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BASIC SCIENCE

Comparison of the efficacy of aflibercept, ranibizumab, and bevacizumab in an RPE/choroid organ culture

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Abstract
Purpose Anti-VEGF treatment is the therapy of choice in age-related macular degeneration and is also applied in diabetic macular edema or retinal vein occlusion. Recently, aflibercept has been approved for therapeutic use. In this study, we investigate the efficacy of aflibercept in comparison with the VEGF-antagonists ranibizumab and bevacizumab in RPE/choroid organ cultures.
Methods RPE/choroid organ cultures were prepared from freshly slaughtered pigs' eyes. Organ cultures were treated with 125 μg/ml aflibercept, ranibizumab, or bevacizumab, and the VEGF content of the supernatant was evaluated over the course of 7 days. Additionally, the minimal concentration of VEGF inhibition was evaluated in organ cultures, measured after 6 h of application.
Results Aflibercept was able to completely inhibit VEGF detection for 6 h at a minimal concentration of 0.031 μg/ml, in contrast to bevacizumab (3.9 μg/ml) and ranibizumab (0.244 μg/ml). A statistically significant VEGF inhibition compared to control could be found for aflibercept and ranibizumab down to and including 0.031 μg/ml, while bevacizumab was significantly reduced compared to control down to a concentration of 0.244 μg/ml and again at 0.061 μg/ml. Inhibition of VEGF after a single aflibercept application of 125 μg/ml could be found over the course of 7 days, with some VEGF detectable at the 7th day. In contrast, VEGF was detectable after 72 h of ranibizumab treatment and some VEGF could already be found 12 h after bevacizumab treatment.
Conclusions In conclusion, aflibercept displays a prolonged VEGF inhibition, confirming its effectiveness but also raising concerns about possible side effects of long-term usage.

Keywords Aflibercept · Ranibizumab · Bevacizumab · VEGF · Efficacy

Introduction

Age-related macular degeneration (AMD) is the prominent cause for legal blindness in developed countries. Until the advent of anti-VEGF medication in 2005, treatment options for exudative AMD were limited, with photodynamic therapy being the only FDA-approved drug [1]. Intravitreal application of anti-VEGF molecules enable physicians to significantly slow down the wet form of AMD, with a possibility of vision gain in a subset of patients [2]. Currently in use for intravitreal anti-VEGF application are the approved ranibizumab, the off-label bevacizumab, and the recently approved aflibercept [2–4]. The current treatment labels request repeated intravitreal application, such as monthly for ranibizumab and bimonthly for aflibercept, even though in clinical practice another treatment regime is most often applied [5]. The cessation of anti-VEGF treatment may result in the reoccurrence of choroidal neovascularizations, bleeding, and vision loss [6].

While all clinically applied anti-VEGF agents have shown a favorable safety profile in patients so far, the effects of prolonged VEGF-inhibition on retinal cells raises concerns. VEGF is an important physiological factor in the retina [7], upholding choriocapillaris fenestration and protecting the endothelium, the retina pigment epithelium (RPE), and the neuroretina [8–11]. Conflicting results exist regarding the effects of long-term inhibition of VEGF in the adult retina. While some authors do not see any impact on the survival and

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physiology of adult retinal cells under VEGF inhibition [12, 13], other authors do describe an effect of VEGF inhibition on cellular integrity and functions, even displaying quite dramatic effects, such as the degeneration of photoreceptor cells [14–16]. Regarding patient data, long-term degenerative developments have been found in retinas that were VEGF-deprived for 24 months [17, 18].

We previously have investigated the efficacy of ranibizumab, bevacizumab, and pegaptanib in an in vitro organ culture [19]. In this study, we investigate the efficacy of aflibercept regarding VEGF inhibition in comparison with bevacizumab and ranibizumab using a RPE/choroid organ culture.

Materials and methods

Perfusion organ culture

Organ culture was prepared as previously described with modifications [19, 20]. In brief, for the preparation of RPE/choroid sheets, freshly slaughtered pigs' eyes were cleaned of adjacent tissue and immersed briefly in antiseptic solution. The anterior part of the eye was removed, RPE/choroid sheets were separated from sclera, and prepared tissue was fixed between the lower and upper parts of a fixation ring. Organ sheets were cultivated in a perfusion 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; PAA, Cölbe, Germany) and Ham F12 medium (PAA) (1:1) supplemented with penicillin/streptomycin (1 %), L-glutamine, HEPES (25 mM), sodium-pyruvate (110 mg/ml), and 10 % porcine serum (PAA). The medium entered the perfusion chamber at the front side, passed between the tissue carriers, and left the chamber at the rear side, where it could be collected. The flow rate was 2 ml/h. The gas exchange took place via the silicone tubes and the pH and CO_2 content of the media was stabilized by HEPES. The perfusion of the tissue allowed a steady-state equilibrium of the tissue [21, 22]. The viability of the organ cultures was evaluated by calcein staining [20].

