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LABORATORY STUDY

Multiphasic changes in systemic VEGF following intravitreal injections of ranibizumab in a child

EH Shao[1,2], V Sivagnanavel[3], A Dabbagh[3], R Dave[3], S Tempest-Roe[1], FWK Tam[1] and SR Taylor[1,2]

Abstract

Purpose **To investigate whether intravitreal ranibizumab injections administered to a child alter systemic plasma levels of total and free VEGF 165.**

Methods **A 9-year-old child sustained a choroidal rupture from blunt trauma. He subsequently developed a secondary choroidal neovascular membrane, which was treated with five ranibizumab injections over a period of 8 months. Peripheral venous blood samples were taken at each visit over a period of 12 months and plasma was extracted. Plasma VEGF 165 levels were determined using enzyme-linked immunosorbent assay and were assayed both pre- and post-immunodepletion to remove complexed VEGF.**

Results **Plasma VEGF 165 levels proved labile following intravitreal injection of ranibizumab. Levels increased by 30% above baseline following the first intravitreal ranibizumab injection, but then returned to baseline despite two subsequent injections. There was then a rebound increase of 67% in total plasma VEGF levels following a further injection, which remained above baseline for 12 weeks despite two further intravitreal ranibizumab injections. Baseline levels were re-attained 26 weeks after the final injection.**

Conclusions **These results suggest intravitreal ranibizumab injections can cause significant, multiphasic changes in systemic VEGF levels. This may be of particular clinical significance in children as VEGF is known to be vital in the development of major organs, in addition to its role in the maintenance of normal organ function in adults.**

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Introduction

Anti-vascular endothelial growth factor (VEGF) agents have become widely used in the treatment of macular degeneration and retinal vascular disease and have revolutionized clinical outcomes.[1] However, VEGF is one of the most potent cytokines and has an essential role in angiogenesis, giving it an important role in the normal development of the brain, lung, and kidney tissue in children,[2] as well as in maintaining normal organ function in adults.[3–5] However, the effects of intraocular administration of anti-VEGF drugs on systemic levels remain controversial.[1] Different anti-VEGF agents also have different molecular structures, different transmissibility across the blood–retinal barrier, and different systemic half-lives, so could potentially have different effects on systemic VEGF levels.[1,6–9]

Ranibizumab is a recombinantly produced, humanized monoclonal antibody fragment (Fab) designed for intraocular use that binds and inhibits all biologically active isoforms of human VEGF.[10] Its transmissibility across the blood–retinal barrier is lower than that of full-length molecules, but systemic bioavailability of ranibizumab has been reported post intravitreal injections.[11] However, the effect of systemic leakage of intraocularly delivered anti-VEGF agents is poorly understood, and unpredictable changes in systemic VEGF levels have been reported.[9,12] Although systemic levels might be thought to decrease on administration of an

[1]Faculty of Medicine, Imperial College London, Hammersmith Hospital Campus, London, UK

[2]Royal Surrey County Hospital NHS Foundation Trust, Egerton Road, Guildford, UK

[3]Kingston Hospital NHS Foundation Trust, Galsworthy Road, Kingston-upon-Thames, Surrey, UK

Correspondence: SR Taylor, Faculty of Medicine, Imperial College London, 5N8B Commonwealth Building, Hammersmith Hospital Campus, London W12 0NN, UK
Tel: +44 20 8383 2394; Fax: +44 20 838 2394.
E-mail: s.r.taylor@imperial.ac.uk

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anti-VEGF agent, studies in the oncology literature frequently report intravenous administration of bevacizumab leading to a counterintuitive increase of plasma VEGF.[13–16] Indeed, one randomized controlled study of 116 patients with renal cell carcinoma showed a consistent rise in plasma VEGF in those taking low-dose (3 mg/kg) bevacizumab, but not in those taking a placebo;[12] the mechanism of this has not yet been elucidated.[12] Although ranibizumab has a shorter systemic half-life than bevacizumab, it binds to the same bioactive isoforms of human VEGF as bevacizumab and with greater affinity.[17]

