Comparison of Bevacizumab, Ranibizumab, and Pegaptanib In Vitro: Efficiency and Possible Additional Pathways

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Purpose. Vascular endothelial growth factor (VEGF) antagonists are the therapy of choice for age-related macular degeneration. Ranibizumab and pegaptanib have been approved by the United States Food and Drug Administration, whereas bevacizumab is used off label. In this study, the authors compare these VEGF inhibitors directly regarding their efficiency to neutralize VEGF in a quantifiable in vitro system.

Methods. Porcine retina–retinal pigment epithelium–choroid organ culture and RPE cell culture were prepared from fresh eyes, cultivated in a perfusion chamber, and treated with clinically relevant concentrations of bevacizumab, ranibizumab and pegaptanib. VEGF content of the supernatant was analyzed with ELISA. Additionally, the influence of bevacizumab and ranibizumab on intracellular VEGF was analyzed with Western blot.

Results. At clinically significant doses, bevacizumab (0.25 mg/mL) and ranibizumab (0.125 mg/mL) neutralized VEGF completely for 6 hours, whereas pegaptanib (0.08 mg/mL) showed no effect. Bevacizumab and ranibizumab neutralized VEGF significantly up to 16 hours. When diluted, bevacizumab lost its inhibiting properties at a concentration of 975 ng/mL, and ranibizumab neutralized VEGF up to a concentration of 120 ng/mL. Both substances significantly diminished VEGF expression in Western blot.

Conclusions. At clinical doses, bevacizumab and ranibizumab are equally potent in neutralizing VEGF. To neutralize VEGF completely in this system, a fraction of the clinical dose is needed. Ranibizumab is more efficient at neutralizing VEGF when diluted. Pegaptanib showed no effect in this system, which might help explain the clinical experience regarding this drug. A direct effect of ranibizumab and bevacizumab on VEGF protein expression indicates additional pathways of VEGF inhibitors. (*Invest Ophthalmol Vis Sci.* 2008;49:4523–4527) DOI:10.1167/iovs.08-2055

Age-related macular degeneration (AMD) is the primary cause of legal blindness in the elderly in the industrialized world. Until recently, photodynamic therapy, subretinal surgery, and laser surgery showed only moderate beneficial effects in selected patients. The development of VEGF antagonists introduced a new treatment regimen that has led to better long-term results. The first VEGF antagonist to be developed was pegaptanib (Macugen, Melville, NY), an aptamer developed to bind specifically to $VEGF_{165}$. It was approved by the United States Food and Drug Administration (FDA) for the treatment of AMD after the VISION study showed a highly significant preservation of vision compared with sham-injected controls.[1] Pegaptanib proved VEGF to be the main angiogenic factor in CNV. The first VEGF antibody used off-label in AMD was bevacizumab (Avastin; Genentech, South San Francisco, CA),[2] a full-length anti-VEGF antibody approved for use in colon cancer.[3] In 2006, ranibizumab (Lucentis; Genentech), an antibody fragment developed from the same antibody as bevacizumab by the same company (Genentech), was approved by the FDA for the treatment of AMD after the MARINA and ANCHOR studies showed not only a stabilizing but, in 30% of the patients, a beneficial effect.[4] Because ranibizumab is highly effective, but considerably expensive, bevacizumab, which costs approximately one-fortieth the price of ranibizumab, is continually used off label, despite the possible legal problems of off-label use. Bevacizumab has shown good effectiveness and tolerability in case studies and retrospective studies but has never been tested in a clinical phase 3 trial.[5]

The rationale of this work was to directly compare VEGF inhibitors regarding their ability to neutralize VEGF in a quantifiable, reproducible in vitro setting. As a model, we chose the perfusion organ culture. In this system, freshly prepared organ sheets of choroid, retinal pigment epithelium, and retina are fixed in a fixation ring and cultivated in a chamber with a continuous flow of medium, where the retinal cytoarchitecture, the retinal pigment epithelium, and the choroid remain well preserved up to 10 days. The chamber is easily accessible for manipulation and ideal for the testing of pharmacologic agents interfering with secreted products[6] (Miura M, et al. *IOVS* 2007;48:ARVO E-Abstract 2540).

