EXTENDED REPORT

Vascular endothelial growth factor C promotes survival of retinal vascular endothelial cells via vascular endothelial growth factor receptor-2

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Aim: To determine vascular endothelial growth factor C (VEGF-C) expression in retinal endothelial cells, its antiapoptotic potential and its putative role in diabetic retinopathy.

Method: Cultured retinal endothelial cells and pericytes were exposed to tumour necrosis factor (TNF) α and VEGF-C expression determined by reverse transcriptase-polymerase chain reaction. Secreted VEGF-C protein levels in conditioned media from endothelial cells were examined by western blotting analysis. The ability of VEGF-C to prevent apoptosis induced by TNF α or hyperglycaemia in endothelial cells was assessed by flow cytometry. The expression of VEGF-C in diabetic retinopathy was studied by immunohistochemistry of retinal tissue.

Result: VEGF-C was expressed by both vascular endothelial cells and pericytes. TNF α up regulated both VEGF-C and vascular endothelial growth factor receptor-2 (VEGFR)-2 expression in endothelial cells in a dose-dependent manner, but had no effect on VEGFR-3. Flow cytometry results showed that VEGF-C prevented endothelial cell apoptosis induced by TNF α and hyperglycaemia and that the antiapoptotic effect was mainly via VEGFR-2. In pericytes, the expression of VEGF-C mRNA remained stable on exogenous TNF α treatment. VEGF-C immunostaining was increased in retinal vessels in specimens with diabetes compared with retinal specimens from controls without diabetes.

Conclusion: In retinal endothelial cells, TNF α stimulates the expression of VEGF-C, which in turn protects endothelial cells from apoptosis induced by TNF α or hyperglycaemia via VEGFR-2 and thus helps sustain retinal neovascularisation.

Vascular endothelial growth factor A (VEGF-A) plays a key part in diabetic retinopathy by increasing retinal vascular permeability and inducing neovascularisation. However, the inhibition of VEGF-A only partially decreases neovascularisation and vessel hyperpermeability,¹ suggesting that other VEGF family members may also be involved in this process.^{2–3}

VEGF-C is a member of the VEGF family that displays a high degree of homology with VEGF-A.⁴ The VEGF-C precursor binds only vascular endothelial growth factor receptor (VEGFR)-3, whereas the fully processed VEGF-C ligand can bind and activate both VEGFR-2 and VEGFR-3.⁵ VEGF-C stimulates proliferation and migration of blood vascular endothelial cells⁵ and promotes release of nitric oxide and plasminogen activator from endothelial cells.^{6–7} In animal models, VEGF-C induces angiogenesis and increases vascular permeability.^{7–8} Furthermore, high expression of the VEGF-C protein and gene has been found in different vascularised tumour tissues.^{9–11} The activation of both VEGFR-2 and VEGFR-3 has been implicated in angiogenesis,^{12–13} and VEGFR-3 is present in different vascular beds including the retinal vasculature.^{14–15}

The pathogenesis of diabetic retinopathy may be correlated with chronic subclinical inflammation,¹⁶ and anti-inflammatory drugs have been shown to prevent early diabetic retinopathy via tumour necrosis factor (TNF) α suppression.¹⁷ TNF α has been found in human retinas with proliferative eye diseases^{18–20} and in animal models of retinal neovascularisation.²¹ Furthermore, hyperglycaemia also plays an important part in the onset and progression of diabetic retinopathy by inducing apoptosis of vascular cells, advanced glycation end product deposition and up regulation of angiogenic factors.^{22–24}

This paper reports that VEGF-C can promote survival of retinal endothelial cells and that this can be regulated by both TNF α and hyperglycaemia.

MATERIALS AND METHODS

Reagents

Recombinant TNF α , an anti-VEGFR-2 neutralising antibody, recombinant VEGF-C wild type (which binds both VEGFR-2 and VEGFR-3) and VEGF-C (Cys156Ser; a selective agonist of VEGFR-3) were obtained from R&D Systems Europe (Abingdon, UK). Anti-VEGF-C antibody was from Santa Cruz (UK). For immunohistochemistry, an affinity-purified goat polyclonal antibody raised against the carboxy terminus of the VEGF-C precursor of human origin (c-20) was obtained from Autogen Bioclear (Calne, Wiltshire, UK). TRIzol was from Invitrogen (Glasgow, UK), and polymerase chain reaction (PCR) Reddy Mix and Master Mix Kit were purchased from Abgene (UK). Vybrant apoptosis Assay Kit was from Molecular Probes (UK). All other materials were from Sigma unless otherwise stated.

