EXTENDED REPORT

Antiproliferative and cytotoxic properties of bevacizumab on different ocular cells

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Aim: To evaluate the antiproliferative and cytotoxic properties of bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF), on human retinal pigment epithelium (ARPE19) cells, rat retinal ganglion cells (RGC5), and pig choroidal endothelial cells (CEC).

Methods: Monolayer cultures of ARPE19, RGC5, and CEC were used. Bevacizumab (0.008–2.5 mg/ml), diluted in culture medium, was added to cells that were growing on cell culture dishes. Cellular proliferative activity was monitored by 5′-bromo-2′-deoxyuridine (BrdU) incorporation into cellular DNA and the morphology assessed microscopically. For cytotoxicity assays ARPE19, RGC5, and CEC cells were grown to confluence and then cultured in a serum depleted medium to ensure a static milieu. The MTT test was performed after 1 day. The ''Live/Dead'' viability/cytotoxicity assay was performed and analysed by fluorescence microscopy after 6, 12, 18, 24, 30, 36, and 48 hours of incubation.

Expression of VEGF, VEGF receptors (VEGFR1 and VEGFR2) and von Willebrand factor was analysed by immunohistochemistry.

Results: No cytotoxicity of bevacizumab on RGC5, CEC, and ARPE19 cells could be observed after 1 day. However, after 2 days at a bevacizumab concentration of 2.5 mg/ml a moderate decrease in ARPE19 cell numbers and cell viability was observed. Bevacizumab caused a dose dependent suppression of DNA synthesis in CEC as a result of a moderate antiproliferative activity (maximum reduction 36.8%). No relevant antiproliferative effect of bevacizumab on RGC5 and ARPE19 cells could be observed when used at a concentration of 0.8 mg/ml or lower. CEC and ARPE 19 cells stained positively for VEGF, VEGFR1, and VEGFR2. More than 95% of the CEC were positive for von Willebrand factor.

Conclusions: These experimental findings support the safety of intravitreal bevacizumab when used at the currently applied concentration of about 0.25 mg/ml. Bevacizumab exerts a moderate growth inhibition on CEC when used in concentrations of at least 0.025 mg/ml. However, at higher doses (2.5 mg/ml) bevacizumab may be harmful to the retinal pigment epithelium.

Vascular endothelial growth factor (VEGF) has been identified as a major angiogenic stimulus in age related macular degeneration (AMD) and diabetic retinopathy.[1 2]

Inhibition of VEGF by either intravitreal ranibizumab (Lucentis, Genentech, San Francisco, CA, USA) or pegaptanib (Macugen, Eyetech Pharmaceutical, New York, NY, USA) resulted in a stabilisation or improvement of vision in some patients with neovascular AMD. Pegaptanib has already been approved for treating exudative AMD and ranibizumab may gain approval soon.

Recently, there have been promising case reports and case series using off-label intravitreal bevacizumab (Avastin, Genentech, San Francisco, CA, USA) in neovascular AMD,[3 4] proliferative diabetic retinopathy,[5] iris neovascularisation,[6 7] macular oedema from central retinal vein occlusion,[8 9] or refractory pseudophakic cystoid macular oedema.[10] Furthermore, a study using off-label intravenous bevacizumab showed improvement of visual acuity in neovascular AMD in some of the treated patients.[11] The same systemic approach has been reported to be beneficial in choroidal neovascularisation (CNV) caused by pathological myopia.[12] Bevacizumab has been approved for the treatment of metastatic colorectal cancer. It has been shown to increase the survival time when it is added to chemotherapy with 5-fluorouracil.[13 14] The intravitreal use of bevacizumab is controversial as this medication is not approved for intraocular use and a controversy exists about whether the antibody may or may not reach the deeper retinal layers where CNV occurs.[15 16] Preliminary electrophysiological studies in humans and rabbits suggest that the intravitreal application of bevacizumab may be safe and does not cause damage to ocular tissues.[16–18]

However, up to now no experimental data or studies about the safety and effect of bevacizumab on ocular cells and tissue have been reported.

