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# Bevacizumab and Ranibizumab on Microvascular Endothelial Cells: A Comparative Study

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## ABSTRACT

Given its broad effects in endothelium, vascular endothelial growth factor (VEGF) represents the primary rate-limiting step of angiogenesis. Therefore, VEGF targeting therapies were soon developed. Bevacizumab and ranibizumab are two of these therapeutic agents already in clinical use. Bevacizumab was first used for cancer treatment, whereas ranibizumab was designed to target choroidal neovascularization, the main cause of blindness in age-related macular degeneration. The present study aims to compare the multiple effects of bevacizumab and ranibizumab in human microvascular endothelial cells (HMECs). HMEC cultures were established and treated during 24 h with the anti-VEGF agents within the intravitreal-established concentration range or excipients. Analyses of VEGF content in cell media and VEGF receptor-2 (VEGFR-2) expression in cell lysates were performed. No cell cytotoxicity (MTS assay) was found in anti-VEGF-treated cultures at any concentration. Apoptosis (TUNEL assay) was significantly increased and cell proliferation (BrdU assay), migration (transwell assay) and assembly into vascular structures were significantly reduced by incubation with both agents at the two doses used. These findings were accompanied by a strong decrease in VEGF release, and in phosphorylated VEGFR-2 and Akt expression for both agents at the clinical concentration. Interestingly, phosphorylated Erk was only significantly reduced upon bevacizumab treatment. In addition, proliferation was more affected by ranibizumab, whereas migration, capillary formation, and phosphorylated VEGFR2 expression were significantly reduced by bevacizumab as compared to ranibizumab. Therefore, although both agents presented anti-angiogenic actions, distinct effects were exerted by the two molecules in HMEC. These findings suggest that a careful confirmation of these effects in clinical settings is mandatory. J. Cell. Biochem. 108: 1410–1417, 2009. 

**KEY WORDS:** BEVACIZUMAB; RANIBIZUMAB; ANGIOGENESIS; ENDOTHELIAL CELLS; VEGF SIGNALING

Angiogenesis, the formation of new blood vessels from pre-existing ones, is essential both in embryonic development, as well as in adulthood [Costa et al., 2004, 2007; Erickson et al., 2007]. Besides physiological angiogenesis, the neovascularization also occurs as an attempt to repair tissue damage in some diseases, such as cancer, myocardial infarct [Erickson et al., 2007], rheumatoid arthritis [Wang et al., 2004] and various vasculo-proliferative ocular diseases [Ferguson and Apte, 2008].

Angiogenesis is mediated by a huge number of stimulatory and inhibitory factors. An imbalance between these molecules occurs in pathological conditions [Damico, 2006]. Vascular endothelial growth factor (VEGF) is a crucial pro-angiogenic factor and the main promotor of endothelial cell (EC) growth, migration, and vessel dilation and permeability. Given its relevance, several studies addressed VEGF as a mediator of pathological vascularization, particularly in the development of anti-angiogenic therapy targeting vascular enhancement in pathological situations, such as cancer and age-related macular degeneration (AMD).

AMD is the leading cause of blindness in individuals over 50 years in developed countries, increasing with age [Wang et al., 2004], and affecting approximately 50 million people worldwide [Ferguson and Apte, 2008]. AMD-related visual loss is a complex process starting by the deposition of debris in the outer retina [Damico, 2006; Andreoli and Miller, 2007]. The deposition of insoluble material, the calcification and increase in thickness of Bruch's membrane, and a less fenestrated and thinner choriocapillaris lead to photoreceptors/retinal pigment epithelium hypoxia resulting in a stimulus for VEGF release [Shibuya, 2001; Adamis, 2005; deJong, 2006; Semenza, 2007]. The development of choroidal neovascularization (CNV) results into devastating visual effects for patients [Damico, 2006; Andreoli and Miller, 2007].

Although VEGF is not the only factor whose expression is increased in AMD, its effect is necessary and sufficient for the

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induction of retinal neovascularization [Damico, 2006]. VEGF is able to initiate a cascade of endothelial signaling that acts specifically in terms of survival, proliferation and migration, both in vitro and in vivo [Costa et al., 2004, 2007]. The formation of CNV has been closely related to an increase in local VEGF levels [Andreoli and Miller, 2007; Hussain et al., 2007]. Thus, there have been excellent results focusing on anti-VEGF therapies lately. Inhibition of VEGF with appropriate antibodies causes a decrease in CNV and vascular permeability, suggesting that it is an effective therapy [Presta et al., 1997; Castellon et al., 2002; Browning et al., 2008].