On porcine models

Regarding anatomy and physiology, the porcine eye is considered to be the closest to the human eye of all mammals, with the exception of the primate. The morphology and size corresponds well with human eyes, retinal and choroidal blood flow are present, and, moreover, retinal organization corresponds well to the human retina, as both have ten layers and are of comparable thickness [23–25]. In addition, considering immunological aspects, the porcine retina can be considered to be similar to humans, e.g., regarding IgG homology [26] or the presence and features of retinal microglia, which is very similar between human and pig, and very different from rat or mouse [27, 28].

Organ culture treatment with VEGF-antagonists

Organ cultures were treated with VEGF-antagonists as described elsewhere with modifications [19]. In brief, on the second day of cultivation, the tissue sheets were exposed to bevacizumab (Roche), ranibizumab (Novartis), or aflibercept (Bayer) at indicated concentrations. For this, the perfusion of the tissue was interrupted and the medium was removed from the chamber with a syringe and transferred to a falcon tube, where the respective VEGF-antagonist was added to the medium. The medium was transferred back into the chamber and incubated for 20 min. The perfusion of the medium, containing the appropriate concentration of the VEGF-antagonist in the perfusion chamber, was restarted. The applied VEGF-antagonist was diluted over time because of medium perfusion as previously described [19]. The flow-through of the growth medium was collected at indicated time points for 1 h.

Evaluation of VEGF content

The VEGF content was measured by a VEGF-ELISA (R&D Systems, Wiesbaden, Germany) following the manufacturer's instructions. The range of detection of the ELISA was between 15 and 1,046 pg/ml. The ELISA detects all isoforms of VEGF-A, and readily detects porcine VEGF-A [19].

Statistics

Each experiment was independently repeated for at least three times. Bar graphs display mean and standard deviation. The scatterplot with regression analysis comparing the potency of the antagonists was calculated with Stastica 7.1. Significance was evaluated with an unpaired, two-tailed Student's *t* test. A p value of <0.05 was considered significant.

Results

Minimal inhibitory concentrations

We have compared the efficacy of VEGF inhibition of aflibercept, ranibizumab, and bevacizumab in RPE/choroid organ cultures, applying different concentrations of the respective inhibitor. We found that all three compounds exhibited a complete inhibition of VEGF after 6 h of application in our system down to a concentration of 3.9 μg/ml. Aflibercept displayed a complete inhibition of VEGF down to a

concentration of 0.031 μg/ml. A statistically significant VEGF inhibition compared to control could be found for aflibercept in all tested concentration down to and including 0.031 μg/ml, while at 0.015 μg/ml (38.8±33.8 pg/ml VEGF) and 0.0076 μg/ml (102.6±43.8 pg/ml), inhibition no longer reached statistical significance (Fig. 1a). Ranibizumab, with the exception of very little VEGF found at 1.95 μg/ml (3.9± 6.7 pg/ml), displayed a complete inhibition of VEGF down to a concentration of 0.244 μg/ml. Starting at a concentration of 0.122 μg/ml, VEGF could be found in the supernatant (19.4± 17.3 pg/ml). The inhibition of VEGF compared to control reached significance down to and including a concentration of 0.031 μg/ml (0.061 μg/ml, 11.1±14.5 pg/ml; 0.031 μg/ml, 37.1±1.9 pg/ml), in lower concentrations (0.015 μg/ml, 48.5 ±13.2 pg/ml; and 0.0076 μg/ml, 40.7±14.5 pg/ml), significance was lost (Fig. 1b). Bevacizumab displayed a less distinct pattern, as starting from the concentration of 1.95 μg/ml, VEGF could be found in the supernatant (11.9±20.7 pg/ml), but the inhibition was significant compared to control down to a concentration of 0.244 μg/ml (39.9±9.9 pg/ml) and again at 0.061 μg/ml (70.5±29.3) (Fig. 1c). To directly compare the potency of the drugs, the inhibition of VEGF in % of untreated control has been plotted against the concentration (Fig. 2). A statistically significant difference in potency between aflibercept and ranibizumab could be found at a concentration of 0.031 μg/ml ($p<0.001$). Both ranibizumab and aflibercept were significantly more potent than bevacizumab (aflibercept: 0.244 μg/ml $p<0.01$; 0.122 μg/ml 0.01; 0.061 μg/$p<0.01$; 0.031 μg/ml $p<0.05$; ranibizumab 0.244 μg/ml $p<0.01$; 0.122 μg/ml $p<0.05$; 0.061 μg/$p<0.01$).

