Relative Contribution of VEGF and TNF-α in the Cynomolgus Laser-Induced CNV Model: Comparing the Efficacy of Bevacizumab, Adalimumab, and ESBA105

Peter Lichtlen,1 Tim T. Lam,2 T. Michael Nork,3 Tim Streit,2 and David M. Urech1

PURPOSE. To compare the relative contribution of VEGF and TNF-α in the development of laser-induced choroidal neovascularization (CNV) in monkeys and to exploit the feasibility of topical use of suitable antibody fragments for the prevention of experimental CNV.

METHODS. To induce experimental CNV, small high-energy laser spots were used to treat several areas of the macula in the retinas of cynomolgus monkeys according to previously published protocols. To prevent abnormalities, bevacizumab (a potent VEGF inhibitor) and adalimumab or ESBA105 (potent TNF-α inhibitors) were given by intravitreal injection 1 week before and 1 week and 3 weeks after laser treatment. ESBA105 was also applied topically in a separate group. Control animals were treated with either intravitreal or topical saline. Eyes were monitored by ophthalmic examination, color photography, and fluorescein angiography.

RESULTS. Inhibition of VEGF by bevacizumab completely blocked the formation of CNV. Both TNF-α inhibitors also significantly reduced laser-induced CNV abnormalities after intravitreal administration. Most important, topical use of the anti-TNF-α single-chain antibody fragment ESBA105 also reduced the formation of CNV.

CONCLUSIONS. TNF-α contributes to laser-induced CNV formation, and its inhibition can be a new therapeutic target for CNV. This study suggests TNF-α as another therapeutic target for the prevention and treatment of CNV and adds to the emerging clinical data suggesting the therapeutic value of TNF-α inhibitors in age-related macular degeneration (AMD). Further, this study shows that topical therapy with suitable antibody fragments has the potential of being introduced to retinal disease treatment regimens.

C
choroidal neovascularization (CNV) is a common feature of retinal diseases and a defining characteristic of exudative age-related macular degeneration (AMD).1 In recent years it has become apparent that antibody-based inhibitors of vascular endothelial growth factor (VEGF) represent effective treatment modalities for AMD, leading to clinically significant improvement of visual acuity in a substantial number of patients. However, overall only 30% to 40% of AMD patients gain three lines in visual acuity, and roughly every sixth patient continues losing visual acuity and progresses to legal blindness even under standard treatment with potent VEGF inhibitors.3–4 In addition, frequent intravitreal injections, required for optimal results with common VEGF inhibitors such as bevacizumab and ranibizumab, represent a significant burden for patients and the retinal specialists who treat them.5 Thus, despite the tremendous change in prognosis current VEGF inhibitor-based therapy has brought to AMD patients, there remains significant potential for further improvement of treatment regimens.

Various links exist between angiogenesis and inflammation,6–9 and an increasing number of studies support a role of inflammatory components and proinflammatory cytokines, in particular tumor necrosis factor-α (TNF-α), in the pathophysiology of AMD.10–14 Most important, small case series of AMD patients treated with intravenous or intravitreal TNF-α inhibitors have recently been published.15,16 Data from these patients suggest remarkable efficacy of TNF-α–directed monoclonal antibodies. Significantly, these effects were seen in patients in whom ranibizumab therapy was contraindicated or had failed.16 Thus, it appears appealing to further investigate the therapeutic potential of TNF-α inhibitors, either as monotherapy or in combination with established anti-VEGF treatment, in future clinical trials of AMD.

In addition to the emerging clinical data, TNF-α inhibitors have shown significant efficacy in several mouse models of ocular angiogenesis.12,17 However, conflicting data for TNF-α inhibitors in mice have also been published.18 In fact, data from various preclinical ocular neovascularization models indicate that the relative contributions of VEGF and TNF-α are strongly dependent on the experimental setup, the species, and, in the case of rodents, even the genetics of inbred strains.19–21

Although the laser-induced CNV model is established in several species, there are conceptual advantages of studying the effects of therapeutic compounds, in particular biologics, in monkeys. First, there exist relevant differences in the ocular and, in particular, the retinal anatomy between rodent and rabbit eyes on one hand and human and primate eyes on the other.22–24 Second, all clinically used monoclonal antibodies inhibiting VEGF (bevacizumab, ranibizumab) or TNF-α (adalimumab, infliximab, golimumab, certolizumab) have narrow species specificity25–30 such that they cannot be used in standard rodent or rabbit efficacy models. Hence, the preclinical pharmacologic effects of all these clinically effective monoclonal antibodies can only be studied in primate models.

