EXTENDED REPORT

VEGF-A regulates the expression of VEGF-C in human retinal pigment epithelial cells

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Aim: To determine the expression and regulation of vascular endothelial growth factor C (VEGF-C), and its receptor VEGFR-3, in human retinal pigment epithelial (RPE) cells and to consider their angiogenic role in choroidal neovascularisation (CNV).

Method: The expression of VEGF-C and VEGFR-3 in cultured human RPE was confirmed by immunostaining, PCR, western blotting, and ELISA. Cultured RPE cells were exposed to VEGF-A and glucose and VEGF-C and VEGFR-3 changes in gene expression determined by RT-PCR. Secreted VEGF-C protein in conditioned media from RPE was examined by western blotting and ELISA analysis. The ability of VEGF-C to elicit tube formation in choroidal endothelial cells was assayed by an in vitro Matrigel model.

Result: VEGF-A and glucose upregulated VEGF-C mRNA expression and increased the secretion of VEGF-C protein into the culture medium. VEGF-A, but not glucose alone, stimulated VEGFR-3 mRNA expression. VEGF-C acted synergistically with VEGF-A to promote in vitro tube formation by choroidal endothelial cells.

Conclusion: VEGF-A has a critical role in the orchestration of VEGF-C expression in RPE cells and the synergistic action of VEGF-C with VEGF-A may play an important part in the aetiology of CNV.

Choroidal neovascularisation (CNV) as seen in age related macular degeneration (AMD) is the most common cause of severe visual loss in patients over the age of 60 in developed countries.[1 2] Neovascularisation originates from choroidal blood vessels that grow through Bruch's membrane into the subretinal space and introduce haemorrhage, resulting in metamorphopsia and central or paracentral scotoma.

The retinal pigment epithelium (RPE) is a monolayer of highly specialised cells located between the neural retina and the choroidal vasculature that influences the structure and function of cells in both the retina and choroid. Increasing evidence suggests that the RPE cells have a key role in the pathogenesis of CNV.[3 4] In established CNV, multiple angiogenic growth factors, such as VEGF-A, have been detected in RPE cells.[5–7] Furthermore, RPE cells express receptors for VEGFR-1, VEGFR-2, and VEGFR-3.[8 9] RPE cells not only contribute to the formation of CNV but also have a role in proliferative diabetic retinopathy (PDR).[10 11] Hyperglycaemia is a feature of diabetic retinopathy and glucose has previously been shown to regulate the expression of VEGF family members in endothelial cells.[12 13]

VEGF-C has structural similarity with VEGF-A and can stimulate proliferation and migration of blood vascular endothelial cells,[14] as well as promoting endothelial cells to release nitric oxide and plasminogen activator. In mice and rabbit models, VEGF-C induces angiogenesis and increases vascular permeability.[15 16] VEGF-C is a ligand for both VEGFR-2 and VEGFR-3[14] and recently was identified in CNV derived RPE cells.[17] Interestingly, VEGFR-2 and VEGFR-3 immunostaining is observed on the side of the choriocapillaris endothelium that faces the RPE,[18] indicating that the growth factors produced by RPE cells may be the main source of CNV and that VEGF-C may be involved in this process.

Surprisingly, the regulation of VEGF-C production in RPE cells has not been investigated. However, several in vivo studies reveal that in the normal eye VEGF-A is produced constitutively by RPE cells.[19–22] Presumably, VEGF-A may need to cooperative with other growth factors, particularly other VEGF family members such as VEGF-C or PlGF, to generate pathological neovascularisation.

In this study, the expression of VEGF-C and VEGFR-3 in RPE cells was confirmed and we show that VEGF-C was regulated by both VEGF-A and high glucose (a predictor of ocular angiogenesis associated with diabetes). Furthermore, VEGF-C was able to promote in vitro angiogenesis and this was enhanced in the presence of VEGF-A.

MATERIALS AND METHODS

Reagents

Recombinant VEGF-A_{165} and VEGF-C enzyme linked immunosorbent assay (ELISA) kit (DVEC00) were purchased from R&D Systems (R&D systems Europe Ltd, Abingdon, UK). VEGF-C was from Calbiochem (Calbiochem, UK). The antibodies against VEGF-C (sc-25783), VEGFR-3 (sc-321), and α tubulin were from Santa Cruz (Santa Cruz, UK). TRIzol was purchased from Invitrogen (Invitrogen, Glasgow, UK), Polymerase chain reaction (PCR) Reddy Mix and Master Mix Kit were purchased from ABgene (ABgene, UK). Matrigel was from BD Biosciences (BD Biosciences, UK). All other materials were from Sigma unless otherwise stated.

