Systemic counterregulatory response of angiopoietin-2 after aflibercept therapy for nAMD: a potential escape mechanism

Reinhard Angermann, Teresa Rauchegger, Yvonne Nowosielski, Christof Seifarth, Stefan Egger, Martina T. Kralinger, Gerhard F. Kieselbach and Claus Zehetner

1Department of Ophthalmology, Medical University Innsbruck, Innsbruck, Austria
2Department of Ophthalmology, Paracelsus Medical University Salzburg, Salzburg, Austria

ABSTRACT.

Purpose: To analyse the effect of intravitreal aflibercept injections on systemic angiopoietin-2 (Ang2) and vascular endothelial growth factor (VEGF)-A levels in patients with neovascular age-related macular degeneration (nAMD).

Methods: In a prospective, randomized study, aflibercept (2.0 mg/50 µl) or ranibizumab (0.5 mg/50 µl) was administered intravitreally to 38 treatment-naive patients. Blood samples were taken before, 7 days after, and 28 days after the first intravitreal therapy. Cytokine levels were measured by enzyme-linked immunosorbent assay. Twenty-two age- and sex-matched individuals served as controls.

Results: At baseline, there were no significant differences of systemic Ang2 and VEGF-A levels among the treatment and control groups. After intravitreal aflibercept administration, median (interquartile range: IQR) systemic Ang2 was significantly upregulated from 1819 pg/ml (1262–3099) to 2123 pg/ml (1441–3769; p = 0.011) 7 days after the drug injection and remained non-significantly elevated at 1944 pg/ml (1431–2546 pg/ml; p = 0.653) 28 days after the drug injection. Median (IQR) systemic VEGF-A levels were significantly reduced from 43 pg/ml (30–57) to 8 pg/ml (8–20; p < 0.0001) 7 days and 16 pg/ml (8–26; p = 0.001) 28 days after the injection in the aflibercept group. There were no significant effects on systemic VEGF-A and Ang2 levels in the ranibizumab group at any time point following the first injection.

Conclusion: In this study, we report significant systemic upregulation of Ang2 after intravitreal aflibercept administration. This counterregulatory response may represent a potential escape mechanism from antiangiogenic therapy.

Key words: aflibercept – age-related macular degeneration – angiopoietin-2 – escape mechanism – vascular endothelial growth factor

Introduction

Neovascular age-related macular degeneration (nAMD) is caused by abnormal angiogenesis, which occurs in the setting of imbalance of proangiogenic cytokines and antiangiogenic regulatory factors (Ozaki et al. 1999; Kijlstra et al. 2005; Campochiaro 2013). The discovery of vascular endothelial growth factor (VEGF) as an important regulator of the pathomoronic development of choroidal neovascularization (CNV) gave rise to antiangiogenic nAMD therapies. However, patients require frequent administration of anti-VEGF medications to maintain their visual acuity; further, some may experience vision loss due to recurrent leakage from persistent CNV despite intensive treatment (Rofagha et al. 2013; Comparison of Age-related Macular Degeneration Treatments Trials (CATT) Research Group et al. 2016). Alternative angiogenic regulation that allows the arousal of escape mechanisms in thepresence of anti-VEGF therapy and leads to reactivation of neovascular tissue has been proposed as a mechanism explaining treatment failure and recurrent leakage in the management of nAMD (de Oliveira Dias et al. 2011; Cabral et al. 2018).

The strong involvement of angiopoietin-2 (Ang2) in the pathogenesis of CNV in nAMD and vessel formation in growing tumours has been frequently reported (Holash et al. 1999; Augustin et al. 2009; Cabral et al. 2018). This cytokine has been implicated in the promotion of angiogenesis and destabilization of the vascular endothelial cell layer via activating the receptor tyrosine kinase (Tie2); thereby, it may contribute to the increased susceptibility of this cell layer.
to VEGF (Holash et al. 1999; Holash et al. 1999; Augustin et al. 2009; Benest et al. 2013). Promising results of selective Ang2 and VEGF neutralization and the superiority of combined suppression in preclinical studies brought Ang2 into focus as an additional treatment target (Regula et al. 2016; Chakravarthy et al. 2017).