Methods

Organ Culture

For the preparation of retina–retinal pigment epithelium–choroid sheets, freshly slaughtered pig eyes were used 2 to 3 hours after enucleation. Eyes were cleaned of adjacent tissue and immersed briefly in antiseptic solution. The anterior part of the eye was removed, as were the lens and vitreous. Retina–retinal pigment epithelium–choroid sheets were separated from sclera using forceps and scissors. Prepared tissue was fixed between the lower and upper part of a fixation ring, excess tissue was removed, and the ring was placed in a six-well culture chamber (Minucells & Minutissue, Bad Abbach, Germany). One eye per fixation ring was used. The chamber was placed on a heating plate and perfused with Dulbecco modified Eagle medium (DMEM; PAA, Cölbe, Germany) and Ham F12 medium (PAA) 1:1 and supplemented with penicillin/streptomycin (1%), L-glutamine, HEPES (25 mM), sodium pyruvate (110 mg/mL), and 10% porcine serum (PAA).

Treatment with VEGF Antagonists

On the second day of cultivation, the tissue sheets were exposed to clinically relevant concentrations of bevacizumab (0.25 mg/mL),

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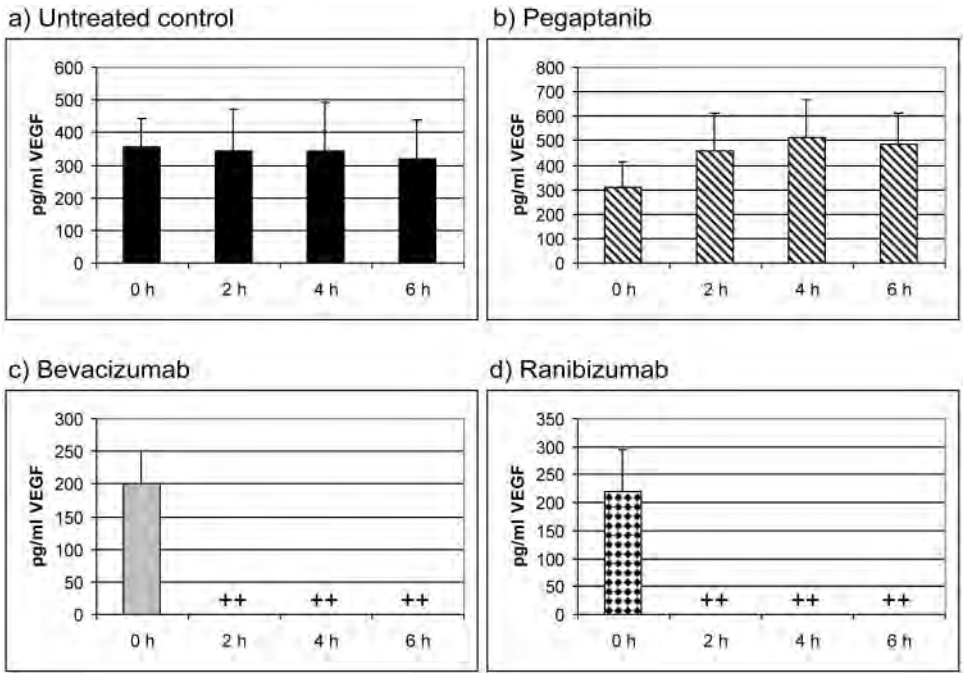

FIGURE 1. VEGF content of supernatant measured by ELISA in untreated control, after 0.08 mg/mL pegaptanib, 0.25 mg/mL bevacizumab, or 0.125 mg/mL ranibizumab. Significance was evaluated with the *t*-test. $++P < 0.01$.

ranibizumab (0.125 mg/mL), or pegaptanib (0.08 mg/mL)[7] or other indicated concentrations. Tissue perfusion was interrupted, and the medium was removed from the chamber with a syringe and transferred to a tube (Falcon; BD Biosciences, Franklin Lakes, NJ) in which the respective VEGF antagonist was added to the medium. The medium was transferred back to the chamber and incubated for 20 minutes. The perfusion was restarted, and the supernatant was collected. Tubes collecting the medium were changed every hour.

