Original article

Vascular morphological and microdensity changes of corneal neovascularization induced by topical bevacizumab and sunitinib in an animal model

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A B S T R A C T

Objective: To evaluate the effects of topical bevacizumab and topical sunitinib on vascular microdensity and morphology of corneal neovascularization (NV).

Methods: A total of 33 rabbits were distributed into 3 groups: group 1 (control; n = 11): saline; group 2 (n = 11): bevacizumab 5 mg/ml; and group 3 (n = 11): sunitinib 0.5 mg/ml. A corneal NV model was used, based on sutures in the right eye of each rabbit. Each treatment was administered topically 3 times daily for 14 days. Corneas were then processed for the study of vascular microdensity (6 eyes) and vascular morphology analysis (5 eyes) using enzymatic staining histological techniques.

Results: The vascular response in group 3 was limited to small-sized tree formations with various vascular axes compared with the extensive, lush and directional corneal NV of groups 1 and 2. In the histological sections near the limb, there were no differences in vascular microdensity studies between the three groups. However, the mean sectional area of vessels (MSAV) in group 3 was 41.88% lower than in group 1 and 19.19% lower than in group 2. In distal sections, there were no differences between groups 1 and 2. However, group 3 was characterized by the absence of vessels.

Conclusions: Bevacizumab produced no changes in the morphology of the vessels or the vascular microdensity. Sunitinib reduced the size of the new vessels and induced changes in the vascular tree.

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Cambios en la morfología y la microdensidad neovascular corneal inducidos tras la administración tópica de bevacizumab y sunitinib en un modelo animal

R E S U M E N

Objetivo: Evaluar los efectos de la administración tópica de bevacizumab y sunitinib sobre la microdensidad vascular y la morfología de la neovascularización (NV) corneal.

Método: Se distribuyeron 33 conejos en 3 grupos: grupo 1 (control; n = 11): suero salino; grupo 2 (n = 11): bevacizumab 5 mg/ml y grupo 3 (n = 11): sunitinib 0,5 mg/ml. Se realizó un modelo de NV corneal mediante suturas en el ojo derecho de cada conejo. Se administró cada tratamiento por vía tópica 3 veces al día durante 14 días. Posteriormente, se procesaron las córneas para el estudio de la microdensidad vascular (6 ojos) y el análisis de la morfología vascular (5 ojos) mediante técnicas histológicas de tinción enzimática.

Resultados: La respuesta vascular del grupo 3 quedó limitada a unas pequeñas formaciones arborescentes con varios ejes vasculares en comparación con la extensa, frondosa y direccional NV corneal de los grupos 1 y 2. Las secciones histológicas proximales al limbo no mostraron diferencias en los estudios de microdensidad vascular entre los 3 grupos. No obstante, la media del área de sección de los vasos (MASV) del grupo 3 fue un 41,88% menor que la del grupo 1 y un 19,19% menor que la del grupo 2. En las secciones distales, no hubo diferencias entre los grupos 1 y 2. Sin embargo, el grupo 3 se caracterizó por la ausencia de neovasos.

Conclusiones: Bevacizumab no produjo cambios en la morfología de los vasos ni en la microdensidad vascular. Sunitinib redujo el calibre de los neovasos e indujo cambios en el árbol vascular.

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Introduction

Corneal neovascularization (NV) produces severe visual damages, compromising the transparency of the cornea and deteriorating its optical quality. Diseases associated to corneal NV include inflammatory corneal disorders, infectious keratitis, hypoxia due to contact lenses, chemical burns, stromal ulcers and limbal stem cell deficiency. In addition, corneal NV worsens corneal transplant prognostic.

In clinical practice, corticoids are the main pillar for treating corneal NV. However they are not always effective and chronic use could induce glaucoma or the formation of cataracts.

Bevacizumab is a humanized recombinant monoclonal antibody anti-vascular endothelial growth factor (VEGF) which links to all VEGF isoforms. Bevacizumab neutralizes the interaction of VEGF with its receptor and subsequently diminishes vascular permeability and angiogenesis. Subconjunctival and topical administration of bevacizumab significantly inhibits corneal NV. However, the elimination of said neovessels is not complete.