Previous studies have shown that intravitreal aflibercept exerts a great systemic inhibitory effect on VEGF-A, whereas no significant effects were observed after treatment with ranibizumab (Avery et al. 2014; Wang et al. 2014). Beside the exponentially greater VEGF-A-binding aflibercept affinity than ranibizumab affinity, aflibercept has the ability to interact with the neonatal FC receptors (FcRn). Aflibercept is able to cross endothelial boundaries like the blood–retina barrier through modulation by the FcRn and possibly mediates off-target effects in the systemic circulation (Powne et al. 2014). These specific pharmacodynamic properties underlie changes of the peripheral angiogenic milieu that can be further investigated as a result of intravitreal aflibercept therapy.

Thus, the purpose of the present study was to analyze and compare the systemic levels of Ang2 and VEGF-A in patients with neovascular AMD treated with intravitreal injections of aflibercept or ranibizumab. To the best of our knowledge, there are no published data from a prospective series on the systemic response of Ang2 to the blockage of key mediators by the currently most potent intravitreal anti-VEGF drugs.

Materials and Methods

Subjects
This prospective, randomized, case series study was conducted according to the tenets of the Declaration of Helsinki. The experimental and informed consent procedures were approved by the institutional review committee of the Medical University of Innsbruck (Innsbruck, Austria). Written informed consent for participation was obtained from all patients after explanation of the nature and possible consequences of the study.

At the time of diagnosis and inclusion, all patients had been treatment-naive for at least 8 months. At patient inclusion, AMD was diagnosed and classified by a retinal specialist by a fundus examination and optical coherence tomography (Heidelberg Spectrals®; Heidelberg Engineering, Heidelberg, Germany). After inclusion, 38 patients with nAMD were randomized by permuted block randomization to aflibercept or ranibizumab therapy. Each participant received a loading dose of three intravitreal injections (IVI) of aflibercept (2.0 mg/50 µl) or ranibizumab (0.5 mg/50 µl) for three consecutive months as a part of the treatment. Blood samples were collected 7 and 28 days after the first injection. Follow-up and clinical evaluations were performed 4 weeks after the third injection. Patients with a reduction in the central macular thickness by ≤50 µm were defined as non-responders.

Exclusion criteria were chorioretinal abnormalities, history of vitreotomacy or uveitis, systemic inflammatory comorbidities, hypertension, diabetes mellitus (DM), systemic vasoproliferative disorders, treatment with anti-inflammatory medications such as steroids or nonsteroidal anti-inflammatory drugs (NSAID), dialysis therapy, a history of cancer or previous treatment of cancer (NSAID), dialysis therapy, a history of cancer or previous treatment of cancer.

Statistical analyses
All statistical analyses were performed using SPSS Statistics 24® (IBM, Armonk, NY, USA). Sample size estimation was carried out using a two-sided test. Previous data of VEGF-A levels in patients receiving aflibercept and ranibizumab were utilized for this purpose. Using type I error of α = 0.05 and type II error of 10% (meaning 1–ß that is power of 90%) at a standard deviation of 22 pg/ml, we estimated a sample size of 20 per treatment group to achieve a medium effect size (r > 0.4).

The Kolmogorov–Smirnov test was utilized to evaluate all variables for normal distribution. All continuous data (Ang2 and VEGF-A) with the exception of age were not normally distributed. Continuously distributed data are reported as means with standard deviation or medians with interquartile range (IQR). To compare baseline characteristics between the treatment groups and control group, the analysis of variance (ANOVA) was used for normally distributed data, whereas the Kruskal–Wallis test was applied for non-normally distributed data. Mann–Whitney U-test was used for comparisons of non-normally distributed data. ANOVA using SPSS Statistics 24®.

Blood sample collection
For the enzyme-linked immunosorbent assay (ELISA), blood samples were collected 1–3 h before as well as 7 and 28 days after the first IVI injection. Blood samples were drawn by venipuncture with minimal stasis and collected into di-potassium-ethylenediaminetetraacetic acid (K2EDTA) tubes. Centrifugation was performed at 1000 g for 20 min, and plasma was stored within 2 h of collection at ~80°C, until it was subjected to ELISA.