Cell culture

RPE cells

Primary RPE cultures from human donor eyes were obtained from the Bristol Eye Bank and used in accordance with the tenets of the Declaration of Helsinki regarding the use of human tissue for research. RPE cells were isolated and cultured as described previously.[23] Briefly, the anterior segment was removed from whole eyes by dissection. The

Abbreviations: AMD, age related macular degeneration; CNV, choroidal neovascularisation; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPKs, mitogen activated protein kinases; PCR, polymerase chain reaction; PDR, proliferative diabetic retinopathy; PKC, protein kinase C; RPE, retinal pigment epithelium; RT-PCR, reverse transcription polymerase chain reaction; VEGF, vascular endothelial growth factor

neurosensory retina was gently peeled away from the underlying RPE. The eyecups were filled with 0.25% trypsin + 0.05% EDTA in phosphate buffered saline (PBS) for 30 minutes to remove RPE cells from Bruch's membrane. The cells were harvested and cultured in Ham's F-10 (containing 5.6 mM glucose) supplemented with 10% fetal calf serum (FCS). The purity of cells was confirmed by positive immunostaining for Pan cytokeratin.[24] The cell strains used in the present study were isolated from cadaveric eyes of three adult individuals (aged 58, 67, 90) without evidence of eye disease. The cells from different individuals were pooled before final culture to minimise the influence of interindividual variation. Cells employed for all the experiments were within five passages. The culture medium was replaced with basal medium containing 1% FCS overnight before experiments.

Choroidal endothelial cells

Choroidal endothelial cells were isolated from fresh bovine eyes obtained from the local abattoir. The eyes were dissected 5 mm posterior to the limbus, retinas were removed, remaining Bruch's membrane and choroid were then dissected from the sclera and inverted. The choroidal vessels were carefully peeled off from Bruch's membrane under a dissecting microscope. Choroidal vessels were rinsed thoroughly with PBS to remove pigment and minced with scissors. The minced tissue was homogenised and the homogenate was filtered through a 210 μm nylon sieve. The filtrate was then further filtered over a 53 μm sieve to retain the microvessels. The microvessels were then incubated with an enzyme cocktail (collagenase 0.5 mg/ml; Dnase 0.2 mg/ml, Pronase 02 mg/ml) in Minimal Essential Medium for 30 minutes to produce a cell suspension. Cells were pelleted, plated into 25 cm^2 culture flasks and maintained in endothelial cell basal medium with growth supplement (TCS Works Ltd, Bucks, UK). Bovine choroidal endothelial cells from the primary culture were further enriched using the different detachment time between endothelial cells and contaminant cells under the procedure of trypsinisation. Choroidal endothelial cells were characterised by their cobblestone appearance and expression of factor VIII antigen.[25] First passage choroidal endothelial cells were used for the tube formation assay.

Immunostaining

The method of immunostaining to identify VEGF-C or VEGFR-3 is as follows: RPE cells were seeded onto coverslips and allowed to attach. Cells were rinsed twice with PBS and fixed with 4% neutral buffered formalin for 20 minutes. After washing with PBS, the fixed cells were permeabilised by 0.1% Triton X-100 for 5 minutes. Non-specific binding sites were blocked by 10% milk/PBS for 30 minutes. Cells were incubated with either goat anti-human VEGF-C or rabbit anti-human VEGFR-3 primary antibody (1:100) in 3% milk/PBS for 2 hours, rinsed in PBS three times, and then incubated with the secondary antibody (1:150) conjugated with TRITC/FITC in 3% milk/PBS for 1 hour. Coverslips were rinsed in PBS three times and mounted on microscope slides using gelvatol containing Hoechst 33345 (2 μl/ml). All the above procedures were performed at room temperature. Images were captured under a Leica DMR microscope using the software of Leica Qfluoro with equal exposure time. Cells exhibiting fluorescence under the TRITC/FITC filter were considered to have stained positive for the antibodies. Substitution of the primary antibodies with an inappropriate goat/rabbit IgG at the same concentration as the primary antibodies acted as a negative control.

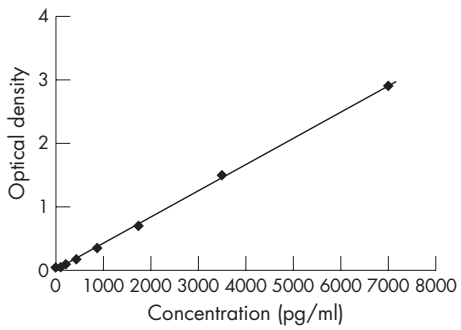

Figure 1 Standard curve for VEGF-C ELISA showing the sensitivity of the assay.

RT-PCR

Confluent human RPE cells were exposed to VEGF-A (10 or 100 ng/ml), glucose (15 mM or 25 mM), or VEGF-A (100 ng/ml) plus glucose (15 mM) for 24 hours. Medium alone containing 5.6 mM glucose served as baseline control. Total RNA was isolated using TRIzol, and then analysed by reverse transcription polymerase chain reaction (RT-PCR) using the First Strand Synthesis Kit and PCR ReddyMix according to the manufacturer's protocol. The RNA concentrations were determined spectrophotometrically and equal quantities of total RNA were used from different samples. The primers for human VEGF-C were designed according to the sequences of VEGF-C mRNA from the GenBank. The oligonucleotide primers used for the amplification of human VEGF-C cDNA were 5′-GTC CGG TTT CCT GTG AGG CTT T-3′ (sense) and 5′-CTG CTC CTC CAG ATC TTT GCT T-3′ (antisense). The resultant PCR product was 426 bp. The primers for human VEGFR-3 were from Yonemura *et al*.[26] The sequences were 5′-AGC CAT TCA TCA ACA AGC CT-3′ (sense) and 5′-GGC AAC AGC TGG ATG TCA TA-3′ (antisense) and the target fragment is 298 bp. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. The sequence were 5′-GGT GAA GGT CGG AGT CAA CG-3′ (sense) and 5′-CAA AGT TGT CAT GGA TGA CC-3′ (antisense) and gave a 500 bp amplimer. The cDNA was amplified using the PCR Master Mix, each cycle