Furthermore, it is unknown whether bevacizumab also exhibits an antiproliferative effect on aberrant proliferating ocular cells of non-endothelial origin, which may make bevacizumab a valuable adjunct that goes beyond its use in neovascular eye diseases.

This study evaluates the antiproliferative and cytotoxic properties of bevacizumab in a wide range of concentrations on human retinal pigment epithelium cells (ARPE19), rat retinal ganglion cells (RGC5), and pig choroidal endothelial cells (CEC).

Abbreviations: AMD, age related macular degeneration; BrdU, 5′-bromo-2′-deoxyuridine; CEC, choroidal endothelial cells; CNV, choroidal neovascularisation; HUVEC, human umbilical vein endothelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide; RGC, retinal ganglion cells; RPE, retinal pigment epithelium; VEGF, vascular endothelial growth factor

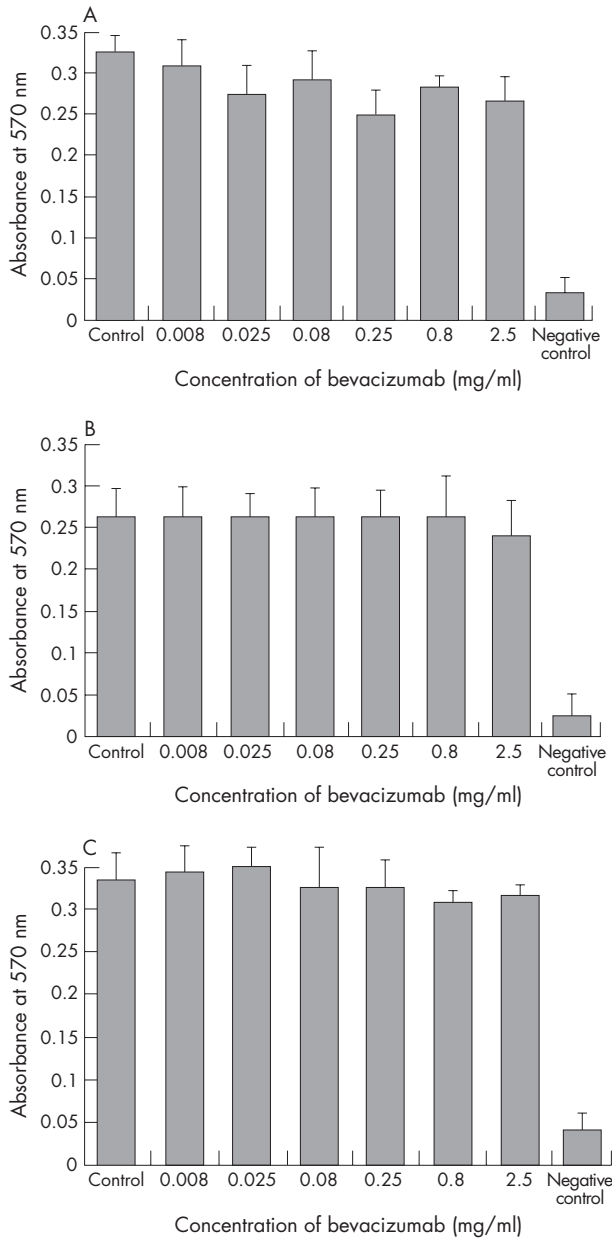

Figure 1 Toxicity determination in a stationary, confluent cell culture using MTT labelling. No significant cytotoxicity of bevacizumab could be seen in choroidal endothelial cells (A), ARPE19 (B), and RGC5 (C). However, a statistically non-significant trend ($p<0.05$) towards less cell viability was seen in ARPE19 cells when a bevacizumab concentration of 2.5 mg/ml was used. At higher bevacizumab concentrations CEC cell viability was slightly reduced (non-significant).

METHODS

Cell culture

The ARPE19 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The rat ganglion cell line (RGC5) was kindly provided by Professor Neeraj Agarwal (UNT Health Science Center, Fort Worth, TX, USA). Pig CEC were isolated as described before[19] and used in the second to third passage.

