



Angiogenesis: A special reference to corneal neovascularization

Angiogeneza: poseban osvrt na neovaskularizaciju rožnjače

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History

Angiogenesis, the term coined in 1935¹ means the development of new blood vessels from the pre-existing capillaries and venules. It is fundamental for reproduction, development and reparation, when strict regulation and short duration prevent the uncontrolled growth of neovascularization². In pathological conditions, represented by solid tumors and a myriad of the neovascular diseases which involve retina, uvea and cornea, the disbalanced regulation leads to the lasting, life- or sight-threatening vascular proliferation.

Algire's³ observation that the tumors actively attract blood vessels, and Michaelson's⁴ conclusion that a diffusible substance, factor X, present in the extravascular retina in various concentrations, is necessary for retinal neovascularization, were the beacons along the path which lead to the right direction: development of the field of angiogenesis research fifty years ago, started by the pioneering work of Folkman et al.⁵. Their hypothesis concerning the potential anticancer effects of angiogenesis inhibitors, based on the observation that the growth of solid tumors depends on their vascularization,⁶ had been questioned until the isolation of a tumor factor responsible for angiogenesis⁷.

Folkman's laboratory introduced new methods necessary for the new field of research: corneal pocket assay, polymers for the sustained release of macromolecules, chorioallantoic membrane, and capillary endothelial cell culture^{8,9}. Matrigel was added later⁹. These new tools helped in the discovery of the first angiostimulators: basic and acid fibroblast growth factor (bFGF, aFGF), and angiogenin¹⁰. Angiomodulators, most notably heparin, were added to the concept of angiogenesis¹¹. Heparin antidote, protamine, was the first angioinhibitor with the known structure¹². Then followed the discovery of a po-

tent angioinhibitory effect of heparin in the presence of cortisone,^{13–15} and of two endogenous angioinhibitors, angiostatin¹⁶ and endostatin¹⁷.

Growth factors

A large number of molecules which stimulate or inhibit angiogenesis are known today. A key role among stimulators, especially in the eye, plays vascular endothelial growth factor (VEGF)^{18,19}. Its inhibition has been used for the therapy of various diseases of different organs, from colorectal cancer to age related macular degeneration, just because they share pathological angiogenesis in common.

VEGF is a potent hemoattractant and endothelial cell mitogen. Its angiogenic action is regulated by hypoxia²⁰. It is a dominant factor of ocular and general angiogenesis, being a perfect match for „Factor X“ postulated by Michaelson⁴ as early as 1948. A single gene is coding for binding VEGF A, B, C, D to the tyrosin-kinase receptors VEGFR^{1–3}. Various isoforms of VEGF, created by alternate splicing,²¹ enable this growth factor to act in more than one way: to stimulate angiogenesis and vascular permeability, participate in organ development and vasculogenesis, maintain small fenestrated blood vessels, and protect nerve cells in the retina and elsewhere^{22,23}.

Basic fibroblast growth factor, although unable to promote neither retinal nor choroidal neovascularization alone, can act in synergism with VEGF²⁴. Due to the lack of a signaling pathway for its release from cells and membranes, bFGF can act only upon their injury. Yet, its proangiogenic role is established by the findings of high levels of bFGF in the vitreous of the eyes with proliferative diabetic retinopathy, and large tumors, as well as the rodent corneal neovascularization after

implantation of bFGF²⁵. Finally, a simultaneous inhibition of both bFGF and VEGF activities in vitro is more efficient than inhibition of only VEGF²⁶.

Findings of the raised vitreous levels of erythropoietin in proliferative diabetic retinopathy, and inhibition of neovascularization in the ischemic murine retina indicate that this, otherwise blood-forming substance has a role in angiogenesis²⁷. This complex process is also influenced by angiopoietins, cyclooxygenases²⁸, platelet derived growth factor (PDF), hepatocyte growth factor (HGF), placenta growth factor (PIGF), transforming growth factor-beta (TGF- β), tumor necrosis factor-alpha (TNF- α), and interleukines²⁹.

Switch to angiogenic phenotype

The sequence of events during angiogenesis is: burst of endothelial cells mitotic activity under the influence of an angiostimulator; creation of a break in the basement membrane; degradation of extracellular matrix; creation of a columnar structure through which blood starts to flow; termination of the process. For this to happen, a large number of receptors and ligands must be successively activated, while keeping a delicate balance of numerous stimulatory and inhibitory signals. Differently put, an angiogenic phenotype is achieved through a switch in the balance of angiogenesis stimulation and inhibition towards the former. As soon as the switch, usually a hypoxic stimulus, is turned on, the activity of angiogenic factors is up-regulated;³⁰ the shape of the cells is changed making cells susceptible to the action of angiogenic factors;³¹⁻³³ growth factors are released from their bound state;³⁴⁻³⁶ pericytes, a barrier to angiogenesis, are lost and macrophages activated;³⁷⁻⁴⁰ genes coding for angioinhibitors are inactivated;⁴¹ proteolysis of large molecules to which angioinhibitors are often bound is halted; soluble, decoy receptors,⁴² like Fit-1, stop to bind angiostimulators, primarily VEGF; and notch signaling⁴³ starts navigating activated cells through a matrix prepared by proteinases⁴⁴ and bi-directional transmembrane receptors, integrins⁴⁵.

