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Potential antiedematous effects of intravitreous anti-VEGF, unrelated to VEGF neutralization

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The intravitreous injection of therapeutic proteins that neutralize vascular endothelial growth factor (VEGF) family members is efficient to reduce macular edema associated with wet age-related macular degeneration (AMD), retinal vein occlusion (RVO) and diabetic retinopathy (DR). It has revolutionized the visual prognosis of patients with macular edema. The antiedematous effect is dependent on an intravitreous dose of drug, which varies between patients and requires frequent and repeated injections to maintain its effects. At the time when optimizing the duration of anti-VEGF effects is a major challenge, understanding how anti-VEGF reduces macular edema is crucial. We discuss herein how anti-VEGF exerts antiedematous effects and raise the hypothesis that mechanisms, unrelated to VEGF neutralization, might have been underestimated.

Introduction

More than 20 years after VEGF was recognized as a major actor in retinal and choroidal neovascularization, anti-VEGFs have revolutionized ophthalmological practice and the visual prognosis of the major retinal blinding diseases. The natural evolution of wet AMD has radically changed after the pivotal Marina study reported in 2006 that ~90% of patients treated with monthly injections of ranibizumab maintained their vision (<15 letters loss) at 1 and 2 years, compared with 60% in the untreated control group. More impressively, ~30% of patients gained 15 letters in the treated group compared with only 5% in the untreated group at 1 year [1]. Twelve years later, several trials have confirmed the efficacy and safety of different proteins designed to neutralize VEGF (ranibizumab, bevacizumab, and aflibercept) in the treatment of wet AMD [2]. Anti-VEGF also efficiently reduced macular edema associated with DR [3] and RVO [4]. Although the clinical benefit of anti-VEGF-neutralizing proteins is undeniable, the exact mechanisms of their antiedematous effects in various forms of macular edema are not fully understood. A deeper clarification of these mechanisms could help identify novel molecular downstream targets, potentially accessible to therapeutic modulation. Here, we discuss these mechanisms and suggest that part of the antiedematous effects of intravitreously injected anti-VEGF proteins could be unrelated to VEGF neutralization.

Anti-VEGF drugs reduce vascular permeability

Most of the antiedematous effects of anti-VEGF in the retina are thought to result from a decrease in VEGF-induced vascular permeability, which, by reducing fluid entry in the retina, subsequently reduces macular edema. Two of the three anti-VEGF agents commonly used for intravitreous injections are approved for ophthalmic indications, ranibizumab and aflibercept, whereas bevacizumab is used off-label to reduce macular edema of various origins, but more commonly associated with choroidal and retinal neovascularization, DR, and RVO. Bevacizumab is a humanized monoclonal 150 kDa antibody that binds to all isoforms of VEGF-A. Ranibizumab is a 48-kDa fab fragment with a 10-

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to 100-fold increased VEGF-binding affinity compared with bevacizumab. Aflibercept is a 115-kDa soluble decoy receptor comprising an all-human amino acid sequence of the second immunoglobulin (Ig) domain of human VEGFR1 and the third Ig domain of human VEGFR2 fused with the constant region (Fc) of human IgG1. Similar to bevacizumab and ranibizumab, aflibercept binds all isoforms of VEGF-A and, in addition, binds placental growth factor (PlGF) and VEGF-B [5]. All anti-VEGFs neutralize the propermeating effects of VEGF on endothelial cells. Indeed, upon binding to VEGFR2, VEGF-A destabilizes the tight junctions through the phosphorylation of occludin via PKC beta activation, through the downregulation of occludin expression by free cytosolic beta catenin increase and, through the internalization of occludin via SRC family kinase activation. VEGF also enhances the *trans*-endothelial permeability through endothelial nitric oxide synthase (eNOS)|-dependent caveola formations [6].

Arguments in favor of additional antiedematous mechanisms, unrelated to VEGF neutralization

Bevacizumab does not reach the choroid but still reduces macular edema in wet AMD

In retinal diseases, including DR or RVO, anti-VEGFs target retinal vasculopathy in the inner retinal layers, which are accessible to drugs injected into the vitreous. In wet AMD, choroidal neovascularization (CNV) grows from the choroidal vessels located under the retinal pigment epithelium (RPE). To target CNV, the drug injected into the vitreous should be able to cross all retinal layers to reach the site of pathologic angiogenesis. For this purpose, Genentech developed ranibizumab (48 kDa) based on the observation that, after intravitreous injection in the monkey eye, a full-length150-kDa anti-VEGF antibody poorly penetrated the RPE and the choroid [7]. Not only the inner limiting membrane, but also the external limiting membrane was a barrier to the antibody penetration; the latter membrane is also a filter to macromolecular diffusion across the retina, limiting further the diffusion of large stoke radius proteins [6]. Although bevacizumab does not reach its target site, it does reduce macular edema resulting from CNV and both ranibizumab and bevacizumab are currently used for intravitreous injections in the treatment of wet AMD with similar efficacy [8].