Platelet-derived growth factor (PDGF) is another important angiogenesis mediator. Its receptors express in pericytes and vascular smooth muscle cells which provide mechanical support for the new vessels and prevent spontaneous regression thereof.

In a previous publication, the authors demonstrated that simultaneous pharmacological interruption of VEGF and PDGF system signals (dual pathway) produces deep antiangiogenics effects. To this end, the authors compared in an experimental animal model the effects of topical administration of bevacizumab (anti-VEGF) and sunitinib (tyrosine-kinase receptor inhibitor with anti-VEGF and anti-PDGF activity). Sunitinib was 2.9 times more potent than bevacizumab at inhibiting corneal NV surface assessed by means of biomicroscopy and fluorescein angiography.

The objective of this study is to research the effects produced by bevacizumab and sunitinib in vascular microdensity and morphology of corneal neovessels with enzymatic staining histological techniques.

Material and methods

Thirty-three New Zealand male rabbits aged 2.5 months and weighing 2.5–3 kg were used. Under general anesthesia induced by intramuscular injections of 20 mg/kg of ketamine HCl and 6 mg/kg of xilazine HCl, supplemented with topi- cal anesthesia (0.4% oxybuprocaine hydrochloride), a suture technique was carried out for inducing corneal NV consisting in 5 stitch points with 8/0 pure silk (LorcaMarin S.A., Murcia, Spain) in the middle stroma of the superior cornea, following a triangular pattern based on the limbus (Fig. 1). After the intervention, erythromycin ophtalmic cream was adminis- tered 3 times a day for 24 h. Only one eye of each rabbit was intervened.

Rabbits were randomly distributed in 3 groups: group 1 (control group; n = 11) which was administered 0.9% topical saline solution; group 2 (n = 11) was given 5 mg/ml beva- cizumab (Avastin, Roche Registration Ltd., Welwyn Garden City, United Kingdom) and group 3 (n = 11) received 0.5 mg/ml
sunitinib (Axon Medchem BV, Groningen, Holland). Treatment was initiated 12 h after the surgical treatment and was administered topically 3 times a day during 14 days. The concentrations of bevacizumab (5 mg/ml) and sunitinib (0.5 mg/ml) were selected on the basis of previous studies.\textsuperscript{4,10,11}

Subsequently, the rabbits of all the groups were sacrificed and the enucleated eyes were processed for determining vascular microdensity (six eyes) and vascular morphology analysis (five eyes) of corneal NV. All the experimental procedures were measured and quantified by a masked examiner for all treatment groups.

For studying vascular microdensity, 2 areas were analyzed as shown in Fig. 2. Zone one, located between the limbus and the first row of suture points, and zone 2 located between the first and second row. The corneal tissue sections were stained with NADPH-diaphorase and 20 μm histological sections were obtained. From every zone, 2 points were selected at 750 μm from the limbus in zone one (sections A and B) and at 2,250 μm of the limbus in zone 2 (sections C and D) centered at the level of the middle of each suture.

As indicated in Fig. 3, photographs were taken with an increase of 100× in TIFF formats with a field of 1.368 μm × 1,094 μm (width × height). In every zone the stromal area of both sections was measured and added to obtain the overall stromal area of each zone being studied. Subsequently, neovessel analysis was carried out for every zone, calculating the mean vessel section area (mean of the areas of all the best since sectioned in each zone) and vascular microdensity, determined by the number of vessels/mm\(^2\), vascular area/mm\(^2\) and percentage of the latter against the corneal stroma area.

The diameter of a capillary is approximately 5–10 μm. Therefore, to avoid false positives, the structures under 5 μm wide were excluded because the NADPH-diaphorase staining is able to stay in the nuclei of some cells that are probably involved in the inflammatory response as well as the individual nervous fibers of the sub-basal plexus which have a diameter between 0.05 and 0.25 μm.