Corneal avascularity

One of the most useful ways towards understanding corneal angiogenesis is to study avascularity of the normal cornea. The avascularity and easy accessibility of cornea made it one of the most frequently used model of angiogenesis research^{9,46}. As Cogan⁴⁷ nicely put it: „Any theory which claims to explain corneal neovascularogenesis must account for the absence of blood vessels in the normal cornea. Unlike most other tissues, except probably cartilage, the cornea has no vessels and yet it is in immediate proximity of structures having blood vessels. No anatomic boundary separates the vascular limbus from the avascular cornea. An adequate explanation for this anatomic paradox would undoubtedly account for neovascularization of the cornea“. He believed that compactness of the normal cornea presented a barrier, while corneal edema was a condition

for the invasion of blood vessels⁴⁸. However, the shape of neovascularization induced by an isolated experimental corneal lesion led Campbell and Michaelson⁴⁹ to postulate the presence of a diffusible stimulator of blood vessel growth⁴⁹. Ashton and Cook⁵⁰, in a lengthy critical review, added one more possible cause, hypoxia, which acted *per se* or by inducing the activity of a diffusible factor. These statements are nowadays incorporated into the growing body of recently accumulated data on angiogenesis.

All corneal layers participate in the maintenance of avascularity. The intact epithelium prevents both from corneal edema and activation of stromal proteinases, active players along the cascade of angiogenesis. This layer also contains a high expression of soluble VEGF receptors,⁵¹ which bind and inactivate this potent mitogen of vascular endothelial cells⁵². Consequently, antagonization of one of these soluble receptors by a tripeptide modulates angiogenesis⁵³. These decoy receptors are considered as the key players in maintenance of corneal avascularity. However, a recent observation of simultaneous suppression of corneal inflammation and neovascularization by netrin,⁵⁴ a member of the family of proteins similar to laminin, previously thought to be involved in neurogenesis only, adds to the complexity of the proposed mechanism. These substances seem to originate from the superficial limbus,^{55,56} possibly from its stem cells, which are likely to have a task more complex than epithelial regeneration⁵⁷. The appearance of new blood vessels in cases with stem cell deficiency supports this line of thinking.

Both epithelial cells and keratocytes show the expression of a potent angioinhibitor, thrombospondin^{58,59}. Thrombospondins induce apoptosis of vascular endothelial cells and shield them from the bFGF activity⁶⁰. Both epithelium and endothelium of the rat cornea show the expression of pigment epithelium derived factor (PEDF), one of the most potent angioinhibitors, which is able to block a VEGF receptor⁶¹.

Matrix metalloproteinases (MMPs) are also expressed in various corneal cells. These zinc-dependent endopeptidases are able either to stimulate (MMP2, MMP14) or to inhibit (MMP3, MMP7) angiogenesis. The latter is achieved by degradation of collagen XIII and plasminogen, leaving active endostatin and angiostatin⁶². It has recently been shown that these enzymes can also have an anti-inflammatory effect by changing a gene expression⁶³.

In conclusion, corneal avascularity is maintained by homeostasis, which includes a well-known edema-preventing balance between corneal swelling pressure and dehydration, as well as an equilibrium of numerous pro- and anti-angiogenic activities.

Corneal neovascularization

Corneal neovascularization (CONV) is formed when blood vessels from the limbus penetrate the avascular corneal tissue (Figure 1).

Subepithelial neovascularization is characterized by direct arborization of blood vessels creating a pannus, which

splits the space between the epithelium and the Bowman's membrane. Interstitial new blood vessels follow the direction of collagen fibers and grow in a brush-like fashion. The deepest stromal neovascularization has an umbilical shape at first, and a membranous shape upon further growth⁶⁴. CONV is essentially a reparatory attempt in response to hypoxia created by infection, trauma, immune reaction, tumor growth and stem cell loss. Accompanying processes and sequellae are: inflammation with cellular infiltration, edema, fibrous scarring, fatty deposit, and the loss of corneal immune privilege^{60,65}. The price of this reparatory process, which occasionally saves the ocular globe, is often high, and can be expressed in visual and aesthetic loss.



Fig. 1 – Corneal neovascularization.

Trachoma and onchocerciasis, both characterized by dramatic CONV, are among the world's most frequent causes of blindness. Their eradication needs measures that belong to economy rather than to angiogenesis research and therapy. But, about four percent of the population of the developed world also suffers from corneal neovascularization, mostly caused by herpes, with almost fifteen hundred thousand new cases every year⁶⁶. The world statistics reports forty thousand new cases of a drastic monocular visual loss or blindness per year⁶⁷. Other infective agents, like *Pseudomonas*, *Chlamidia* and fungi are less frequent causes of visual loss. The non-infective causes of CONV are: contact lens wear, ocular surface diseases, corneal graft rejection, eye drops with preservative, and trauma, especially chemical burns. The socioeconomic significance of CONV is not negligible, and the new treatment modalities can lessen the burden carried by many individuals and the society.