The laser-induced CNV model in cynomolgus monkeys has shown positive predictive value for the efficacy of clinically effective VEGF inhibitors.19 Despite the fact that experimental laser-induced CNV does not truly reflect the pathophysiology of age-related macular degeneration (AMD),31 these models are attractive to study the feasibility of topical administration of antibodies.

From 1ESBA Tech, an Alcon Biomedical Research Unit, Schlieren, Switzerland; 2Covance Laboratories Inc., Madison, Wisconsin; and 3Comparative Ophthalmic Research Laboratories, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin. Supported by ESBA Tech, an Alcon Biomedical Research Unit, Schlieren, Switzerland.

Submitted for publication November 10, 2009; revised February 5, 2010; accepted March 17, 2010.

Disclosure: P. Lichtlen, ESBA Tech, an Alcon Biomedical Research Unit (E); T.T. Lam, None; T.M. Nork, None; T. Streit, None; D.M. Urech, ESBA Tech, an Alcon Biomedical Research Unit (E), P

Corresponding author: David M. Urech, ESBA Tech, an Alcon Biomedical Research Unit, Wagistrasse 21, CH-8952 Schlieren, Switzerland; david.urech@esbatech.com

Copyright © Association for Research in Vision and Ophthalmology

4738
of AMD, this monkey model represents the only available animal model for the human disease that allows assessment of the relative contribution of VEGF and TNF-α using marketed anti-VEGF and anti-TNF-α antibodies. For this reason the first goal of the study presented here was to investigate the relative roles of TNF-α and VEGF in the development of laser-induced CNV lesions in cynomolgus monkeys. Consequently, we studied the efficacy of two potent TNF-α inhibitors, adalimumab and ESBA105, in comparison with bevacizumab after intravitreal administration.

ESBA105 is a single-chain antibody fragment (scFv) directed against TNF-α that shows novel and distinct pharmacologic properties and is in phase I/II clinical trials. Indeed, we have recently shown the suitability of topically applied ESBA105 to achieve therapeutic intraocular levels in rabbits. Because topical administration of therapeutic antibodies in AMD therapy would represent a highly attractive adjuvant or even alternative treatment modality to current intravitreal injections, we assessed the efficacy of topically administered ESBA105 in the same laser-induced monkey CNV model as the second part of our study.

**Materials and Methods**

**Antibodies**

Bevacizumab (Avastin; Genentech/Roche, South San Francisco, CA) and adalimumab (Humira; Abbott Laboratories, Abbott Park, IL) are commercially available monoclonal antibodies that were purchased in a local pharmacy and prepared for injection according to the manufacturer's instruction. ESBA105 was expressed in and purified from *Escherichia coli* and used in 20 mM sodium citrate buffer (115 mM NaCl, pH 6.0), as previously described.

**Animals**

Forty cynomolgus monkeys (*Macaca fascicularis*) (Covance Research Products Inc, Alice, TX) were included in the study. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare, with approval by the Covance Institutional Animal Care and Use Committee. Anesthesia for all procedures was dosed by bilateral intravitreal injection on days 1, 15, and 29 of the study (three independent dosing events). Group 1 animals were treated continuously with 10 daily instillations of either 50 μg per dose) ESBA105 eyedrops to both eyes, applied at hourly intervals from day 1 to day 36 of the study.

Intravitreal injections were made as previously described. Topical instillations were made by application of one eyedrop to each eye using a pipette at a volume of approximately 50 μL. The upper eyelid was gently extended away from the eyeball. The eyedrop was instilled by placing the dose material directly onto the cornea.