Bevacizumab (Avastin®) is a recombinant humanized monoclonal anti-VEGF-A antibody, produced in mice with the ability to neutralize all isoforms of this important endothelial growth factor, blocking its interaction with the appropriate receptors. This molecule targets several stages of the angiogenic process, such as proliferation, migration, and survival [Erickson et al., 2007], resulting in inhibition of CNV [Presta et al., 1997]. It was approved by the FDA for intravenous administration in patients with metastatic colon cancer [Andreoli and Miller, 2007; Carneiro et al., 2008]. It is also used off-label for the treatment of exudative AMD [Carneiro et al., 2008; Melamud et al., 2008]. The clinical intravitreal dose is 1.25 mg, which results in a concentration of 0.31 mg/ml considering an average vitreous cavity of 4 ml [Carneiro et al., 2008]. Ranibizumab (Lucentis®, Genetech and Novartis) is a Fab fragment derived from the bevacizumab antibody. It was especially designed by genetic manipulation for intravitreal application in patients with exudative AMD, as its small size molecule allows easy access to the outer retina. Ranibizumab is also specific for every isoform of VEGF and received FDA approval in June 2006 for the treatment of neovascular AMD [Andreoli and Miller, 2007; Hussain et al., 2007; Melamud et al., 2008]. It is, however, an expensive therapy [Spitzer et al., 2007]. The clinical intravitreal dose of this antibody is 0.5 mg, leading to a concentration of 0.125 mg/ml in the vitreum [Hussain et al., 2007].

Despite the lack of comparative studies, a few reports regarding the clinical use of these agents in series of patients suggest that treatment with bevacizumab has similar efficacy in the treatment of neovascular AMD to ranibizumab, but with less cost involved [Nagpal et al., 2007]. The cost issue is a relevant feature for both patients and health services considering the increasing prevalence of the disorder, which renders them a public health problem. In the present work, we compared the effect of bevacizumab and ranibizumab in the different steps of the angiogenic process, including cell viability, proliferation, migration, invasion, and formation of tubular structures using human microvascular endothelial cells (HMECs). We also highlighted the effect of the two agents in VEGF release to cell supernatant and the expression of the active (phosphorylated) form of VEGFR-2 as well as of their downstream effectors in HMEC cultures.

## MATERIALS AND METHODS

### CELL CULTURE EXPERIMENTS

Human microvascular endothelial cells (HMECs) (kindly provided by Dr João Nuno Moreira, Coimbra University) were used between passages 13 and 22. HMECs were cultured in RPMI 1640 medium (Invitrogen Life Technologies, UK) supplemented with 10% FBS (Invitrogen Life Technologies), 1% penicillin/streptomycin (Invitrogen Life Technologies), 1.176 g/L of sodium bicarbonate, 4.76 g/L of HEPES, 1 ml/L of EGF and 1 mg/L of hydrocortisone >98% (Sigma, Portugal), and maintained at 37°C in a humidified 5% $CO_2$ atmosphere. Treatments were performed for 24 h in serum-free conditions. Bevacizumab and ranibizumab were diluted in serum-free culture medium and added to cell cultures at final concentrations ranging the doses used in clinics: 0.125–2.5 mg/ml for bevacizumab, and 0.06–0.5 mg/ml for ranibizumab. Controls were performed using identical volume of excipients as previously described [Gaudreault et al., 2005].

### MTS TOXICITY ASSAY

HMECs ($2 \times 10^5$ cells/ml) were allowed to grow until 70–90% confluence and incubated with each treatment for 24 h. Cells were washed twice with PBS and their viability was assessed using Cell Titer 96® Aqueous ONE Solution Reagent (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] colorimetric assay (Promega, Madison, EUA), according to the instructions provided by the manufacturer. Optical density was measured at 492 nm. Results are expressed as percentage of control, which was considered to be 100%.

### TUNEL ASSAY

HMECs ($6 \times 10^4$ cells/ml) were grown on glass coverslips and incubated with the different treatments for 24 h. TUNEL assay (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instruction and as previously reported [Rocha et al., 2007]. The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nuclei observed, at a 200× magnification field. DAPI was purchased in Roche Diagnostics. One thousand nuclei were evaluated and three independent experiments were performed.

### BRDU PROLIFERATION ASSAY

HMECs ($6 \times 10^4$ cells/ml) were cultured following standard conditions or the treatment procedures for 24 h. Cells were also incubated with a 5′-bromodeoxyuridine (BrdU) solution at a final concentration of 0.01 mM for 24 h and then the in situ detection was performed using *In-Situ* Detection Kit (BD Biosciences Pharmigen, USA), according to manufacturer's instructions and as previously recorded [Rocha et al., 2007]. The results are given as mean ± SEM and are expressed as percentage of proliferating cells. This percentage was evaluated as a 200× magnification field. One thousand nuclei were examined and three independent experiments were performed.

### MIGRATION ANALYSIS

Injury assay was performed as previously described [Rocha et al., 2007]. Cells were grown to 90% confluence. Using a pipette tip, cells were scrapped from the culture dish leaving a void space. Cells were then incubated for 24 h following the standard treatments. After incubation cells were washed with PBS and cell migration to the

damaged area was visualized and photographed on a phase contrast microscope (Nikon, UK) at a magnification of 200×.

Migration capacity of HMEC was then quantified by counting the number of cells that migrated through matrigel-coated transwell BD-matrigel basement membrane matrix inserts (BD-Biosciences, Belgium). Transwell inserts containing an 8 μm pore-size PET membrane coated with a uniform layer of matrigel basement membrane were used. HMECs ($5 \times 10^4$ cells/ml) were harvested on inserts in serum-free medium, and placed on wells containing medium complemented with FBS (10%) and the different treatments. After incubation for 24 h membranes were removed from inserts, stained with DAPI-methanol for 5 min and visualized under a fluorescence microscope (Olympus, BH-2, UK). Twenty-five random fields of each membrane were counted, at a magnification of 200×.