A key player in the ocular as well as corneal angiogenic cascade, like everywhere in the body, is VEGF. Its richest corneal resources are the epithelium, vascular endothelial cells, macrophages, and fibroblasts⁶⁰. VEGF expression is significantly upregulated in inflamed and vascularized corneas⁶⁸. On the other hand, CONV stops when VEGF or its receptors are inhibited, or when signals for VEGF release are blocked^{69,70}.

Other minor factors involved in CONV are bFGF, released from basement membranes after injury;^{34,35,71} PDGF, which stimulates VEGF transcription and brings pericytes to block apoptosis of new vascular buds;⁷² and angiopoietin²⁹. Recent observations add epoxyeicosanoids to this list. These products of arachidonic acid metabolism control inflammatory and angiogenic response to injury, as a part of tissue and organ reparation and regeneration⁷³.

Treatment of CONV

Corticosteroids are still the mainstay of the therapy for CONV, sometimes aided by non-specific anti-inflammatory agents (NSAID) or cyclosporine. Physical methods include diathermy and photodynamic therapy, while transplantation of the limbus is beyond the scope of this review.

Corticosteroids

Corticosteroids act mainly against inflammation by prevention of neutrophil and macrophage accumulation (a hallmark of the late sensitivity reaction), their adhesion to the capillary endothelial cells, and formation of plasminogen activator⁷⁴. As Professor Claes Dohlman used to teach, this is why these medications helped the success of keratoplasty more than any surgical minutia.

Antiangiogenic effects of corticosteroids do not depend on their gluco- or mineralo-corticoid action. It seems to be achieved by capillary basement membrane degradation⁷⁵, and is enhanced in the presence of heparin or its pentasaccharide fragment^{14,15}. Unfortunately, these potent drugs have many side-effects: they are associated with masking of the signs of bacterial infection, progression of herpetic keratitis, and corneal melt if given later than a week after a chemical burn⁷⁶. Prolonged topical corticosteroid therapy may cause cataract⁷⁷ and glaucoma⁷⁸.

Physical methods

Photodynamic therapy can occlude larger blood vessels. It includes an intravenous injection and the use of argon or diode laser beam. It is a costly procedure, and the injected substance may be potentially harmful⁷⁹.

Fine needle diathermy is quite easy to perform. Its best indication is occlusion of one or few larger blood vessel prior to keratoplasty. A long-lasting effect in a bunch of small vessels is hard to achieve^{80,81}.

NSAID

Topical NSAID inhibits angiogenesis in rat cornea⁸². These medications inhibit cyclooxygenases, and the consequence is a low level of prostaglandines produced from arachidonic acid. Their use is limited to the early stages of angiogenesis, until accumulation of a large quantity of VEGF is created. Occasional corneal melts have been reported during the use of NSAID. Even one drop of a preservative-free NSAID can result in intense burning sensation. Therefore, caution and close observation are advised during their use⁸³⁻⁸⁵.

Other medications

Well-known angioinhibitors, cyclosporine A,⁸⁶ methotrexate,⁸⁷ and tacrolimus⁸⁸ are mainly used as a substitution of corticosteroids, when a prolonged therapy after complicated keratoplasty is needed. Angioinhibitory action of thalidomide was unknown until it produced a tragic effect of inborn phocomelia^{89,90}. Thalidomide has recently been found useful in the treatment of some malignant tumors and uveitis,⁹¹ but its use in CONV inhibition has been checked only experimentally. Amiloride, a competitive inhibitor of urokinase type-plasminogen activator system, has been shown to inhibit CONV in various experimental models,^{92,93} but without a clinical use. It is interesting to speculate whether the concentrations of this drug, widely used as a diuretic, can produce inconspicuous angioinhibition.

Anti-VEGF therapy

According to a recent meta analysis,⁹⁴ a few studies have shown that a VEGF blockade may compete with corticosteroids as the therapy of choice in cases of CONV. One of the jewels in the crown of a half a century long angiogenesis research⁹⁵ was USA Food and Drug Administration (FDA) approval of bevacizumab, a humanized monoclonal antibody against VEGF A (Avastin, Genentech, Roche), for the adjuvant treatment of metastatic colorectal cancer first, and some other solid malignant tumors later^{96,97}. Intravitreal injections of this drug have also revolutionized the therapy of the wet form of age-related macular degeneration, helped in the resolution of diffuse diabetic macular edema, and tried in various other ocular angiogenic diseases^{98,99}. However, the effect of topical bevacizumab in experimental CONV is only partial¹⁰⁰. It is possible that a humanized antibody cannot exert a full effect in experimental animals, while the inhibition of CONV in humans is significant¹⁰¹⁻¹⁰⁵. Subconjunctival and deep stromal injections of bevacizumab have been given in hope for a better effect on the deep CONV¹⁰⁶. A better drug penetration into the cornea has been tried with a fragment of bevacizumab, ranibizumab, which has a small molecule and a higher affinity for VEGF. Its effect, more on the vessel diameter than on the involved surface area, has been only slightly better than the effect of bevacizumab¹⁰⁷. The cost/effectiveness ratio of these two drugs calls for further investigations⁶⁹.