**Grading of Fluorescein Angiograms**

Laser lesions were graded by a masked retinal specialist for the presence of CNV based on the characteristics of fluorescein dye leakage, as determined subjectively from black-and-white fluorescein angiograms. Grades 1 through 4 were assigned to each laser lesion according to the grading scheme described by Krzsystolik et al., using standardized angiograms for comparison. To further refine this grading system, a new clock-hour grading method was used as an adjunct to the present study. Each grade 4 lesion (containing either one spot or two adjacent spots) was visually inspected. The center of the “clock” was defined as either the center of the single-spot lesions or the midpoint between the lesions with the two adjacent spots. The total number of clock hours (each clock hour subtending an angle of 30°) of late fluorescein leakage that extended beyond the borders of the treated area was then estimated visually and recorded for each grade 4 lesion.

**Image Analysis of Fluorescein Angiography Leakage**

Fluorescein angiogram images taken approximately 5 minutes after fluorescein injection were scanned and digitized into black-and-white .jpg files. For image analysis, the positive image (fluorescence as white) was used. Parameters determined included cumulative leakage area and relative mean intensity of the cumulative leakage area. Analysis was conducted using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). To calculate the cumulative leakage area and its relative mean intensity per eye, the observer first manually traced the perimeter of each laser spot to include any leakage. The borders of each laser spot/leakage area were established as the point at which the leakage area was indistinguishable from the background. The software then provided direct readings of cumulative area and mean intensity in accordance with the tracings. Relative mean intensity of the cumulative laser spots/leakage area was calculated using the formula $RI = (L - [(B1 + B2 + B3)/3])/V$, where $RI$ represents relative mean intensity, $L$ represents mean intensity of the cumulative laser spots/leakage area, $B1$, $B2$, and $B3$ represent mean intensity of the three background areas, and $V$ represents mean intensity of the brightest area of the vasculature.

Background areas were defined as ovals approximately the size of the optic disc that did not include any of the defined laser spot/leakage areas. The brightest area of vasculature was used for normalizing the measurement. Typically, the mean intensity of the optic disc was measured, but if the optic disc was underdeveloped, the mean intensity of a bright blood vessel was measured.

Cumulative area and relative mean intensity of the cumulative areas were averaged between the eyes of each animal. Group comparisons were made between animals treated by intravitreal injection or topical instillation and their respective controls.

**Statistical Analysis**

All statistical testing was performed using statistical software (Prism version 4.03; GraphPad, San Diego, CA). Differences between treatment groups and controls for assessment of data resulting from masked lesion grading were determined using the nonparametric Mann-Whitney U test. Differences between treatment groups and controls applying image software analysis were determined using the parametric paired *t* test. $P < 0.05$ was considered significant.
RESULTS

Intravitreal Adalimumab, Intravitreal ESBA105, and Topical ESBA105 All Reduced the Number of Grade 4 CNV Lesions Compared with Saline

The study described here had two major goals: to characterize the pathophysiological role of TNF-α versus VEGF in the development of laser-induced CNV in cynomolgus monkeys and to explore the feasibility of preventing such CNV formation by topical administration of a TNF-inhibitory scFv antibody fragment.