Analysis of VEGF-A and Ang2 levels in plasma samples by ELISA
Systemic concentrations of free Ang2 and VEGF-A were determined by ELISA (Quantikine ELISA Kit, R&D Systems Europe, Abingdon, OX14 3NB, UK, DANG20 for Ang2 and DVE00 for VEGF-A) as described by the manufacturer. Briefly, 100 µl assay diluent was added to each well of 96-well polystyrene microplates; then, a standard, control or sample (EDTA-plasma, diluted 1:5 for Ang2 or undiluted for VEGF-A) was added and incubated for 2 h at room temperature on a horizontal orbital microplate shaker. Afterwards, washing was performed three times followed by the addition of 200 µl substrate solution to each well and incubation for 25 min. Finally, the enzymatic substrate reaction was terminated by stop solution. The concentration was measured by a Multiskan Multisoft ELISA reader (Labsystems Diagnostics Oy, Helsinki, Finland) at 450 nm with wavelength correction at 540 nm. The concentrations were calculated on the basis of the standard curve.
distributed continuous variables between the treatment groups. Categorical data were compared utilizing chi-square and Fisher’s exact tests between treatment groups and control group. Pearson’s correlation or Spearman rank correlation coefficients were calculated to analyze correlations between parameters. Within treatment group comparisons were performed using the Friedman and Wilcoxon signed-rank tests for non-normally distributed variables and paired sample t-test for normally distributed data between treatment groups. A p value of <0.05 was considered statistically significant in all analyses.

Results
The treatment and control groups did not differ significantly with respect to age (p = 0.445, ANOVA) and sex distribution (p = 0.489, chi-squared test). Further, there were no significant differences between the pretreatment systemic levels of VEGF-A (p = 0.086, Kruskal–Wallis test) and Ang2 (p = 0.280) of patients randomized to aflibercept or ranibizumab and the control group. Further details on the demographic and cytokine levels are shown in Table 1.

Ang2 concentration

The median (IQR) systemic concentration of Ang2 was 1375 (1134–2007) pg/ml in the control group. In the ranibizumab group, a statistically non-significant increase of systemic Ang2 to 1662 pg/ml was observed. It remained stable at 7 and 28 days with 1806 pg/ml (p = 0.472, Wilcoxon signed-rank test) and 1787 pg/ml (p = 0.653), respectively (Fig. 1).

At 7 days after the aflibercept injection, a significant feedback upregulation of systemic Ang2 was observed with a median (IQR) of 2123 (1441–3769) pg/ml. An increase of Ang2 was noted in 73% (14 of 19) of patients (p = 0.011). After 28 days, the systemic concentration of Ang2 remained increased (median 1944; IQR 1431–2546 pg/ml), but no statistical significance was observed compared to the pretreatment concentration (p = 0.660). Systemic Ang2 concentrations did not differ significantly across the treatment groups at either 7 days (p = 0.463, Mann–Whitney U-test) or 28 days (p = 0.557) (Fig. 1).

A post hoc subgroup analysis of responders and non-responders who received intravitreal aflibercept therapy revealed no differences in the baseline characteristics (Table 2 and Fig. 1). The Ang2 levels of these two groups differed significantly at 28 days (median 1601; IQR 1262–2194 pg/ml, 2546; IQR 1900–3464 pg/ml respectively) (p = 0.037) after therapy. There was no difference of systemic Ang2 levels between responders and non-responders who received ranibizumab (p = 0.365).

VEGF-A concentration

In the ranibizumab group, the median (IQR) VEGF-A baseline concentration was 61 (39–81) pg/ml, and it did not change significantly at 7 and 28 days after the first injection with 55 (42–77) pg/ml (p = 0.740) and 59 (43–96) pg/ml (p = 0.670), respectively.

In the aflibercept group, a significant decrease of systemic VEGF-A levels was observed from a median (IQR) VEGF-A of 40 (30–57) pg/ml at baseline to a concentration below the minimal detection level of 9 pg/ml (p < 0.0001 – 99% CI 0.0–0.0) at 7 days and 16 (8–26) pg/ml (p < 0.001) at 28 days after the first injection. There was a significant difference across treatment groups at 7 days (p < 0.0001 – 99% CI 0.0–0.0) and 28 days (p < 0.0001 – 99% CI 0.0–0.0) after the first injection.