All the measurements were taken using image analysis and processing computer application (Image-Pro Plus V.6.0., Media Cybernetics Inc., Bethesda, MD, USA).

Five eyes out of every group were allocated to vascular morphology study. For this purpose, the corneas were sectioned to obtain a block of triangular tissue containing the area covered by the sutures and therefore the corneal NV surface. The resulting histological samples were processed by enzymatic staining using the NADPH-diaphorase technique. Subsequently, the corneal neovessels of every sample were drawn on paper using a camera lucida included in an optical microscope (Leika Microsystems Ltd., Heerbrugg, Switzerland). For every sample the neovessels visualized in the microscope (10× increases) in areas close to the limbus measuring approximately 2 mm × 1.5 mm were drawn.

All the experimentation procedures with the animals were carried out in accordance with the ARVO (Association for Research in Vision and Ophthalmology) animal manipulation and maintenance regulations and with Royal Decree 1201/2005 dated October 10 on the protection of animals utilized for experimentation and other scientific purposes.

For the statistical analysis, SPSS version 15.0 for Windows was utilized (SPSS Incorporated, Chicago, IL, USA). The parametric statistical analysis was carried out after verifying the normal distribution of data by means of the Kolmogorov-Smirnov sample test. The microdensity changes between the different areas of every group were assessed using the T for student test for paired samples. Differences between groups for every variable were assessed by means of factor variance analysis (one factor ANOVA). The homogeneity of group variances in the one factor ANOVA test was determined by means of Levene’s statistic. When the variances were similar the Tukey test was utilized for multiple comparisons between the groups. When the variances were unequal, the Tamhane test was utilized. The correlation between the 2 quantitative variables was analyzed using Pearson’s correlation coefficient (normal distribution). The differences were considered to be statistically significant when the value of \(p < 0.05\).

Graphic representations were made with Microsoft Office Excel 2007.
Analysis of each zone

Fig. 3 – The corneal stroma area to be studied is calculated adding the corneal stroma areas of the 2 sections of every zone. On the basis of this stromal area sum, the vessels are analyzed and the vascular microdensity is studied.

Results

The mean vessel section area (MVSA), as well as vascular microdensity data (mean number of vessels/mm², vascular area/mm² and vascular area percentage) of zone one and zone 2 for every group are shown in Table 1, while vascular microdensity changes between both zones is illustrated in the graphs of Fig. 4.

In group 1 (physiological serum), MVSA was of 621.45 ± 48.29 µm² in zone 1 and of 593.75 ± 209.37 µm² in zone 2, without finding statistically significant differences between both zones (p = 0.74). The number of corneal stroma vessels/mm² diminished from 47.35 ± 13.91 in zone 1 to 28.79 ± 10.11 in zone 2 although the differences were not significant either (p = 0.06). In what concerns the stromal area occupied by the vascular tissue (vascular area) in zone 1 it was of 2.93 ± 0.88% and diminished significantly to 1.68 ± 0.65% in zone 2 (p = 0.012) (Fig. 5).

In group 2 (bevacizumab) no significant differences in MVSA were found between zones 1 and 2 (477.63 ± 115.15 µm² in zone 1 and 357.47 ± 85.77 µm² in zone 2; p = 0.08). Likewise, the reduction in the number of vessels between the 2 zones was not significant (62.81 ± 10.29 vessels/mm² and 36.94 ± 28.26 vessels/mm² in zones 1 and 2 respectively;
p = 0.06). The vascular area diminished significantly from 3.01 ± 0.84% in zone 1 to 1.41 ± 1.28% in zone 2 (p = 0.03) (Fig. 5).

In all the corneas of group 3 (sunitinib), corneal NV did not exceed the first row of stitches and therefore the zone to did not exhibit neovessels. Zone one exhibited MVSA of 361.17 ± 46.89 μm², 52.92 ± 33.02 vessels/mm² and 1.84 ± 1.17% of vascular area in relation to the stromal area (Fig. 5).