Inhibition of VEGF by intravitreal ranibizumab injection is highly effective in preventing CNV formation in this model.19 As opposed to bevacizumab, a full-size antibody; ranibizumab is a Fab antibody fragment of approximately 48 kDa molecular weight.20 This may translate to a reduced intravitreal half-life of the molecule compared with conventional full-length monoclonal antibodies that have a molecular weight of approximately 150 kDa.21 Such pharmacokinetic differences between antibody formats may possibly influence duration of effect after intravitreal administration.22 When the experiment was conducted, all marketed anti-TNF-α antibodies were of the full-length format, (infliximab, adalimumab). Consequently, we decided to use the full-length anti-VEGF antibody bevacizumab (which is highly effective in the treatment of AMD as well) instead of ranibizumab as a positive VEGF inhibitor control in our experiment. Because infliximab has an extremely narrow species specificity, neutralizing only human and chimpanzee TNF-α,23 we used adalimumab to characterize the role of TNF-α in this cynomolgus model. This choice allowed exclusion of potential antibody format-specific effects when assessing the pathophysiological role of TNF-α because two potent molecules with comparable intravitreal pharmacokinetics (bevacizumab, adalimumab) but different target specificity were used. Hence, monkeys were treated with intravitreal injection of 1 mg bevacizumab or adalimumab, respectively, 1 week before (day 1) and 1 week (day 15) and 3 weeks (day 29) after argon green laser photocoagulation to induce CNV lesions. Development of grade 4 lesions was assessed by fluorescence angiography 2, 3, and 4 weeks (days 22, 28, and 36) after laser treatment, as described previously.19 Intravitreal bevacizumab completely blocked the formation of grade 4 lesions at all times. Intravitreal adalimumab was clearly less effective than bevacizumab but reduced the number of laser-induced grade 4 lesions by >50%, demonstrating an important contributing pathophysiological role of TNF-α in the model (Fig. 1).

We also injected ESBA105,3,37 a potent anti-TNF-α single-chain (scFv) antibody, intravitreally into the eyes of monkeys. ESBA105, based on its low molecular weight of 27 kDa, carries entirely distinct pharmacokinetic properties and represents the first topically applied antibody in clinical trials in ophthalmology.37,38 Intravitreal ESBA105 was injected at the same time points as bevacizumab and adalimumab. Given that the intravitreal half-life of ESBA105 in rabbits was determined to be in the range of 1 day only,31 whereas the intravitreal half-life of full-length antibodies (such as bevacizumab and adalimumab) was determined to be in the range of 5.5 to 8.5 days34,35,40 ESBA105 (2 mg) was injected at each of the dosing events to partially compensate for the anticipated lower exposure. Similar to adalimumab, intravitreal ESBA105 reduced the formation of grade 4 CNV lesions at all times by approximately half (Fig. 1), confirming previous data demonstrating that ESBA105 shows in vivo potency similar to that of marketed TNF-α inhibitors in suitable animal models.35

Most important, these data on intravitreal adalimumab and ESBA105 define the anticipated maximal pharmacologic effect size for the prevention of grade 4 lesion formation that a TNF-α inhibitor can possibly achieve in this model because the expected minimal concentration (Cmin) in the vitreous throughout this study is at least equal to that of adalimumab during steady state in rheumatoid arthritis therapy.11 This definition was crucial because the second objective of this study was to assess the feasibility of topical administration of a suitable scFv antibody fragment in this model.

Thus, another group of animals was treated with 10 daily eyedrops of ESBA105 for the entire study duration. We found that topical ESBA105 reduced the number of developing grade 4 lesions by approximately 30% in this experiment compared with saline-treated control animals (Fig. 1).

TNF-α Significantly Contributes to Grade 4 Lesion Formation

Development of individual laser-induced CNV lesions in this model is variable and is influenced by small experimental differences within the focus of individual laser beams causing the choroidal damage and by the number of arbitrarily hit large choroidal vessels in a given lesion.32 In addition, the number of monkeys that can be used per dose group in this species is limited for ethical reasons, resulting in generally small group sizes. However, pooling of data from intravitreal and topical controls or ESBA105-treated animals, respectively, results in statistically significant differences when individual eyes are considered the randomization unit. This further supports the role of TNF-α in the monkey CNV model (Fig. 2). Indeed, ESBA105 significantly reduced the formation of grade 4 lesions on day 22 and day 28. On day 36, ESBA105 treatment approached statistical significance (P = 0.078; Fig. 2).

