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RETINAL DISORDERS

Different properties of VEGF-antagonists: Bevacizumab but not Ranibizumab accumulates in RPE cells

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Abstract
Background Vascular endothelial growth factor (VEGF) antagonists are currently the therapy of choice for age-related macular degeneration. Here we compared the effects of FDA-approved Ranibizumab and off-label used Bevacizumab on RPE cells, investigating their respective uptake by RPE cells over time.
Methods Primary porcine RPE cells were treated with Bevacizumab or Ranibizumab, respectively. Uptake of the respective VEGF-antagonists was assessed with confocal laser scanning microscopy and flow cytometry. Cell death was assessed with MTT assay and VEGF secretion was measured with ELISA.
Results When clinical doses were applied for 1 h, Bevacizumab was taken up by RPE cells as assessed by confocal laser scanning microscopy and flow cytometry. After 24 h of incubation, and further assessed after 1d, 5d, and 7d, Bevacizumab was detected in RPE cells where it accumulated over time. The presence of Bevacizumab within RPE cells after 7d was confirmed by flow cytometry. While some Ranibizumab was found in RPE cells after 1 h of incubation when assessed with confocal laser microscopy but not by flow cytometry, no signal above control was detected after 1d, 5d, or 7d. Neither substance induced significant cell death after 7 days and no inhibitory effect on VEGF secretion was observed after day 3 of culture.
Conclusions Bevacizumab, but not Ranibizumab, accumulates in RPE cells over time, implying substantial differences between these two drugs.

Keywords Age-related macular degeneration (AMD) · Bevacizumab · Ranibizumab · VEGF-antagonists

Grants DOG Forschungsförderung

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The authors have full control of all primary data and they agree to allow Graefe's Archive for Clinical and Experimental Ophthalmology to review their data if requested.

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Introduction

Age-related macular degeneration (AMD) is the major cause for legal blindness in the industrialized world. The development of VEGF antagonists has revolutionized the treatment for the wet form of this devastating disease, even though the current therapies cannot heal AMD. Together with Pegaptanib (Macugen), which is not discussed in this study, Ranibizumab (Lucentis) is approved for the treatment of AMD, while Bevacizumab (Avastin) is used off label.

Bevacizumab is a full-length humanized antibody developed from a murine anti-VEGF antibody, A.4.6.1 [1, 2]. It is

an anti-angiogenic drug approved for the treatment of colon cancer. Ranibizumab is a 48-kDa humanized Fab-fragment, also developed from the mouse monoclonal anti-VEGF antibody A.4.6.1 [3]. Experiments with an antibody against human epidermal growth factor suggested that a full length antibody, in contrast to a Fab-fragment, cannot penetrate the retina [4]. In a monkey model, however, Heiduschka et al. proved Bevacizumab to be capable of penetrating the retina [5]. Bevacizumab has been used off-label to treat AMD since 2005 [6]. Retrospective studies demonstrated good results with Bevacizumab [7]; yet Bevacizumab has not been tested in phase 3 clinical trials. While Bevacizumab shows little toxicity, RPE tears have occurred [8] and alteration on a cellular level was observed [9].

Bevacizumab and Ranibizumab differ, apart from their molecular weight and the fact that Ranibizumab does not contain an Fc part, in six amino acids [3]. Additionally, Bevacizumab is produced in a eukaryotic cell line (CHO cells) and is glycosylated at Asn 303 [10], while Ranibizumab is produced in prokaryotic *E. coli*, and thus does not carry any glycosylation sites.

Our group previously compared the efficacy of Bevacizumab and Ranibizumab in vitro, and found Ranibizumab to be about six times more efficient in inhibiting VEGF. We also observed intriguing effects, as both substances influenced on VEGF expression and appear to still have an effect even when no longer present in the culture medium [11]. Bevacizumab and Ranibizumab are generally considered to be clinically equivalent; however, their respective effects on RPE cells have not been directly compared so far. In this study, we compared effects of Bevacizumab and Ranibizumab on cultured RPE cells over time, focusing on the uptake of the VEGF antagonists by RPE cells.

