· Original article ·

The effects of anti-VEGF drugs on the retinal pigment epithelium and inner segment after intravitreal injection in the monkeys

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猴眼玻璃体腔内注射抗 VEGF 药物对视网膜色素上皮层和光感受器内节 VEGF 含量的影响

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摘要

目的:研究比较抗血管内皮生长因子(VEGF)药物(贝伐单抗、雷珠单抗、阿柏西普)行猴眼玻璃体腔内注射后对视网膜色素上皮层和眼内节 VEGF 的作用效果。

方法:选取 14 只健康猕猴,4 只双眼注射贝伐单抗,4 只双眼注射雷珠单抗,4 只双眼注射阿柏西普,每种注射药物的 4 只猴双眼分别于注射药物后第一天和第七天分别摘除 2 只猴的眼球,剩余 2 只未注射任何药物猴的双眼作为对照。所有摘除后的眼球福尔马林固定,石蜡包埋,切片后给予抗 VEGF 抗体,用光学显微镜观察,经 Image-Pro Plus 软件处理图片,用 JMP10.0 进行统计学分析。

结果:这三种药物均能降低视网膜色素上皮(RPE)和光感受器内节(inner segment)的 VEGF 水平,贝伐单抗作用在三种药物中作用最强,雷珠单抗在注射后第一天与阿柏西普注射后第一天相比,雷珠单抗作用较强,但二者在注射后第七天,作用基本相似。

结论:这三种药物均能降低 RPE 和 inner segment 的 VEGF 水平。

关键词:抗血管内皮生长因子药物;贝伐单抗;雷珠单抗;阿柏西普;视网膜色素上皮;光感受器内节;血管内皮生长因子

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Abstract

• AIM: To compare the effects on the retina inner segment and retinal pigment epithelium (RPE) of intravitreally injecting bevacizumab, ranibizumab and aflibercept into monkey eyes.

• METHODS: Fourteen healthy cynomolgus monkeys (Macaca fascicularis, aged 3-8y, 10 males, 4 females) were raised at the Covance Laboratories under standard conditions. The 14 monkeys were grouped into 4 groups. Three of the groups with 4 monkeys each were injected intravitreally with one of the drugs, either bevacizumab, ranibizumab or aflibercept, while the 4th group with 2 monkeys served as a negative control. On 1d and 7d of injection, 2 monkeys from each drug treatment group were sacrificed under general anaesthesia and the 4 eyes were enucleated. All the enucleated eyes were fixed in formalin, embedded in paraffin wax, cut into 4.0 μm sections and deparaffinized according to standard procedures. Image-Pro Plus was used for all the photos to measure the content of vascular endothelial growth factor (VEGF) in the inner segment and RPE. The ANOVA test from JMP10.0 statistical program was used to evaluate the results.

• RESULTS: Retinal sections were checked for their anti-VEGF immune reactivity. The untreated control samples had the highest level of VEGF in the RPE and inner segment. All of these three drugs can reduce the level of VEGF in the RPE and inner segment, but Avastin seems to be more effective than Eylea in this regard. Lucentis treatment at 1d seems to be more effective than Eylea at VEGF 1d. But at 7d, both Lucentis and Eylea have the same effect on reducing VEGF expression level in the RPE and inner segment.

• CONCLUSION: All of these three drugs can reduce the level of VEGF in the RPE and inner segment.

• KEYWORDS: anti-vascular endothelial growth factor drugs; bevacizumab; ranibizumab; aflibercept; retinal pigment epithelium; inner segment; vascular endothelial growth factor
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INTRODUCTION

Ocular neovascularization forms one of the most common pathological changes and clinical manifestations of a variety of eye diseases, such as age-related macular degeneration (AMD), diabetic retinopathy in adults, and retinopathy of prematurity in infants. The blinding complications of the disease make it a major cause of global visual morbidity in many countries[1-2]. Among these diseases, AMD is the leading cause of blindness in patients over the age of 65y in developed countries, with choroidal neovascularization (CNV) being the main factor responsible for vision loss in AMD. It accounts for approximately 90% of AMD-related blindness[3]. Although the pathogenesis of AMD is not completely clear, several growth factors have been implicated in the disease process as they induce the development of blood vessels [i.e. basic fibroblast growth factor, transforming growth factor β, insulin-like growth factor-1, epidermal growth factor, interleukins and vascular endothelial growth factor (VEGF)]. However, only VEGF appears to be sufficient and essential for both physiological and pathological angiogenesis[4]. Furthermore, VEGF has been considered an important stimulus for CNV formation and persistence[5]. Thus VEGF has been found to be an important therapeutic target in neovascular AMD[6].