Topical ESBA105 Significantly Reduces CNV Lesion Development Using the Conventional CNV Grading Scheme

Influenced by the overwhelming efficacy of anti-VEGF antibodies, only grade 4 CNV lesions were previously defined to be of

![Figure 1. Prevention of grade 4 CNV lesion formation induced by laser photocoagulation. Intravitreal injection of 1 mg of bevacizumab (stars) 1 week before (day 1) and 1 week (day 15) and 3 weeks (day 29) after laser treatment completely prevented the formation of grade 4 lesions. Intravitreal treatment with 1 mg adalimumab (filled circles) and 2 mg ESBA105 (filled triangles) injected at the same time reduced the number of grade 4 lesions by at least 50% compared with intravitreal saline (open triangles). Daily topical treatment with ESBA105 (filled squares) throughout the study period reduced the number of grade 4 lesions by approximately 30% compared with topical saline (open squares).](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932963/ on 06/24/2017)
clinical significance in this model. However, after laser beam damage, there is increasing pathologic impairment of choroidal structures from grade 2 (normal) to grade 4 lesions resulting in increasing hyperfluorescence and leakage in FA.19 Thus, grades 2 and 3 lesions also represent pathologic FA findings, which are of obvious additional value when assessing the efficacy of compounds that do not lead to complete protection from grade 4 lesions in this model. For this reason, we analyzed the grading distribution of all CNV lesions within the different treatment groups (Fig. 3). Again, bevacizumab was most effective in CNV pathology prevention (P < 0.001), resulting primarily in grade 1 lesions, a median lesion grade of 1, and a range of lesions from grade 1 to grade 3. All groups treated with a TNF-α inhibitor showed a wider distribution of lesions ranging from grade 1 to grade 4. However, all TNF-α inhibitor treatments also resulted in statistically significant reductions in CNV lesion pathology and in the median lesion grade at all times (Fig. 3). Most important, topical ESBA105 treatment also resulted in significant reductions in pathology.

Although CNV pathology in control animals improved slightly over the three assessment times during which the conventional grading scheme was applied (Figs. 1–3), treatment with the TNF-α inhibitors ameliorated CNV pathology at all times, indicating that not only was the inhibition of TNF-α effective at preventing the formation of laser-induced CNV, as was implied by previous data,19 it also had a beneficial effect on individual lesions during the treatment phase of this experiment.

**TNF-α Inhibition Significantly Reduces Mean Fluorescence Intensity on Image Analysis**

The conventional CNV lesion grading system introduced by Krzystolik et al.19 results in nonparametric data. Such data are limited in that ethically justified group sizes do not generate sufficient statistical power to demonstrate effects with non-complete CNV inhibitors. For this reason we tried to analyze our data by a parametric method. Fluorescence intensity of CNV lesions was quantified using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html), and the mean fluorescence intensity was determined for individual eyes (Fig. 4). Again, intravitreal bevacizumab was most effective in reducing relative mean intensity of eyes. TNF-α inhibition significantly reduced mean fluorescence intensity after topical application, though to a lesser extent than did bevacizumab.

**Introducing a New, More Refined Grade 4 Lesion Subscore: The Clock Hour Grading Method**

The conventional CNV lesion grading system introduced by Krzystolik et al.19 was adequate to assess the efficacy of highly effective compounds such as VEGF inhibitors in this model. However, to assess the relative efficacy of different classes of inhibitors, a more refined analysis of grade 4 lesions was needed. Consequently, we attempted to introduce a grade 4 lesion subscore by subdividing grade 4 lesions into 12 (clock hour) areas (see Materials and Methods). Indeed, this method allows for more detailed assessment of effects of compounds on grade 4 lesions (Fig. 5). Again, intravitreal administration of both TNF-α inhibitors significantly reduced the severity of leakage at all times, which is important to note, because clock hour scoring only considers conventional grade 4 lesions. The effect of topical ESBA105 approached statistical significance at the end of the study (P = 0.064).

Whereas overall clock hour grading scores did not improve in control animals over time, there was continued improvement with the use of anti-TNF-α therapy (Fig. 5). Again, these data suggest that TNF-α is important not only in the early stages of laser-induced CNV pathophysiology, it also plays a relevant role at later stages.