Methods

Cell culture

RPE were isolated as described elsewhere [11]. In brief, eyes of freshly slaughtered pigs were cleaned of adjacent tissue, briefly incubated in antiseptic solution, and the anterior part of the eye was removed. In each eye cup, trypsin was added, and incubated for 5 min at 37°. Trypsin solution was removed and substituted with trypsin-EDTA for 45 min at 37°. RPE cells were gently pipetted of the choroid, collected in media and washed. RPE of three eyes were collected and seeded in a 60-mm dish. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, PAA, Cölbe, Germany) and Ham F12 medium (PAA, Cölbe, Germany) (1:1) supplemented with penicillin/streptomycin (1%), L-glutamine, amphotericine B (0.5 µg/ml), HEPES (25 mM), sodium-pyruvate (110 mg/ml) and 10% porcine serum (PAA, Cölbe, Germany). For microscopy, cells were cultivated to confluence on collagen-coated (Collagen A, Biochrome, Berlin, Germany) cover slips (21×26 mm, Menzel GmbH, Braunschweig Germany).

Organ culture

In order to investigate the impact of Bevacizumab and Ranibizumab on VEGF secretion, experiments were conducted in Retina-RPE-choroid organ culture. Organ culture was prepared as described elsewhere [11]. In brief, eyes of freshly slaughtered pig were cleaned of adjacent tissue and the anterior part of the eye was removed and Retina-RPE-choroid sheets were separated from sclera. Prepared tissue was fixed between the lower and upper part of a fixation ring and the ring was placed in a six-well culture chamber (Minucells & Minutissue, Bad Abbach, Germany). The chamber was placed on a heating plate and perfused with medium, (Dulbecco's modified Eagle's medium (DMEM) and Ham F12 medium (1:1) supplemented with penicillin/streptomycin (1%), L-glutamine, HEPES (25 mM), sodium-pyruvate (110 mg/ml) and 10% porcine serum.

On the second day of cultivation, the tissue sheets were exposed to clinically relevant concentrations of Bevacizumab (250 µg/ml Roche, Grenzach-Wyhlen, Germany) or Ranibizumab (125 µg/ml Novartis, Nürnberg, Germany) [12]. The supernatant was collected for 1 hour every 24 h for 7 days.

Dot blot

In order to ensure the detection of the Fab-fragment by the goat-anti-human antibody, a dot blot was conducted. For the dot blot, 10% of the clinical concentration (25 µg/ml and 12,5 µg/ml) was applied directly on the PVDF membrane. An antibody against human IgG served as a positive control, distilled water served as a negative control. The blot was blocked by 4% skim milk in Tris buffered saline with 0,1% Tween for 1 h at room temperature. Goat-anti-human antibody (Invitrogen Karlsruhe, Germany, 1:2000) was applied and incubated overnight at 4°. After washing with TTBS, blots were incubated with peroxidase-conjugated donkey-anti-goat secondary antibody for 30 min at RT. Following the final wash, the blot was incubated with Immobilon chemiluminscence reagent (Millipore) and the signal was detected with Amersham Hyperfilm.

Laser scanning microscopy

RPE cells of second passage were cultivated on collagen-coated cover slips until confluence. The cells were treated with clinical concentration of Bevacizumab (250 µg/ml) or Ranibizumab (125 µg/ml) or indicated concentration. The

cells were treated with respective substance for 1 h or 24 h and fixed after 1 h, 1 day, 5 days, or 7 days. For the 5-d and 7-d fixation, the medium was discarded and replaced with fresh medium 24 h of treatment with respective substance and treated as normal cell culture. For fixation, the cells were briefly washed with PBS 0.1% azide, and fixed in 2.5% paraformaldehyde at RT followed by acetone-ethanol for 10 min at −10°. Cells were blocked with 0.1% BSA 0.2% glycine in TBS, washed, and incubated with goat-anti-human antibodies coupled with AlexaFluor 555 (Invitrogen, Karlsruhe, Germany) at 37° for 1 h. Nuclear staining was performed using Hoechst stain. Cells were washed and mounted using Slow Fade Mounting Medium (Invitrogen, Karlsruhe, Germany). Uptake of Bevacizumab and Ranibizumab was visualized using confocal laser scanning microscopy (LSM510UV, Carl Zeiss MicroImaging, Jena, Germany).

Flow cytometry

Cells of 1×10^6 RPE were trypsinized, washed, and incubated with Bevacizumab (25 or 250 µg/ml) or Ranibizumab (12,5 µg or 125 µg/ml) for 1 h. Additionally, 1×10^6 RPE cells were cultured with either 250 µg/ml Bevacizumab or 125 µg/ml Ranibizumab, respectively, for 24 h and analyzed after 1 week. For cell-surface staining, the cells were washed with 400 mM NaCl. For intracellular staining, cells were permeabilized using the Cytofix/Cyxtoperm Fixation/Permeabilization Kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's protocol. RPE cells were washed twice and surface bound or internalized Bevacizumab or Ranibizumab were detected with AlexaFluor 488 conjugated goat-anti-human IgG (Invitrogen, Karlsruhe, Germany). All analyses were measured on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences, Heidelberg, Germany). Results are presented as histogram plots or bar plots representing the geometric mean fluorescence intensity.