There are few controlled studies examining systemic VEGF levels following intravitreal ranibizumab administration, and uncontrolled studies have reported mixed results. The randomized controlled trial of alternative treatments to Inhibit VEGF in Age-related choroidal Neovascularization (IVAN) study, the largest study to date of serum VEGF levels in age-related macular degeneration (AMD) patients, reported a decrease in serum VEGF levels with both intravitreal ranibizumb and intravitreal bevacizumab at 1 year compared with baseline, and a greater reduction with continuous treatment with both ranibizumab and bevacizumab versus discontinuous treatment. However, by measuring serum rather than plasma VEGF, a significant portion of the VEGF measured would include VEGF released by platelets, which artificially increases systemic levels of VEGF measured and may mask subtle changes in VEGF levels.[7,18] Subsequent studies measuring plasma VEGF have also reported significant decreases in plasma VEGF following intravitreal bevacizumab, and non-statistically significant decreases in plasma VEGF following intravitreal ranibizumab,[5–9] which supports the IVAN findings, but remains at odds with data from the oncology literature.

One of the reasons why results might differ is that VEGF biology is inherently complex and measuring techniques have limitations. ELISAs are unable to differentiate between bevacizumab–VEGF and ranibizumab–VEGF complexes and free VEGF, so it is possible for functional uncomplexed VEGF levels to fall while total VEGF levels remain the same or increased.[19] In this study, we analysed changes in both total (bound and unbound) and free VEGF levels in a child with a choroidal rupture from blunt trauma who developed a secondary choroidal neovascular membrane and was treated with a course of ranibizumab injections.

Materials and methods

A 9-year-old child sustained a choroidal rupture from blunt trauma and subsequently developed a secondary subfoveal choroidal neovascular membrane at the macula with intraretinal haemorrhage and oedema causing visual loss. He was treated with five ranibizumab injections over a period of 8 months, reinjection being guided by either the loss of visual acuity or the return of intraretinal fluid on optical coherence tomography (OCT) scanning.

Intravitreal injections were performed under general anaesthesia by a Consultant Ophthalmologist. Ranibizumab was given at a dose of 0.5 mg in 0.05 ml through a 30-gauge needle under sterile conditions after flushing of the conjunctival sac with 5% povidone-iodine. The injection site was compressed for several seconds to avoid reflux when the needle was removed, and no anterior chamber paracentesis was performed. Peripheral venous blood samples were either taken at the same time or under local anaesthesia using EMLA cream. The blood samples were immediately centrifuged at 3000 r.p.m. for 10 min to separate out plasma. Plasma fractions were divided in two aliquots and stored at –70 °C until analysis.

One-half of the split samples were subjected to an immunodepletion protocol using Protein G-Sepharose 4 Fast Flow beads (Pharmacia Biotech, Uppsala, Sweden) as previously described.[19,20] Protein G-Sepharose 4 Fast Flow beads were washed three times in phosphate buffered Saline (PBS) before being reconstituted to 50% (v/v) protein G-Sepharose in PBS. To deplete plasma samples of the ranibizumab immunoglobulin antibody and ranibizumab-bound VEGF, 100 μl of protein G slurry (50% v/v protein G-Sepharose in phosphate-buffered saline) was added to 200 μl of plasma and incubated with gentle mixing at 10 °C for 10 h. After centrifugation (2 min at 10 000 r.p.m.), 200 μl of plasma supernatants were removed.

Both plasma samples were then assayed in triplicate for VEGF 165 levels using a human ELISA kit purchased from R&D Systems (Duoset; R&D systems, Minneapolis, MN, USA). This kit detects VEGF 165, VEGF 165b, and VEGF 121 in cell culture supernants and plasma with a lower limit of detection of 16 pg/ml.

Results are reported as the mean and SD. Statistical significance was assessed using the Student's paired two-tailed *t*-test (SPSS version 20, IBM, New York, NY, USA) with the level of significance being set at $P < 0.05$.

This study followed the Declarations of Helsinki. Parental informed consent for this study was obtained, and this study was approved by the R&D department of the Royal Surrey County Hospital (RSCH/DEV/0018).

Results

Total and free VEGF 165 levels are shown in Figure 1.