As previously stated, VEGF has more than one function. Possible late complications of huge doses of this drug given in oncology have not been excluded, but they are not within the scope of ophthalmology. However, corneal epithelial defects have been observed after topical application of bevacizumab^{103,108-110}. Pegaptanib, which binds only one VEGF isoform, offers less probability of complications which is unfortunately associated with a lesser effect¹¹¹. A novel therapeutic approach, VEGF trap, is the use of a soluble receptor molecule, aflibercept, that includes sequences from VEGFR 1 and 2, and possesses a high binding affinity for VEGF-A and B, as well as for PlGF. It prevents VEGF from binding to its natural receptor and from promoting proliferation and migration of vascular endothelial cells. The effect of VEGF trap lasts twice as long as the effect of VEGF blockade by monoclonal antibodies^{112,113}.

Another approach is silencing of a gene for VEGF production by one of ribonucleic acids, which inhibits the post-transcriptional processing¹¹⁴ and signals from tyrosin-kinase receptors¹¹⁵.

Targets other than VEGF

Among the substances which have also been tried for CONV inhibition are topical netrins;⁵⁴ infliximab, a monoclonal antibody against TNF- α ;¹¹⁶ and doxycycline. Doxycycline is a tetracycline and a potent MMP inhibitor¹¹⁷. It seems to be one of the smart drugs, and has recently been used in oncology,¹¹⁸ cardiology,¹¹⁹ and neurology¹²⁰. Its topical administration inhibits CONV in both experimental animals,¹²¹ and in patients¹²² by a MMP-independent mechanism¹²³. Doxycycline acts in synergism with bevacizumab, and can additionally protect the corneal epithelium from untoward effects of the latter¹²⁴.

Conclusion

It appears that the ocular anti-angiogenic therapy in the years ahead will find more use of vascular endothelial growth factor trap and integrins, and less of corticosteroids and monoclonal antibodies against vascular endothelial growth factor. Inexpensive therapy with a well-known drug, doxycycline, might be used when corneal neovascularization is associated with disturbed epithelium. In the distant future treatment of corneal neovascularization will probably be based upon targeting a specific gene.

R E F E R E N C E S

1. Hertig AT. Angiogenesis in the early human chorion and in the placenta of the macaque monkey. *Contrib Embryol Carnegie Inst* 1935; 25(146): 37-81.
2. Folkman J, Shing Y. Angiogenesis. *J Biol Chem* 1992; 267(16): 10931-4.
3. Algire GH. Microscopic studies of the early growth of a transplantable melanoma of the mouse, using the transparent-chamber technique. *J Natl Cancer Inst* 1943; 4(1): 13-20.
4. Michaelson IC. The mode of development of the vascular system of the retina with some observations on its significance in certain retinal diseases. *Trans Ophthalmol Soc UK* 1948; 68: 137-80.
5. Folkman J, Long D, Becker FF. Growth and metastasis of tumor in organ culture. *Cancer* 1963; 16: 452-67.
6. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; 285(21): 1182-6.