ARPE19 and RGC5 cells were maintained in Dulbecco's modified Eagle's medium containing 3 mM L-glutamine, 1% glucose, 10% fetal bovine serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulphate. The CEC were maintained in EGM MV-Microvascular Endothelial Cell Medium (Cambrex Clonetics, Wokingham, UK).

Cell suspensions (5×10^3 cells/ml) were seeded onto 96 well tissue culture plates. For proliferation assays using bevacizumab Invitrogen-Gibco Advanced MEM medium containing 1% fetal bovine serum, 1% glucose, VEGF at 2 ng/ml, 50 U/ml penicillin G, and 50 U/ml streptomycin was used. Stock solutions of bevacizumab were serially diluted with culture medium to obtain 0.008 mg/ml, 0.025 mg/ml, 0.08 mg/ml, 0.25 mg/ml, 0.8 mg/ml, and 2.5 mg/ml, respectively.

MTT stationary toxicity assay

To assess the cytotoxicity of bevacizumab on CEC, RGC5, and ARPE19 cells the amount of cell proliferation was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay. Bevacizumab in serum free medium was added to the CEC, RGC5, and ARPE19 cells. After 24 hours the cells were washed with phosphate buffered saline (PBS), and either fresh serum free medium without bevacizumab or MTT at 0.5 mg/ml in serum free medium were added to the cells. After 2 hours of incubation formazan extraction was performed and the quantity was measured colorimetrically with an ELISA reader (SLT Spectra 400 ATX, Salzburg, Austria) at 570 nm.

''Live/Dead'' viability/cytotoxicity kit

To assess the cytotoxicity of bevacizumab on the three cell lines under none starving conditions, cell viability was assessed using the ''Live/Dead'' Viability/Cytotoxicity Kit No 1 (L-7013) (Molecular Probes, Eugene, OR, USA). Bevacizumab or the vehicle alone in the concentrations specified above and medium containing 5% fetal calf serum were added to the cells. Staining was performed according to the manufacturer's instructions. Adequate negative (cells without bevacizumab) and positive controls for cell death (cells treated with the detergent Triton X-100 (Serva, Heidelberg, Germany) 0.3% in PBS) were run with each set of experiments. Cell viability was analysed by fluorescence microscopy at 6, 12, 18, 24, 30, 36, and 48 hours of incubation.

BrdU ELISA

Cellular proliferative activity under none starving conditions was directly monitored by quantification of 5'-bromo-2'-deoxyuridine (BrdU) incorporation into the genomic DNA during cell growth. DNA synthesis was assessed by a colorimetric cell proliferation ELISA assay (Calbiochem, LaJolla, CA, USA) according to the manufacturer's instructions. Absorbance was analysed at dual wavelengths of 450–540 nm.

Immunohistochemistry

Immunohistochemistry for VEGF was performed on all three cell lines. CEC and ARPE19 cells were stained for VEGF receptor 1 (Flt-1) and 2 (Flk-1) and von Willebrand factor staining was performed on the CEC cells.

The following primary antibodies were used:

(1) monoclonal mouse antibody to VEGF (VEGF C-1: sc-7269, Santa Cruz Biotechnology, Santa Cruz, CA, USA)
(2) polyclonal goat antibody to VEGF-Receptor 1 (Flt-1 C-17: sc-316, Santa Cruz Biotechnology, Santa Cruz, CA, USA)
(3) monoclonal mouse antibody to VEGF-Receptor 2 (Flk-1 A-3: sc-6251, Santa Cruz Biotechnology, Santa Cruz, CA, USA)
(4) monoclonal mouse antibody to von Willebrand factor (M 0616; F8/86, Dako, Glostrup, Denmark)