Since 2004, anti-VEGF therapy has become the standard treatment for wet AMD and has revolutionized the management of this disease. Between 2004 and 2006, three anti-VEGF drugs were introduced to ophthalmology after receiving regulatory approval for the treatment of AMD, ranibizumab (Lucentis, Genentech/Novartis), aflibercept (VEGF Trap-Eye/Eylea, Regeneron/Bayer) and bevacizumab (Avastin, Genentech/Roche). Among the most well-known medications injected are the anti-VEGF agents. Bevacizumab is a humanized full-length monoclonal antibody that binds to and inhibits VEGF. It is widely used in an off-label manner to inhibit VEGF in the eye[7]. Ranibizumab is a recombinantly produced humanized antibody (Fab) fragment that binds all active forms of VEGF-A, and is Food and Drug Administration (FDA) approved for ophthalmic use[8]. Aflibercept is a new anti-VEGF agent recently approved by the Food and Drug Administration. It is a fully human, recombinant fusion protein composed of the second immunoglobulin (Ig) binding domain of VEGFR1 and the third Ig-binding domain of VEGFR2, fused to the fragment crystallizable (Fc) region of human IgG1[9].

The aim of this study was to compare the effects of bevacizumab, ranibizumab and aflibercept on the monkey retina inner segment and retinal pigment epithelium (RPE). The monkey is the ideal model for this *in vivo* study, in contrast to rodents, the interactions between the Fc domain and the Fc receptors mimic those present in humans[10].

MATERIALS AND METHODS

Animals and Study Protocol Fourteen healthy cynomolgus monkeys (Macaca fascicularis, aged 3-8y, 10 males, 4 females) were raised at the Covance Laboratories (Muenster, Germany) under standard conditions. The 14 monkeys were grouped into 4 groups. Three of the groups with 4 monkeys each were injected intravitreally with one of the drugs, either bevacizumab, ranibizumab or aflibercept, while the 4th group with 2 monkeys served as a negative control. At 1d and 7d of injection, 2 monkeys from each drug treatment group were sacrificed under general anaesthesia and the 4 eyes were enucleated.

All animals were housed and handled in strict accordance with good animal practice under supervision of veterinarians and were monitored for evidence of disease and changes in attitude, appetite or behaviour suggestive of illness. Handling and housing of the animals at Covance Laboratories GmbH was done in accordance with the German Animal Welfare Act. Before the experiments were started, all monkeys were approved as experimental animals by a veterinarian of the site veterinary service and local authorities. Moreover, all animals underwent a range of ophthalmic examinations (described in 'Ophthalmic examinations') during the predose phase in order to detect possible ocular disorders that would have not been related to the injection of these three drugs. For each monkey, one eye was fixed for light and electron microscopy and the other eye for immunohistochemistry. For the notice of approval by the appropriate institutional animal care and use committee, please see Covance Studies 8260977 and 8274007.

Intravitreal Injection of Bevacizumab, Ranibizumab and Aflibercept The animals were sedated by an intramuscular injection of medetomidine (Domitor) and ketamine hydrochloride, the eyes having previously been examined for any signs of inflammation. Pupils were dilated (Mydriasis with 1% tropicamide) and anaesthetised (proxymetacaine; Proparakain-POS 0.5%; Ursapharm). The conjunctival and corneal surface was disinfected (povidone iodine 10%). After sterile coating and insertion of a lid speculum, 1.25mg (25mg/ml) bevacizumab, 0.5 mg (10mg/ml) ranibizumab or 2 mg (40mg/ml) aflibercept were injected into the vitreous cavity using a 27-gauge canula. When removing the canula, the injection site was compressed with forceps to prevent reflux and a topical antibiotic (gentamicin) was administered. Animals were monitored for signs of inflammation until sacrificed.

Ophthalmic Examinations For all ophthalmic examinations, a mydriatic agent (tropicamide) and a local ophthalmic anaesthetic (proxymetacaine) were instilled in the eyes of the sedated monkeys before examinations.