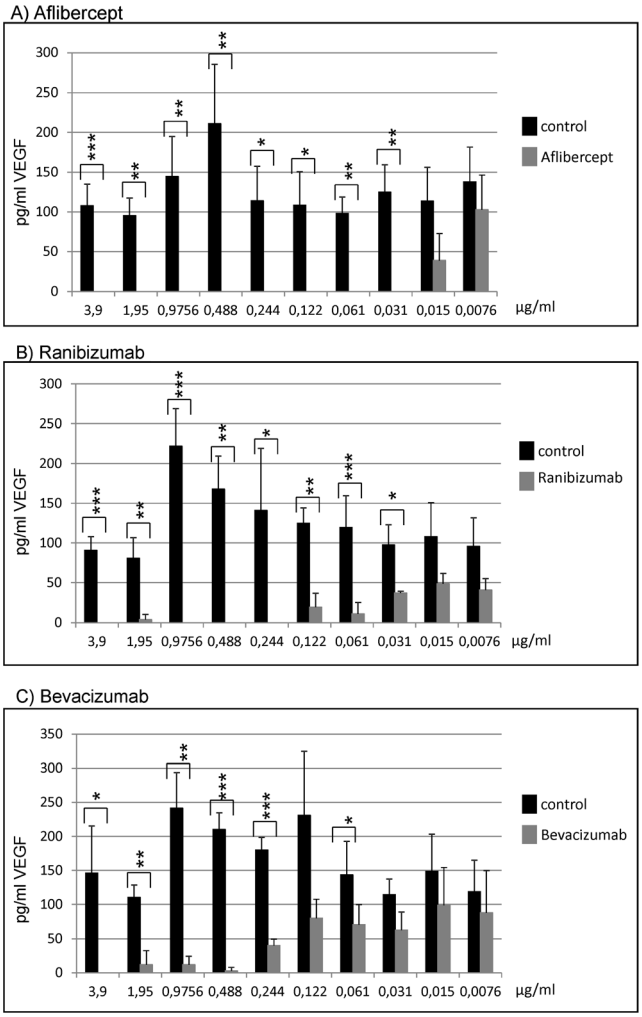

Fig. 1 Minimal concentration of VEGF inhibition. Organ cultures were stimulated with VEGF-antagonist and VEGF content of the supernatant was detected after 6 h. **a** Aflibercept displayed a complete inhibition of VEGF down to a concentration of 0.031 μg/m. A statistical significance of VEGF inhibition compared to control could be found in all tested concentration down to and including 0.031 μg/ml. **b** Ranibizumab, with the exception of very little VEGF found at 1.95 μg/ml, displayed a complete inhibition of VEGF down to a concentration of 0.244 μg/ml. The inhibition of VEGF compared to control reached significance down to and including a concentration of 0.061 μg/ml. **c** Bevacizumab displayed a less distinct pattern, as starting from the concentration of 1.95 μg/ml, VEGF could be found in the supernatant, but the inhibition was significant compared to control down to a concentration of 0.244 μg/ml and again at 0.061 μg/ml. Significance was determined by Student's *t* test. "+" $p<0.05$; "++" $p<0.01$, "+++" $p<0.001$