Anti-VEGF drugs do not act primarily as anti-angiogenic agents in wet AMD

All anti-VEGF drugs bind VEGF165 with high affinity and prevent VEGF proliferating activity on endothelial cells with IC_{50} values of 0.088 ± 0.032, 0.090 ± 0.009, and 0.500 ± 0.091 nM for ranibizumab, aflibercept, and bevacizumab, respectively [9]. *In vitro*, anti-VEGF molecules not only prevent endothelial cell proliferation [9] and vessel growth, but also reverse the angiogenic processes towards normal vasculature [10].

However, in AMD, despite repeated anti-VEGF injections, 80% of type 1 CNV (located under the RPE) continue to grow when evaluated by optical coherence tomography angiography (OCT-A) [11], although CNV lesion remodeling occurs shorty after intravitreous injections of anti-VEGF. Given that anti-VEGF agents do not eliminate the CNV despite years of intravitreous anti-VEGF injections, maintenance of treatment is required to reduce macular edema.

Correlation between anti-VEGF 'drying' effects and VEGF levels is variable

In a pure vascular disease, such as branch RVO (BRVO), VEGF vitreous levels poorly correlate with macular thickness and ~30% of patients do not have increased VEGF levels [12], although VEGF neutralization efficiently reduces macular edema associated with BRVO in >80% of cases [4]. In patients with diabetic macular edema, various studies have shown that intravitreous injections of anti-VEGF drugs efficiently neutralize VEGF and also significantly reduce other proedematous cytokines [intercellular adhesion molecule 1 (ICAM-1), interleukin (IL)6, and IL8] [13]. The decrease of such cytokines appears important because glucocorticoids that have limited or no effect on VEGF-A levels [14] significantly reduce proinflammatory and proedematous cytokines and chemokines through their transcriptional regulation of nuclear factor (NF)-κB, and have stronger 'drying' effects than anti-VEGFs in diabetic macular edema.

Systemic and intravitreous antiedematous effects of bevacizumab in wet AMD

The systemic injection of bevacizumab, which directly targets CNV, takes longer to reduce macular edema compared with the direct intravitreous injection of bevacizumab [15], which was shown to have poor penetration in the retina and limited access to the CNV. This suggests that the route of administration impacts the effect of bevacizumab.

Although bevacizumab does not neutralize rodent VEGF, its injection in rodent vitreous exerts ocular effects

Various biological effects of bevacizumab are constantly reported in rodent models of retinal diseases. Two recent publications reported antiangiogenic effects of intravitreous bevacizumab in the rat eye [16,17] although bevacizumab does not bind to rat VEGF and does not neutralize rat VEGF [18] suggests that non-VEGF-mediated effects result from the intravitreous injection of bevacizumab.

Proposed mechanisms

Change in oncotic pressure gradients

VEGF controls the fenestration of choroidal endothelial cells and anti-VEGF drastically reduces the number of fenestrations [19]. Given that fenestrations regulate the passage of proteins into the choroidal stroma [20], anti-VEGFs might reduce the protein concentration in the choroidal stroma and, thus, reduce its interstitial pressure and, subsequently, the volume of water in the choroid. This is evidenced by the reduction in choroidal thickness after anti-VEGF treatments.

By contrast, the vitreous is a low-protein-concentration media (~0.5 mg/ml) and injection of therapeutic proteins in the vitreous, from 0.3 mg for ranibizumab and up to 2 mg for aflibercept, creates a significant increase in the vitreous oncotic pressure. Given that the retina is permeable to water [21], it is expected that water accumulated in the retina might follow protein gradients, as in any other biological environment. Thus, the modification of protein gradients might have impact retinal thickness. This could explain the rapid change in retinal thickness and recurrence of macular edema when the concentration of therapeutic proteins in the vitreous decreases below a threshold, which can vary between different individuals, and with the underlying disease mechanisms.