Evaluation of VEGF Content

VEGF content was measured by VEGF ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. ELISA detected all isoforms of VEGF(A).

RPE Isolation and Cell Culture

For RPE isolation, freshly slaughtered pig eyes were cleaned of adjacent tissue and immersed briefly in antiseptic solution. The anterior part of the eye was removed, as were the lens, vitreous, and retina. In each eyecup, trypsin was added and incubated for 5 minutes at 37°C. Trypsin solution was removed and substituted with trypsin-EDTA for 45 minutes at 37°C. RPE cells were gently pipetted of the choroid, collected in media, and washed. Retinal pigment epithelia of three eyes were collected and seeded in a 60-mm dish. Cells were cultivated in DMEM and Ham F12 medium (1:1) supplemented with penicillin/streptomycin (1%), L-glutamine, amphotericin B (0.5 μg/mL), HEPES (25 mM), sodium pyruvate (110 mg/mL), and 10% porcine serum.

Treatment of Cells

For all experiments, confluent RPE cells of passage 1 or 2 were used. Cells were treated with 0.25 mg/mL bevacizumab or 0.125 mg/mL ranibizumab and incubated for the indicated period.

Whole Cell Lysate

Whole cell lysate of retinal pigment epithelium was generated from treated RPE cells as described elsewhere.[8] In brief, the cells were scraped off in PBS and centrifuged, and the pellet was resuspended in DLB-buffer (Tris [pH 7.4], 10 mM. 1% SDS, protease inhibitor, phosphatase inhibitor). Samples were heated at 95°C for 5 minutes, sonicated by ultrasound, and centrifuged. The protein concentration of the supernatant was determined by the Bio-Rad (Hercules, CA) protein assay with BSA as standard.

Western Blotting

Whole cell lysate was separated under reducing conditions on 15% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The blot was blocked by 4% skim milk in Tris-buffered saline with 0.1% Tween for 1 hour at room temperature and incubated overnight at 4°C with a primary antibody against VEGF (R&D Systems). After washing with TTBS, blots were incubated with peroxidase-conjugated donkey-anti–goat secondary antibody for 30 minutes at room temperature. After the final wash, the blot was incubated with chemiluminescence reagent (Immobilon; Millipore, Billerica, MA), and the signal was detected with Amersham film (Hyperfilm; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Biotechnology software (GeneTool; Syngene, Frederick, MD) was used to evaluate Western blots. The density of each band was measured and related to a nonspecific protein control band that was detected by the secondary antibody alone and that correlated only to the protein content visualized by Ponceau staining. Each band was displayed as a percentage of untreated control.

Dilution Formula

The dilution process can be described with a differential equation, $A_{(t)} = A_{(0)} \times e^{-k \times t}$, where $A_{(t)}$ = concentration at given time point, $A_{(0)}$ = initial concentration, k = dilution constant ($-1/84$), and t = time. Its solution yields an exponential function with the dilution constant as the exponential decay value. The given number of the dilution constant refers to the experimental boundary conditions.

Statistical Analysis

Every experiment was independently repeated at least three times. Significance was calculated with *t*-tests and graphing software (Sigma Plot; Systat, Chicago, IL). $P < 0.05$ was considered significant.