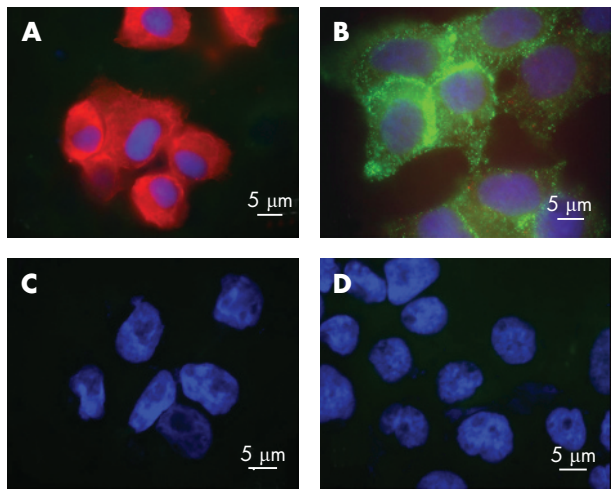

Figure 2 Immunostaining for VEGF-C and VEGFR-3 in human RPE cells. RPE cells demonstrated positive staining for both VEGF-C (A) and VEGFR-3 (B). VEGF-C and VEGFR-3 stained red and green respectively while Hoescht stained nuclei appear blue. (C) and (D) are representative negative controls. The images are representative of at least three independent experiments.

consisting of 20 seconds at 94℃, 30 seconds at 55℃ for amplifier VEGF-C cDNA at 51℃ for VEGFR-3 and GAPDH cDNA, and 60 seconds at 72℃. 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 onto a 1.2% agarose gel containing ethidium bromide and visualised under ultraviolet irradiation.

Immunoprecipitation and western blotting

As VEGF-C mRNA expression was greatest at VEGF-A 100 ng/ml, glucose 15 mM and VEGF-A 100 ng/ml plus glucose 15 mM, the cells were incubated under these conditions in Ham F-10 basal culture medium for 24 hours. The conditioned media from the cell cultures were collected and centrifuged at 200 *g* for 5 minutes to remove cell debris. The protein concentrations were determined by the BCA protein assay (Pierce, UK) and equal quantities of total proteins were subjected to immunoprecipitation. Target protein was immunoprecipitated by incubation with an anti-VEGF-C antibody and then protein A/G-agarose and the resultant immunoprecipitates were subjected to SDS-PAGE. The proteins were then transferred onto nitrocellulose membranes. The membranes were probed with an anti-VEGF-C antibody followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The ECL system (Santa Cruz, UK) was used to visualise the bands and resultant bands were normalised against α tubulin.

ELISA

The RPE cells were incubated under different concentrations of VEGF-A (1, 10, 50, or 100 ng/ml) in Ham F-10 culture medium for 24 hours. The conditioned media from the cell cultures were collected and centrifuged at 200 *g* for 5 minutes to remove cell debris. VEGF-C was determined by an ELISA kit. The assay was performed according to the manufacturer's instructions. Briefly, standards or samples (50 µl) were pipetted into an antibody coated 96 well plate containing 100 µl of assay diluent and incubated for 2 hours at room temperature on a shaker. The wells were then washed four times with wash buffer, 200 µl of VEGF-C conjugate was added, and the samples were again incubated for 2 hours at room temperature. Samples were washed four times, 200 µl substrate buffer was added, the samples were incubated for 30 minutes at room temperature, the reaction was stopped, and the absorption was measured with an ELISA reader at 450 nm wavelength, and 570 nm as a correction. All measurements were performed in triplicate. The data were recorded and the average background absorbances obtained from blank wells were subtracted. The minimum sensitivity of ELISA was 13.3 pg/ml. The sample concentration was calculated from the standard curve and corrected for total protein (fig 1).

Tube formation by choroidal vascular endothelial cells

Tube formation by endothelial cells was assessed using the Matrigel model as previously described.[27] Basement Matrix was diluted in ice cold Dulbecco's modified Eagle's medium at a ratio of 1:3. Choroidal endothelial cells (2×10^4 cells/well) were seeded onto Matrigel coated wells of a 96 well plate in MCDB131 (containing 5.6 mM glucose) supplemented with 10% FCS. After cells had attached to the Matrigel (overnight at 37℃), the medium was removed and cells were exposed to VEGF-A (50 ng/ml), VEGF-C (50 ng/ml), VEGF-A (50 ng/ml)/VEGF-C (50 ng/ml), VEGF-A (50 ng/ml)/glucose (15 mM), VEGF-C (50 ng/ml)/glucose (15 mM), or the vehicle alone in medium containing 1% FCS. For the observation of low dose VEGF-C in capillary-like tube formation, choroidal endothelial cells were stimulated with 3 ng/ml of VEGF-C. Capillary-like tube structures formed by choroidal endothelial cells were photographed digitally in four random microscope fields in each well at different time points. Tubular length was quantified using the software of Spot Advance and given in mm/mm^2.