Discussion

Besides VEGF-A, various cytokines have been implicated in the promotion of neovascularization (Campochiaro 2015). Although great success has been achieved in the suppression of pathologic neovascularization since the introduction of anti-VEGF drugs, critical reports of proangiogenic escape mechanisms after VEGF therapy have emerged (Itatani et al. 2018). Recent translational research in oncology found an upregulation of Ang2 and vascular Tie2 after anti-VEGF treatment in therapy-resistant or recurrent tumours (Rigamonti et al. 2014; Labussiere et al. 2016). These studies suggested evasive tumour resistance to anti-VEGF drugs, which may involve the adaptive enforcement of Ang2/Tie2 signalling and promote a reversal by Ang2 neutralization. Further studies, describing high expression of Ang2 in

Table 1. Baseline characteristics and systemic cytokine levels determined using enzyme-linked immunosorbent assay.

|                      | Aflibercept | Ranibizumab | p Value | Control | p Value |
|----------------------|-------------|-------------|---------|---------|---------|
| Age (years)          | 77 ± 9.8    | 77 ± 9.8    | 1.0     | 74 ± 7.7 | 0.481   |
| Sex (m/f)            | 6/12        | 6/12        | 1.0     | 11/8    | 0.162   |
| BCVA, logMAR         | 0.7 (0.4–1.1) | 0.9 (0.6–1.05) | 0.294 | –       | –       |
| CMT, mm              | 396 (312–489) | 413 (365–534) | 2.84    | –       | –       |
| VEGF-A at baseline, pg/ml | 40 (30–57)     | 61 (39–81)    | 0.03*   | 47 (33–110) | 0.083   |
| VEGF-A after 7 days, pg/ml | 8 (8–8; p < 0.001*) | 55 (42–77; p = 0.816) | <0.001* | –       | –       |
| VEGF-A after 28 days, pg/ml | 16 (8–26; p = 0.001*) | 59 (43–96; p = 0.670) | <0.001* | –       | –       |
| Ang2 at baseline, pg/ml | 1819 (1262–3099) | 1662 (1360–2471) | 0.707 | 1375 (1134–2007) | 0.280   |
| Ang2 after 7 days, pg/ml | 2123 (1441–3769; p = 0.011*) | 1806 (1507–2718; p = 0.472) | 0.463 | –       | –       |
| Ang2 after 28 days, pg/ml | 1944 (1431–2546; p = 0.660) | 1787 (1358–2373; p = 0.653) | 0.557 | –       | –       |

Ang2 = angiopoietin-2, BCVA logMAR = best corrected visual acuity given as a logarithm of the minimum angle resolution; CMT = central macular thickness.

Values are presented as means with standard deviations or medians with interquartile ranges.

* Statistical significance (p < 0.05) between groups and within groups.
pathological neovascularization (Moon et al. 2003; Wang et al. 2007; Labussière et al. 2016) indicated that Ang2 may be strongly involved in an escape mechanism from anti-VEGF therapy. The systemic change of Ang2 after intravitreal anti-VEGF therapy has not been studied, yet. Therefore, we examined systemic levels of this cytokine after intravitreal exposure to anti-VEGF therapy.

In the present study, a significant systemic upregulation of Ang2 was found 7 days after an intravitreal aflibercept injection. In a further subgroup analysis of the aflibercept group, non-responders showed significantly higher systemic Ang2 levels than responders at 28 days after the first injection. The upregulation of Ang2 upon anti-VEGF therapy could represent a systemic counterregulatory response to antiangiogenic therapy. No changes of systemic Ang2 and VEGF-A were observed in the ranibizumab group. The different pharmacokinetics, molecular weight and structure of intravitreal aflibercept and ranibizumab provide a rationale for the lack of systemic response in patients treated with ranibizumab. Ranibizumab is a recombinant humanized IgG1 antibody fragment designed for intraocular use, which lacks a Fc region. In contrast, aflibercept was

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**Table 2.** Comparison of responders and non-responders after receiving intravitreal aflibercept therapy.