7. Folkman J, Merler E, Abernathy C, Williams G. Isolation of a tumor factor responsible for angiogenesis. *J Exp Med* 1971; 133(2): 275–88.
8. Nikolic Lj. Angiogenesis. In: Nikolic Lj, Danjo S, editors. Diabetic retinopathy. Beograd: Zavod za udžbenike i nastavna sredstva; 1999. p. 75–98. (Serbian)
9. Auerbach R, Lewis R, Shimmers B, Kubai L, Akhtar N. Angiogenesis assays: a critical overview. *Clin Chem* 2003; 49(1): 32–40.
10. Folkman J, Klagsbrun M. Vascular physiology. A family of angiogenic peptides. *Nature* 1987; 329(6141): 671–2.
11. Folkman J, Klagsbrun M. Angiogenic factors. *Science* 1987; 235(4787): 442–7.
12. Taylor S, Folkman J. Protamine is an inhibitor of angiogenesis. *Nature* 1982; 297(5864): 307–12.
13. Folkman J, Langer R, Linhardt R, Haudenschild C, Taylor S. Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. *Science* 1983; 221(4612): 719–25.
14. Crum R, Szabo S, Folkman J. A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science* 1985; 230(4732): 1375–8.
15. Nikolic L, Friend J, Taylor S, Thoft RA. Inhibition of vascularization in rabbit corneas by heparin: cortisone pellets. *Invest Ophthalmol Vis Sci* 1986; 27(4): 449–56.
16. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994; 79(2): 315–28.
17. O'Reilly MS, Boehm T, Shing Y, Fukui N, Vasios G, Lane WS, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997; 88(2): 277–85.
18. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995; 146(5): 1029–39.
19. Ferrara N, Gerber H, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; 9(6): 669–76.
20. Shima DT, Adamis AP, Ferrara N, Yeo KT, Yeo TK, Allende R, et al. Hypoxic induction of endothelial cell growth factors in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen. *Mol Med* 1995; 1(2): 182–93.
21. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 1991; 266(18): 11947–54.
22. Yamazaki Y, Morita T. Molecular and functional diversity of vascular endothelial growth factors. *Mol Divers* 2006; 10(4): 515–27.
23. D'Amore PA. Vascular endothelial cell growth factor-a: not just for endothelial cells anymore. *Am J Pathol* 2007; 171(1): 14–8.
24. Ljubimov AJ. Growth factor synergy in angiogenesis. In: Penn JS, editor. Retinal and choroidal angiogenesis. Dordrecht, Netherlands: Springer; 2008. p. 289–310.
25. Nugent MA, Iozzo RV. Fibroblast growth factor-2. *Int J Biochem Cell Biol* 2000; 32(2): 115–20.
26. Stahl A, Paschek L, Martin G, Felten N, Hansen LL, Agostini HT. Combinatory inhibition of VEGF and FGF2 is superior to solitary VEGF inhibition in an in vitro model of RPE-induced angiogenesis. *Graefes Arch Clin Exp Ophthalmol* 2009; 247(6): 767–73.
27. Watanabe D, Suzuma K, Matsui S, Kurimoto M, Kiyu J, Kita M, et al. Erythropoietin as a retinal angiogenic factor in proliferative diabetic retinopathy. *N Engl J Med* 2005; 353(8): 782–92.
28. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem* 2000; 69: 145–82.
29. Kwanta A. Ocular angiogenesis: the role of growth factors. *Acta Ophthalmol Scand* 2006; 84(3): 282–8.
30. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992; 359(6398): 843–5.
31. Balsara RD, Merryman R, Virjee F, Northway C, Castellino FJ, Ploplis VA. A deficiency of uPAR alters endothelial angiogenic function and cell morphology. *Vascular Cell* 2011; 3(1): 10.
32. Singhi R, Kumar A, Lopez GP, Stephanopoulos GN, Wang DI, Whitesides GM, et al. Engineering cell shape and function. *Science* 1994; 264(5159): 696–8.
33. Ingber DE. Fibronectin controls capillary endothelial cell growth by modulating cell shape. *Proc Natl Acad Sci U S A* 1990; 87(9): 3579–83.
34. Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I. A heparin-binding angiogenic protein–basic fibroblast growth factor–is stored within basement membrane. *Am J Pathol* 1988; 130(2): 393–400.
35. Nikolic LB. Angiogenesis in rabbit cornea after disruption of descemet's membrane in the presence of heparin. *Vestn Oftalmol* 1998; 114(4): 35–8.
36. Wang F, Sloss C, Zhang X, Lee SW, Cusack JC. Membrane-bound heparin-binding epidermal growth factor like growth factor regulates E-cadherin expression in pancreatic carcinoma cells. *Cancer Res* 2007; 67(18): 8486–93.
37. Speiser P, Güttelsohn AM, Patz A. Studies on diabetic retinopathy. 3. Influence of diabetes on intramural pericytes. *Arch Ophthalmol* 1968; 80(3): 332–7.
38. Orledge A, D'Amore PA. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol* 1987; 105(3): 1455–62.
39. Ramsauer M, D'Amore PA. Getting Tie(2)d up in angiogenesis. *J Clin Invest* 2002; 110(11): 1615–7.
40. Gerhardt H, Betsholtz C. Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res* 2003; 314(1): 15–23.
41. Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* 1994; 265(5178): 1582–4.
42. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci U S A* 1993; 90(22): 10705–9.
43. Phng LK, Gerhardt H. Angiogenesis: a team effort coordinated by notch. *Dev Cell* 2009; 16(2): 196–208.
44. Pepper MS. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscler Thromb Vasc Biol* 2001; 21(7): 1104–17.
45. Barczyk M, Carracedo S, Gullberg D. Integrins. *Cell Tissue Res* 2010; 339(1): 269–80.
46. Nikolic LJ. Experimental keratoplasty: a model for corneal vascularization research. *Srp Arh Celok Lek* 1986; 113: 135–44.
47. Cogan D. Corneal vascularization. *Invest Ophthalmol* 1962; 1: 253–61.
48. Cogan DG. Vascularization of the cornea: its experimental induction by small lesions and a new theory of its pathogenesis. *Arch Ophthalmol* 1949; 41(4): 406–16.
49. Campbell FW, Michaelson IC. Blood-vessel formation in the cornea. *Br J Ophthalmol* 1949; 33(4): 248–55.
50. Ashton N, Cook C. Mechanism of corneal vascularization. *Br J Ophthalmol* 1953; 37(4): 193–209.
51. Ambati BK, Nozaki M, Singh N, Takeda A, Jani PD, Suthar T, et al. Corneal avascularity is due to soluble VEGF receptor-1. *Nature* 2006; 443(7114): 993–7.
52. Cursiefen C, Chen L, Saint-Geniez M, Hamrah P, Jin Y, Rashid S, et al. Nonvascular VEGF receptor 3 expression by corneal epithelium maintains avascularity and vision. *Proc Natl Acad Sci U S A* 2006; 103(30): 11405–10.
53. Ponticelli S, Marasco D, Tarallo V, Albuquerque RJ, Mitola S, Takeda A, et al. Modulation of angiogenesis by a tetrameric