Cell culture

Primary cultures of bovine microvascular retinal endothelial cells (MECs) and pericytes were isolated as described previously.²⁵ Endothelial cells were maintained in an endothelial

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEC, microvascular retinal endothelial cell; PCR, polymerase chain reaction; PDR, proliferative diabetic retinopathy; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

cell basal medium with growth supplement (TCS Works, Buckingham, UK). Cells were characterised by their cobblestone appearance and expression of factor VIII antigen.²⁵ Pericytes were cultured in Eagle's minimal essential medium (GibcoBRL, Paisley, UK) containing 10% fetal calf serum. Pericytes were identified and distinguished from endothelial cells by their size, irregular morphology and negative staining for factor VIII.²⁵ Both cell types were used between passages 1 and 3 for all experiments.

To ensure cross species recognition of VEGFRs, primary cultures from human donor eyes were obtained from the Bristol Eye Bank, Bristol, UK, and used in accordance with the tenets of the Declaration of Helsinki. The cultures were isolated and maintained as described above and used for the apoptosis studies within five passages.

TNF α treatment

For gene and protein expression studies, cells were treated with different concentrations of TNF α for up to 6 h. For time-dependent studies of TNF α treatment, cells were incubated with 10 ng/ml TNF α at different time points.

Reverse transcriptase-polymerase chain reaction

To investigate gene expression of VEGF-C and its receptors, total RNA was isolated from endothelial cells and pericytes exposed to different experimental conditions, using the isolation kit TRIzol, and then analysed by reverse transcriptase-polymerase chain reaction (RT-PCR) using the First Strand Synthesis Kit and PCR ReddyMix according to the manufacturer's protocol. Equal quantities of total RNA were used from different samples. The primers for VEGF-C were according to the sequences of bovine VEGF-C from GenBank. The oligonucleotide primers for amplification of VEGF-C cDNA were 5'-GAA CAA GGC TTA TGC AGG CAA AG -3' and 5'-CCA CAT CTG TAG ACG GAC ACA C-3'. The resultant PCR product was 348 bp long. The primers for VEGFR-2 were from Berisha *et al.*²⁶ and VEGFR-3 was from Pepper *et al.*⁶ Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The sequences were 5'-TGT TCC AGT ATG ATT CCA CCC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3', and gave an 850 bp amplicon. The cDNA was amplified using the PCR Master Mix, each cycle consisting of 20 s at 94°C, 30 s at 55°C for amplifying VEGF-C and GAPDH cDNA, 51°C for VEGFR-2 cDNA, 56°C for VEGFR-3 cDNA and 60 s at 72°C. All the samples were amplified in a linear amplification range established using a serial cDNA dilution and varying the number of cycles. PCR products were electrophoresed on to a 1.2% agarose gel containing ethidium bromide and visualised under ultraviolet light. The relative intensities of the bands were quantified by densitometric analysis.

Immunoprecipitation and western blotting

To measure VEGF-C protein, confluent MECs were starved overnight in basal medium containing 1% fetal calf serum, after which either 1 or 10 ng/ml TNF α was added to the basal medium. De novo protein synthesis was blocked by the addition of 3.6 μ M cycloheximide. Cells were exposed to different conditions for 24 h, the conditioned media was collected and centrifuged to remove cell debris. The protein concentrations were determined by the BCA protein assay (Pierce, UK). The medium with equal quantity of proteins was immunoprecipitated by incubation with an anti-VEGF-C antibody, and then protein A/G-agarose (Santa Cruz Biotechnology, USA). The immunoprecipitates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and proteins transferred on to nitrocellulose membranes. The membranes were probed with an anti-VEGF-C antibody followed by incubation

with secondary antibody conjugated with horseradish peroxidase. The enhanced chemiluminescence reaction system (Santa Cruz, UK) was used to visualise the bands.