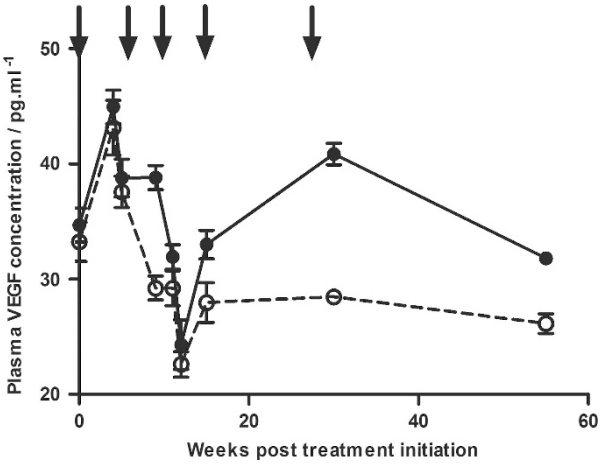

Figure 1 Changes in total plasma VEGF levels (solid line) and free plasma VEGF levels (broken line) as measured by ELISA on non-immunodepleted and immunodepleted plasma, respectively (pg/ml). Arrows indicate the time points of intravitreal injections of 0.5 mg ranibizumab in 0.05 ml.

Total VEGF 165 levels

Total plasma VEGF 165 levels (including both ranibizumab–VEGF complexes and free VEGF 165) significantly increased following the first intravitreal ranibizumab injection from 34.7 ± 2.5 pg/ml to 44.9 ± 2.5 pg/ml ($P = 0.02$) but subsequently declined despite two further injections to 24.3 ± 3.7 pg/ml. There was a statistically significant rebound increase in total plasma VEGF levels at week 12 to 40.9 ± 1.6 pg/ml ($P = 0.008$) before a return to baseline at 12 months. There was no difference between initial and final VEGF levels (34.7 ± 2.5 pg/ml *vs* 31.8 ± 0.7 pg/ml; $P = 0.14$).

Free VEGF 165 levels

Free VEGF 165 levels initially followed a similar pattern, with a statistically significant increase of 30% from 33.2 ± 2.9 pg/ml to 43.1 ± 4.1 pg/ml ($P = 0.02$) followed by a decline to a below baseline level of 22.6 ± 1.9 pg/ml. However, there was no subsequent rebound increase, and free VEGF 165 levels did not exceed baseline levels by the end of the study. There was no difference between initial and final free VEGF levels (33.2 ± 2.9 pg/ml *vs* 26.1 ± 1.5 pg/ml; $P = 0.10$).

There was no significant difference between VEGF levels measured in depleted and undepleted plasma at baseline (34.7 ± 2.5 pg/ml *vs* 33.2 ± 2.9 pg/ml; $P = 0.287$). The difference was the greatest at week 30 and reached statistical significance at this stage (40.9 ± 1.6 pg/ml *vs* 28.5 ± 0.3 pg/ml; $P = 0.005$).

No ocular or systemic adverse events were reported during the treatment course.

Discussion

Our study suggests that intravitreal ranibizumab injections can result in changes in systemic VEGF levels in children, and that these changes are complex and multiphasic. It is unlikely that these effects were due to variation in drug-dose delivery, as studies have shown that 30-gauge needles result in negligible reflux.[21] Studies of normal VEGF levels in children have been limited, but VEGF 121 levels have been shown to increase around the time of puberty.[22]

The importance of potential systemic leakage of intravitreally delivered anti-VEGF agents has driven some of the debate regarding the use of intravitreal ranibizumab and bevacizumab. Ranibizumab is less able to cross the blood–retinal barrier and has a shorter systemic half-life, which was hoped to reduce its systemic side-effect profile.[8] Indeed, the CATT and IVAN studies of bevacizumab *vs* ranibizumab in patients with age-related macular degeneration showed smaller reductions in systemic VEGF levels with intravitreal ranibizumab than with bevacizumab.[23] However, systemic VEGF levels still decreased with intravitreal ranibizumab, with greater reductions in continuous versus discontinuous therapy, indicating that there is still systemic leakage of ranibizumab and the possibility of systemic side effects.[24] Although there were fewer numbers of serious adverse events in the ranibizumab groups, these proved not to be the side effects such as arterial thromboembolism, which were predicted by both the drugs' mechanism of action and the cancer literature, but instead events such as gastrointestinal haemorrhages.