Statistical analysis

All the experiments were repeated at least three times. Statistical analysis was carried out using an unpaired Student's *t* test. Statistical significance was defined as $p<0.05$.

RESULTS

Expression of VEGF-C and VEGFR-3 in human RPE cells

Cultured human RPE cells stained positive for both VEGF-C (fig 2A) and VEGFR-3 (fig 2B). Staining for VEGF-C tended to be localised within the perinuclear region while VEGFR-3 was diffusely distributed across the cell. The negative control demonstrated no immunostaining for either VEGF-C or VEGFR-3 (fig 2C, D). PCR confirmed the expression of VEGF-C and VEGFR-3 in human RPE cells maintained under baseline conditions (fig 3).

The effect of VEGF-A and glucose on VEGF-C and VEGFR-3 mRNA expression in human RPE cells

Both VEGF-A (10 ng/ml and 100 ng/ml) and high glucose (15 mM and 25 mM) significantly increased VEGF-C mRNA expression compared to control (5.6 mM glucose) (fig 3A). The expression of VEGF-C mRNA was substantially increased in cells exposed to 100 ng/ml VEGF-A with 10 ng/ml VEGF-A being lower but significantly greater than control. VEGF-C expression was similar in cells exposed to either 15 mm or 25 mm glucose. Moreover, 100 ng/ml VEGF-A and 15 mM glucose in combination further stimulated VEGF-C mRNA expression. To decide whether the increase of VEGF-C mRNA expression induced by high glucose was due to the change of osmolarity, the RPE cells were exposed to 15 mM mannitol in control medium (containing 5.6 mM glucose) for 24 hours. The result demonstrated that mannitol did not increase the VEGF-C mRNA expression compared to control and this expression was significantly lower than that of glucose 15 mM (fig 3B). The expression of VEGFR-3 mRNA was upregulated by VEGF-A but not high glucose (fig 3C). The expression of VEGFR-3 mRNA was greater at 10 ng/ml VEGF-A when compared to 100 ng/ml VEGF-A (fig 2C).

The effect of VEGF-A and glucose on VEGF-C protein expression in human RPE cells

To determine whether the increase of VEGF-C mRNA was accompanied by an increase in VEGF-C protein by human RPE cells, the conditioned medium was collected after exposure to VEGF-A 100 ng/ml, glucose 15 mM, or VEGF-A 100 ng/ml plus glucose 15 mM for 24 hours. VEGF-C protein expression profiles were similar to those for VEGF-C mRNA expression. The results showed that the secreted peptide of VEGF-C was significantly elevated following exposure to VEGF-A and high glucose conditions when compared to that of control cultures and VEGF-A 100 ng/ml plus glucose 15 mM further stimulated this secretion (fig 4A). To decide if there is an increasing expression of VEGF-C protein under high osmotic condition, RPE cells were exposed to 15 µM mannitol in control medium (containing 5.6 mM glucose) for 24 hours. The result demonstrated that 15 µM mannitol did not increased the expression of VEGF-C in culture medium compared to controls and this expression was also significantly lower than that of 15 µM glucose (fig 4B).