### CAPILLARY-LIKE STRUCTURES FORMATION

Matrigel assay was performed to investigate the effect of the anti-VEGF agents in preventing formation of capillary-like structures. Cells were incubated with the distinct treatments and immediately added to matrigel-coated plates. The next day, the number of capillary-like structures was counted in each well in a phase-contrast microscope (Nikon), at a magnification of 200×.

### ELISA ASSAYS

VEGF protein concentrations in cell media were quantified using a commercially available Quantikine Human VEGF ELISA kit (R&D Systems, USA) in accordance with manufacturer's instructions. ODs were measured at 450–570 nm and VEGF was calculated as protein adjusted amount of VEGF (pg/μg protein), according to the standards used.

### WESTERN BLOTTING ASSAY

Proteins were isolated from HMEC lysates using Tripure (Roche Diagnostics). Proteins were quantified using a spectrophotometer (Jenway, 6405 UV/vis, Essex, UK) and equal amounts of protein were subjected to 8% SDS–PAGE with a 5% stacking gel. After electrophoresis, proteins were blotted into a Hybond nitrocellulose membrane (Amersham, Arlington, USA), using a mini-transblot electrophoretic transfer cell (Amersham Biosciences, USA). Immunodetection for active (phosphorylated) forms of VEGFR-2 (Santa Cruz Biotechnology, CA, USA), Akt and Erk (both purchased at Cell Signaling, MA, USA) and for β-actin (Santa Cruz Biotechnology) was accomplished with enhanced chemiluminiscence (ECL kit, Amersham Biosciences). The VEGFR-2 antibody recognizes the tyr-951/996 kinase insert domain, which is a major site of phosphorylation of this receptor, primarily involved in angiogenic signaling pathways. The relative intensity of each protein blotting analysis was measured using a computerized software program (Biorad, CA, USA) and normalized with β-actin bands to compare the expression of proteins in the different treatment groups. Experiments were repeated three times.

### STATISTICAL ANALYSES

All experiments were performed in triplicate. Quantifications are expressed as mean ± SEM. Samples were evaluated by the analysis of variance test. A difference between experimental groups was analyzed by Student's *t*-test, and was considered statistically significant whenever *P*-value was less than 5%. Statistical analyses were performed between each treatment and respective control (excipient-treated cells), and between the clinical doses used for bevacizumab and ranibizumab (0.25 mg/ml bevacizumab/control vs. 0.125 mg/ml ranibizumab/control), as well as half of the dose used in the clinic, that is, 0.125 mg/ml bevacizumab/control versus 0.06 mg/ml ranibizumab/control.

## RESULTS

### ANTI-VEGF AGENTS WERE NOT CYTOTOXIC FOR HMEC

In order to investigate whether bevacizumab and ranibizumab presented any cytotoxic effects on HMECs, cells were incubated with five concentrations of each agent within the clinical dose used. After 24 h incubation period, cell viability was assessed by MTS. Although a slight decrease in viability was found for increasing concentrations of the anti-VEGF agents, cell viability of HMECs was not significantly affected either by bevacizumab or ranibizumab at any concentration tested, as compared to excipient-treated cultures (controls) (Fig. 1). MTS assay was performed in HMECs at a confluency of 70–90%, thus, presenting already a slow replication rate. This ensures that the possible toxic effect was not masked by the decrease in HUVEC proliferation induced by the antibodies used. Our findings showed that EC maintain their metabolic activity and are not suffering a direct toxic effect by this anti-VEGF molecule at the concentrations tested. In contrast, proliferation and apoptosis were examined using rather lower cell concentration (40–50% confluency) to evaluate the effects of the agents on actively proliferating cells.

### PROLIFERATION, APOPTOSIS, AND MIGRATION OF HMECS

We next examined the effects of the two pharmacological agents in cell growth, apoptosis, and migration capacity at concentrations similar to the established clinical dose (0.25 mg/ml bevacizumab or 0.125 mg/ml ranibizumab). In addition, in order to determine whether concentrations lower than the established clinical dose were able to affect cell behavior, 0.125 mg/ml bevacizumab and 0.06 mg/ml ranibizumab (corresponding to half of the clinical dose used), were also tested and compared to excipient-treated HMEC.

The effect of the anti-VEGF agents in cell apoptosis and proliferation was tested by TUNEL and BrdU incorporation assays respectively. As illustrated in Figure 2A, apoptosis was increased after incubation of cells with bevacizumab or ranibizumab in a dose-dependent manner. However, significant differences were only observed for the doses established in the clinic ($^*P < 0.05$ vs. excipient-treated cells). At these concentrations, the percentage of apoptotic cells tripled the one observed in controls for both antibodies used. This was confirmed by statistical analysis. No significant differences were observed among the two agents at the therapeutic concentrations (0.25 mg/ml bevacizumab with 0.125 mg/ml ranibizumab), indicating a similar effect of these two agents in enhancing HMEC apoptosis.