Intravitreous injection of proteins induces rapid transcriptional regulations in the retina

In clinical studies, for obvious ethical reasons, subjects included in the control group are sham injected (they do not receive any injection). However, in all preclinical studies, control animals are treated with the injection of a 'vehicle' solution and also do not receive a neutralized form of the protein or an isotype control antibody or a control fab. Thus, the effect of a 'control' protein is never tested. Yet, important changes might be induced by the injection of a macromolecule into the vitreous, the physiological composition of which is low in protein. To explore this question, the effects of ranibizumab (Lucentis® 0.5 mg/m, Novartis, Switzerland) were compared with the same dose of a control fab (50 kDa, OAMA04119, Aviva Systems Biology) in 0.01% polysorbate 20 (to match the

concentration of polysorbate 20 used in Lucentis®) or to water at 24 h after injection in the vitreous of 8-week-old male Lewis rats. The volumes of injections were calculated to match the volume injected in a human vitreous, resulting in a final vitreous concentration of 0.1 mg/ml. A full pan-transcriptomic analysis of retinal transcripts was performed after 24 h, a time that is too short to observe a potential immune reaction of the rat to a human protein. All experiments were performed in accordance with the European Communities Council Directive 86/609/EEC and approved by local ethical committees. Total neuroretina RNA was extracted using the RNeasyMini Kit (Qiagen) including DNase I treatment. RNA integrity was checked on the Agilent 2100 Bioanalyser. At least three independent biological replicate samples were sequenced and used for downstream analysis. RNA sequencing was performed on Illumina HiSeq2000 platform. The average number of reads per sample was 27 M. Reads from each sample were processed as follows. First, reads were trimmed using an in-house Perl script with a minimum phred quality of 20 per vase and a minimum read length of 30 base pairs. On average, 24% of reads per sample were discarded. The resulting reads were later aligned to the Rattus norvegicus genome assembly 3.4 (from Ensembl) using Tophat version 2.0.10. At least 12 million reads were aligned to the genome for each sample. Gene expression was then quanitifed to obtain read count and FPKM values. The nonadjusted read counts for each gene were used for statistical calculation of global differential expression using DESeq2. Differentially expressed genes were selected at an adjusted P values of $\leq$0.05 and fold changes $>$1.5 [22].

At 24 h, 139 genes were significantly regulated by ranibizumab (six downregulated and 133 upregulated) and 225 genes were significantly regulated by the control fab (20 downregulated and 205 upregulated) [22]. As expected, 88% of the genes (122/139) regulated by ranibizumab were also regulated by the fab, given that ranibizumab does not neutralize rat VEGF. The genes regulated in the neuroretina were unrelated to VEGF neutralization. Among the regulated genes, 4% have known roles in angiogenesis (Fig. 1). *Angptl4*, encoding angiopoietin-like 4, was downregulated. Its aqueous level was recently shown to correlate with the response to ranibizumab [23]. The gene encoding angiotensin, an important player in retinal diseases [24], was also downregulated. By contrast, *Thbs1*, encoding thrombospondin 1, was upregulated by the fab; this is an important antiangiogenic factor in retinal diseases, and is decreased in eyes with wet AMD [25]. Several genes involved in the maintenance of tight junctions were upregulated, such as *Sphk1*, encoding sphingosine phosphate kinase 1 [26], and the gene encoding clusterin [27]. Expression of *Cav1*, encoding caveolin 1, was also downregulated, which could protect the blood–retinal barrier [28]. Another important finding was that injection of proteins in the vitreous induced the upregulation of podoplanin, a known