Apoptosis assay

Apoptosis was evaluated using the Vybrant Apoptosis Assay Kit based on annexin-V binding to phosphatidylserine exposed on the outer leaflet of the plasma membrane lipid bilayer of cells. MECs were treated with either 100 ng/ml TNF α or 30 mM glucose in the presence or absence of 200 ng/ml VEGF-C for 48 h in culture medium. The cells from different treatments were subjected to the apoptosis assay according to the manufacturer's instructions. The samples were analysed using a fluorescent activated cell sorting 440 Flow Cytometer (Becton Dickinson, Oxford, UK). Viable cells were double-negative stained, early apoptotic cells stained positive for annexin V and negative for propidium iodide, whereas, late apoptotic/necrotic cells were double-positive stained for annexin V and propidium iodide. To define the role of VEGFR-2 in the anti-apoptotic effect of VEGF-C, 60 ng/ml anti-VEGFR-2 antibody was added to the culture medium for 1 h before incubation with 100 ng/ml TNF α and 200 ng/ml VEGF-C, or 30 mM glucose and 200 ng/ml VEGF-C. Anti-VEGFR-2 antibody alone acted as a control. To observe whether VEGFR-3 had an anti-apoptotic function, 200 ng/ml VEGF-C (Cys156Ser; a selective agonist of VEGFR-3) was administered together with 100 ng/ml TNF α for 48 h.

To block the basal secretion of VEGF-C, cells were transfected with either VEGF-C small interfering RNA (siRNA) or scrambled siRNA for 72 h and then incubated with 100 ng/ml TNF α for 48 h. siRNA duplexes were designed and synthesised by Dharmacon Research (Lafayette, Colorado, USA) to target the bovine sequence of VEGF-C 5'-ACA GAG ATC TTA AGA AGT A-3'. The premade siRNA (scramble II; Dharmacon) was used as a negative control. Cells were transfected with siRNA duplexes using DharmaFECT 1 (Dharmacon) at a final RNA concentration of 100 nmol/l according to the manufacturer's protocol. To determine the efficiency of transfection, the medium from parallel samples was collected and subjected to immunoprecipitation and western blotting after 24-h of incubation.

Immunohistochemistry

A total of 47 eyes enucleated and fixed in 10% neutral-buffered formalin within 10 h after death were obtained from the National Disease Research Interchange, Philadelphia, USA. All procedures were performed according to the Declaration of Helsinki. Eyes were categorised by an ophthalmologist based on fundus appearance as normal (no known ophthalmic disease, no history of diabetes, no abnormalities on biomicroscopy), diabetic with no overt retinopathy, diabetic with intraretinal changes but no evidence of proliferative diabetic retinopathy (PDR), diabetic with preretinal PDR and diabetic with scatter laser photocoagulation but no evidence of residual PDR.¹⁴

Immunohistochemistry was performed on 5 μ m sections as described previously.¹⁴ Sections were incubated overnight at 4°C with a polyclonal VEGF-C antibody (2 μ g/ml). The negative control was the substitution of the primary antibody with an inappropriate rabbit IgG. After washing, sections were incubated for 30 min with biotinylated rabbit anti-goat IgG, then for a further 30 min with alkaline phosphatase reaction mixture (Dako) and incubated with Fast Red TR/naphthol AS-MX substrate. Slides were counterstained with Mayer's haematoxylin. The degree and pattern of immunostaining was assessed by two blinded observers. The intensity of staining was graded qualitatively as background, weak, moderate or intense (corresponding to the highest level of immunoreactivity).

Figure 1 The regulation of vascular endothelial growth factor-C (VEGF-C) mRNA expression by tumour necrosis factor (TNF) α in microvascular endothelial cells and pericytes. (A) Dose-response of mRNA induction of VEGF-C by TNF α in microvascular retinal endothelial cells (MECs). MECs were stimulated with the indicated concentrations of TNF α for 6 h. (B) Time dependence of VEGF-C mRNA induction by TNF α . MECs were stimulated with TNF α (10 ng/ml) for 0–24 h. (C) Expression of VEGF-C mRNA in bovine retinal pericytes after exposure to different concentrations of TNF α for 6 h. The isolated total RNA from different treatments was subjected to reverse transcriptase-polymerase chain reaction and polymerase chain reaction products were analysed by agarose gel electrophoresis. Band intensities were quantified by laser densitometry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal control. Representative results from three separate experiments are shown. Vertical bars represent mean (standard error of the mean); ** $p < 0.01$, indicating significant difference between treatment and controls.

Statistical analysis

The results represent the mean of at least three separate experiments. Statistical analysis was carried out using an unpaired Student's *t* test. Significance was defined as $p < 0.05$. All numerical results are expressed as mean (standard error of the mean).