**DISCUSSION**

Monoclonal antibodies inhibiting VEGF (ranibizumab, bevacizumab) have proven to be very effective in neovascular ocular disorders, in particular AMD.1–3 VEGF-induced signaling is crucial for the development of pathology in laser-induced CNV models as well.19 Because of the anatomic similarity between the cynomolgus monkey eye and the human eye, applying the model in this species provides conceptual advantages over other commonly used ocular neovascularization models. In addition, clinically approved monoclonal antibodies targeting VEGF, as well as most marketed antibodies targeting other potential drug targets in ocular neovascularization, have very narrow species specificities,25–30 which excludes their pharmacologic use in murine or rabbit models. Finally, the use of species cross-reactive surrogate antibodies in rodent models does not allow one to draw entirely valid conclusions for antibodies used in the clinic for both pharmacodynamic and pharmacokinetic reasons.25–28 Thus, monkeys remain the favored species in which to assess pharmacologic effects of clinically applied antibodies on the pathophysiology of laser-induced CNV.

Indeed, ranibizumab, the anti-VEGF monoclonal Fab antibody fragment approved for the treatment of AMD, almost completely blocks the development of grade 4 CNV lesions in this model.19 However, clinical success rates in AMD patients are much lower than these dramatic effects of ranibizumab in the experimental model would suggest.5–4 Thus, in established exudative AMD, disease factors other than VEGF are likely to play important roles as well.10,14,33 For this reason, a more detailed understanding of the pathophysiological events and a
A set of refined scoring systems and objective assessment methods to interpret data would be of great practical value with which to conduct preclinical efficacy studies of promising non-VEGF antibodies in the model. Methods such as the proposed clock hour grading scheme and the objective, parametric fluorescence intensity assessment may present promising approaches.

TNF-\alpha\,/H9251 represents an attractive potential drug target in AMD.\textsuperscript{15,16} This proinflammatory master cytokine is known to be a potent inducer of angiogenesis in vivo.\textsuperscript{44} VEGF and TNF-\alpha\,/H9251 interact during several steps of the neoangiogenic process, and CNV formation and TNF-\alpha\,/H9251 may even act upstream of VEGF in certain vascular beds.\textsuperscript{14,20,45} In fact, TNF-\alpha\,/H9251 has been suggested as a molecular player in ocular angiogenesis in a variety of studies and animal models.\textsuperscript{11–14,17} However, the relevance of TNF-\alpha\,/H9251 has also been questioned based on data in mouse models.\textsuperscript{18} In humans, TNF-\alpha\,/H9251 is expressed at significant levels in the retina of patients with neovascular ocular diseases.\textsuperscript{10,46,47} Most important, emerging clinical data with TNF-\alpha inhibitors suggest significant clinical efficacy in various intraocular disorders, including CNV and chronic cystoid macular edema.\textsuperscript{48–51} Small clinical case studies have shown that TNF-\alpha inhibitors, administered by both the systemic and the intravitreal route, can be remarkably effective in AMD, even when ranibizumab failed or was contraindicated.\textsuperscript{15,16} For this reason, treatment with TNF-\alpha inhibitors has been proposed to be further evaluated as adjuvant, or in some cases even alternative, treatment to VEGF inhibitors.\textsuperscript{51,52} Indeed, several groups are preparing for or are conducting clinical trials with TNF-\alpha inhibitors in AMD.

To assess the feasibility of the laser-induced CNV model for the assessment of clinically active anti–TNF-\alpha antibodies, it was

**FIGURE 3.** Distribution of individual lesion grades. Data are presented as box plot. Boxes: 25th to 75th percentiles. Horizontal line with circle: median. Vertical lines: full spectrum of data. All treatments, including topical ESBA105, significantly reduced the severity of laser-induced CNV compared with respective control treatment. Interestingly, treatment with TNF-\alpha inhibitors was beneficial throughout the entire study period (*\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\)).

**FIGURE 4.** Relative MFI in individual eyes on day 22, quantified by ImageJ software analysis (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Circles: individual eyes. Intravitreal bevacizumab, adalimumab, and topical ESBA105 significantly reduced MFI. Linear combination of all ESBA105-treated eyes (gray circles) compared with all saline-treated eyes (open circles) also results in statistically significant reduction (*\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\)).
important to exclude a potential impact of the full-length antibody format on experimental results. The marketed full-length anti-VEGF antibody bevacizumab rather than the Fab antibody fragment ranibizumab was used as a positive control for the full-length anti-TNF-α antibody adalimumab.