Enucleation On 1d and 7d after the intravitreal injection, the animals were sacrificed under general anaesthesia, that is, intramuscular injection of ketamine hydrochloride followed by an intravenous sodium pentobarbitone (Lethabarb, Virbac, Australia) overdose. The eyes were enucleated 5min postmortem, cleaned of orbital tissue and were slit carefully at the limbus without damaging the ora serrata. Then 25 μL of the fixative were carefully injected into the vitreous before the eyes were fixed at 4℃ by immersion into 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, Sigma, St. Louis, Missouri, USA) overnight for electron microscopy or into

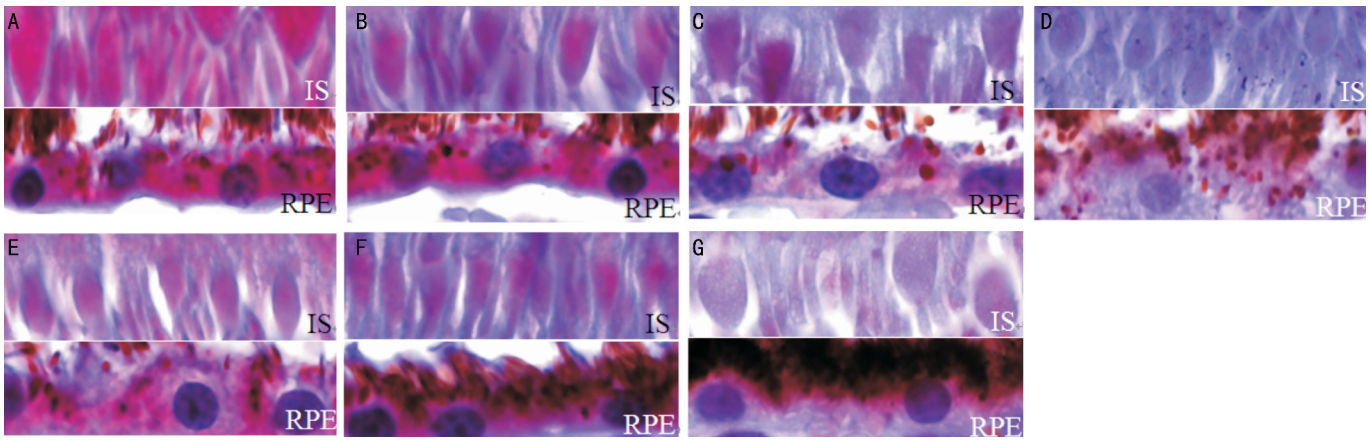

Figure 1 Light micrographs of untreated control eye (A), eye treated with Eylea on 1d (B), eye treated with Lucentis on 1d (C), eye treated with Avastin on 1d (D), eye treated with Eylea on 7d (E), eye treated with Lucentis on 7d (F), eye treated with Avastin on 7d (G) This can be seen by the reduction in the red staining after treatment with Avastin, Lucentis and Eylea. The red staining in Avastin 1d and Avastin 7d are more reduced than the staining in others. Compare Eylea 1d and Lucentis 1d, the red staining in Lucentis 1d is more reduced than the staining in Eylea 1d. IS: Inner segment; RPE: Retinal pigment epithelium.

formalin (Carl Roth, Karlsruhe, Germany) for immunohistochemistry. The eyes of two healthy monkeys without treatment were handled in the same manner.

Immunohistochemistry All the enucleated eyes were fixed in formalin, embedded in paraffin wax, cut into 4.0 μm sections and deparaffinized according to standard procedures. Bevacizumab, ranibizumab and aflibercept were detected respectively.

Using the first antibody Monoclonal Mouse Anti-Human Vascular Growth Factor, Clone VG1, the antibody labels the VEGF-165, and VEGF-121, and VEGF-189 isoforms of VEGF. (Dako Cytomation Denmark, Code No. M7273) dilution 1:140; and REAL™ Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse (Code k5005, Dako) is the second antibody, dilution 1:25. and inspected with a light microscope (Axioskop; Carl Zeiss, Oberkochen, Germany).

Semi-quantification of VEGF Staining in Inner Segment of the Retina We obtained images from each section of the specimens, both from untreated eyes and treated eyes at 1d and 7d, at a magnification of 630-fold using Zeiss Axioskop with AxioVision. For each sample there were two eyes coming from different monkeys (except for one of the samples treated with Avastin on 1d, because the monkey had endophthalmitis after the injection, so we did not take a photo of that sample). Almost every sample came from the same place (*i.e.* the layer which included the optic nerve) for each eye. One section was selected for photography, and we then took 6 photos in different areas of the inner segment and RPE in each eye. All images were made using Image-Pro Plus, in order to measure the content of VEGF in the inner segment. The first step in these photos was to define all the inner segment and RPE as the area of interest (AOI). We took 5 AOIs for each image, and each one we selected was isolated using Image-Pro Plus software. The next step was to stain each AOI area using a color cube base which absorbed the red color in the AOI.