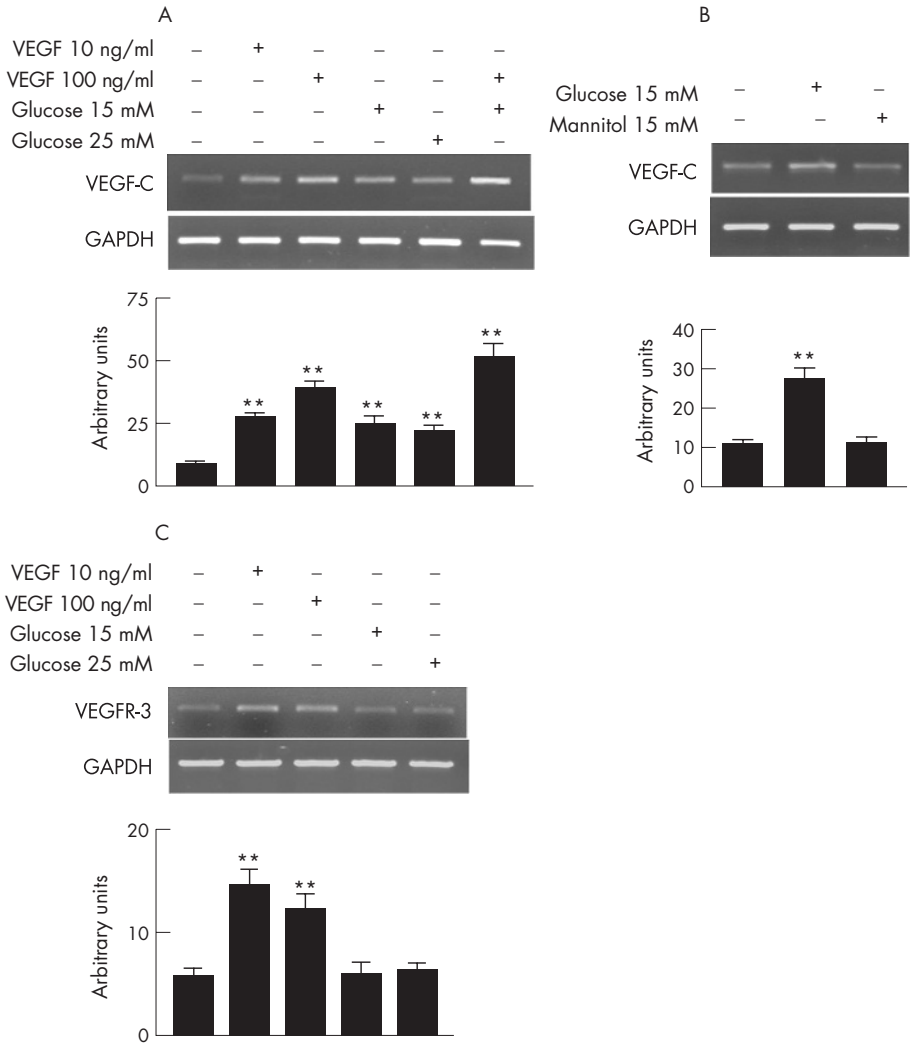

Figure 3 The expression of VEGF-C and VEGFR-3 mRNA in human RPE cells exposed to VEGF-A or/and high glucose for 24 hours. Total RNA was extracted and RT-PCR performed to determine changes in VEGF-C and VEGFR-3 mRNA expression. PCR products were analysed by agarose gel electrophoresis. The signal intensity was determined by densitometry, and the amount of VEGF-C and VEGFR-3 mRNA was normalised for the amount of GAPDH present. Representative results from at least there separate experiments are shown. (A) The expression of VEGF-C mRNA by cells exposed to VEGF-A, high glucose, VEGF-A plus glucose, or medium alone (5.6 mM glucose). (B) The expression of VEGF-C mRNA after the cells were exposed to high glucose or 15 mM mannitol. (C) The expression of VEGFR-3 mRNA after addition of VEGF-A or high glucose. Vertical bars represent SEM; p values (**$p<0.01$) indicate the significant difference between the different treatments and untreated control.

Quantification of the expression of VEGF-C stimulated by VEGF-A in human RPE cells

To further quantify the expression of VEGF-C in culture supernatant of RPE cells after exposure to different concentrations of VEGF-A, ELISA was performed. Consistent with the results of western blotting, the expression of VEGF-C increased after stimulation with VEGF-A. The upregulation of VEGF-C induced by VEGF-A occurred in a dose dependent manner and the expression of VEGF-C was greatest (3057 (SEM 112) pg/ml) at 100 ng/ml VEGF-A stimulation (fig 5).

VEGF-C acts synergistically with VEGF-A in a choroidal endothelial cell angiogenesis model

The Matrigel assay was used to determine the action of VEGF-C, VEGF-A, VEGF-C + VEGF-A, VEGF-A + glucose, and VEGF-C + glucose on capillary-like tube formation in vitro. The results showed that at a concentration of 50 ng/ml, VEGF-C had a stimulatory effect on tube formation compared to control although this effect was relatively weak compared to that of VEGF-A. However, VEGF-A and VEGF-C in combination had a synergistic effect. At 72 hours, the tube length (mm/mm^2) under different conditions were: control: 0.61 (0.03); VEGF-A: 2.20 (0.38); VEGF-C: 1.71 (0.21); VEGF-A + VEGF-C: 4.93 (0.53); VEGF-A + glucose: 2.23 (0.36); VEGF-C + glucose: 1.69 (0.24). Tubular formation stimulated by VEGF-A + VEGF-C was 124% higher compared to that of VEGF-A alone and greater than the summative effects of VEGF-A and VEGF-C alone. Neither VEGF-A + glucose nor VEGF-C + glucose had further stimulatory effect on tube formation compared to that of VEGF-A or VEGF-C alone (fig 6A, B). In addition, to confirm the physiological relevance of VEGF-C levels released in the supernatant of RPE cells following the stimulation with VEGF-A, 3 ng/ml of VEGF-C was added into Matrigel. The result demonstrated that this dose of VEGF-C also had a significant effect on tube formation at different time points (fig 6C).

DISCUSSION

The RPE cells have a paradoxical relation with the choriocapillaris. In normal healthy conditions, the RPE appears to have a positive survival effect on the maintenance of the highly vascularised, highly permeable fenestrated choriocapillaris.