Full-length monoclonal antibodies have been shown to have intravitreal half-lives in the range of 5.5 to 8.5 days after intravitreal injection in rabbits. The anti-TNF scFv antibody fragment ESBA105 was previously shown to have an intravitreal half-life of only approximately 1 day in rabbits. Nevertheless, in our study intravitreal injection of 2 mg (less than expected to compensate for the shorter half-life) of this antibody resulted in an overall efficacy similar to that of adalimumab. Improved penetration characteristics of this small antibody fragment might have further compensated for the likely relative underdosing of ESBA105 compared with adalimumab and might have explained the highly similar efficacy of the two intravitreally administered TNF-α inhibitors. Generally, however, to a lesser extent, topical administration of ESBA105 also resulted in the reduction of laser-induced pathology. Indeed, and in contrast to full-length antibodies, topically applied ESBA105 was previously shown to penetrate the retinal layers in rabbits. The fact that topical ESBA105, despite the comparably high dosing frequency of 10 drops/day, resulted in less pronounced effects than intravitreal injections may be readily explained by the fact that the antibody was applied in a simple saline buffer. Use of the antibody in such a preliminary formulation is expected to result in considerable interanimal variation. Thus, the efficacy of topical treatment may be significantly improved by ongoing formulation development. In addition, topical monotherapy with ESBA105 might have missed a threshold level required for full efficacy. Topical treatment may be sufficiently effective for use as adjuvant therapy, such as in combination with intravitreal injection of a conventional VEGF inhibitor, possibly allowing for dose reduction or prolongation of dosing intervals during maintenance therapy. In any case, our data speak very much in favor of the feasibility of the model for testing such low-dose combinations of VEGF and TNF-α inhibitors. Furthermore, adjuvant topical administration of scFv antibody fragments directed against other promising targets that act independently of VEGF, or even the use of a topical scFv antibody directed against VEGF itself, should be evaluated to identify more effective and more convenient therapeutic regimens for AMD patients.

Previous pathologic characterization of this cynomolgus model suggested that inflammatory contribution was restricted to the early stages of CNV. However, our data with both adalimumab and ESBA105 suggest that the inhibition of TNF-α is beneficial in the early stages of CNV formation and is sustained (Figs. 3, 5) through the later stages. Indeed, at the later assessments we found that amelioration of already established lesions was significantly and increasingly improved by TNF-α inhibitor treatment (days 28 and 36; Figs. 3, 5). This may indicate that at later stages, cells other than infiltrated macrophages and leukocytes generate TNF-α. Choroidal endothelial cells may be candidates for such production.

**Figure 5.** Distribution of clock hour gradings within individual grade 4 lesions. Treatment with intravitreal bevacizumab, adalimumab, and ESBA105 significantly reduced the clock hour grading score at all times. Interestingly, the significance of treatment with intravitreal ESBA105 continuously increased over time. Topical ESBA105 approached statistical significance at the end of the study period. Data are presented as box plot. Boxes: 25th to 75th percentiles. Horizontal line within box: median. Vertical lines: full spectrum of data (*P < 0.05; **P < 0.01; ***P < 0.001).
A limitation of this monkey model remains the low number of animals that can be ethically used. This limitation generally results in studies with low statistical power. However, in our view, the value of testing the pharmacologic properties of clinically used monoclonal antibodies in a model with the closest possible anatomic similarity to the human eye clearly outweighs this limitation.

The results of our experiments validate TNF-\(\alpha\) as a complementary drug target in the laser-induced CNV model in monkeys and demonstrate that topical therapy with a suitable antibody fragment appears feasible. These data are of high interest for future development of improved treatment regimens in AMD therapy.

Acknowledgments

The authors thank Anne Schmidt and Tea Gunde for excellent logistical support, Colin Hughson for critical review of the manuscript, and Lea Noser for help in developing the image analysis method.

References