RESULTS

Regulation of VEGF C mRNA expression by TNF α in microvascular endothelial cells and pericytes

RT-PCR analysis showed that VEGF-C mRNA was expressed in MECs. TNF α stimulated the expression of VEGF-C mRNA in a dose-dependent manner, with a maximal 4.6 (0.5)-fold increase with 10 ng/ml TNF α (fig 1A). Stimulation of cells with 10 ng/ml TNF α increased VEGF-C mRNA expression in a time-dependent manner with a maximum at 6 h. Beyond 6 h, VEGF-C expression decreased, but even after 24 h stimulation, the expression of VEGF-C mRNA was still higher than that with no stimulation (fig 1B). Pericytes expressed VEGF-C mRNA, but TNF α had no regulatory effect on this expression (fig 1C).

Increased expression of VEGFR-2, but not VEGFR-3 mRNA in MECs challenged with TNF α

RT-PCR results showed that MECs expressed both VEGFR-2 and VEGFR-3 mRNA (fig 2). TNF α induced an increase of VEGFR-2 mRNA in a dose-dependent manner. The levels of VEGFR-2 mRNA began to increase at 1 ng/ml TNF α and reached a maximum level at 50 ng/ml TNF α (fig 2A). By contrast, the expression of VEGFR-3 was not modified by exposure to TNF α (fig 2B). The expression of VEGFR-2 and VEGFR-3 mRNA was not detectable in pericytes (data not shown).

TNF α increases VEGF C protein synthesis and secretion in MECs

Western blotting showed that the secreted peptide was present at high amounts, with a maximum 4.1 (0.4)-fold increase in the medium under TNF α conditions compared with the medium of control cultures (fig 3A). Treatment with cycloheximide considerably reduced the amount of VEGF-C in the conditioned medium. This result indicates that increased amounts of VEGF-C released by MECs in response to TNF α treatment are due to

Figure 2 Tumour necrosis factor (TNF) α stimulates the expression of vascular endothelial growth factor receptor (VEGFR)-2 mRNA, but not VEGFR-3 in microvascular retinal endothelial cells (MECs). Total RNA was isolated 6 h after stimulation by different concentrations of TNF α . Reverse transcriptase-polymerase chain reaction was performed and polymerase chain reaction products were analysed by agarose gel electrophoresis. The signal intensity was determined by densitometry, and the amount of VEGFR-2/VEGFR-3 was normalised for the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) present. Representative results from at least three separate experiments are shown. Vertical bars represent mean (standard error of the mean); * $p < 0.05$ and ** $p < 0.01$, indicating significant difference between treatment and controls. (A) Expression of VEGFR-2 mRNA; (B) expression of VEGFR-3 mRNA.

increased protein synthesis rather than an increased release of VEGF-C from cell storage (fig 3B)

VEGF C prevents TNF α and hyperglycaemia-induced apoptosis in MECs and this effect occurs mainly via VEGFR-2

Flow cytometry showed that TNF α induced apoptosis/necrosis and that this was markedly inhibited by VEGF-C. The cell population at the late stage of apoptosis/necrosis reduced from 56.5% (1.38%) to 28.1% (0.7%) when VEGF-C was present (fig 4A–C). The apoptotic/necrotic population increased to 82.7% (2.8%) after exposure to TNF α in cells treated with VEGF-C siRNA compared with 57.9% (1.72%) in cells treated with scrambled siRNA (fig 4D,E). The efficiency of knockdown of VEGF-C expression in culture medium with RNAi was confirmed using immunoprecipitation and western blotting (fig 4L).

To identify which of the two VEGF-C receptors was responsible for the anti-apoptotic effect of VEGF-C, VEGFR-2 was blocked by neutralising antibody or cells were treated with a VEGFR-3 agonist. After neutralising VEGFR-2, the protective effect of VEGF-C on TNF α -induced apoptosis was considerably

Figure 3 Western blotting analysis of de novo vascular endothelial growth factor-C (VEGF-C) protein synthesis. (A) Microvascular retinal endothelial cells (MECs) were treated with 1 or 10 ng/ml tumour necrosis factor (TNF) α for 24 h. (B) MECs were treated with 10 ng/ml TNF α and 3.6 μ M cyclohexamide (CHX) for 24 h, and 10 ng/ml TNF α alone was the control. Conditioned media were immunoprecipitated using a polyclonal antibody raised to VEGF-C and the immunoprecipitates electrophoresed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by blotting on a nitrocellulose membrane. Positive bands were visualised by an enhanced chemiluminescence reaction detection system. Band intensity for VEGF-C was quantified by laser densitometry from at least three separate experiments. Vertical bars represent mean (standard error of the mean); ** $p < 0.01$, indicating significant difference between treatment and controls.