- tripeptide that antagonizes vascular endothelial growth factor receptor 1. *J Biol Chem* 2008; 283(49): 34250–9.
54. Han Y, Shao Y, Lin Z, Qu Y, Wang H, Zhou Y, et al. Netrin-1 simultaneously suppresses corneal inflammation and neovascularization. *Invest Ophthalmol Vis Sci* 2012; 53(3): 1285–95.
 55. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature* 1971; 229(5286): 560–1.
 56. Ebato B, Friend J, Thoft RA. Comparison of central and peripheral human corneal epithelium in tissue culture. *Invest Ophthalmol Vis Sci* 1987; 28(9): 1450–6.
 57. Ti S, Grueterich M, Espana EM, Toubami A, Anderson DF, Tseng SC. Correlation of long term phenotypic and clinical outcomes following limbal epithelial transplantation cultivated on amniotic membrane in rabbits. *Br J Ophthalmol* 2004; 88(3): 422–7.
 58. Armstrong DJ, Hiscott P, Batterbury M, Kaye S. Corneal stromal cells (keratocytes) express thrombospondins 2 and 3 in wound repair phenotype. *Int J Biochem Cell Biol* 2002; 34(6): 588–93.
 59. Sekiyama E, Nakamura T, Cooper LJ, Kanwasaki S, Hamuro J, Fullwood NJ, et al. Unique distribution of thrombospondin-1 in human ocular surface epithelium. *Invest Ophthalmol Vis Sci* 2006; 47(4): 1352–8.
 60. Azar DT. Corneal angiogenic privilege: angiogenic and antiangiogenic factors in corneal avascularity, vasculogenesis, and wound healing (an American Ophthalmological Society thesis). *Trans Am Ophthalmol Soc* 2006; 104: 264–302.
 61. Cai J, Jiang WG, Grant MB, Boulton M. Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. *J Biol Chem* 2006; 281(6): 3604–13.
 62. Pepper MS. Extracellular proteolysis and angiogenesis. *Thromb Haemost* 2001; 86(1): 346–55.
 63. Khamis ZI, Zorio DA, Chung LW, Sang QA. The Anti-inflammatory Role of Endometase/Matrilysin-2 in Human Prostate Cancer Cells. *J Cancer* 2013; 4(4): 296–303.
 64. Mann I, Pirie A, Pullinger BD. An experimental and clinical study of the reaction of the anterior segment of the eye to chemical injury, with special reference to chemical warfare agents. *Br J Ophthalmol* 1948; 13(Suppl): 171.
 65. Chang JH, Gabison EE, Kato T, Azar DT. Corneal neovascularization. *Curr Opin Ophthalmol* 2001; 12(4): 242–9.
 66. Lee P, Wang CC, Adamis AP. Ocular neovascularization: an epidemiologic review. *Surv Ophthalmol* 1998; 43(3): 245–69.
 67. Farooq AV, Shukla D. Herpes simplex epithelial and stromal keratitis: an epidemiologic update. *Surv Ophthalmol* 2012; 57(5): 448–62.
 68. Philipp W, Speicher L, Humpel C. Expression of vascular endothelial growth factor and its receptors in inflamed and vascularized human corneas. *Invest Ophthalmol Vis Sci* 2000; 41(9): 2514–22.
 69. Stevenson W, Cheng S, Dastjerdi MH, Ferrari G, Dana R. Corneal neovascularization and the utility of topical VEGF inhibition: ranibizumab (Lucentis) vs bevacizumab (Avastin). *Ocul Surf* 2012; 10(2): 67–83.
 70. Kim LA, D'Amore PA. A brief history of anti-VEGF for the treatment of ocular angiogenesis. *Am J Pathol* 2012; 181(2): 376–9.
 71. Hajrasouliha AR, Sadrai Z, Chanban SK, Dana R. b-FGF induces corneal blood and lymphatic vessel growth in a spatially distinct pattern. *Cornea* 2012; 31(7): 804–9.
 72. Song S, Ewald AJ, Stallcup W, Werb Z, Bergers G. PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. *Nat Cell Biol* 2005; 7(9): 870–9.
 73. Panigrahy D, Kalish BT, Huang S, Bielenberg DR, Le HD, Yang J, et al. Epoxyeicosanoids promote organ and tissue regeneration. *Proc Natl Acad Sci U S A* 2013; 110(33): 13528–33.
 74. Haynes RC, Murad F. Adrenocorticotrophic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of adrenocortical steroid biosynthesis. In: Gilman AG, Goodman LS, Rall TW, Murad F, editors. *Goodman and Gilman's The Pharmacological Basis of Therapeutics*. New York: Macmillan Publishing Company; 1985. p. 1459–89.