In zone one no significant differences were found between the 3 groups of drugs in what concerns vascular microdensity in terms of the number of vessels/mm² (one factor ANOVA; p = 0.47), vascular area measured in μm²/mm² (p = 0.09) and percentage of vascularized stromal surface (p = 0.09). Group 2 (bevacizumab) MVSA was lower compared to the control group (physiological serum; p = 0.014). In group 3 (sunitinib), MVSA significantly lower than in group 1 (physiological serum; p < 0.01) and group 2 (bevacizumab; p = 0.048). The correlation between the number of vessels and MVSA for every group was not significant (p = 0.86; p = 0.74 and p = 0.22 for groups 1, 2 and 3, respectively).

In zone 2, group 3 (sunitinib) was characterized by the absence of neovessels and therefore the differences were significant for all the studied variables against groups 1 and 2. Between group 1 (physiological serum) and group 2 (bevacizumab) no significant differences were found in relation to MVSA and vascular microdensity. Similarly, a significant correlation between the number of vessels and MVSA was not found (p = 0.75 and p = 0.36 for groups 1 and 2, respectively).

In what concerns vascular morphology, in group 1 arterial vessels exhibited darker stain, uniform gauge and straight pathways with some ramifications for angiogenic stimulation. Venous vessels that met the pericorneal veins were generally located in deeper planes and exhibited a soft and even profile with soft staining and greater sinuosity. As regards NV, an immature, non-hierarchical node was observed made up by a labyrinth of short and ramified anastomosing channels. In the more distant areas the vascular buds were identified. The source of these protruding structures were mainly venules, possibly reflecting a greater surface of venous vascular area in this tissue.

The corneal NV of group 2 (bevacizumab) exhibited morphological characteristics similar to those of group 1 (physiological serum), the only difference being surface extension (Fig. 6).

In group 3 (sunitinib) the vascular response was limited to small formations with several vascular axes which acquired a tree-shaped arrangement (Fig. 7). Each vascular axis was made up of a narrow gauge central artery which issued short branches with less defined orientation than in the other groups. On the edges of the plexus, the vessels exhibited the characteristics of venules and capillaries with a high number of anastomosis and small size vascular birds. The morphological differences between the 3 groups are illustrated in detail in the graphs made with the camera lucida (Fig. 8).

Discussion

The prominent role played by VEGF in angiogenesis pathophysiology due to its action on the VEGFR-1 and mainly VEGFR-2 tyrosine-kinase receptors has been demonstrated in experimental corneal vascularization models and in human cornea. In fact, most antiangiogenic drugs for treating ocular NV act upon the VEGF system.

Bevacizumab is a monoclonal antibody that blocks all the VEGF isoforms, preventing bonding to the biological receptors present on the surface of vascular endothelial cells. However, VEGF system blockage prevents or reduces corneal NV but this is not enough for deep inhibition of angiogenesis. This finding indicates that the angiogenesis mediators are also relevant and could act independently of the VEGF system or in conjunction with it.

PDGF is an important mediator of angiogenesis. Newly formed vessels can remit spontaneously unless they are surrounded by wall cells constituted by pericytes and smooth muscle cells. For endothelial cells to recruit wall cells, the PDGF-B produced by vascular endothelial cells is required together with their signaling through the PDGFR-β receptor, which is expressed by pericytes and smooth vascular muscle cells.

Tyrosine-kinase inhibitors are a new type of drugs that block the tyrosine-kinase receptors, including VEGF and PDGF receptors. Sunitinib is a small inhibitor of tyrosine-kinase receptor which is administered orally and exhibits powerful antiangiogenic and antitumoral activity. Sunitinib inhibits VEGFR-1, VEGFR-2 and PDGFR-β receptors.
demonstrated that orally administered sunitinib was able to significantly reduce the volume of experimental choroidal neovascular membranes in rats.\textsuperscript{20}

In a previous paper, the authors compared the effect of topical administration of bevacizumab (anti-VEGF) and sunitinib (anti-VEGF and anti-PDGF) on the corneal NV surface in an animal model. Sunitinib was 9 times more potent than bevacizumab for corneal angiogenesis inhibition, demonstrating that the combined blockage of VEGF as well as PDGF is more efficient for treating corneal neovascular diseases than the single VEGF system inhibition.\textsuperscript{19}