Long-term VEGF inhibition

We compared the long-term inhibitory effect of a single application of 125 μg/ml aflibercept, ranibizumab, or bevacizumab in organ cultures up to 7 days. Aflibercept displayed a significant inhibition of VEGF over the whole period of 7 days, with a complete inhibition of VEGF up to 6 days and only little VEGF found at day seven (11.2± 25.0 pg/ml). Ranibizumab displayed a significant inhibition of VEGF up to 72 h (29.2±5.7 pg/ml) with a complete inhibition of VEGF up to 48 h. Bevacizumab was already detectable at 12 h post-stimulus (5.5±9.4 pg/ml) and lost inhibitory potency starting at 48 h (33.4± 31.4 pg/ml). However, the inhibition reached significance up to 4 days (91.1±9.3 pg/ml). Aflibercept displayed a significantly stronger effect on VEGF inhibition compared to ranibizumab at 72 h, 4 days, 5 days, 6 days (all $p<0.001$) and 7 days ($p<0.01$) and was significantly more effective than bevacizumab at 14 h ($p<0.05$), 48 h ($p<0.05$) 72 h ($p<0.001$), 4 days ($p<0.001$), 5 days ($p<0.001$), 6 days ($p<0.001$), and 7 days ($p<0.001$). Ranibizumab was more efficient than bevacizumab at 72 h ($p<0.05$), 5 days ($p<0.01$), and 7 days ($p<0.05$) (Fig. 3).

We additionally tested aflibercept at a concentration of 500 μg/ml, which resembles the clinical concentration, and found the same effects as with an application of 125 μg/ml, with no VEGF found up to 6 days and little VEGF found at day seven (6.2±15.3 pg/ml), and a significant inhibition compared to control at all time points tested.

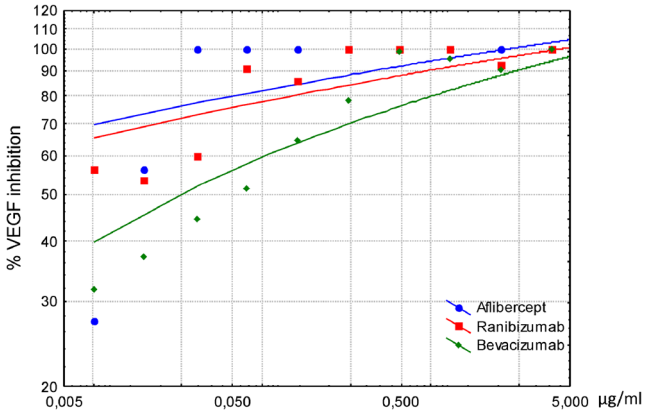

Fig. 2 Comparison of the potency of the three drugs; values depicted as % VEGF inhibition in logarithmic scale

Discussion

The biological activity of aflibercept in comparison with ranibizumab is controversially debated [29, 30]. Depending on the models used and on the availability of external applied VEGF, different results considering the efficacy of these two drugs were found; the same is true for the comparison with bevacizumab. Our approach utilizes an in vitro system that is considerably close to the in vivo situation and hence may be an appropriate model for the biological efficiency of the drugs. Our model is well characterized [21, 22], and cultivates RPE/choroid tissue sheets in a perfusion chamber, which allows the interaction of the RPE and the underlying choroid in a steady-state equilibrium with a stable VEGF secretion by the tissue [19, 22], which not only includes the RPE but also the choroidal structures, which may participate in VEGF secretion under pathological conditions [31–33]. As the cultures are obtained from the eyes of genetically diverse pigs, the amount of secreted VEGF varies between individual cultures, a situation closer to the in vivo situation than the use of cell lines or inbred mouse strains. In this model, we have previously compared VEGF-inhibitors [19], and also studied the effect of various factors on VEGF secretion [20, 34]. We found that, considering the minimal concentration used in order to inhibit VEGF, both ranibizumab and aflibercept were superior to bevacizumab. Moreover, aflibercept was superior to ranibizumab, but only in one of the tested concentrations (0.031 µg/ml), displaying a stronger efficacy of the factor two. Considering the published papers of Yu et al. [29], which claimed a similar efficacy of aflibercept and ranibizumab,