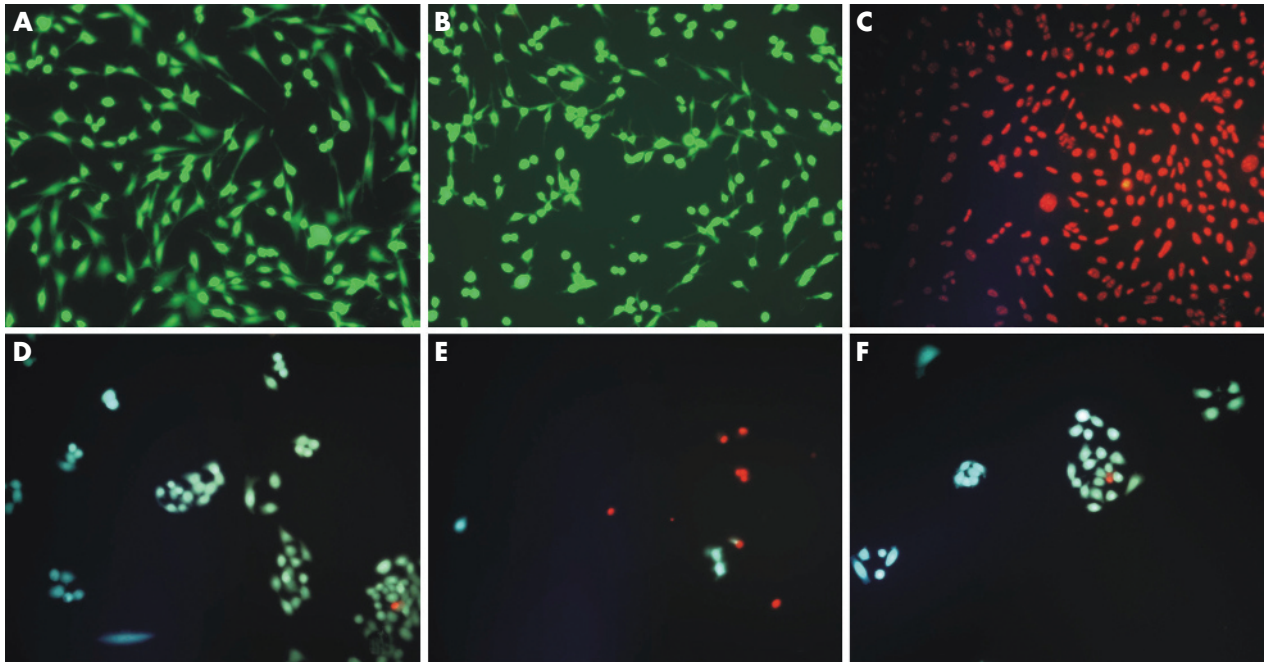

Figure 2 Toxicity determination using the Life/Dead viability/cytotoxicity assay. (A) RGC5 cells and bevacizumab 2.5 mg/ml after 48 hours of incubation. (B) RGC5 cells: negative control. (C) RGC5 cells cytotoxic control: treatment with Triton X-100. (D) ARPE19 cells and bevacizumab 0.25 mg/ml. (E) ARPE19 cells and bevacizumab 2.5 mg/ml: showing a decreased number of viable ARPE19 cells after 2 days of incubation. (F) negative control (magnification ×200).

All antibodies were diluted in ChemMate antibody diluent (Dako, Glostrup, Denmark). After incubation with the primary antibody for 12 hours at 4°C the cells were rinsed with PBS and the secondary antibody was introduced using the ChemMate detection kit, alkaline phosphatase/RED, rabbit/mouse (Dako, Glostrup, Denmark) for immunocytochemistry. For the primary goat antibody a biotinylated rabbit anti-goat antibody was used.

Expression of results and statistics

Results were expressed as units of mean absorbance (SD) for MTT and BrdU assays. Ten individual samples per group were measured in triplicate. The relative difference between the control and drug treated groups was analysed with ANOVA using JMP statistical software (version 4.0, SAS Institute Inc, Cary, NC, USA). In all experiments, $p<0.05$ was considered to indicate a statistically significant difference and marked with an asterisk.