MTT-assay

Cell survival was evaluated with methyl thiazolyl tetrazolium (MTT) (Sigma, Deisenhofen, Germany) assay**.** Twenty-four hours after treatment with 250 µg/ml Bevacizumab or 125 µg/ml Ranibizumab, respectively, culture media was discarded and the cells were cultivated for six additional days. One week after treatment, the cells were washed three times with PBS and incubated for 2 h with 0.5 mg/ml MTT in DMEM at 37°C. After incubation, the MTT solution was discarded and DMSO was added to the cells. Cells were shaken at 200 rpm for 5 min on an orbital shaker, the DMSO was collected and the absorbance was measured at 555-nm wavelength. Untreated control was defined as 100% survival.

ELISA

Organ culture was treated as described above, the supernatant was collected for 1 h every 24 h for 7 days and the VEGF-content was measured by a VEGF-ELISA (R&D Systems, Wiesbaden, Germany) following the manufacturer's instructions.

Statistics

Each experiment was independently repeated at least three times. Significances were calculated with Student's *t*-test, calculated with SigmaPlot 9.0 software. A *p*-value of less than 0.05 was considered significant.

Results

The goat-anti-human antibody detects Ranibizumab

The goat-anti-human antibody used in the experiments detects Bevacizumab and Ranibizumab in the applied concentrations, tested in dot blot (Fig. 1)

Bevacizumab (but not Ranibizumab) is taken up by RPE cells

Laser scanning microscopy, short term

When applied in clinically relevant concentration (250 µg/ml) for 1 h, laser scanning microscopy revealed a staining pattern for Bevacizumab suggesting that Bevacizumab binds to and is taken up by the cells (Fig. 2a). Some Bevacizumab was localized on the cell surface and seemed to have been taken up into vesicle-like structures. A similar pattern was observed when 25 µg/ml of Bevacizumab were applied, but not when 1 µg/ml was used (Fig. 2c,e).

When Ranibizumab was applied in clinically relevant concentration (125 µg/ml) for 1 h, Ranibizumab exhibited a staining pattern suggesting that Ranibizumab was taken up by the cells to a certain extent. Some intracellular staining was observed (Fig. 2b). However, the staining was

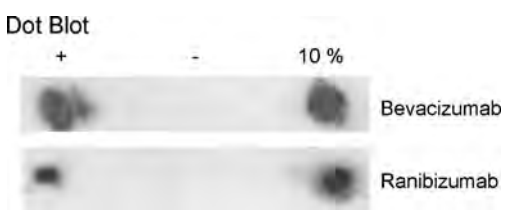

Fig. 1 Dot blot conforming the specificity of the goat-anti-human antibody. The antibody detects Bevacizumab and Ranibizumab in 10% of the clinical concentration (25 µg/ml and 12,5 µg/ml). +: positive control, −: negative control, 10%: 10% of the clinical concentration (25 µg/ml Bevacizumab, 12,5 µg/ml Ranibizumab)