|                | Aflibercept Responders, n = 13 | Non-responders, n = 6 | p Value |
|----------------|--------------------------------|-----------------------|---------|
| BCVA, logMAR   | 0.52 (0.40–1.05)                | 0.70 (0.41–1.10)      | 0.898   |
| CMT, µm        | 426 (310–490)                   | 349 (310–429)         | 0.462   |
| Ang2 at baseline, pg/ml | 1422 (1223–3203)               | 1979 (1692–3932)      | 0.301   |
| Ang2 after 7 days, pg/ml | 1878 (1204–3694)               | 2600 (1871–5421)      | 0.216   |
| Ang2 after 28 days, pg/ml | 1601 (1262–2194)               | 2546 (1900–3464)      | 0.037*  |

Ang2 = angiopoietin-2, BCVA logMAR = best corrected visual acuity given as logarithm of minimum angle resolution, CMT = central macular thickness. Values are presented as mean with standard deviation or median with interquartile range. * Statistical significance (p < 0.05) between the groups.
designed for intraocular injection and systemic infusion (Stewart 2011). It combines the VEGF receptors 1 and 2 and an Fc antibody fragment, which is likely to interact with FcRn. The FcRn has the ability to actively transport aflibercept across the blood–retina barrier leading to higher aflibercept concentration in the systemic circulation than the concentration of ranibizumab. Upon reaching the blood stream, the systemic half-life of ranibizumab is approximately 2 h, whereas aflibercept recycling and rescue from catabolism by the FcRn extends the half-life of aflibercept up to 6 days (Holash et al. 2002; Roopenian & Akiles 2007). These specific pharmacodynamic properties underlie the possibility to estimate changes in the intraocular angiogenic milieu by investigating off-target effects in the systemic circulation (Avery et al. 2014).

Tie2 was identified as a receptor tyrosine kinase expressed exclusively on endothelial cells of blood and lymphatic vessels. The receptor is specifically activated by phosphorylation of the angiopoietin ligand family, of which Ang1 and Ang2 are best known (Eklund & Saharinen 2013). The angiopoietin/Tie2 pathway is indispensable for vascular development and function. Activation of Tie2 signalling by the growth factor Ang1 exerts vasoprotective effects by inhibition of endothelial cell apoptosis (Kim et al. 2000), anti-inflammatory activity associated with prevention of plasma leakage (Gamble et al. 2000) and maturation and stabilization of vessels by promotion of vascular endothelial cell migration and remodelling (Baffert et al. 2004). Under hypoxia or oxidative stress, the proangiogenic cytokine Ang2 that competitively binds to the Tie2 receptor on endothelial cells is upregulated (Benest et al. 2013). The dominant model proposes that Ang2 competes with Ang1 to bind to the Tie2 receptor and thereby inhibit the Tie2 pathway, leading to loss of endothelial cell junction integrity, vascular destabilization, disruption of the blood–retinal barrier, leakage and facilitation of VEGF-dependent angiogenesis (Holash et al. 1999a; Holash et al. 1999b; Augustin et al. 2009). The increased expression of Ang2 in the presence of VEGF suggests a close interaction of these cytokines in the development of pathological neovascularization (Minami et al. 2013).

In fact, preclinical oncological studies described an additional benefit of tumour vessel regression by the blockade of Ang2 under conditions of complete or specific VEGF blockade. Intriguingly, combined therapy resulted in a more dramatic regression of tumour vascularity than treatment with either agent alone (Hashizume et al. 2010; Daly et al. 2013). These findings are further supported by a recent clinical report that showed a worse response to anti-VEGF treatment in the presence of high Ang2 serum levels (Goede et al. 2010). Preclinical ophthalmologic models also confirmed a more effective reduction of vessel leakage and CNV numbers by a combined therapy with anti-VEGF and Ang2 than by either agent alone (Regula et al. 2016; Wolf & Langmann 2019). A close interaction of VEGF and Ang2 is further supported by recent findings of elevated Ang2 levels in the vitreous bodies of eyes with nAMD and their association with disease severity, increased central macular thickness and reduced best corrected visual acuity (Regula et al. 2016; Ng et al. 2017). In addition, a study by Cabral et al. reported increased vitreous Ang2 levels after intravitreal therapy with bevacizumab, suggesting a maintained angiogenic stimulus despite VEGF blockade (Cabral et al. 2018). Persistent disease activity after nAMD treatment, as reflected by recurrent leakage, may be attributed to compensatory mechanisms of other proangiogenic factors that promote an escape and ensure a survival of the neovascular tissue in the presence of VEGF inhibition. A selective therapeutic interference of a member of the proangiogenic circuit during neovascularization treatment is likely to result in the compensatory upregulation of other factors involved in this disease. This may involve converse regulatory effects that contribute to a limited therapeutic efficacy. The benefit of anti-VEGF therapy could be compromised through aberration of vessel normalization by Ang2, thereby contributing to a rebound mechanism of vascular leakage in concordance with the renewed rise of VEGF levels in the vitreous body after therapy (Chae et al. 2010). The potential of Ang2 as a target in nAMD therapy has led to randomized clinical trials simultaneously targeting VEGF-A and Ang2.