A reduction in the percentage of proliferating cells was found with increasing concentrations of both bevacizumab and ranibi-

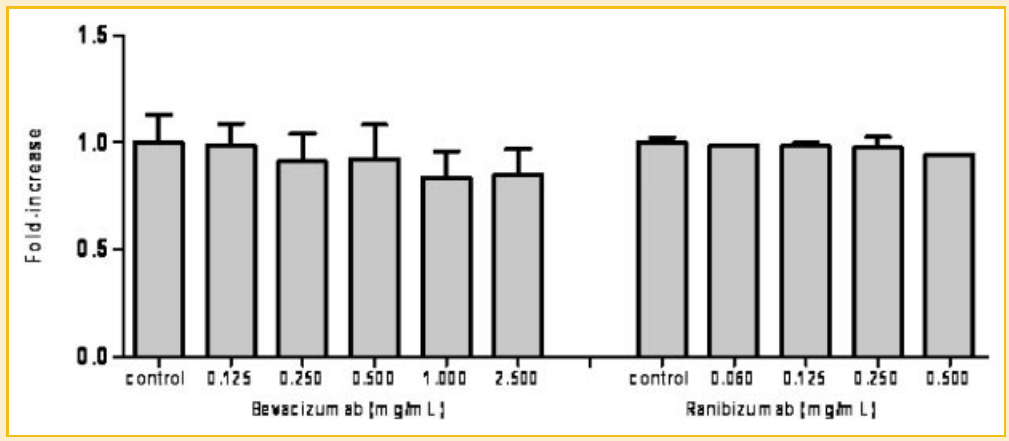


Fig. 1. Cytotoxicity evaluation in confluent HMEC cultures using MTS assay. No significant cytotoxicity was found either for bevacizumab or ranibizumab at any of the concentrations tested. Results are expressed in absorbance values at 540 nm and are fold-increase relative to control cell cultures. Control bar refers to incubation with excipients ($n = 14$).