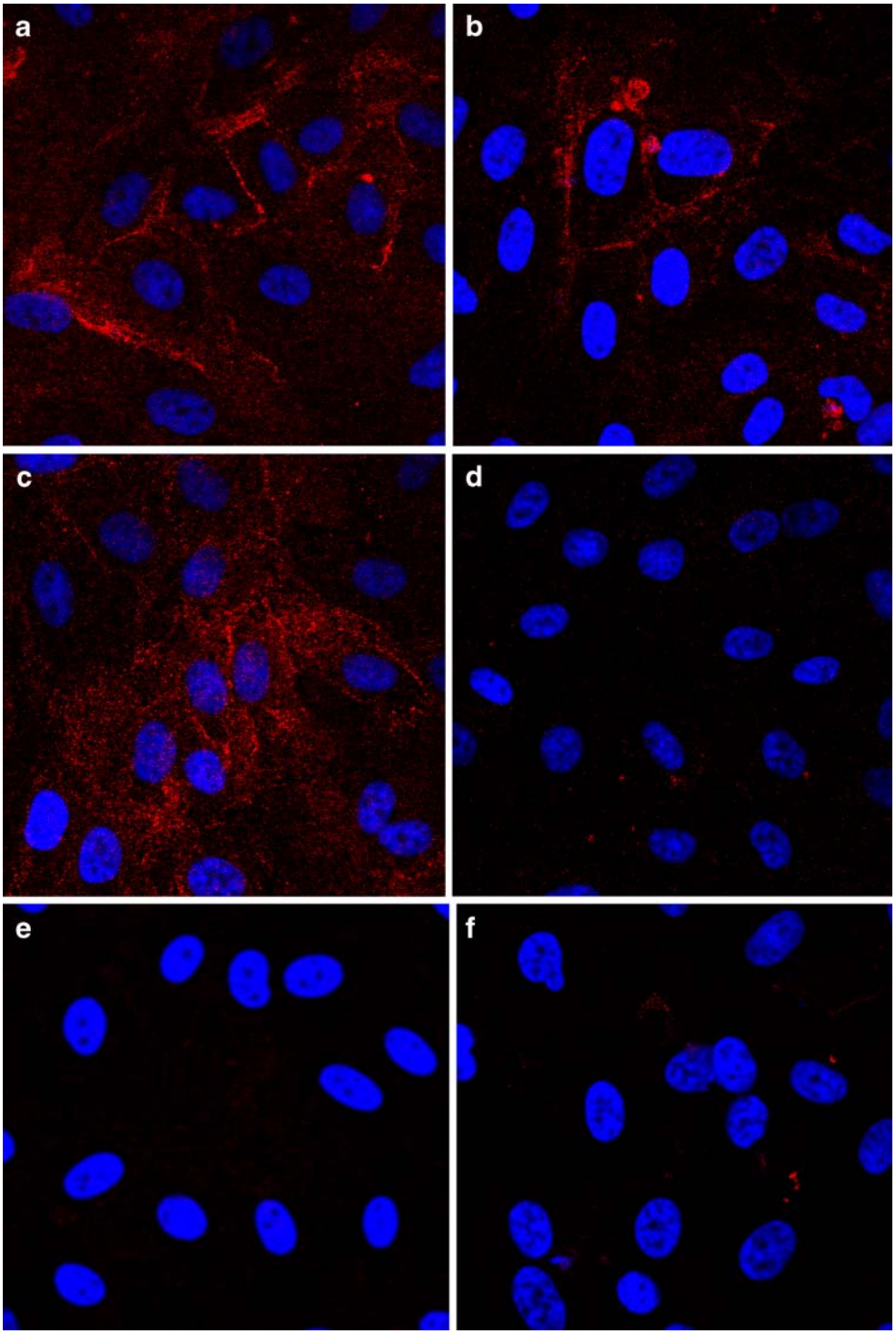

Fig. 2 Uptake of Bevacizumab and Ranibizumab, respectively, by RPE cells after 1 h. When clinically relevant concentrations are applied for 1 h, Bevacizumab (250 µg/ml) (**a**), and to a lesser extent Ranibizumab (125 µg/ml) (**b**) can be detected. No staining can be detected in untreated control (**f**). When 10% of the clinical concentration is applied, Bevacizumab (25 µg/ml) (**c**) but not Ranibizumab (12.5 µg/ml) (**d**) can be seen. When 1 µg/ml Bevacizumab is applied, no signal can be detected (**e**). Representative images are shown. Nuclear staining was performed using Hoechst stain (*blue*), Bevacizumab and Ranibizumab were stained with goat-anti-human antibodies coupled with AlexaFluor 555 (*red*). Experiments were conducted in RPE cell culture

completely lost when Ranibizumab was applied in a lower concentration (12.5 µg/ml) (Fig. 2d). All experiments were conducted in RPE cell culture.

Laser scanning microscopy, long term

When Bevacizumab was added for 24 h, removed, and cells then fixed for microscopy after 1, 5, and 7 days, the staining intensity appears to intensify over time, and Bevacizumab staining appeared to intensify in the cells (Fig. 3c,e). After 7 days, some cells appeared to be thoroughly filled with Bevacizumab in vesicle-like structures (Fig. 3e). Staining was also observed at the cell surface. When Ranibizumab was given for 24 h, removed, and cells then fixed for microscopy after 1, 5, and 7 days, no staining above threshold was detected