Faricimab (formerly known as RG7716) is a bispecific humanized immunoglobulin G (IgG) monoclonal antibody, selectively blocking VEGF-A and Ang2. A phase II clinical trial was initiated for nAMD (STAIRWAY) and compared 6.0 mg faricimab administered in masked fashion every 12 or 16 weeks after a loading dose to 0.5 mg ranibizumab administered every 4 weeks. Promising results were observed of increased faricimab durability up to 16 weeks and a comparable central subfoveal thickness in both treatment arms. These findings demonstrated the potential role of simultaneous Ang2 and VEGF-A neutralization and led to the initiation of pivotal phase III clinical trials (Chakravarthy et al. 2017; Khandani et al. 2020).

There are several limitations to the study. First, the concentrations of Ang2 and VEGF-A were obtained from blood samples and not from aqueous humour or neovascular tissue. Since systemic aflibercept concentration is tremendously exceeded by intraocular aflibercept concentration after an intravitreal injection (Avery et al. 2014; Do et al. 2019), we expect the results of the present study to underestimate the intraocular response of Ang2 to anti-VEGF therapy. Nonetheless, the current data should be interpreted with caution. First, there are no published data analysing the association between intraocular and systemic Ang2 levels after intravitreal anti-VEGF therapy. Second, as the first blood sample was collected 7 days after therapy, early changes in the systemic angiogenic milieu could be overseen. However, recent results have shown that the total systemic aflibercept concentration peaks 7 days after an intravitreal injection and steadily declines afterwards. Thus, we expect a maximum effect on systemic cytokines 7 days after therapy (Kaiser et al. 2019). Third, K$_2$EDTA was used for blood sample collection. K$_2$EDTA could potentially cause a bias towards higher VEGF-A levels by incomplete suppression of VEGF release from thrombocytes, which raises the possibility of VEGF-A contamination (Walz et al. 2016). Nevertheless, systemic VEGF-A was below the minimal detection level at 7 days and remained significantly reduced at 28 days after an intravitreal injection of aflibercept. However, this limitation does not apply
to the determination of systemic Ang2, which is not affected by platelet activation, as it is derived from endothelial cells (Brouwers et al. 2013). The strengths of the study are its prospective study design and randomized antiangiogenic treatment allocation.

Although current anti-VEGF therapy provides transient control of neovascular AMD, there is still an urgent need to better understand VEGF-independent pathways leading to recurrent disease activity in nAMD. Increased Ang2 levels upon an intravitreal injection of aflibercept could potentially exert a countervailing trophic effect on the neovascular membrane, increasing its susceptibility to VEGF and thus limiting the therapeutic effect duration. Promising new substances are currently emerging driven by the demand to further decrease vascular AMD, there is still an urgent disease activity in nAMD. Potentially exert a countervailing counteracting potential for angiogenic agents in nAMD treatment. We are looking forward to the results of the upcoming phase III trials TENAYA (NCT03823287) and LUCERNE 8 (NCT03823300) of the bispecific anti-VEGF/Ang2 inhibitor faricimab.

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Correspondence:
Claus Zehetner, MD
Medical University Innsbruck
Department of Ophthalmology
Anichstrasse 35
6020 Innsbruck
Austria
Tel: +43 512 504 82284
Fax: +43 512 504 23722
Email: claus.zehetner@i-med.ac.at

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