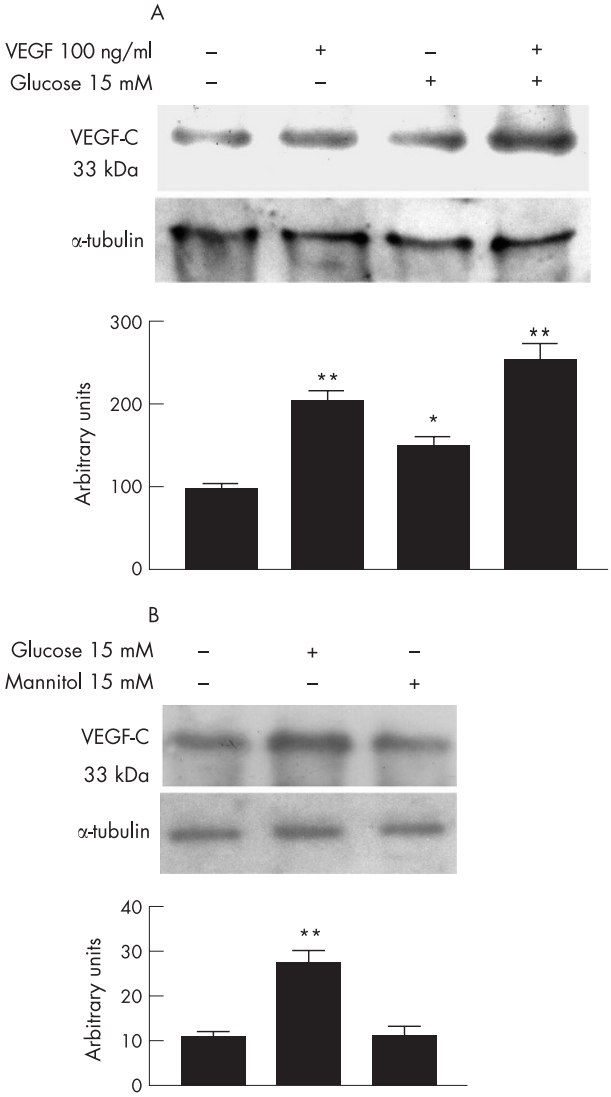

Figure 4 Western blotting analysis of de novo VEGF-C protein synthesis. Human RPE cells were treated with VEGF 100 ng/ml, glucose 15 mM, VEGF-A 100 ng/ml plus glucose 15 mM, or 15 mM mannitol for 24 hours. Conditioned media were collected and immunoprecipitated using a polyclonal antibody raised against VEGF-C. The immunoprecipitates were electrophoresed on a 12% SDS-PAGE followed by blotting on a nitrocellulose membrane. Positive bands were visualised by an ECL detection system. The band intensity was quantified by laser densitometry and the amount of VEGF-C was normalised against the amount of α tubulin present. (A) Cells were exposed to VEGF 100 ng/ml, glucose 15 mM, or VEGF-A 100 ng/ml plus glucose 15 mM. (B) Cells were exposed to glucose 15 mM or mannitol 15 mM. The vertical bars represent SEM; p value (*p<00.5, **p<0.01) indicates the significant different between the different treatments and untreated control.

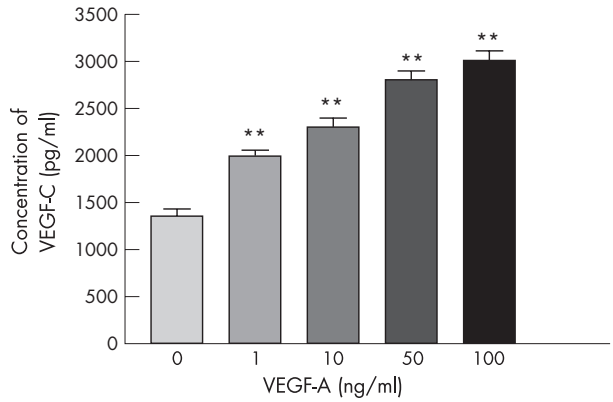

Figure 5 Quantification of the expression of VEGF-C after stimulation with VEGF-A in human RPE cells. Human retinal pigment epithelial cells were exposed to serial concentrations of VEGF-A for 24 hours. The conditioned media were collected and ELISA was performed. The VEGF-C concentrations were calculated from the standard curve and normalised to total protein. The vertical bars represent SEM; p values (**p<0.01) indicate the significant difference between VEGF-A stimulation and untreated control.

Clinical observations in AMD and experimental animal studies show that absence or dysfunction of the RPE can cause secondary atrophy of the choriocapillaris.[22 28 29] However, RPE cells have been observed to arrest CNV growth by encapsulation.[30] It appears that whether the RPE cells have a role in angiogenesis depends on the temporal expression of growth factors and cytokines in specific microenvironments.