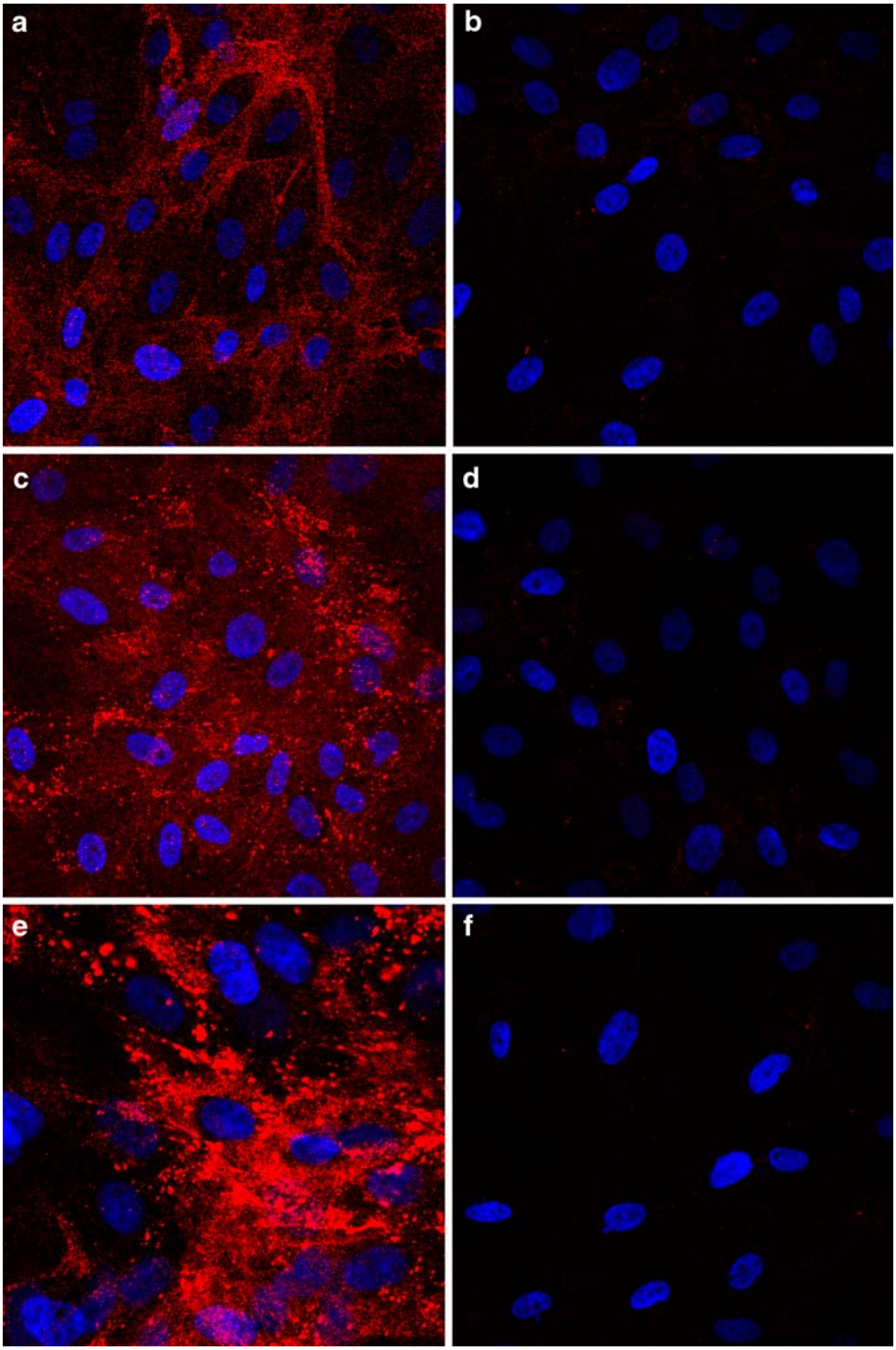

Fig. 3 When clinical concentrations of the VEGF Inhibitors are given for 24 h, Bevacizumab can be strongly detected after 1 day (**a**), 5 days (**c**) and 7 days (**e**), with the signal accumulating in vesicle-like structures. No Ranibizumab can be detected after 1 day (**b**), 5 days (**d**) or 7 days (**f**). Representative images are shown. Nuclear staining was performed using Hoechst stain (*blue*), Bevacizumab and Ranibizumab were stained with goat-anti-human antibodies coupled with Alexa-Fluor 555 (*red*). Experiments were conducted in cell culture

(Fig. 3b,d,f). All experiments were conducted in RPE cell culture.