RESULTS

Cytotoxicity assays

A stationary, confluent cell culture is better suited to detect a toxic drug effect than a proliferating culture and is more comparable to the natural situation within the eye (fig 1). No cytotoxicity was detectable for bevacizumab in concentrations up to 2.5 mg/ml on ARPE19 cells and RGC5 cells. A slightly decreased cell viability of CEC compared to controls was seen in the MTT assay when bevacizumab was added to the cell culture. Using the Live/Dead kit in a proliferating cell culture no increased cell death rate could be observed when bevacizumab was added to CEC and RGC5 cells (fig 2). Moreover, no cytotoxicity on ARPE19 cells was observed in a proliferating cell culture in the presence of bevacizumab in a concentration of up to 0.8 mg/ml. However, at a concentration of 2.5 mg/ml, a moderate decrease of cell number and increase of dead ARPE19 cells was noted when cells were incubated for more than 24 hours (fig 2). The number of viable ARPE19 cells decreased by up to 30% compared to control after 48 hours. The majority of the dead cells were detached from the bottom of the cell culture well (fig 2). No cytotoxic effect of the vehicle could be observed.

Antiproliferative activity of bevacizumab

The BrdU assay is a sensitive tool to investigate whether a drug effect inhibits cellular proliferation as it monitors the cellular proliferative activity directly at the level of the DNA (fig 3). Quantitative analysis of BrdU positive nuclei showed that exposure of CEC to concentrations of bevacizumab of 0.08 mg/ml moderately reduced the amount of DNA synthesis. The proliferation rate of CEC was reduced to about 65% during exposure to 2.5 mg/ml bevacizumab. No relevant inhibition of cell proliferation of ARPE19 or RGC5 cells was seen when concentrations of bevacizumab of up to 0.8 mg/ml were used. Even when VEGF in concentrations from 10 ng/ml to 50 ng/ml was added to ARPE19 or RGC5 culture, no relevant antiproliferative effect of bevacizumab could be seen (data not shown). Furthermore, the antiproliferative effect of bevacizumab on CEC was not different when a longer incubation time was used (3 days; data not shown).

Immunohistochemistry results

CEC and ARPE19 stained positively for VEGF whereas the RGC5 cells only showed a slight mottled stain which was considered to be non-specific (fig 4).

A strong VEGFR1 expression in ARPE19 but only a weak one in CEC cells could be observed. However, VEGFR2, the main mediator for VEGF induced mitogenesis and vascular permeability was strongly expressed in the cytoplasm of ARPE19 cells and CEC (fig 5).

More than 95% of the CEC cells displayed immunoreactivity for the von Willebrand factor and thus were considered to be purely endothelial cells.