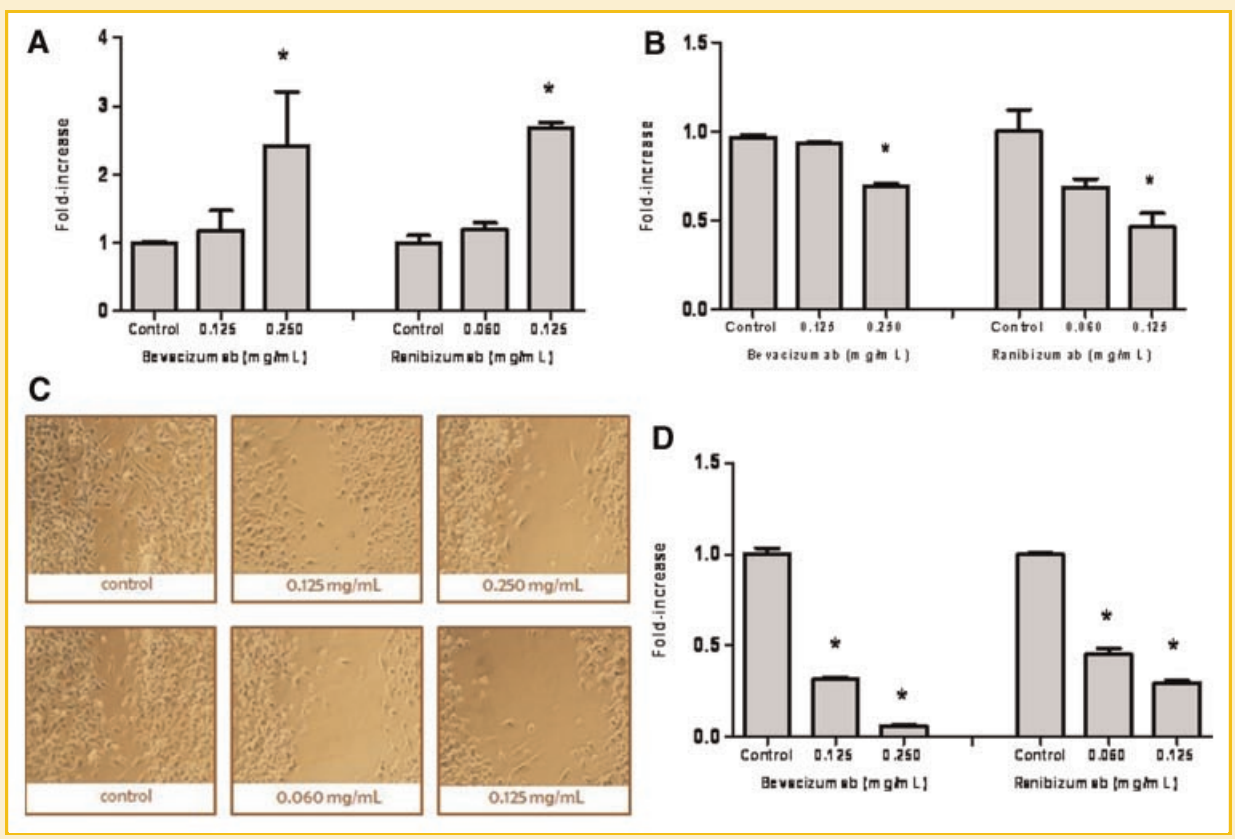


Fig. 2. Apoptosis, proliferation and migration of HMEC were evaluated after incubation with 0.125 or 0.25 mg/ml bevacizumab or with 0.06 and 0.125 mg/ml ranibizumab. A: Apoptosis was increased at every incubation as evaluated by TUNEL assay, reaching statistical significance for clinical dose of both agents ($^*P < 0.05$ vs. controls). Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture. Experiments were repeated three times with identical results ($n = 6$). B: Cell proliferation was assessed by BrdU incorporation assay. Cell proliferation was only effectively reduced by the two concentrations of bevacizumab and ranibizumab at the clinical dose. Bars represent the percentage of BrdU-stained cells in 1,000 hematoxylin-stained nuclei. Three independent experiments were performed in triplicate with identical results. $^*P = 0.05$ versus control ($n = 9$). C: Cell migration was visualized by injury assay after 24 h incubations. A significant abrogation of cell migration to the damaged areas was found after incubation with both bevacizumab and ranibizumab in a dose-dependent manner. Pictures are representative of three independent studies. Magnification (200×). D: Quantification of cell migration was performed by double-chamber assay. Both concentrations of the two agents resulted in a significant and dose-dependent reduction in the number of migrating cells ($^*P < 0.05$ vs. control). Bars represent the number of invasive cells and are fold-increase relative to control cell cultures. Assays were repeated three times and performed in triplicate ($n = 9$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

zumab, showing statistically significant differences for the clinical dose when compared to controls ($^{*}P < 0.05$ vs. Controls) (Fig. 2B). A tendency towards significant results was found for ranibizumab at 0.06 mg/ml concentration ($P = 0.06$ vs. Control). Interestingly, 0.25 mg/ml bevacizumab led to a decrease in the percentage of cell proliferation to $13.01 \pm 0.62$ in comparison to $18.84 \pm 0.75$ in excipient-treated controls (69% of control values), whereas the clinical dose of ranibizumab resulted in a more efficient decrease to 50% of the control values ($9.41 \pm 1.24$ in ranibizumab vs. $18.7 \pm 0.56$ in controls). Accordingly, statistical comparison of the effects of the two agents confirmed that incubation with ranibizumab resulted in a significant reduction in the number of proliferating cells at the two concentrations used ($P = 0.004$ for 0.125 mg/ml bevacizumab vs. 0.06 mg/ml ranibizumab; $P = 0.03$ for 0.25 mg/ml bevacizumab vs. 0.125 mg/ml ranibizumab).

The ability of cell migration was first evaluated by injury assay. Treatment with bevacizumab at the two concentrations tested caused a decrease in migration of HMEC to the damaged area in comparison to excipient-treated cells (Fig. 2C). Similarly, treatment with ranibizumab resulted in impairment of migratory capacity as compared to controls. This decrease was more pronounced when cells were incubated with the highest concentration of the drug. Cell migration was then quantified by double chamber assay. A strong decrease in the capacity of invasion after incubation with bevacizumab and ranibizumab at the two concentrations tested was observed again in a dose-dependent manner ($^{*}P < 0.05$ vs. controls) (Fig. 2D). Interestingly, this effect seemed to be more successful for bevacizumab, since the percentage of invasive cells decreased to 6% of the control values, whereas the clinical dose of ranibizumab led to 30% of the control values. Comparison between the effect of the two antibodies revealed the stronger effect of bevacizumab at the clinical dose in migration ($P = 0.03$ for 0.25 mg/ml bevacizumab vs. 0.125 mg/ml ranibizumab). A tendency to significant decrease was also observed for bevacizumab when the lower concentrations were compared ($P = 0.06$ for 0.125 mg/ml bevacizumab vs. 0.06 mg/ml ranibizumab).

## ASSEMBLY INTO CAPILLARY-LIKE STRUCTURES IN VITRO WAS AFFECTED BY BEVACIZUMAB AND RANIBIZUMAB IN HMEC

HMECs are able to organize into capillary-like structures in an appropriate matrix, such as Matrigel™, a model of in vitro angiogenesis. To address the effect of bevacizumab and ranibizumab in the assembly of these structures, cells were cultured in matrigel-coated wells and incubated with different concentrations of the drugs or excipient (control). The number of cord structures formed was quantified 24 h after incubations on an inverted microscope. A significant reduction in the number of cord-like structures formed was found after incubation with both bevacizumab and ranibizumab at any of the concentrations in comparison to excipient-treated cells (control), as illustrated in Figure 3. However, comparison between the two anti-VEGF agents showed that the number of cord structures was significantly decreased by treatment with 0.25 mg/ml bevacizumab when compared with ranibizumab at the intravitreal dose ($P = 0.02$ for 0.25 mg/ml vs. 0.125 mg/ml ranibizumab).

