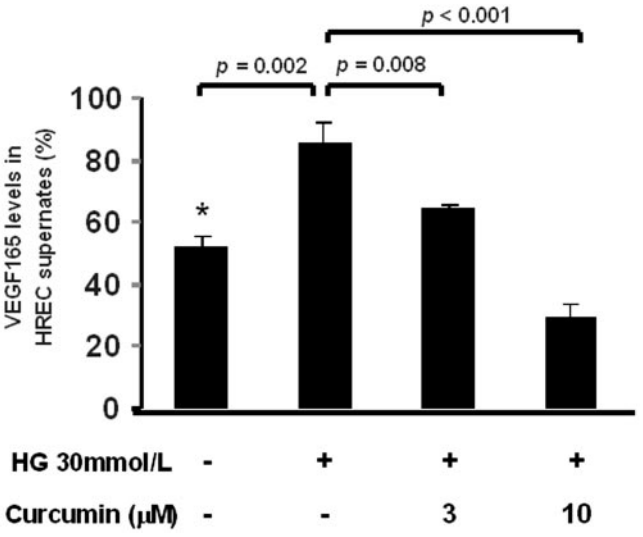

FIGURE 6. Effect of curcumin on VEGF release in HRECs. The conditioned media from HRECs treated with or without curcumin (3 and 10 μM) cultured in high glucose concentrations were subjected to mouse VEGF-ELISA analysis in triplicate. The OD at 450 nm was measured, and the results are presented as the mean ± SD; $n = 3$; $P < 0.05$.

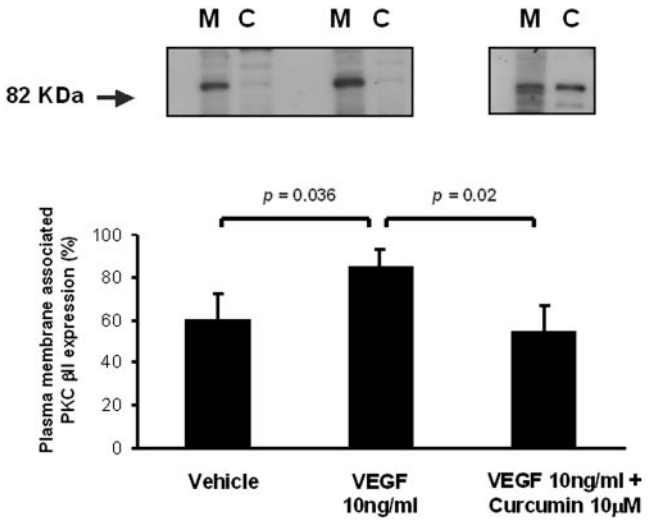

FIGURE 7. Curcumin on VEGF-induced PKC βII activation in HRECs. PKC βII expression in the plasma membrane of HRECs shown by Western blot analysis. The results are presented as percentage of PKC βII translocation to the plasma membrane. M, plasma membrane fractions; C, cytosolic fractions of HRECs. The band intensity was measured using molecular analyst software, and the levels of expression were plotted ($n = 4$).