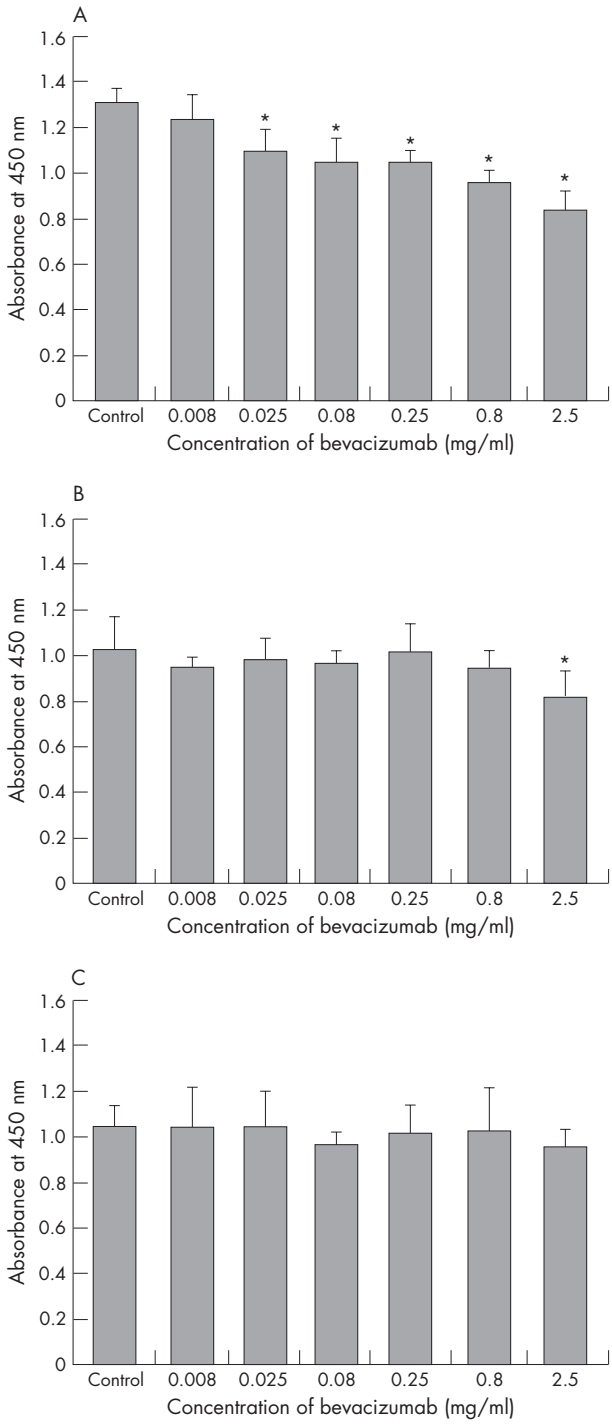

Figure 3 Proliferation assay using quantitative ELISA analysis of BrdU incorporation into choroidal endothelial (A), ARPE19 (B), and RGC5 (C) cells during exposure to various concentrations of bevacizumab. The anti-proliferative activity was determined after 1 day of treatment. The assay revealed a significant antiproliferative effect of bevacizumab on choroidal cells. Apart from that a decrease of BrdU incorporation into ARPE19 cells was noted in the presence of 2.5 mg/ml bevacizumab. The proliferation rate of RGC5 cells was not affected by bevacizumab. Asterisk indicates a statistically significant difference ($p<0.05$).

DISCUSSION

The aim of our study was to examine the effect of bevacizumab on certain ocular cells that may be likely to respond to treatment with this VEGF inhibitor.

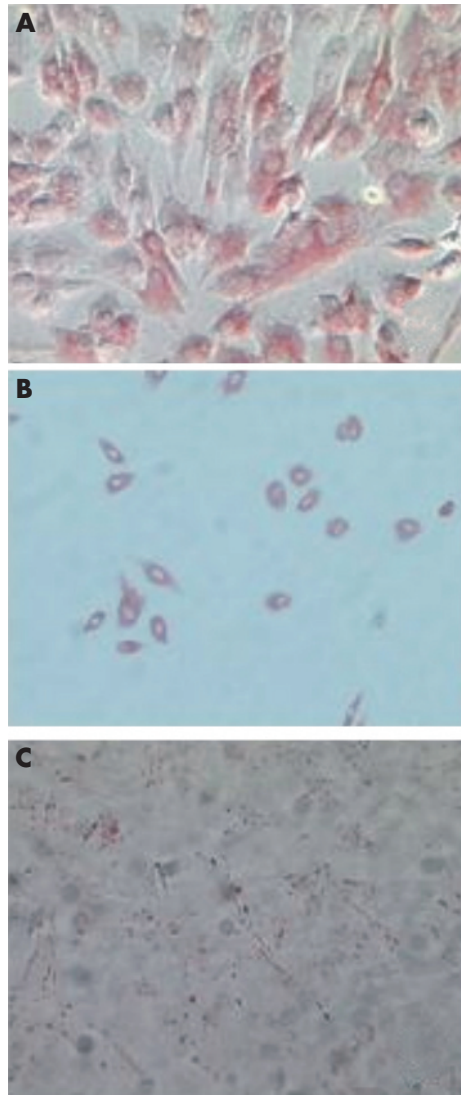

Figure 4 Immunohistochemistry for VEGF. A strong expression of VEGF in CEC (A) and ARPE19 (B) cells was observed. RGC5 (C) cells showed a weak mottled staining for VEGF.

Since bevacizumab penetrates the retina from the vitreal side, among the first and probably most vulnerable cells to be exposed to the drug are the retinal ganglion cells.