RESULTS

Efficacy of Bevacizumab, Ranibizumab, and Pegaptanib

In one perfusion organ culture containing six fixation rings, approximately 350 pg/mL VEGF per hour was produced (0

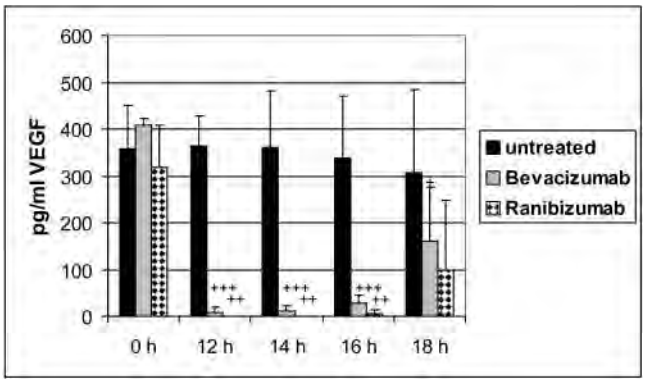

FIGURE 2. VEGF content of supernatant measured by ELISA in untreated control, 0.25 mg/mL bevacizumab, or 0.125 mg/mL ranibizumab up to 18 hours. Significance was evaluated with the *t*-test. $+P < 0.05$; $++P < 0.01$; $+++P < 0.001$.

hour, 354 ± 90 pg/mL; 2 hours, 343 ± 125 pg/mL; 4 hours, 344 ± 149 pg/mL; 6 hours, 317 ± 129 pg/mL). This amount of VEGF is completely neutralized in the evaluated period (6 hours) by bevacizumab (0.25 mg/mL) and ranibizumab (0.125 mg/mL) but not by pegaptanib, which showed no effect on VEGF content (Fig. 1).

VEGF Neutralization: Maximal Time Period

We wanted to evaluate the maximal time period in which bevacizumab and ranibizumab are effective in neutralizing VEGF. Both inhibitors were effective in neutralizing VEGF significantly up to 16 hours after application and did not significantly differ in their effects (Fig. 2). Given that each substance was given only at the beginning of the experiment, it was continually diluted by perfusion, according to the formula $A_{(t)} = A_{(0)} \times e^{-1/84k \times t}$. After 14 hours of perfusion, the calculated concentrations of bevacizumab and ranibizumab were 12 ng/mL and 6 ng/mL, respectively. When applying these concentrations directly, no neutralization could be seen, indicating additional pathways of action (Fig. 3).

VEGF Neutralization: Minimal Concentration

Bevacizumab and ranibizumab are highly efficient in neutralizing VEGF, and they neutralize VEGF completely when used in clinical concentrations. When diluted, both substances are highly efficient at neutralization, but ranibizumab is more efficient than bevacizumab. When bevacizumab is used, 1 μg/mL is the threshold at which VEGF is detectable in considerable amounts (>50 pg/mL), whereas ranibizumab at 60 ng/mL is the threshold after which VEGF is clearly detectable (>100 pg/mL; Fig. 4).

VEGF Expression: Influence of Ranibizumab and Bevacizumab on VEGF Expression

Because ranibizumab and bevacizumab were effective when diluted for 14 hours in the perfusion chamber but not when the appropriate concentrations were applied directly to the organ culture, we investigated the effect of ranibizumab and bevacizumab on VEGF in RPE cells in primary cell culture. When given to RPE cells, cellular VEGF protein expression declined significantly after 6 hours of incubation with ranibizumab or bevacizumab (Fig. 5).

DISCUSSION

To our knowledge, this is the first time that the efficiency of VEGF sequestering of bevacizumab, ranibizumab, and pegaptanib has been directly compared. One striking result is the failure of pegaptanib to perform in this system. This failure can be explained by the design of this VEGF antagonist and of this study and might also help explain the clinical experience with pegaptanib.

To understand this intriguing finding, the molecular properties of VEGF and the binding sites of its antagonists must be considered. $VEGF_{165}$ consists of a receptor-binding domain (AS8–109) and of the heparin-binding domain (AS111–165). The receptor-binding domain binds to VEGFR-2 and induces signal transduction and, consequentially, migration and proliferation. The primary purpose of the heparin-binding domain is to enhance VEGF-induced signaling by binding to the cell