 75. Ingber DE, Madri JA, Folkman J. A possible mechanism for inhibition of angiogenesis by angiostatic steroids: induction of capillary basement membrane dissolution. *Endocrinology* 1986; 119(4): 1768–75.
 76. Renfro L, Snow JS. Ocular effects of topical and systemic steroids. *Dermatol Clin* 1992; 10(3): 505–12.
 77. Thorne JE, Woreta FA, Dunn JP, Jabs DA. Risk of cataract development among children with juvenile idiopathic arthritis-related uveitis treated with topical corticosteroids. *Ophthalmology* 2010; 117(7): 1436–41.
 78. Bremer F. Origin of corticosteroid glaucoma. *Bull Soc Belge Ophthalmol* 2007; (304): 111–6. (French)
 79. Sheppard JD, Epstein RJ, Lattanzio FA, Marantonio D, Williams PB. Argon laser photodynamic therapy of human corneal neovascularization after intravenous administration of dihematoporphyrin ether. *Am J Ophthalmol* 2006; 141(3): 524–9.
 80. Pillai CT, Dua HS, Hossain P. Fine needle diathermy occlusion of corneal vessels. *Invest Ophthalmol Vis Sci* 2000; 41(8): 2148–53.
 81. Maguire MG, Stark WJ, Gottsch JD, Stulting RD, Sugar A, Fink NE, et al. Risk factors for corneal graft failure and rejection in the collaborative corneal transplantation studies. Collaborative Corneal Transplantation Studies Research Group. *Ophthalmology* 1994; 101(9): 1536–47.
 82. Takahashi K, Saishin Y, Saishin Y, Mori K, Ando A, Yamamoto S, et al. Topical nepafenac inhibits ocular neovascularization. *Invest Ophthalmol Vis Sci* 2003; 44(1): 409–15.
 83. Zanini M, Savini G, Barboni P. Corneal melting associated with topical diclofenac use after laser-assisted subepithelial keratectomy. *J Cataract Refract Surg* 2006; 32(9): 1570–2.
 84. Asai T, Nakagami T, Mochizuki M, Hata N, Tsuchiya T, Hotta Y. Three cases of corneal melting after instillation of a new nonsteroidal anti-inflammatory drug. *Cornea* 2006; 25(2): 224–7.
 85. Moisseien E, Varssano D. Comparison of Ocular Tolerability Between Preserved and Preservative-Free Diclofenac Sodium Drops. *J Ocul Pharmacol Ther* 2011; 27(4): 333–7.
 86. Lipman RM, Epstein RJ, Hendricks RL. Suppression of corneal neovascularization with cyclosporine. *Arch Ophthalmol* 1992; 110(3): 405–7.
 87. Jousseaume AM, Kruse FE, Völcker HE, Kirchhof B. Topical application of methotrexate for inhibition of corneal angiogenesis. *Graefes Arch Clin Exp Ophthalmol* 1999; 237(11): 920–7.
 88. Sloper CM, Powell RJ, Dua HS. Tacrolimus (FK506) in the management of high-risk corneal and limbal grafts. *Ophthalmology* 2001; 108(10): 1838–44.
 89. D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci U S A* 1994; 91(6): 4082–5.
 90. McBride WG. Thalidomide and congenital anomalies. *Lancet* 1961; 2: 1358.
 91. Parentin F, da Pozzo S, Lepore L, Perissutti P. Thalidomide effectiveness for bilateral chronic idiopathic anterior uveitis in a three-year-old child. *Ophthalmologica* 2001; 215(1): 70–3.
 92. Avery RL, Connor TB, Farazdaghi M. Systemic amiloride inhibits experimentally induced neovascularization. *Arch Ophthalmol* 1990; 108(10): 1474–6.
 93. Ignjatović Z, Nikolić L. Inhibition of angiogenesis in the cornea with amiloride. *Srp Arh Celok Lek* 1996; 124(5-6): 120–3. (Serbian)
 94. Papatheanassiou M, Theodoropoulou S, Analitis A, Tzgonou A, Theodosiadis PG. Vascular endothelial growth factor inhibitors for treatment of corneal neovascularization: a meta-analysis. *Cornea* 2013; 32(4): 435–44.
 95. Cao Y, Arbiser J, D'Amato RJ, D'Amore PA, Ingber DE, Kerbel R, et al. Forty-year journey of angiogenesis translational research. *Sci Transl Med* 2011; 114(3): 114rv3.