Flow cytometry

After 1 h of stimulation with Bevacizumab in clinical concentrations, Bevacizumab was detected by flow cytometry intracellularly (which combines the intra- and extracellular signal), but not extracellularly. In lower concentrations, no significant staining was detected (Fig. 4a–b). After 7 days, however, a significant shift of the signal was detected both intracellularly and extracellularly (Fig. 5). After 1 h of incubation with Ranibizumab, clinical or lower (12.5 µg/ml) concentrations of Ranibizumab did not induce a significant shift in the signal when compared to untreated control, neither extra- nor intracellularly (Fig. 4b). Also, 7 days after

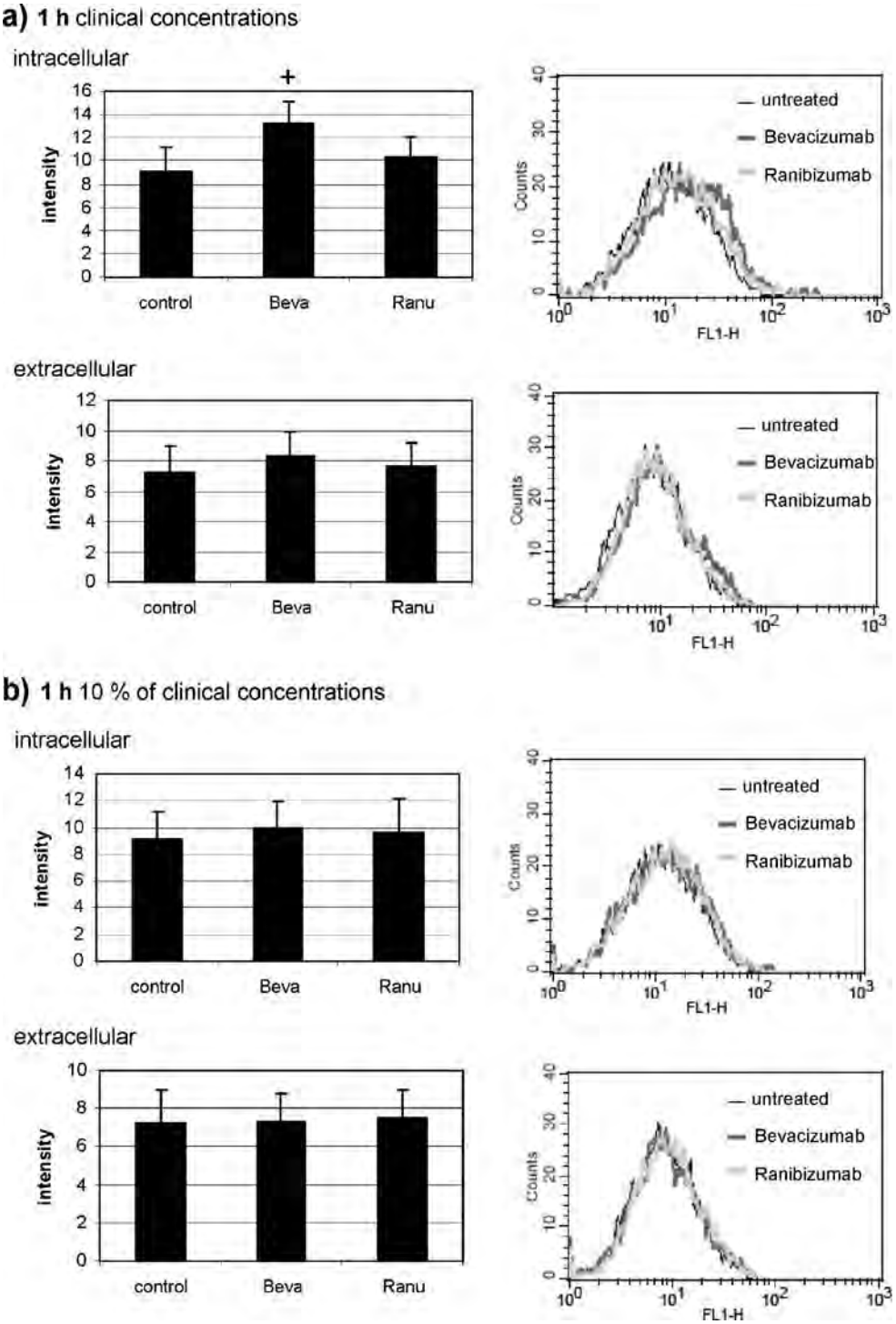

Fig. 4 Flow cytometry. **a** 1-h incubation with clinical concentrations of Bevacizumab (250 μg/ml) induced a significant shift in the intracellular (which combines the extra- and intracellular signal), but not in the extracellular signal. Clinical concentrations of Ranibizumab did not induce a shift. **b** Neither 10% of the clinical concentration of Bevacizumab (25 μM) nor of Ranibizumab (12.5 μM) induced a shift in the detected signal. Experiments were conducted in cell culture. Results are presented as bar blots (mean and standard deviation) and histogram plots, representing the geometric mean fluorescence intensity. Significance was calculated with *t*-test. + $p<0.05$

application of Ranibizumab, no significant staining was detected (Fig. 5). All experiments were conducted in RPE cell culture.