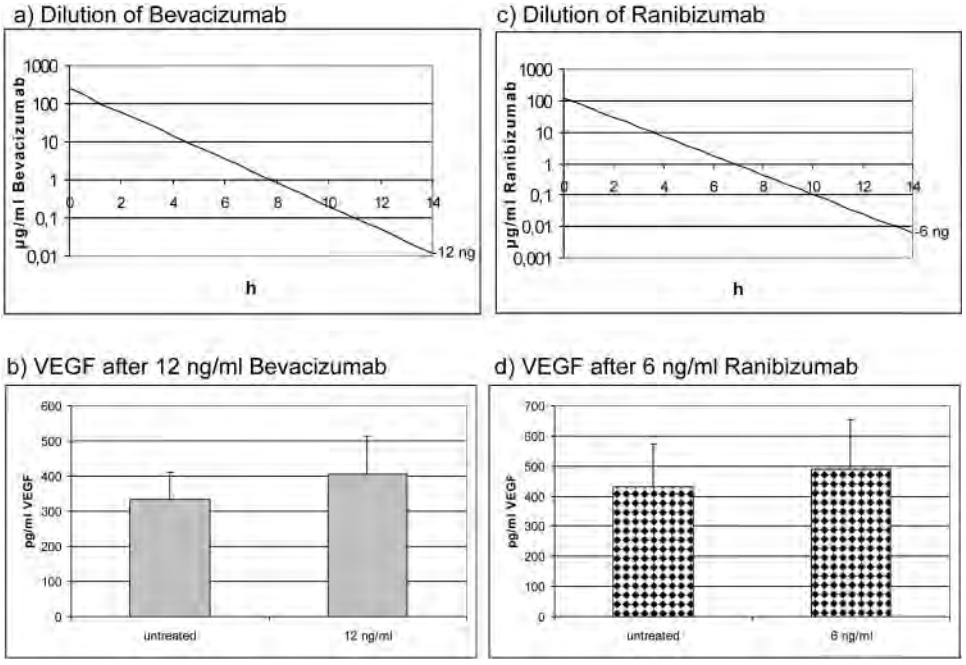

FIGURE 3. (**a**) Dilution of bevacizumab in the perfusion chamber. (**b**) VEGF content of supernatant measured by ELISA in untreated control or 12 ng/mL bevacizumab. (**c**) Dilution of ranibizumab in the perfusion chamber. (**d**) VEGF content of supernatant measured by ELISA in untreated control or 6 ng/mL ranibizumab. Significance was evaluated with the *t*-test.

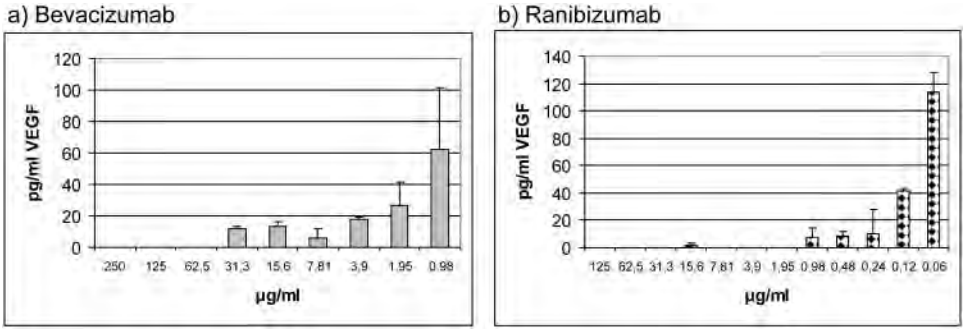

FIGURE 4. Minimal concentration of (**a**) bevacizumab and (**b**) ranibizumab needed to neutralize VEGF. VEGF content of the supernatant was measured by ELISA after the indicated concentration of either substance.