VEGF-A, a primary ligand for the endothelial tyrosine kinase receptors VEGFR-1 and VEGFR-2, has been strongly implicated in neovascularisation within the eye. However, transgenic mice with inducible expression of either VEGF-A alone or VEGF-A and Ang-2 in RPE did not develop CNV,[31] indicating that VEGF-A may need to cooperate with other VEGF family members in the formation of CNV. The presence of VEGFR-3 in the vessel segments of the choriocapillaris[18] that contain VEGFR-1 and VEGFR-2 suggests that cooperation may occur between different types of VEGF molecules. In addition, PlGF mRNA expression is present in the intact choroid and significantly upregulated during the course of experimental CNV.[32]

VEGF-C was originally thought to be a potent inducer of lymphangiogenesis.[33 34] Later evidence shows that it is also important in vascular angiogenesis.[35 36] VEGF-C induces angiogenesis in some animal models,[15 16] which is the result of its capacity to bind to and activate VEGFR-2, in addition to VEGFR-3.[14] The expression of VEGFR-3 in blood vessels of tumours appears to be important for blood vessel integrity.[37 38] In the present study, we demonstrate the expression of VEGF-C and VEGFR-3 in normal primary RPE culture. Furthermore, high concentrations of VEGF-A, glucose, or VEGF-A + glucose have an upregulatory effect on VEGF-C expression while only VEGF-A stimulates the expression of VEGFR-3 mRNA. While we cannot definitively conclude whether the increased levels of VEGF-C in the conditioned medium are due to an upregulation in production or simply secretion, the mRNA data support production.

The mechanism by which VEGF-A stimulates the expression of VEGF-C is not clear. However, mitogen activated protein kinases (MAPKs) are ubiquitous enzymes involved in signal transduction. Their activation is essential in numerous cellular functions and it is possible that these enzymes are also involved in the expression of VEGF-C induced by VEGF-A just like TGF-β induces expression of VEGF-A in RPE cells.[39] Previous observations show that RPE cells exposed to 16.5 mM glucose for 1-3 days do not increase their VEGF-A production although exposure for 10 days significantly elevates the expression of VEGF-A.[40] It can therefore be concluded that in our experimental system the upregulatory effect of glucose on the production of VEGF-C is independent of VEGF-A. This hypothesis is reconciled with our data for detection of VEGFR-3 in RPE cells. The upregulaton of VEGF-A by glucose is independent of osmolarity through protein kinase C (PKC) activation.[41] To decide whether the increase of VEGF-C induced by high glucose is correlated with the change of osmolarity, 15 mM mannitol was administered and the result showed that the addition of mannitol did not change the expression of VEGF-C at either gene or protein