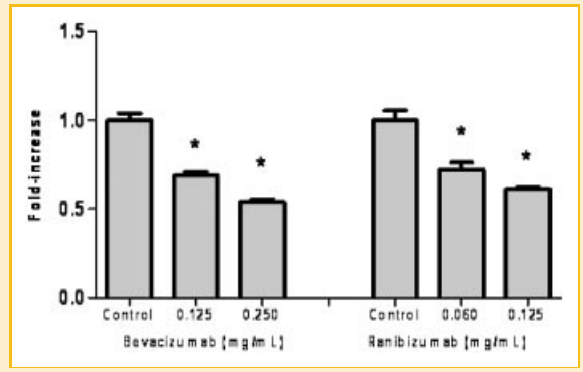


Fig. 3. Assembly of capillary-like structures after incubation with bevacizumab, ranibizumab, or excipient (controls). The number of cord structures was quantified on an inverted microscope. Vascular assembly was reduced in a significant manner after incubation with any agent as compared to excipient-treated cells ($^{*}P < 0.05$ vs. control) (n = 9).

## BOTH ANTI-VEGF AGENTS REDUCED VEGF RELEASE BY HMEC

To confirm the inhibitory effects of the anti-VEGF agents, we then quantified VEGF protein in the supernatant of HMEC cultures after incubation with each agent or respective excipient using ELISA assays. Bevacizumab and ranibizumab were able to reduce VEGF protein released by these cells (Fig. 4A), reaching statistical significance after treatment with 0.25 mg/ml bevacizumab, as well as after 0.125 mg/ml ranibizumab ($P < 0.001$ vs. controls). A tendency towards significance was found for the lower concentrations of both agents ($P < 0.06$ vs. controls). No significant difference was obtained when the two anti-VEGF antibodies were compared, indicating an identical inhibitory effect on VEGF among the two agents.

## EFFECT OF ANTI-VEGF TREATMENT IN VEGF SIGNALING PATHWAY IN HMEC

To ensure that the treatment with anti-VEGF pharmacological agents was effective in HMEC, we confirmed the expression of the phosphorylated form of the VEGF receptor-2 (VEGFR2) in these cell lysates upon anti-VEGF treatment. As illustrated in Figure 4B the expression of VEGFR2 was detected in every cell culture. The active form of this receptor was decreased with increasing bevacizumab and ranibizumab concentrations, resulting in a significant reduction for both agents at the dose established in the clinic. Furthermore, a tendency towards significant decreased values was found whenever cells were incubated with the smaller dose of both agents ($P < 0.10$ vs. controls) (Fig. 4B). A significant decrease in the expression of the active receptor was observed after incubation with bevacizumab at the two doses, when compared with ranibizumab ($P = 0.04$ for 0.125 mg/ml bevacizumab vs. 0.06 mg/ml ranibizumab; $P = 0.001$ when 0.25 mg/ml bevacizumab was compared with 0.125 mg/ml ranibizumab).

To further evaluate the effects of the two anti-VEGF agents on VEGF signaling, we next examined the expression of the phosphorylated forms of Akt and Erk, two established downstream molecules known to be implicated in cell proliferation, migration, and invasion. Expression of p-Akt was significantly reduced by bevacizumab at both concentrations tested. Incubation with