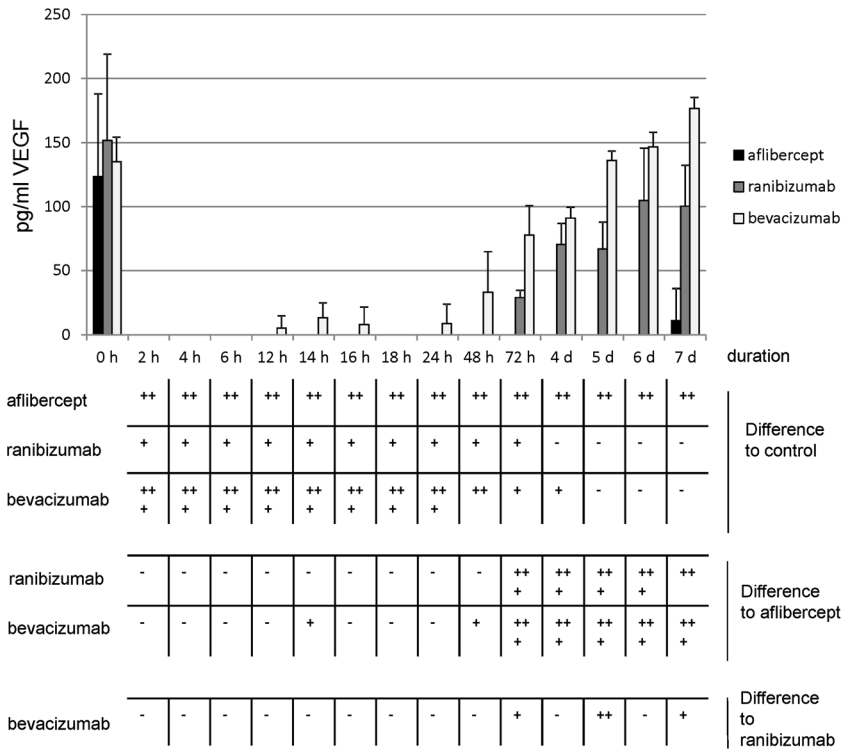

Fig. 3 Long-term inhibition of VEGF by aflibercept, ranibizumab, and bevacizumab (125 µg/ml). Aflibercept displayed a significant inhibition of VEGF over the whole period of 7 days, with a complete inhibition of VEGF up to 6 days and only little VEGF found at day seven. Ranibizumab displayed a significant inhibition of VEGF up to 72 h with a complete inhibition of VEGF up to 48 h. Bevacizumab was already detectable at 12 h post-stimulus and lost inhibitory potency starting at 48 h; however, the inhibition reached significance up to 4 days. Aflibercept displayed a significantly stronger effect on VEGF inhibition compared to ranibizumab at 72 h, 4 days, 5 days, 6 days, and 7 days, and was significantly more effective than bevacizumab at 14 h, 48 h, 72 h, 4 days, 5 days, 6 days, and 7 days. Ranibizumab was more efficient than bevacizumab at 72 h, 5 days, and 7 days. Significance was determined by Student's *t*-test. "+" $p<0.05$; "++" $p<0.01$, "+++" $p<0.001$

being superior to bevacizumab, and Papadopoulos et al. [30], which claimed a stronger efficacy of aflibercept compared to ranibizumab and bevacizumab, which were considered to be equally potent, our findings may indicate that in a biological setting, the effect seems to be in between these published efficacies, with aflibercept being slightly more potent than ranibizumab and both being more potent than bevacizumab when it comes to minimal concentrations for VEGF inhibition under the conditions of the organ culture.

When looking at the long-term effects of either inhibitor, aflibercept proves to be more potent than ranibizumab, which proves to be more potent than bevacizumab. Noteworthy, aflibercept completely abolishes VEGF up to 6 days in culture and even after 7 days, only little VEGF is detectable. In comparison, when bevacizumab is applied, VEGF displays increasing concentrations after 48 h, while ranibizumab displays increasing VEGF concentrations after 72 h. These time lines differ from results previously published by our group [19]; however, in those experiments, the organ cultures were cultivated with the retina, which is not the case in the experiments presented here. The presence of the retina changes the microenvironment of the perfusion culture; furthermore, it induces VEGF in the culture over time [34] (and non-published results).

While the inhibition of VEGF is the aim of the anti-VEGF therapy, a prolonged inhibition of the physiologically needed VEGF may induce adverse effects that could culminate over the treatment years and result in an atrophy that may hide behind the natural disease progression [7]. Experimental data on this subject are controversial, as some studies do not see any damage after 12 weeks or 7 month of VEGF inhibition in mice [12, 13]; however, changes in the anatomy of the retina can be found under VEGF inhibition, such as a decrease of choriocapillaris fenestration as seen in the monkey [8]. Moreover, the deletion of VEGF in adult mice has been shown to have dramatic effects, inducing photoreceptor atrophy and vision loss [14]. Recent publications have indicated RPE and photoreceptor atrophy after 2 years of ranibizumab treatment, and the loss of VEGF retinal protection has been discussed as a possible underlying mechanism [17, 18]. The effect of a prolonged VEGF inhibition needs to be closely monitored in patients, even more so if the long-term efficacy of the inhibitors increases.

In conclusion, aflibercept displays a prolonged VEGF inhibition, confirming its effectiveness but also raising concerns about possible side effects of long-term usage.

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