In this paper, the authors propose to evaluate the changes produced by both drugs in vascular microdensity and vessel morphology. In zone one, close to the limbus, no significant differences were observed between the 3 groups in what concerns vascular microdensity in terms of number of vessels/mm\textsuperscript{2} of stromal surface, vascular area/mm\textsuperscript{2} and percentage of vascular area vis-à-vis the stromal surface. In
### Table 1 – Mean area of vessels and vascular microdensity measures.

<table>
<thead>
<tr>
<th>Zone 1</th>
<th>Zone 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td><strong>Group 2</strong></td>
</tr>
<tr>
<td>Mean area of vessels (μm²)</td>
<td>621.45 ± 48.29</td>
</tr>
<tr>
<td>Number of vessels/mm²</td>
<td>47.35 ± 13.91</td>
</tr>
<tr>
<td>Vascular area (μm²/mm²)</td>
<td>29.37 ± 8.815.66</td>
</tr>
<tr>
<td>% of vascular area</td>
<td>2.93 ± 0.88</td>
</tr>
</tbody>
</table>

Mean area of vessels and results of vascular microdensity in zone one (close to the limbus) and in zone 2 (distal, between the first and second row of sutures) for all the groups. Group 1 = physiological serum; group 2 = bevacizumab; group 3 = sunitinib.

**Fig. 6** – Microscopic photography of the corneal neovascularization surface stained with NADPH-diaphorase technique in an eye treated with bevacizumab, showing the intensely stained arteries, uniform gauge and straighter lines. At a deeper plane, the veins exhibit softer staining, uneven profile and sinuous path. The vascular buds can be seen at the neovascularization front. Not all the vessels are in focus due to the large thickness of the cornea (NADPH-diaphorase, 10x).

**Fig. 7** – Tree Shaped vascular formation in an eye treated with sunitinib (NADPH-diaphorase, 100x).
comparison with group 1 (physiological serum), MVSA was 27.97% lower in group 2 (bevacizumab). In group 3 (sunitinib), the mean section area was 41.88% smaller than in group 1 and 19.19% smaller than in group 2.

The inhibiting capacity of sunitinib is demonstrated in zone 2, characterized by total absence of neovessels in all the evaluated corneae. In contrast, bevacizumab did not demonstrate significant differences vis-à-vis physiological serum or MVSA or in vascular microdensity.

In group 3 corneae (sunitinib), the vascular response was limited to small formations with several vascular axes which acquired a tree-shaped arrangement in contrast to the vascular morphology of group 1 (physiological serum) and group 2 (bevacizumab) corneae, which exhibited an extensively and abundantly distributed direct channel. This finding suggests that the PDGF pathway blockade plays an important role in remodeling newly formed vessels.

As described by the authors in said preceding paper, sunitinib has a yellow-golden color and deposits in the inferior quadrant of the iris, which indicates that the molecule reaches the anterior chamber after topical application. This could be highly useful in clinical practice for treating intraocular neovascular diseases.\(^\text{10}\)

In summary, the above described findings illustrate the additional changes induced by the PDGF pathway blockade when associated to the VEGF system blockage. Even though bevacizumab produces an inhibition of 28% on the corneal NV surface,\(^\text{10}\) it does not produce significant changes in the morphology of vessels or in the vascular microdensity. In addition to exhibiting a potent inhibition of 82.2% of the corneal NV surface,\(^\text{10}\) sunitinib markedly reduces the gauge of vessels and produces changes in the vascular tree architecture. These results point to the need of carrying out clinical research with topical sunitinib for treating neovascular eye diseases.

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**Conflict of interest**

The authors declare no conflict of interest.

**REFERENCES**

12. Bock F, Onderka J, Dietrich T, Bachmann B, Pyrowski B, Cursiefen C. Blockade of VEGFR3-signalling specifically inhibits lymphangiogenesis in inflammatory corneal...