reduced. The population of apoptotic/necrotic cells was increased from 28.1% (0.7%) to 53.2% (1.41%) (fig 4C,F). VEGFR-2-neutralising antibody alone had no effect on promoting apoptosis/necrosis (fig 4G). After addition of VEGF-C (Cys156Ser), there was no protective effect on TNF α -induced apoptosis. The population of apoptotic/necrotic cells was 55.7% (2.1%), and showed no statistically significant change from cells treated with TNF α alone (fig 4B,H). After addition of VEGF-C, the percentage of apoptosis/necrosis induced by high glucose was reduced from 47.1% (1.6%) to 23.6% (1.2%) (fig 4I,J) and this rescue effect was abolished when VEGFR-2 was blocked by its neutralising antibody (fig 4K). Results were similar for both human and bovine MECs.

Expression of VEGF-C in diabetic retinopathy

Weak to moderate staining for VEGF-C was observed in the vessels of non-diabetic retinas and in retinas without overt retinopathy; staining was increased (moderate to intense) compared with non-diabetic retinas once intraretinal changes became obvious (4 of 5 eyes) and markedly increased in PDR retinas (6 of 6 eyes; table 1, fig 5). Intense staining was also

Figure 4 (A–K) Vascular endothelial growth factor (VEGF)-C prevents tumour necrosis factor (TNF) α -induced apoptosis via vascular endothelial growth factor receptor (VEGFR)-2. Microvascular retinal endothelial cell (MECs) were given different treatments for 48 h. The cells were then stained with annexin V-fluorescein isothiocyanate conjugate (FITC) containing propidium iodide (PI). Data show two-parameter analysis of fluorescence intensity of annexin V and PI. Annexin V/PI-negative cells were counted as viable cells (lower left quadrant). All measurements were performed in triplicate. Representative results from at least three separate experiments are shown. (A) Control; (B) TNF α ; (C) TNF α +VEGF-C; (D) TNF α +VEGF-C siRNA; (E) TNF α +scrambled siRNA; (F) TNF α +VEGF-C+anti-VEGFR-2 antibody; (G) anti-VEGFR-2-neutralising antibody alone; (H) TNF α +VEGF-C (Cys156Ser); (I) high glucose; (J) high glucose+VEGF-C; (K) high glucose+VEGF-C+anti-VEGFR-2 antibody. Numbers in the quadrant are the percentage of FITC⁺/PI⁺ cells. (L) Knockdown of the expression of VEGF-C in culture medium from small interfering (si)RNA-treated MECs. Culture medium from either VEGF-C siRNA or scrambled siRNA-treated cells was collected after 24 h of incubation and equal quantity of proteins was subjected to immunoprecipitation and western blotting. Positive bands were quantified by laser densitometry from at least three separate experiments. Vertical bars represent mean (standard error of the mean); ** $p < 0.01$, indicating significant difference between VEGF-C siRNA treatment and scrambled siRNA.

observed in the vessels of preretinal membranes. After laser treatment, the levels of VEGF-C in the retinal vessels were reduced to weak staining in 11 of 14 eyes. In addition, increased staining was observed in the ganglion cell layer of diabetic retinas both with and without intraretinal changes as compared with the minimal staining in non-diabetic retinas. Staining was weak or absent in the choroidal vessels, the RPE, the photoreceptors, and the outer and inner retinal layers (table 1). The variability of staining within retinas of the same group did not show a correlation with donor age, the type of glycaemic control in the case of the diabetic groups or time after death.

DISCUSSION

In this study, we showed that $\text{TNF}\alpha$ strongly up regulates the expression of VEGF-C in MECs and that this induction is both

time dependent and dose dependent. The dose-response study showed that the stimulatory effect of $\text{TNF}\alpha$ was produced at a concentration as low as 0.1 ng/ml, which is within the range of $\text{TNF}\alpha$ concentrations in vitreous fluid from patients with active PDR.¹⁸ VEGFR-2 was also up regulated by $\text{TNF}\alpha$, whereas the expression of VEGFR-3 mRNA remained stable with various $\text{TNF}\alpha$ treatments, suggesting that in our experimental system VEGF-C may exert its angiogenic effect mainly via increasing VEGFR-2 rather than VEGFR-3. This increase in VEGFR-2 may also be important in enhancing VEGF-A-induced angiogenesis. After blockade of VEGFR-2, the antiapoptotic effect of VEGF-C was abrogated, whereas the activation of VEGFR-3 by VEGF-C (Cys156Ser) did not attenuate $\text{TNF}\alpha$ -induced apoptosis showing that VEGFR-2 is the dominant receptor for VEGF-C action in MECs. Our data support the observations that VEGF-C, which was originally thought to be a potent inducer of