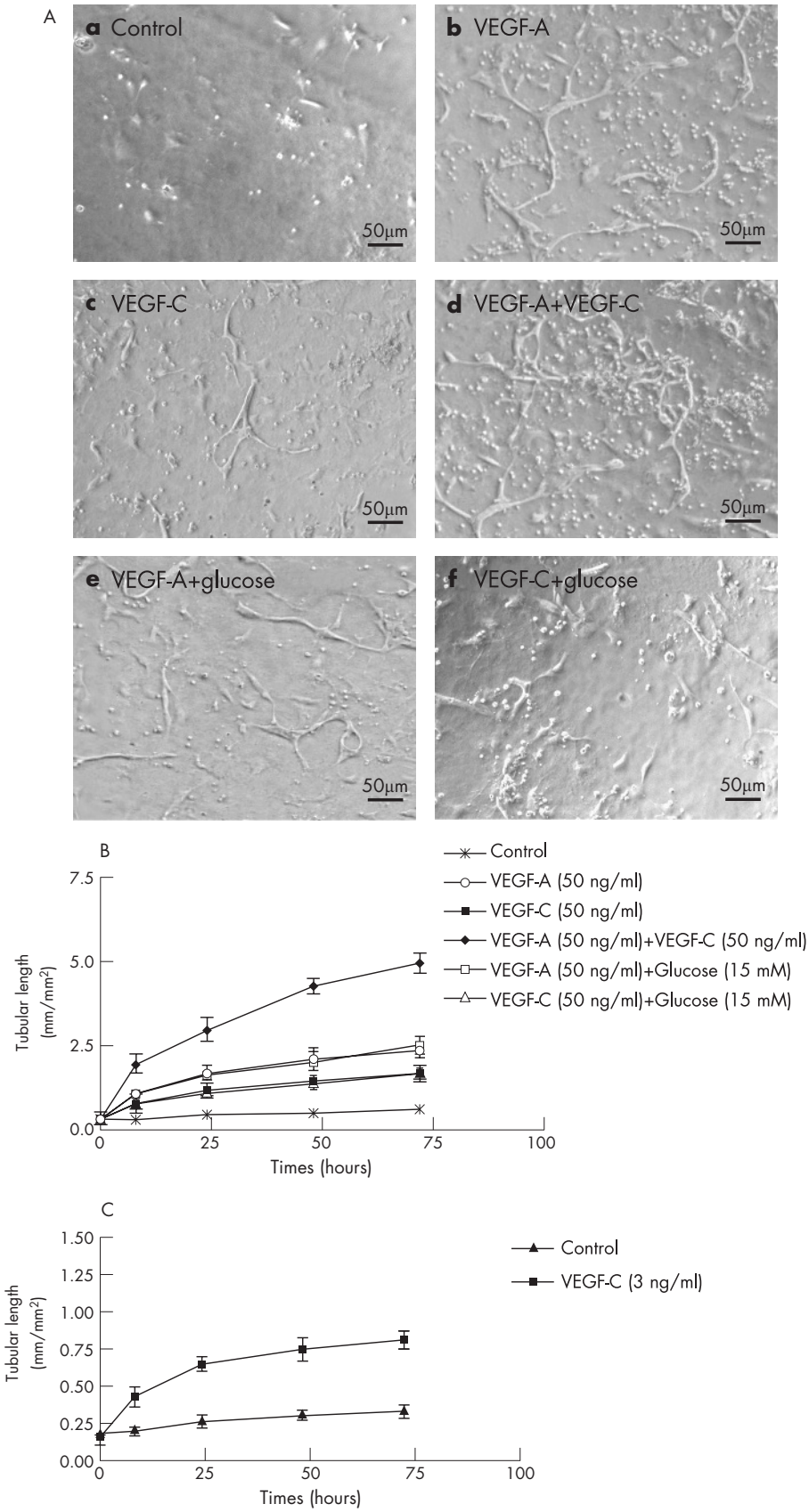

Figure 6 Capillary-like tube formation by choroidal endothelial cells. Choroidal endothelial cells were cultured on Matrigel and exposed to different treatments. (A) Photographs demonstrating tube formation are representative of results after 48 hours stimulation. (a) Control; (b) VEGF-A 50 ng/ml; (c) VEGF-C 50 ng/ml; (d) VEGF-A 50 ng/ml + VEGF-C 50 ng/ml; (e) VEGF-A 50 ng/ml + glucose 15 mM; (f) VEGF-C 50 ng/ml + glucose 15 mM. (B) The graph depicts quantification of tubular length (mm/mm^2) at different time points and different conditions. (C) The graph depicts a low dose VEGF-C induced capillary-like tube formation by choroidal endothelial cells at different time points. Vertical bars represent SEM from at least three experiments.

levels, indicating that the increase of VEGF-C expression under high glucose conditions is independent of high osmolarity. The result of VEGF-A upregulating VEGFR-3 expression is consistent with in vivo observations that the expression of VEGFR-3 is also upregulated in the retinal microvasculature of monkey eyes injected with VEGF-A.[42]

The high expression of VEGF-C following exposure of RPE cells to VEGF-A and glucose may also have a role in diabetic retinopathy. RPE is known to form an outer blood-retinal barrier, and its breakdown is the earliest pathological change in diabetic retinopathy.[43] In the diabetic rat model, this breakdown is due to alteration of membrane permeability rather than to a loss of tight junctions.[44] The overexpression of VEGF-C may be involved in this process through an autocrine pathway, as VEGF-C is known to markedly increase vascular permeability.[16] The autocrine role of VEGF-A in renal glomerular epithelial cells and breast carcinoma cells has been demonstrated in previous studies.[45 46] Furthermore, vitreous haemorrhage in proliferative diabetic retinopathy attracts numerous cell types including the RPE to form fibrovascular membranes, in which RPE cells produce a variety of growth factors including VEGF-A.

The process of neovascularisation can be divided into several stages including capillary basement membrane breakdown, endothelial cell migration, tube formation, anastomosis, and vessel remodelling,[47 48] in which tube formation is an important step in angiogenesis. Our results showed that choroidal endothelial cells elicited a capillary tube response to exogenous VEGF-C. Furthermore, VEGF-C and VEGF-A had a synergistic effect on tube formation. Moreover, we also demonstrate that VEGF-C promotes tube formation at a concentration equivalent to that found in the supernatant of RPE cells stimulated with VEGF-A. Our study clearly demonstrates that VEGF-C is not only a lymphangiogenic factor but also an angiogenic factor. The finding that hyperglycaemia did not exert a synergistic or additive effect with VEGF-A or VEGF-C on choroidal endothelial cell tube formation suggests that hyperglycaemia may act differently in different vascular beds.

Apart from tube formation function, VEGF-C may also act to sustain CNV. VEGF-C supports uterine microvascular endothelial cell growth and removal of VEGF-C from culture medium caused a marked reduction in cell number due to massive apoptosis.[49] Human choroidal neovascular membranes show strong expression of Fas and Fas ligand,[50] and a functional study revealed that Fas ligand on RPE cells controls experimental CNV by Fas mediated killing of choroidal endothelial cells.[51] Thus, apoptotic endothelial cell death is one important mechanism by which to control CNV. The sustained expression and overexpression of VEGF-C by RPE cells may promote stabilisation of CNV by protecting choroidal endothelial cells against apoptosis in cooperation with VEGF-A.

In conclusion, VEGF-C expression is upregulated by high concentrations of VEGF-A in RPE cells and it acts synergistically with VEGF-A in choroidal endothelial cell tube formation. VEGF-C may have an important role in pathological choroidal neovascularisation associated with AMD.

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