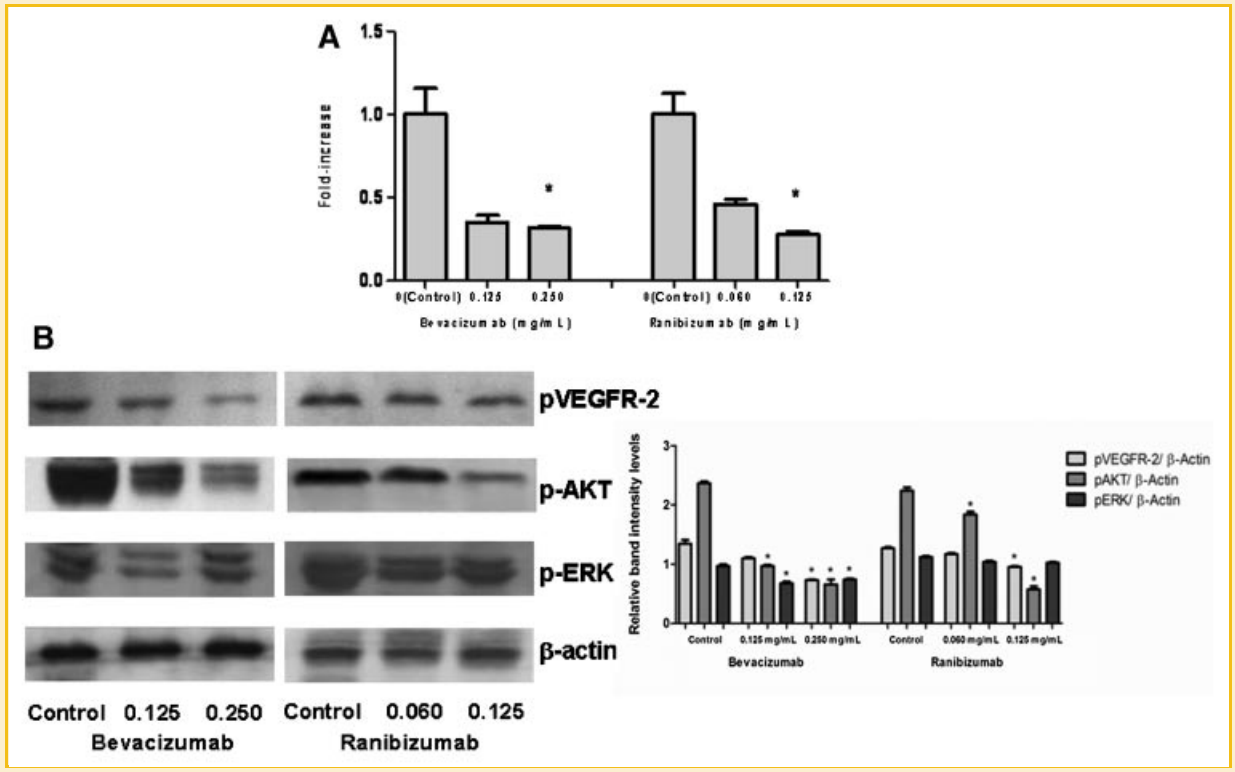


Fig. 4. Evaluation of VEGF signaling in HMEC upon treatment with bevacizumab or ranibizumab. A: VEGF present in supernatant was examined by ELISA assay. A decrease in VEGF release was found after incubation with both agents in comparison to control cells, reaching significant values for 0.25 mg/ml bevacizumab and 0.125 mg/ml ranibizumab. A tendency towards significant decrease was observed upon incubation with the lower concentrations of both anti-VEGF agents evaluated ($P < 0.06$ vs. controls). Four independent experiments were performed ($n = 8$). Statistical analysis was performed using ANOVA ($^*P < 0.05$ vs. controls). B: Expression of phosphorylated-VEGFR2 (p-VEGFR2), p-Akt and p-Erk in HMEC lysates after incubation with clinical doses of bevacizumab or ranibizumab during 24 h. Graph illustrates the relative band intensity ratio after normalization with β-actin. P-VEGFR-2 and p-Akt expression was reduced after each treatment at the clinical dose, in comparison to excipient-treated cell cultures (controls) ($P < 0.05$ vs. controls). P-Erk immunostaining was decreased only upon bevacizumab incubation ($^*P < 0.05$ vs. control). A tendency towards significant reduction for p-VEGFR-2 expression was present upon treatment with 0.125 mg/ml bevacizumab and 0.06 mg/ml ranibizumab. Equivalent protein loading was confirmed by probing stripped blots for β-actin as shown. A representative Western blotting is shown from three independent experiments ($n = 6$).

ranibizumab also led to a strong decrease in Akt signaling, but only at the clinical (highest) concentration. In contrast, p-Erk was only affected by incubation with bevacizumab (Fig. 4B), whereas no significant decrease in the phosphorylated-molecule was observed upon ranibizumab treatment. These findings indicate that bevacizumab exhibits a stronger inhibitory effect on VEGF signaling activation in HMEC.

A comparison of the whole effects observed after incubation with each anti-VEGF antibody on HMEC behavior is illustrated in Table I.

## DISCUSSION

The present paper aims to compare the effects of two anti-VEGF agents already used in the clinic. Bevacizumab is a humanized antibody designed to bind and prevent VEGF signaling. Ranibizumab is a Fab portion derived from bevacizumab. Its smaller size rendered this molecule the advantage for better penetration reaching the outer retina after intravitreal application. In fact, ranibizumab is also being tested worldwide to treat several vascular proliferative ocular pathologies [Spitzer et al., 2007]. Nevertheless, the cost of ranibizumab is much higher than that of bevacizumab. Therefore, a comparison study between these two is of paramount importance, in order to accurately establish the clinical potential of each of the pharmacological compounds.

Herein, we showed that bevacizumab and ranibizumab incubation did not result in decreased cell viability in comparison to

TABLE I. Comparison of the Effects Observed After Incubation of HMEC With the Clinical Dose of Bevacizumab and Ranibizumab in the Different Steps of the Angiogenic Process

| Cell behavior | Bevacizumab | Ranibizumab |
|---|---|---|
| Cell viability | ND | ND |
| Cell apoptosis | ↑ | ↑ |
| Cell proliferation | ↓ | ↓↓ |
| Cell migration | ↓↓ | ↓ |
| Cord-like structures formation | ↓↓ | ↓ |
| VEGF release | ↓ | ↓ |
| VEGFR-2 activity | ↓↓ | ↓ |
| Akt activity | ↓ | ↓ |
| Erk activity | ↓ | ND |

ND = no significant difference relative to control; ↑ = significant increase relative to control; ↓ = significant decrease relative to control; ↓↓ = significant decrease relative to the other anti-VEGF agent.

controls for any of the concentrations tested. These findings confirm that no cytotoxic effects on EC were triggered by these two agents. Furthermore, the anti-VEGF molecules exerted effective actions on distinct steps of the angiogenic process, namely, EC apoptosis, proliferation, migration, and invasive capacity and assembly into tubular-like structures. However, these processes were not identically affected by the two antibodies. A lesser effect on the reduction of proliferation was found after incubation with bevacizumab. In contrast, cell migration and assembly into capillary-like structures were both more effectively reduced by incubation with bevacizumab than by its counterpart. The broad effects of VEGF indicate that this growth factor is able to activate a huge number of cascades inside the cell. Accordingly, the angiogenic role of VEGF is mainly associated with the activation of VEGFR2, which is known to cross-talk with many other transduction pathways, namely PI3K and MAPK [Witmer et al., 2003; Chen et al., 2009]. Therefore, we anticipated that the distinct inhibitory cell responses triggered by the two anti-VEGF agents result in inactivation of different signaling cascades inside the cells.