surface or the neuropilin-1 coreceptor.[9–11] The binding sites of bevacizumab and ranibizumab are in the receptor-binding region (AS82–91).[12] Pegaptanib, in contrast to bevacizumab and ranibizumab, has been developed to bind to $VEGF_{165}$. $VEGF_{165}$ and $VEGF_{121}$ differ in the heparin-binding domain (AS111–165); this is the only region in which pegaptanib can bind to $VEGF_{165}$ without inhibiting other isoforms.[13] Consequently, it does not affect the receptor-binding domain (AS8–109) because the domains are identical in $VEGF_{165}$ and $VEGF_{121}$. Bevacizumab and ranibizumab, on the other hand, bind to all isoforms.[12,14,15] Bevacizumab and ranibizumab bind only weakly to rodent VEGF, as in rodent VEGF, the glycine at AS88 is exchanged with a serine.[10] They do bind to porcine VEGF, which does not carry this mutation. The company that produces the ELISA (R&D Systems) could not provide the exact epitope to which their VEGF antibody binds (personal communication, November 16, 2007), but the antibody does bind to $VEGF_{165}$ and $VEGF_{121}$. Therefore, it must be in the receptor-binding domain. It is reasonable that the binding of bevacizumab or ranibizumab to VEGF sterically inhibits the recognition of VEGF by the ELISA antibody; hence, neutralization is seen. Pegaptanib binds VEGF at a different epitope that does not impede the binding of ELISA. (The heparin-binding domain of porcine and human VEGF is identical.) Although one could argue that the difference between pegaptanib and the other VEGF antagonists is related to the experimental design, this does in fact offer an explanation as to why pegaptanib is not as effective in vivo as ranibizumab. The heparin-binding site assists the association of $VEGF_{165}$ with the cell membrane, ameliorating receptor activation, and binds to NP-1, enhancing the effect of $VEGF_{165}$,[13,16] and it has some importance for the mitogenic potency of VEGF,[11] which explains why pegaptanib shows clinical effects. Binding to the heparin-binding site, however, as indicated in this article, does not efficiently prevent the binding of $VEGF_{165}$ to its receptor site. Hence, it does not inhibit VEGF-induced angiogenesis. Pegaptanib inhibits the amplification of VEGFR signaling, whereas bevacizumab and ranibizumab inhibit VEGFR signaling itself (Fig. 6).

This study showed no difference in efficiency between bevacizumab and ranibizumab when they were used in clinical concentrations. Their effects on VEGF seemed to be identical. Considering their natures as the antibody and the antibody fragment of the same murine precursor,[17] this result may not be surprising. However, ranibizumab shows greater efficiency when it is highly diluted. Therefore, VEGF neutralization can be held to a concentration of 60 ng/mL, where VEGF is detectable again in considerable amounts. Bevacizumab is efficient up to a concentration of 1 μg/mL, indicating a 17-fold higher binding capacity of ranibizumab compared with bevacizumab. On a molar basis, a 6-fold higher binding affinity can be calculated, in accordance with published observations.[17]

Our data raise the question whether either substance is highly overdosed at the current clinical dosage. These findings may explain clinical findings seen in the contralateral eyes when using bevacizumab in AMD or PDR[18] (Giuliano A, et al. *IOVS* 2007;48:ARVO E-Abstract 3365). In addition, as little as 6.2 μg bevacizumab injected in an eye with PDR showed a beneficial effect that correlated well with the data obtained in this study.[18] Further studies regarding minimal and optimal clinical concentrations would be beneficial; some approaches have already been made.[19] Knowledge of the optimal dosage would be interesting with regard to different application methods such as microspheres,[20] which may replace regular intravitreal injections in the future.

A caveat of our system is the fact that the substance applied can reach all the tissue easily and does not have to penetrate the retina. Ranibizumab is a 48-kDa Fab fragment that, unlike a full-length antibody, can easily penetrate the retina.[21] Any difference between ranibizumab and bevacizumab regarding penetration cannot be detected in our system. On the other hand, recent studies[22,23] show that bevacizumab is indeed able to penetrate the retina, possibly rendering the ranibizumab