The intensity of the red staining from the control was set as a standard value, and then every other section needing color was set against this standard to ensure that each film used the same standard and parameters.

Statistical Analysis The ANOVA test from JMP10.0 statistical program (SAS, Heidelberg, Germany) was used to evaluate the results.

RESULTS

Immunohistochemistry Retinal sections were checked for their anti-VEGF immune reactivity. The red color depicting VEGF in the RPE and inner segment was most intense in the untreated controls. On 1d after the injection, it seemed that all the drugs could reduce VEGF in the RPE and inner segment, if depending only on the basis of color. This was most obvious with bevacizumab on 1d compared to the other drugs and other days. The second most effective on 1d was ranibizumab.

Light Microscopy All sections of the untreated eyes, and the eyes treated with bevacizumab, ranibizumab and aflibercept were examined under identical conditions using 630 magnification, the focus being on the RPE and inner segment.

On 1d after the injection, it seemed that all the drugs could reduce VEGF in the RPE and inner segment, if depending only on the basis of color. This was most obvious with bevacizumab on 1d compared to the other drugs and other days. The second most effective on 1d was ranibizumab (Figure 1).

We used the application Image-Pro Plus to measure integral optical density (IOD) in all sections, and the area of VEGF in the inner segment and RPE of the retina. After calculation, we obtained the optical density of the inner segment and RPE. The ANOVA test from JMP10.0 statistical program was used to evaluate the results.

A difference of $P<0.001$ between the controls and all the treated monkey eyes was considered statistically significant. In the comparison of all treated monkeys on 1d and 7d, we compared all the 6 databases with each other, Eylea 1d and Lucentis 1d, Eylea 1d and Avastin 1d, Eylea 1d and Avastin 7d, and when P values were <0.05, the difference was considered statistically significant. But other P values of them >0.05 when considering the difference between the treated

Table 1 *P* value of untreated and after injection

Parameters	Avastin 1d	Avastin 7d	Lucentis 1d	Lucentis 7d	Eylea 1d	Eylea 7d
untreated	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$
Avastin 1d	$P=1$	$P>0.05$	$P>0.05$	$P>0.05$	$P<0.05$	$P>0.05$
Avastin 7d	$P>0.05$	$P=1$	$P>0.05$	$P>0.05$	$P<0.05$	$P>0.05$
Lucentis 1d	$P>0.05$	$P>0.05$	$P=1$	$P>0.05$	$P<0.05$	$P>0.05$
Lucentis 7d	$P>0.05$	$P>0.05$	$P>0.05$	$P=1$	$P>0.05$	$P>0.05$
Eylea 1d	$P<0.05$	$P<0.05$	$P<0.05$	$P>0.05$	$P=1$	$P>0.05$
Eylea 7d	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P=1$

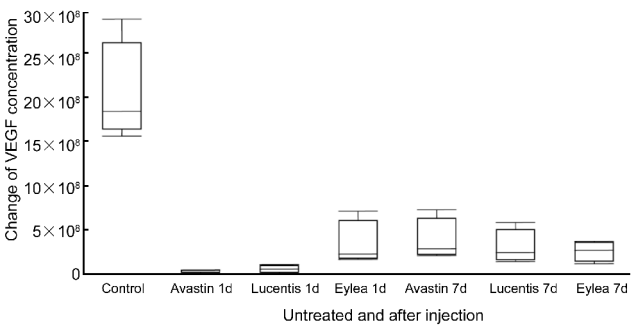

Figure 2 Box-plot of semi-quantification of VEGF in the inner segment and RPE of the retina.

monkey eyes on other days were not taken to be statistically significant (Table 1, Figure 2).

DISCUSSION

Our results show that in the comparison between the controls and the treated monkeys, the difference was considered statistically significant when $P<0.001$. This demonstrates that the VEGF-A inhibitors bevacizumab, ranibizumab and aflibercept can all reduce the level of VEGF in the RPE and inner segment.