However, we did not see any adverse effect of bevacizumab on retinal ganglion cells even when the substance was used at a dose 10-fold of the concentration that is usually injected into the vitreous.

In addition, we examined the effect of bevacizumab on cultured retinal pigment epithelium (RPE) as several investigators have observed important interactions between the choriocapillaris, CNV, and the RPE.[20]

We did not see any toxic or antiproliferative effect on ARPE19 when bevacizumab was used in concentrations up to 0.8 mg/ml.

However, when bevacizumab at a concentration of 2.5 mg/ml was used, we observed an increased rate of cell death after 48 hours of incubation. ARPE19 cells exhibit many properties of freshly isolated RPE cells,[21] however, they may be more robust than normal RPE cells. Thus, the RPE may be more sensitive to higher concentrations of bevacizumab than our experimental results with ARPE19 cells may be able to show.

We also investigated whether our cell lines expressed VEGF since RPE cells and also endothelial cells are known to

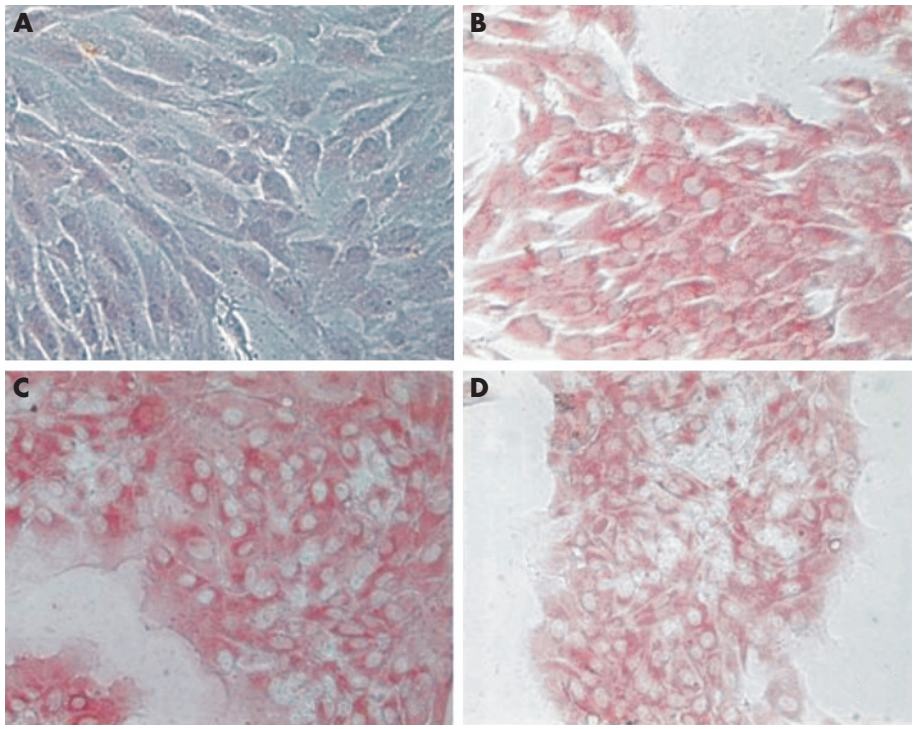

Figure 5 Immunohistochemistry for VEGF receptors. CEC cells showed a low expression of VEGFR1 (Flt-1) (A) and a strong expression of VEGFR2 (Flk-2) (B). ARPE19 cells stained positively for VEGFR1 (Flt-1) (C) and VEGFR2 (Flk-2) (D).

express VEGF.[22 23–25] CEC and ARPE 19 cells stained positively for VEGF; however, the VEGF expression in the CEC cell culture may represent labelled VEGF bound to its receptor as VEGF was present in the endothelial cell medium. No exogenous VEGF was added to the other cell types.

The production of VEGF by the RPE may also have a role in the development of CNV.[26]

On the one hand it is desirable that neovascularisation is inhibited or even reverted by a VEGF antagonist like bevacizumab, but on the other hand existing normal vasculature should not be harmed by the drug.