In the current study, activity of VEGFR2, which is the primary receptor involved in every angiogenic feature analyzed in the current study, was observed to be down-regulated by the two agents. Interestingly enough, a stronger down-regulation of the active form of this receptor was obtained upon bevacizumab at the two concentrations examined, whenever compared to ranibizumab. The major sites of VEGFR-2 autophosphorylation are located at the tyr-951/996 kinase insert domain. However, there are also other kinase domains reported to play crucial roles in VEGF signaling activation. For instances, tyr-1175 stimulates PI3K signaling pathway, which is involved in angiogenic process as well [Kroll and Waltenberger, 1997]. Nevertheless, both agents were able to prevent VEGF release into cell medium within the same extent, implying that the discrepant effects on VEGFR-2 exerted by the two anti-VEGF molecules were not due to differences in their capacity to differentially abrogate VEGF levels at least at 24 h time-point. Instead, they might be due to the action of these agents on VEGF specific receptor tyrosine kinase activity, resulting hence in distinct cell behavior. Accordingly, our findings revealed that incubation with bevacizumab led to decreased activity of Akt and Erk signaling transduction pathways, whereas ranibizumab had no significant effect on Erk signaling. Given the well-established role of Erk signaling in cell fate decisions [Witmer et al., 2003; Chen et al., 2009], these findings may explain the differences in extent on HMEC observed upon treatment with bevacizumab and ranibizumab.

Another possible explanation for the different events observed with the two compounds is the fact that the effect of ranibizumab might be affected by its shorter half-life. Due to the absence of an Fc portion, ranibizumab is much more rapidly degraded than bevacizumab. Conversely, the full antibody characteristics of bevacizumab render this molecule a much more stable one, which probably results in longer clinical effects. In our culture assay, experiments were performed at 24 h incubation. This approach cannot easily be extrapolated to the clinic. However, our findings indicate that further clinical trials comparing the effects of these two agents are necessary.

In addition, diverse side effects have been associated with the use of both agents in ophthalmology. The development of endophthalmitis, uveitis, and stroke has been observed after ranibizumab treatment, though in a small-scale [Fintak et al., 2008]. In turn, inflammation, cataracts progression, hypertension and stroke were also reported in a small number of patients treated with bevacizumab [Spitzer et al., 2007].

Previous studies of our group comparing the effects of bevacizumab, ranibizumab, and pegaptanib in human umbilical vein endothelial cell cultures also showed distinct effects when the concentration used in the clinic for these molecules was used [Carneiro et al., 2009]. Interestingly, the differences observed in the other cell culture were not exactly identical to the ones found in the current study. For instances, ranibizumab did not affect cord structures formation in a significant manner in the former study. In contrast, both concentrations of bevacizumab were actually highly effective in reducing the ability of HMEC to assemble into capillary structures. These differences in cell behavior are likely due to the fact that these endothelial cells are actually different. Accordingly, although EC from distinct types of vessels exhibit identical morphological characteristics, a few reports demonstrated that they present quite different molecular patterns [Nanobashvili et al., 2003]. Taking these findings together, HMEC is probably a more accurate model to study the effects on the angiogenic process, given their capillary background, characteristic of angiogenic vessels. Conversely, HUVECs present features of a more differentiated endothelial cell culture [Nanobashvili et al., 2003].

Another important purpose of the current paper was to examine whether lower doses of the anti-VEGF molecules would present benefic effects as well. To examine this, the effects of bevacizumab and ranibizumab were compared at half the concentrations used in the clinic (0.125 mg/ml bevacizumab and 0.06 mg/ml ranibizumab). Significant reductions were only observed for both treatments in the number of capillary-like structures formation and for bevacizumab in the HMEC migration capacity. Accordingly, these smaller concentrations did not result in effective reduction of VEGF concentration released to cell media, although a tendency towards significant reduction was observed. In agreement, only a tendency towards significance was found for phosphorylated-VEGFR-2 expression at the lower concentration of both agents. These findings suggest that the concentrations tested might not be strong enough to prevent VEGF-induced angiogenesis.

In conclusion, the present study showed that both anti-VEGF agents evaluated were able to prevent VEGF release to HMEC culture medium, preventing, therefore, VEGF signaling through VEGFR2 activation. Incubation with both agents led to impairment of several steps of the angiogenic process. Nevertheless, ranibizumab seemed to have a stronger effect on HMEC proliferation than bevacizumab, whereas this latter agent resulted in an extensive inhibition of VEGF signaling, resulting in HMEC inability to migrate and assemble into tubular structures. The present paper clearly highlighted the effects of both anti-VEGF agents using microvascular EC, an appropriate model for studying angiogenesis. Nevertheless, further studies are required in order to confirm these findings in the clinic, and

to investigate the action of these agents on different types of cells as well.

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