The differences between Eylea 1d and Lucentis 1d, $P=0.0080$; Eylea 1d and Avastin 1d, $P=0.0099$; Eylea 1d and Avastin 7d, $P=0.0172$, $P<0.05$ were considered statistically significant. When these results are combined with the images (Figure 1), Avastin appears to be more effective than Eylea in reducing VEGF in the RPE and inner segment. Moreover, if we only compare Lucentis 1d and Eylea 1d, Lucentis 1d also appears more effective than Eylea 1d. The other $P>0.05$ values indicate that between Eylea 7d and Lucentis 7d; Eylea 1d and Lucentis 7d; Lucentis 7d and Lucentis 1d; Lucentis 7d and Avastin 1d; Eylea 1d and Eylea 7d; Lucentis 7d and Avastin 7d; Avastin 7d and Lucentis 1d; Avastin 7d and Avastin 1d; Avastin 1d and Lucentis 1d, there are not any obviously different effects in reducing VEGF in the RPE and inner segment. Thus according to these results, Avastin appears to be the strongest of the three drugs, Lucentis is stronger than Eylea on 1d, but on 7d these two drugs have the same effect in reducing VEGF in the RPE and inner segment.

Medications injected into the vitreous cavity reach the systemic circulation and can even exert an effect on the contralateral eye[11]. So this is the reason why we chose monkey eyes which had not been injected in either eye as the control.

Monoclonal antibodies (anti-VEGF agents) have been used to target VEGF[12]. VEGF was originally identified as an endothelial cell-specific growth factor that can stimulate angiogenesis and enhance vascular permeability. VEGF-A is the most well-studied member of the VEGF family and is a key target for antiangiogenic therapy[13]. VEGF was also shown to be a survival factor for photoreceptor cells, Müller cells and ganglion cells[14-15].

All of these three drugs can reduce VEGF in the RPE and inner segment of retina. But the current study reports a continuous increase in sub-retinal fibrosis during the first year of bevacizumab treatment. At the beginning of the study, it was present in approximately 33% of analyzed scans and after 12mo of treatment in 52% of scans in 3D imaging mode with visible sub-retinal fibrosis increased, mean central retinal thickness decreased[16].

Furthermore, other studies confirm that even if there is an incredible short-term gain in visual acuity after anti-VEGF drugs, this effect diminishes after at least 24mo of treatment[17]. Moreover, RPE is also responsible for the passage of nutrients and oxygen from the choroid towards the retina, but if damaged, the RPE may not be able to fulfil this task, which would lead to disturbance in photoreceptor renewal. The choriocapillaris is dependent on RPE-derived VEGF. Mouse studies showed that aggressively destroying VEGF could unintentionally destroy the choriocapillaris and furthermore produce geographic atrophy[14,18-19]. In addition, evidence exists for long-term retinal complications including atrophy with anti-VEGF use in AMD, and the drugs are not freely available in a sterile form in many parts of the world[20].

Until very recently the efficacy of ranibizumab seemed greatest, and remains accompanied by a large body of evidence, and a good ocular safety profile. Very recent evidence has emerged from a large randomized controlled trial (RCT) that aflibercept may be more efficacious in patients with poor vision at baseline[21].

In vitro, the same constellation with ranibizumab instead of bevacizumab did not induce formation of protein complexes[22]. Strong haemolysis in the choriocapillaris and in deeper choroidal vessels as well as the presence of extracellular haemoglobin known to be toxic were other important aspects in which aflibercept differs from ranibizumab and the controls[23]. Ranibizumab-treated eyes showed weaker haemolysis[24].

Aflibercept, like bevacizumab, is produced in Chinese hamster ovary (CHO) cells and therefore contains sugar residues in contrast to ranibizumab, which is produced in Escherichia coli. Thus, it is possible that the Fc domain and

sugar residues[25-26] of aflibercept (and on bevacizumab)[27] facilitate interference of the VEGF trap with the physiological metabolism or functioning of cells and can result in complement-mediated red blood cell death[24].

In this study, one case had endophthalmitis after injection of Avastin. Further research needs to be done in the future as to whether this is the natural course of an injection complication or a side-effect of Avastin.

To summarize, Avastin might be more effective than Lucentis and Eylea for treating wet AMD and ocular neovascular diseases, but excessively reducing VEGF might induce geographic atrophy, sub-retinal fibrosis haemolysis *etc*. In this study there seems to be no obvious difference in the effectiveness of Lucentis and Eylea, so this must be further investigated.

It should be noted that this study has examined only limited samples, and antibodies were used to see the effects of the anti-VEGF drugs. We have to point out that we do not think this is sufficient. Unfortunately, we cannot determine the effect from this data alone. But without standing its limitation, this study does suggest that all these three drugs can reduce VEGF in the inner segment and RPE. Furthermore, they should have some different effects, because they have different structures. In the future, these problems could be solved if we also studied the level of platelet-derived growth factor (PDGF), the antilog of VEGF; and analyzed the agents in the inner segment and RPE etc. Apart from this, we would like to obtain more information on how the anti-VEGF drugs influence the cellular structures through electron microscopy studies.

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