VEGF is not inhibited over a prolonged period of time

We tested the VEGF content of the supernatant of perfusion organ culture during the time course of 1 week after application of Bevacizumab or Ranibizumab, respectively. The serum-containing medium does not contain detectable amounts of VEGF. While individual differences of maximal VEGF inhibition time were observed, a significant VEGF inhibition could not be detected later than 3 days after stimulation. Also, no rebound effects were seen with either substance (Fig. 6).

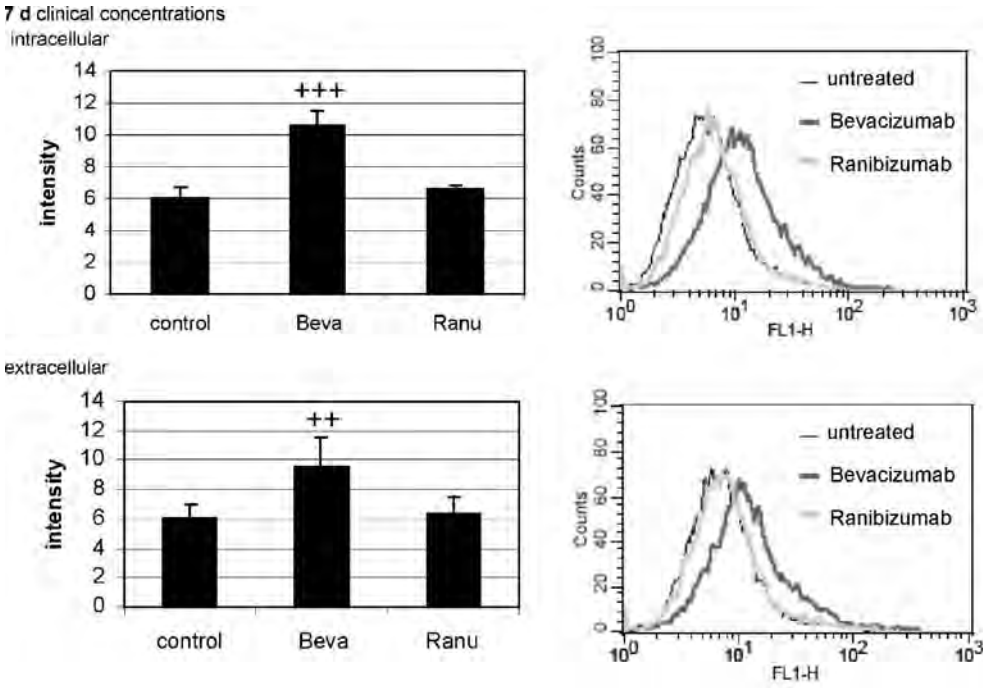

Fig. 5 Flow cytometry. 7 days after a 24-h treatment with clinical concentrations, Bevacizumab (250 μM) induces a significant shift in the detected signal both intracellularly (which combines the intra- and extracellular signal) and extracellularly. Clinical concentrations of Ranibizumab (125 μM) show no effect. Results are presented as bar blots (mean and standard deviation) and histogram plots, representing the geometric mean fluorescence intensity. Significance was calculated with *t*-test, ++ $p<0.01$, +++ $p<0.001$. Experiments were conducted in RPE cell culture

Accumulation does not induce cell death

In order to detect possible acute toxic effects of Bevacizumab accumulation, we assessed cell viability in RPE cell culture with MTT assay 7 days after application of clinical concentration of Bevacizumab (250 μg/ml) or Ranibizumab (125 μg/ml). In this assay, no significant induction of cell death was detected with either substance (Fig. 7).