These findings may be of particular significance in some groups of patients—VEGF is known to be important in the development of organs during childhood and also has a role in renal function, which is likely to be impaired in patients with diabetic retinopathy. Animal studies have shown that vascular endothelial growth factor-receptor 1 (VEGFR1) inhibition in db/db mice aggravated diabetic nephropathy, including albuminuria, glomerular mesangial matrix expansion, inflammatory cell infiltration, profibrotic TGF-β1 expression, and increase in number of apoptotic glomerular cells, in comparison with control db/db mice.[25]

Diabetic retinopathy and nephropathy are both microvasular complications of diabetes mellitus, and the concurrence of nephropathy and diabetic retinopathy in type I diabetes mellitus is well reported

in literature,[26,27] including a study of 285 patients in which significant association between diabetic retinopathy and preclinical morphologic changes of diabetic nephropathy in type 1 diabetic patients was demonstrated.[28] Recently, a study involving over a thousand type 2 diabetes patients with sight threatening diabetic retinopathy indicated a hazard ratio of 2.5 for coexisting microalbuminuria and of 14 for macroalbuminuria.[29,30] Thus, significant changes in systemic VEGF as a result of intravitreal ranibizumab delivery may be of potential clinical significance, given that ranibizumab is NICE approved and licensed for the treatment of diabetic maculopathy.[31]

It is possible that ranibizumab–VEGF complexes act in a similar manner systemically in comparision with bevacizumab–VEGF complexes. Interestingly, our results are in keeping with a previous study of cancer patients by Gordon *et al*[13] who reported that intravenous injection of bevacizumab leads to an increase in serum total VEGF, but a reduction in free VEGF concentration, presumably due to circulating bevacizumab–VEGF complexes. This mechanism is supported by the work of Hsei *et al*,[32] who found that systemic VEGF clearance in rats decreases threefold in the presence of systemic bevacizumab, despite the clearance and terminal half-life remaining unchanged. Our findings of a delayed increase in total VEGF levels in the presence of normal free VEGF levels are consistent with these observations.

However, the phenomenon of counterintuitive increases in total VEGF levels is also seen following treatment with VEGF tyrosine kinase inhibitors,[32,33] suggesting that antibody complex formation may not be the only mechanism for such rebound total VEGF increases. It has been theorized that alternate pathways are activated by the injection of bevacizumab, such as the accumulation of hypoxia-inducible factor[32] or a decrease in VEGF degradation by proteases.[12]

Taken together, our results suggest intravitreal ranibizumab injections can cause significant, multiphasic and prolonged alterations in systemic VEGF levels. Our study is clearly limited by being based on a single paediatric case, but does suggest that systemic exposure needs to be taken into account when considering intravitreal therapy in high-risk groups. An obvious case in point would be the potential systemic side-effects of intravitreal anti-VEGF drugs are delivered to neonates with retinopathy of prematurity, but caution would be needed before extrapolating our results to this group as the pharmacokinetics are likely to differ owing to the differing anti-VEGF levels achieved in the vitreous of neonatal eyes.

Summary

What was known before

- The oncology literature has found a counterintuitive rebound increase in systemic VEGF levels with the administration of bevacizumab, whereas the IVAN trial reported a decrease in systemic VEGF levels in contrast to the oncology literature. The measuring of systemic VEGF levels is complex as ELISAs are unable to differentiate between bound (complexed) VEGF and free VEGF. Similarly, measuring serum rather than plasma VEGF, as in the IVAN trial, also includes VEGF released by platelets, which may affect the results.

What this study adds

- We used immunodepleted plasma samples with the aim of measuring free and unbound VEGF without the confounding effects of VEGF release by platelets, to enable a more accurate understanding of systemic VEGF changes from intraocular VEGF blockade. Plasma VEGF 165 levels proved labile following intravitreal injection of ranibizumab. Levels increased by 30% above baseline following the first intravitreal ranibizumab and remained above baseline for 12 weeks despite two further intravitreal ranibizumab injections. Baseline levels were re-attained 26 weeks after the final injection. These results suggest intravitreal ranibizumab injections can cause significant, multiphasic changes in systemic VEGF levels.

Conflict of interest

SRJT is supported by the UK National Institute of Health Research. SRJT has received advisory board fees from Novartis AG. ST-R receives funding from BBSRC and GlaxoSmithKline. FWKT receives funding from Baxter.

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