References

1. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature*. 2001;414:813–820.
2. King GL, Kunisaki M, Nishio Y, Inoguchi T, Shiba T, Xia P. Biochemical and molecular mechanisms in the development of diabetic vascular complications. *Diabetes*. 1996;45:S105–S108.
3. Bussolino F, Mantovani A, Persico G. Molecular mechanisms of blood vessel formation. *Trends Biochem Sci*. 1997;22:251–256.
4. Dawson DW, Volpert OV, Gillis P, et al. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science*. 1999; 285:245–248.
5. Jimenez B, Volpert OV. Mechanistic insights on the inhibition of tumor angiogenesis. *J Mol Med*. 2001;78:663–672.
6. Rena JG, Jie C, Talbot C. How PEDF prevents angiogenesis: a hypothesized pathway. *Med Hypotheses*. 2005;64:74–78.
7. Marme D. Tumour angiogenesis: the pivotal role of vascular endothelial growth factor. *World J Urol*. 1996;14:166–174.
8. Hagedorn M, Bikfalvi A. Target molecules for antiangiogenic therapy; from basic research to clinical trials. *Crit Rev Oncol Hematol*. 2000;34:89–110.
9. Arbiser JL, Klauber R, Rohan R, et al. Curcumin is an *in vivo* inhibitor of angiogenesis. *Mol Med*. 1998;4:376–383.
10. Sreejayan, Rao MN. Nitric oxide scavenging by curcuminoids. *J Pharm Pharmacol.* 1997;49:105–107.
11. Strasser EM, Wessner B, Manhart N, Roth E. The relationship between the anti-inflammatory effects of curcumin and cellular glutathione content in myelomonocytic cells. *Biochem Pharmacol*. 2005;70:552–559.
12. Singh AK, Sidhu GS, Deepa T, Maheshwari RK. Curcumin inhibits the proliferation and cell cycle progression of human umbilical vein endothelial cell. *Cancer Lett*. 1996;107:109–115.
13. Matsubara TA, Murata TA, Wu GS, Barron EA, Rao NA. Isolation and culture of rat retinal microvessel endothelial cells using magnetic beads coated with antibodies to PECAM-1. *Curr Eye Res*. 2000;20:1–7.
14. Lecomte M, Paget C, Ruggiero D, Wiernsperger N, Lagarde M. Docosahexaenoic acid is a major n-3 polyunsaturated fatty acid in bovine retinal microvessels. *J Neurochem*. 1996;66:2160–2167.
15. Scott PA, Bicknell R. The isolation and culture of microvascular endothelium. *J Cell Sci*. 1993;105:269–273.
16. Balasubramanyam M, Koteswari AA, Kumar RS, Monickaraj SF, Maheswari JU, Mohan V. Curcumin-induced inhibition of cellular reactive oxygen species generation: novel therapeutic implications. *J Biosci*. 2003;28:15–21.
17. Mohan R, Sivak J, Ashton P, et al. Curcuminoids inhibit the angiogenic response stimulated by fibroblast growth factor-2, including expression of matrix metalloproteinase gelatinase B. *J Biol Chem*. 2000;275:10405–10412.
18. Wei SC, Lin YS, Tsao PN, Wu-Tsai JJ, Wu CH, Wong JM. Comparison of the anti-proliferation and apoptosis-induction activities of sulindac, celecoxib, curcumin, and nifedipine in mismatch repair-deficient cell lines. *J Formosa Med Assoc*. 2004;103:599–606.
19. Nishikawa T, Edelstein D, Du XL, et al. Normalising mitochondrial superoxide production blocks three pathways of hyperglycemic damage. *Nature*. 2000;404:787–790.
20. Inoguchi T, Sonta T, Tsubouchi H, et al. Protein kinase C-dependent increase in reactive oxygen species (ROS) production in vascular tissue in diabetes: role of vascular NAD(P)H oxidase. *J Am Soc Nephrol*. 2003;14:S227–S232.
21. Dandona P, Thusu K, Cook S, et al. Oxidative damage to DNA in diabetes mellitus. *Lancet*. 1996;347:444–445.
22. Ceriello A, Quagliaro L, D'Amico M, et al. Acute hyperglycemia induces nitrotyrosine formation and apoptosis in perfused heart from rat. *Diabetes*. 2002;51:1076–1082.
23. Kakimoto M, Inoguchi T, Sonta T, et al. Accumulation of 8-hydroxy-2′-deoxyguanosine and mitochondrial DNA detection in kidney of diabetic rats. *Diabetes*. 2002;51:1588–1595.
24. Park KS, Kim JH, Kim MS, et al. Effects of insulin and antioxidant on plasma 8-hydroxy-guanine and tissue 8-hydroxydeoxyguanosine in streptozotocin–induced diabetic rats. *Diabetes*. 2001;50:2837–2841.
25. Williams B, Gallacher B, Patel H, Orme C. Glucose-induced protein kinase C activation regulates vascular permeability factor mRNA expression and peptide production by human vascular smooth muscle cells in vitro. *Diabetes*. 1997;46:1497–1503.
26. Duyndam MC, Hulscher TM, Fontijn D, Pinedo HM, Boven E. Induction of vascular endothelial growth factor expression and hypoxia-inducible factor 1α protein by the oxidative stressor arsenite. *J Biol Chem*. 2001;276:48066–48076.
27. Brauchle M, Funk JO, Kind P, Werner S. Ultraviolet B and H2O2 are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes. *J Biol Chem*. 1996;271:21793–21797.
28. Xia P, Aiello LP, Ishii H, et al. Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. *J Clin Invest*. 1996;98: 2018–2026.
29. Wang Y, Pampou S, Fujikawa K, Varticovski L. Opposing effect of angiopoietin-1 on VEGF-mediated disruption of endothelial cell-cell interactions requires activation of PKC beta. *J Cell Physiol*. 2004;198:53–61.
30. Sone H, Deo BK, Kumagai AK. Enhancement of glucose transport by vascular endothelial growth factor in retinal endothelial cells. *Invest Ophthalmol Vis Sci*. 2000;41:1876–1884.