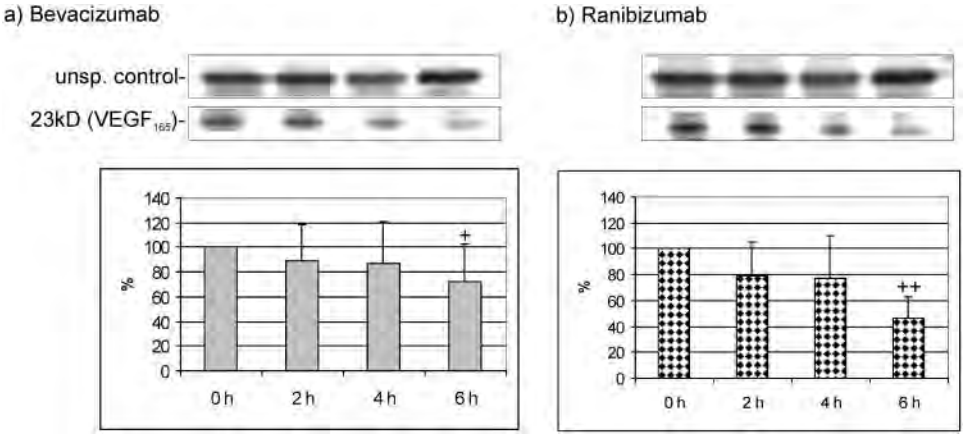

FIGURE 5. VEGF expression in Western blot after (**a**) bevacizumab or (**b**) ranibizumab. One representative blot is shown. Densitometric evaluation of Western blot analysis, using Gene tool software. 0, control set as 100%. Significance was evaluated using the *t*-test. $+P < 0.05$; $++P < 0.01$.

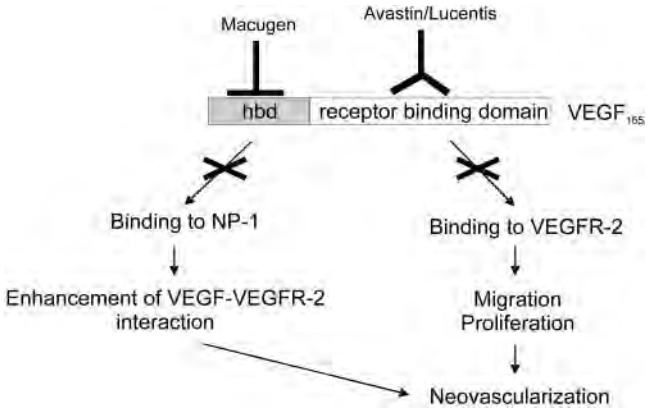

FIGURE 6. Schematic model of different pathways inhibited by VEGF antagonists. Bevacizumab and ranibizumab directly inhibit the binding of VEGF to its receptor VEGFR-2 and abrogate VEGF-induced neovascularization. Pegaptanib inhibits the binding to the coreceptor NP-1 and modulates VEGF-induced neovascularization.

advantage obsolete. Whether ranibizumab or bevacizumab should be the drug of choice, however, must be evaluated in prospective clinical trials.

Another intriguing finding is the indication of possible additional pathways of action of the VEGF inhibitors. The inhibition of VEGF lasts longer than the persistence of VEGF inhibitors in the medium, and protein blots show a significant alleviation of $VEGF_{165}$ expression by bevacizumab and ranibizumab. This result, however surprising, correlates well with data obtained by Heiduschka et al.,[22] who reported that VEGF expression in the inner retinal neurons disappears after intravitreal injection and retinal penetration of bevacizumab and does not reappear for 14 days after injection. An additional pathway of VEGF suppression would suggest a more complex regulation of VEGF expression and secretion than what is known so far. The exact mechanisms through which VEGF antagonists influence VEGF must be elucidated. VEGF expression might be modulated directly through unknown pathways, such as internalization and consequent intracellular effects. Bevacizumab has indeed been shown to be internalized by RPE cells.[22] In addition, indirect pathways could contribute to additional effects. For example, the inhibition of VEGF binding to its receptor might cause feedback mechanisms that influence VEGF expression. Different VEGF isoforms have been shown to regulate the expression of other isoforms.[24]

In conclusion, we were able to show that in clinical concentrations, bevacizumab and ranibizumab, but not pegaptanib, are similarly efficient in vitro and might act through additional pathways.

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