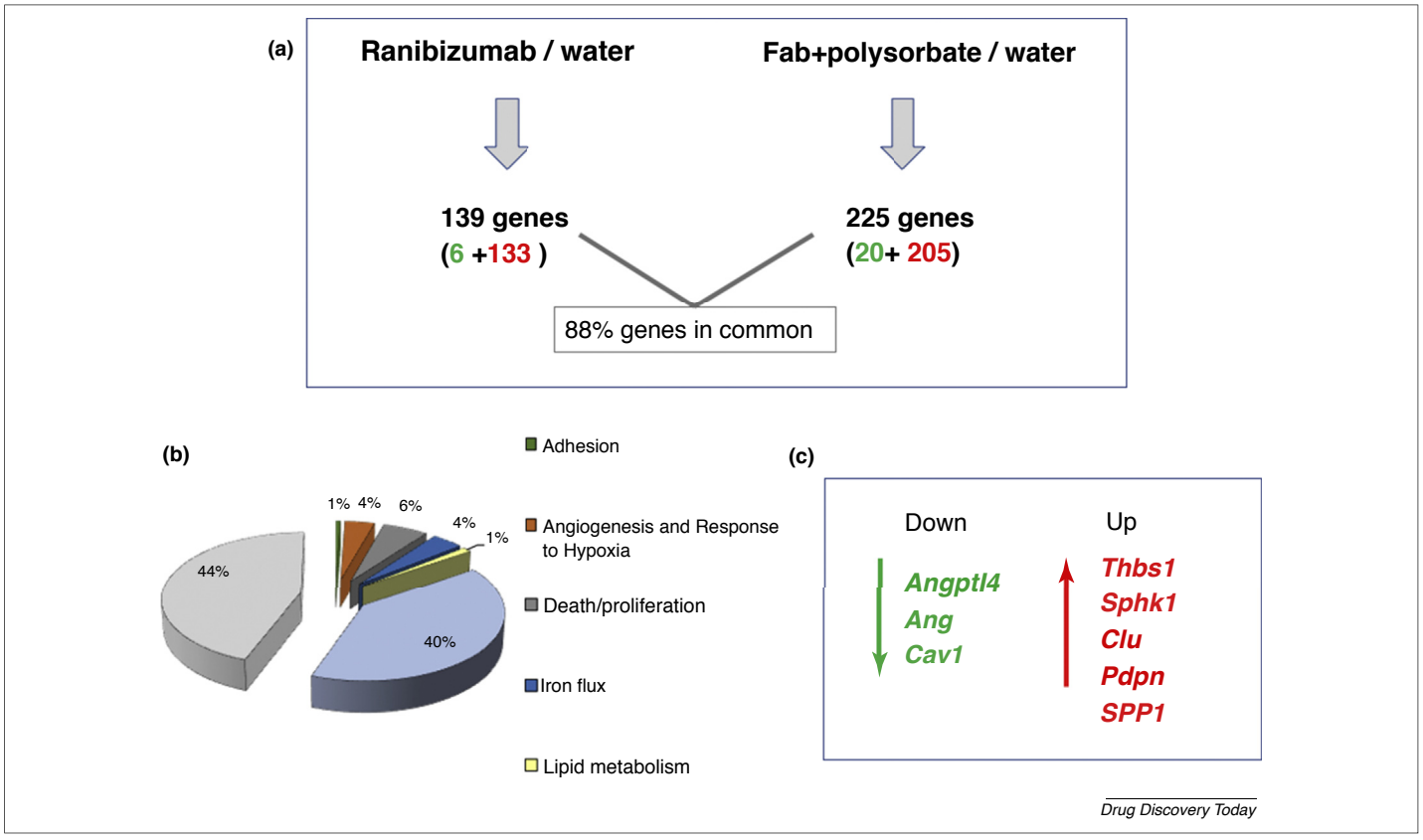

FIGURE 1

Transcriptional effects of ranibizumab or control fab on the rat neuroretina at 24 h after intravitreous injection. **(a)** Representation of the experimental scheme. The retinal transcriptional regulation of ranibizumab (Lucentis®) and of the control Fab +0.01% polysorbate 20 was compared with water at 24 h after injection (0.1 mg/ml final vitreous concentration). The list of genes significantly regulated by ranibizumab was then compared with the list of genes regulated by the control fab + 0.01% polysorbate 20. Green: number of genes downregulated, red: number of genes upregulated. **(b)** Group of genes regulated by the fab + polysorbate 20, classified by their biological functions. **(c)** Restricted list of the most regulated genes, by fab or ranibizumab, known to have a role in macular edema, retinal angiogenesis, or the control of the blood–retinal barrier. Abbreviations: ang, angiotensin; Angptl4, angiopoietin-like 4; cav1, caveolin 1; clu, clusterin; pdpn, podoplanin; sphk1, sphingosine kinase 1; SPP1, osteopontin; thbs1, thrombospodin 1.

marker of lymphatic vessels [29]. Osteopontin, which inhibits the osmotic swelling of retinal muller glia [30], was upregulated, which could directly influence the retinal thickness (Fig. 1).

There are obviously many limitations to this experiment that should be interpreted with caution. The rat retina differs from the human retina because it lacks a macula, and the different formulations were tested on the short term on a normal eye and not on an eye with a retinal disease. Nevertheless, these simple experiments show that the expression of numerous genes, known to have significant effects on macular edema in humans, is regulated shortly after injection into the rat eye of a protein that does not neutralize rat VEGF (ranibizumab or the control fab). Such gene regulations were measured in the neuroretina, meaning that a drug that would not penetrate deeply in the outer retina could exert such effects.

Concluding remarks and perspectives

At a time when, to prolong the duration of their antiedematous effects, higher doses of anti-VEGF proteins are injected in the vitreous, it is important to understand the biological consequences of increasing protein concentrations in the vitreous. Similar to any other compartment in the body, the concentration of proteins in the different compartments of the eye is highly regulated and governs the metabolism of adjacent tissues. A better understanding of the exact mechanisms of the antiedematous effects of anti-VEGF therapeutic proteins, directly related to neutralization of VEGF or to other properties of the physicochemical structure of the molecules, could help design optimized compounds with enhanced antiedematous effects [22].

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