Figure 5 Immunolocalisation of vascular endothelial growth factor (VEGF)-C in the diabetic retina. VEGF-C staining is shown for representative retinal sections. Weak to moderate VEGF-C immunostaining was localised to non-diabetic retina (A) and diabetic retina with no obvious intraretinal vascular changes (B). By contrast, moderate to intense VEGF-C staining was observed in the diabetic retina with obvious intraretinal vascular changes but no evidence of proliferative diabetic retinopathy (PDR; C) and in the diabetic retina with PDR (D). After laser treatment, the only weak immunostaining for VEGF-C was observed (E). Immunostaining was raised in later-stage diabetic retinopathy and was generally confined to the intraretinal vessels (IRV). Immunoreactivity was abolished in the control retina processed with omission of primary antibody (F). Magnification $\times 200$. PRM, polynomial regularisation method.

Table 1 Mean (SD) intensity of vascular endothelial growth factor-C in the retina and choroid

	Choroid	RPE	Photoreceptors	Outer nuclear layer	Inner nuclear layer	Ganglion cell layer	Retinal vessels
Non-diabetic (n = 14)	0.6 (0.8)	0.1 (0.4)	0.4 (0.8)	0 (0)	0.1 (0.4)	0.3 (0.4)	1.1 (1.2)
No overt retinopathy (n = 12)	0.8 (0.6)	0.1 (0.3)	0.3 (0.7)	0 (0)	0.1 (0.3)	1.2 (1.0)	1.6 (1.1)
Intraretinal changes (n = 5)	0.8 (0.8)	0 (0)	0.6 (0.9)	0.2 (0.5)	0.6 (0.9)	1.2 (1.1)	2.4 (0.9)
PDR (n = 6)	0.8 (0.8)	0.3 (0.5)	0.3 (0.5)	0.2 (0.4)	0.3 (0.5)	0.3 (0.5)	2.5 (0.5)
Laser, no residual PDR (n = 14)	0.6 (0.6)	0 (0)	0.4 (0.6)	0.4 (0.9)	0.3 (0.5)	0.4 (0.5)	0.8 (1.2)

PDR, proliferative diabetic retinopathy; RPE, retinal pigment epithelium.
0, background; 1, mild; 2, moderate; 3, intense staining.

lymphangiogenesis,^{27, 28} may also act as a survival factor to suppress apoptosis in vascular endothelial cells. VEGF-C has been shown to be important in vascular angiogenesis in other vascular beds,^{7, 8, 11, 29} and the response can be robust and indistinguishable from that observed using VEGF-A.^{30, 31} The existence of VEGF-C in human retinas and the expression of VEGF-C in the retinal vasculature that increases in diabetic retinopathy further support a role for VEGF-C in diabetic retinopathy. Interestingly, laser photocoagulation, a proved treatment for reversing neovascularisation in PDR, resulted in a marked reduction in VEGF-C protein expression.

Our data showed that mRNA coding for VEGF-C was present not only in endothelial cells but also in pericytes. However, the regulatory effects differ between the two principal types of microvascular cells. The VEGF-C gene remained constitutively expressed in pericytes on adding various concentrations of TNF α , but VEGF-C is unlikely to signal in an autocrine fashion as only VEGFR-1 is expressed in pericytes^{32, 33}; TNF α is a macrophage/monocyte-derived pluripotent mediator. Whether TNF α plays a part in angiogenesis may be highly dependent on its concentration.³⁴ TNF α has been shown to be a powerful activator of angiogenesis in vivo in several animal models when used at appropriate doses.^{35–37} Previous studies show that high glucose or advanced glycation end products induce the expression of proinflammatory cytokines, including TNF α from monocytes and macrophages,^{38, 39} and TNF α may have an important role in mediating angiogenesis in diabetic retinopathy.^{18–20} The angiogenic effect of TNF α may be due to the generation of secondary mediators.^{40, 41}

In conclusion, increased expression of VEGF-C protein in the retinal vasculature of diabetic retinopathy suggests that VEGF-C may have an important role in its pathogenesis.

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