Discussion

We have previously shown that Ranibizumab has a higher efficacy to inhibit VEGF than does Bevacizumab. Here, we compared the uptake of the VEGF antagonists into RPE cells. Our data clearly shows an uptake and accumulation of Bevacizumab into RPE cells over time. While Bevacizumab can already be found after 1 h within the cells, the accumulation over a prolonged period of time was striking, resulting in cells vastly filled with Bevacizumab, even though Bevacizumab had been removed after 24 h in culture. This uptake is not accompanied by a prolonged VEGF inhibition. Also, a strong extracellular binding was observed by microscopy, which was confirmed by flow cytometry analysis after 7 days, but not after 1 h of incubation.

Ranibizumab exhibited a different pattern. While some Ranibizumab may be found in RPE cells after 1 hour (as seen in laser scanning microscopy but not confirmed by flow cytometry), no Ranibizumab can be detected in RPE cells after a prolonged period of time. RPE cells do not take up Ranibizumab and do not accumulate it in the cells.

The clinical consequence of an uptake of Bevacizumab by the RPE is not known. As we used porcine cells in this study, an extrapolation to the human situation has to be viewed with

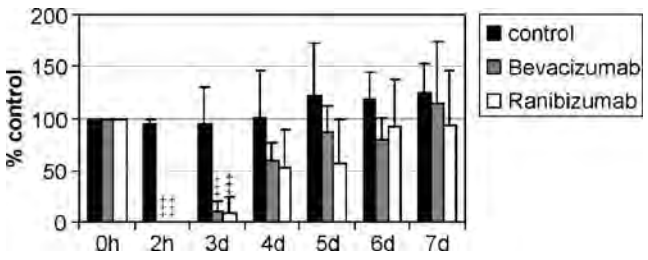

Fig. 6 Long-term effect on VEGF secretion. In clinical concentrations, both Bevacizumab and Ranibizumab significantly inhibited VEGF secretion for 3 days, but not for a longer time period. Significance was calculated with *t*-test, +++ $p<0.001$. Experiments were conducted in organ culture

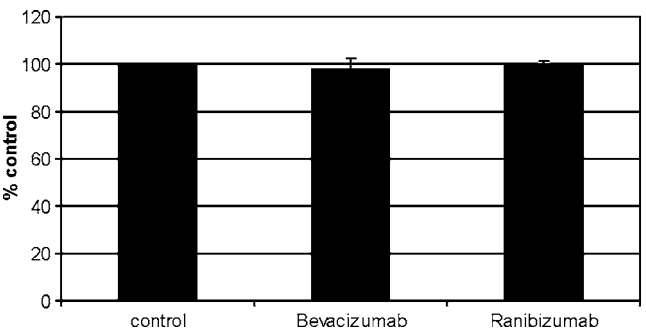

Fig. 7 Cell death. Clinical concentrations of Bevacizumab (250 μM) or Ranibizumab (125 μM) did not induce significant cell death in RPE cells as evaluated with MTT assay. Mean with standard deviation is shown. Significance was calculated with *t*-test. Experiments were conducted in RPE cell culture

caution, especially as in cell culture, the antibody does not have to penetrate the retina, rendering it more available for the cells. Also, intravitreal Bevacizumab has exhibited a good tolerance so far [13], even though some adverse effects as tears of the RPE have occurred [14, 15]. If the intracellular accumulation can be confirmed in patients, however, long-term effects on the RPE are possible and need to be closely observed.

Heiduschka et al. previously observed an uptake of Bevacizumab in RPE cells after intravitreal injection of Bevacizumab in monkey eyes, which strongly indicates that the uptake observed in our study was not an artifact of our in vitro system. In their work, the authors suggested that the staining of the RPE results from a phagocytosis of photoreceptor outer segment that contained Bevacizumab [5]. In our system, no photoreceptor outer segments are present, so at least in vitro, Bevacizumab has to be taken up by a different mechanism. Bevacizumab, but not Ranibizumab, carries an Fc domain and sugar moieties. Fc-receptor mediated phagocytosis is a well-known phenomenon in phagocytic cells of the immune system [16] and Fc-mediated phagocytosis has been described for RPE cells [17]. The data of expression of Fc receptors of RPE cells is, however, controversial [18]. Also, RPE cells have been described to express mannose receptors [19] and galectins [20, 21]; therefore Bevacizumab might be taken up via sugar receptors.

In conclusion, Bevacizumab but not Ranibizumab is taken up by and accumulates over time in RPE cells, possibly inducing long-term effects. These results demonstrate that there is a substantial difference in the uptake of the drugs by RPE cells. Presently, it remains unclear whether this difference has clinical consequences. Taking this into account, long-term follow up of patients receiving repeated doses of Bevacizumab might be advisable.

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