96. *Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al.* Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004; 350(23): 2335–42.
97. *Shib T, Lindley C.* Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. *Clin Ther* 2006; 28(11): 1779–802.
98. *Gunther JB, Altaweel MM.* Bevacizumab (Avastin) for the treatment of ocular disease. *Surv Ophthalmol* 2009; 54(3): 372–400.
99. *Ristic D, Vukosanljovic M, Draganic B, Cerovic V, Petrovic N, Janicijevic-Petrovic M.* The effect of intravitreal administration of bevacizumab on macular edema and visual acuity in age-related macular degeneration with subfoveal choroidal neovascularisation. *Vojnosanit Pregl* 2013; 70(7): 660–3.
100. *Manzano RP, Peyman GA, Khan P, Carvounis PE, Kivilkim M, Renet M, et al.* Inhibition of experimental corneal neovascularisation by bevacizumab (Avastin). *Br J Ophthalmol* 2007; 91(6): 804–7.
101. *de Stefano J, Kim T.* Topical bevacizumab therapy for corneal neovascularization. *Arch Ophthalmol* 2007; 125(6): 834–6.
102. *Bock F, Koenig Y, Kruse F, Baier M, Cursiefen C.* Bevacizumab (Avastin) eye drops inhibit corneal neovascularization. *Graefes Arch Clin Exp Ophthalmol* 2008; 246(2): 281–4.
103. *Kim SW, Ha BJ, Kim EK, Tshab H, Kim TI.* The effect of topical bevacizumab on corneal neovascularization. *Ophthalmology* 2008; 115(6): e33–8.
104. *Dashterdi MH, Al-Arafaj KM, Nallasany N, Pineda R, Pavan-Langston D, Dana R.* Topical bevacizumab in the treatment of corneal neovascularization; results of a prospective, open-label, non-comparative study. *Arch Ophthalmol* 2009; 127(4): 381–9.
105. *Cheng SF, Dashterdi MH, Ferrari G, Okanobo A, Bower KS, Ryan DS, et al.* Short-term topical bevacizumab in the treatment of stable corneal neovascularization. *Am J Ophthalmol* 2012; 154(6): 940–8e1.
106. *Young SN, Lichtinger A, Kim P, Mohammadpour M.* Combined use of subconjunctival and intracorneal bevacizumab injection for corneal neovascularization. *Cornea* 2011; 30(10): 1110–4.
107. *Ferrari G, Dashterdi MH, Okanobo A, Cheng S, Amparo F, Nallasamy N, et al.* Topical ranibizumab as a treatment of corneal neovascularization. *Cornea* 2013; 32(7): 992–7.
108. *Kim TI, Chung JL, Hong JP, Min K, Seo KY, Kim EK.* Bevacizumab application delays epithelial healing in rabbit cornea. *Invest Ophthalmol Vis Sci* 2009; 50(10): 4653–9.
109. *Kim EC, Ryu HW, Lee HJ, Kim MS.* Bevacizumab eye drops delay corneal epithelial wound healing and increase the stromal response to epithelial injury in rats. *Clin Experiment Ophthalmol* 2013; 41(7): 694–701.
110. *Koenig Y, Bock F, Horn F, Kruse F, Straub K, Cursiefen C.* Short- and long-term safety profile and efficacy of topical bevacizumab (Avastin) eye drops against corneal neovascularization. *Graefes Arch Clin Exp Ophthalmol* 2009; 247(10): 1375–82.
111. *Pieramici DJ, Rabena MD.* Anti-VEGF therapy: comparison of current and future agents. *Eye* 2008; 22(10): 1330–6.
112. *Oliveira HB, Sakimoto T, Javier JA, Azar DT, Wiegand SJ, Jain S, et al.* VEGF Trap (R1R2) suppresses experimental corneal angiogenesis. *Eur J Ophthalmol* 2010; 20(1): 48–54.
113. *Stewart MW.* Aflibercept (VEGF Trap-Eye) for the treatment of exudative age-related macular degeneration. *Expert Rev Clin Pharmacol* 2013; 6(2): 103–13.
114. *Dykechoorn DM, Novina CD, Sharp PA.* Killing the messenger: short RNAs that silence gene expression. *Nat Rev Mol Cell Biol* 2003; 4(6): 457–67.
115. *Hos D, Bock F, Dietrich T, Onderka J, Kruse FE, Thierach KH, et al.* Inflammatory corneal (lymph)angiogenesis is blocked by VEGFR-tyrosine kinase inhibitor ZK 261991, resulting in improved graft survival after corneal transplantation. *Invest Ophthalmol Vis Sci* 2008; 49(5): 1836–42.
116. *Kim JW, Chung SK.* The effect of topical infliximab on corneal neovascularization in rabbits. *Cornea* 2013; 32(2): 185–90.
117. *Moses MA, Harper J, Folkman J.* Doxycycline treatment for lymphangioliomyomatosis with urinary monitoring for MMPs. *N Engl J Med* 2006; 354(24): 2621–2.
118. *Golub LM, Lee HM, Ryan ME, Giannobile WV, Payne J, Sorsa T.* Tetracyclines inhibit connective tissue breakdown by multiple non-antimicrobial mechanisms. *Adv Dent Res* 1998; 12(2): 12–26.
119. *Cerisano G, Buonamici P, Valenti R, Sciagra R, Raspanti S, Santini A, et al.* Early short-term doxycycline therapy in patients with acute myocardial infarction and left ventricular dysfunction to prevent the ominous progression to adverse remodelling: the TIPTOP trial. *Eur Heart J* 2014; 35(3): 184–91.
120. *Malik YS, Sheikh MA, Zhu X.* Doxycycline can stimulate cytoprotection in neural stem cells with oxygen-glucose deprivation-reoxygenation injury: a potential approach to enhance effectiveness of cell transplantation therapy. *Biochem Biophys Res Commun* 2013; 432(2): 355–8.
121. *Aydin E, Kivilkim M, Peyman GA, Esfahani MR, Kazji AA, Sanders DR.* Inhibition of experimental angiogenesis of cornea by various doses of doxycycline and combination of triamcinolone acetonide with low-molecular-weight heparin and doxycycline. *Cornea* 2008; 27(4): 446–53.
122. *Jovanovic V, Nikolic L.* The effect of topical doxycycline on corneal neovascularization. *Curr Eye Res* 2014; 39(2): 142–8.
123. *Gilbertson-Beadling S, Powers EA, Stamp-Cole M, Scott PS, Wallace TL, Copeland J, et al.* The tetracycline analogs minocycline and doxycycline inhibit angiogenesis in vitro by a non-metalloproteinase-dependent mechanism. *Cancer Chemother Pharmacol* 1995; 36(5): 418–24.
124. *Su W, Li Z, Li Y, Lin M, Yao L, Liu Y, et al.* Doxycycline enhances the inhibitory effects of bevacizumab on corneal neovascularization and prevents its side effects. *Invest Ophthalmol Vis Sci* 2011; 52(12): 9108–15.

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