According to the literature retinal neovascularisation seems to be more dependent on VEGF than CNV.[25] This may explain why we only saw a moderate inhibition of CEC proliferation by bevacizumab.

Previous investigations observed an inhibition of the proliferation rate of human umbilical vein endothelial cells (HUVEC) of up to 50% in the presence of bevacizumab. However, far lower concentrations of bevacizumab were needed to evoke an antiproliferative effect in HUVEC.[27] Thus, rather high concentrations of bevacizumab may be needed to inhibit CNV. But the findings of Wang *et al* are difficult to compare with our results since the authors used VEGF in a concentration of 50 ng/ml. This concentration by far exceeds the VEGF levels that are usually encountered in proliferative diabetic retinopathy or other neovascular ocular diseases.[28] We used a VEGF concentration of only 2 ng/ml because in neovascular eye diseases such as proliferative diabetic retinopathy the VEGF concentration in the vitreous usually does not exceed 1–2 ng/ml.[28]

Moreover, the medium we used for the maintenance of the endothelial cells (before the cells were seeded onto 96 well plates) contained VEGF in a concentration of about 2 ng/ml (according to information provided by the manufacturer).

Furthermore, we studied porcine CEC and therefore it may be possible that human choroidal endothelial cells respond differently to bevacizumab.

Our CEC were strongly positive for VEGFR2 but only showed mild staining for VEGFR1. VEGFR2 is the major mediator of mitogenesis, migration, and growth of endothelial cells. Furthermore, increased vascular permeability is mediated through VEGFR2[29] whereas it still has not been clearly elucidated whether the activation of VEGFR1 through VEGF has a significant role in the development of neovascular eye disease. Thus, the action of VEGF through VEGFR2 seems to be the most relevant for the development of CNV.[22]

Although ARPE19 cells were positive for VEGFR1 and VEGFR2, no significant antiproliferative effect of bevacizumab was observed. These findings suggest that the growth of ARPE19 cells is mainly mediated by other growth factors than VEGF. Even when higher concentrations of VEGF were added (up to 50 ng/ml, data not shown) no stronger inhibition of ARPE19 cell growth by bevacizumab could be achieved.

Apart from neoangiogenesis VEGF is responsible for increasing vascular permeability (in fact, VEGF originally has been named vascular permeability factor). It was not the purpose of our study to investigate the effect of bevacizumab on vascular permeability. However, other investigators reported a rapid decrease of endothelial cell permeability in the presence of bevacizumab.[27] The rapid improvement some patients experience after treatment with bevacizumab may be the result of decreased vascular permeability and thus resolution of macular oedema.

Monoclonal antibodies generally have been viewed to exhibit only limited toxicity. However, toxicities do occur, and can be grouped into mechanism independent and mechanism dependent categories.

Mechanism independent toxicity usually relates hypersensitivity reactions caused by a protein containing xenogeneic sequences. Hypersensitivity reactions can occasionally be sufficiently severe (for example, anaphylactoid reactions) to require aggressive management and discontinuation of therapy.[14]

Given the immune privileged situation in the vitreous, the risk for serious hypersensitivity reaction appears to be low when bevacizumab is administered intravitreally. However, in many neovascular eye diseases the blood-retina border is

disturbed and lymphocytes and plasma cells may enter the vitreous or intravitreally administered bevacizumab may get into the systemic circulation and evoke an immune reaction. Further clinical and experimental data are necessary to determine whether the effect of repeatedly intravitreally administered bevacizumab may be hampered by the development of anti-bevacizumab antibodies.

Mechanism dependent toxicities result from the binding of a therapeutic antibody to its target antigen. No such toxicity has been reported for bevacizumab so far.

Our experimental findings support the safety of intravitreal bevacizumab when used at the currently established dose of 1–1.25 mg. However, concentrations higher than 0.8 mg/ml may be harmful to the retinal pigment epithelium. As a consequence we suggest bevacizumab should not be used in concentrations higher than 0.25–0.3 mg/ml (equalling a total dose of bevacizumab of 1.25 mg in 4 ml vitreal volume).

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Conflict of interest: none.

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