GENERAL REVIEW

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Angiogenesis: A special reference to corneal neovascularization

Angiogeneza: poseban osvrt na neovaskularizaciju rožnjače

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History

Angiogenesis, the term coined in 1935 [1] means the development of new blood vessels from the pre-existing capillaries and venulae. It is fundamental for reproduction, development and reparation, when strict regulation and short duration prevent the uncontrolled growth of neovascularization [2]. 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's [3] observation that the tumors actively attract blood vessels, and Michealson's [4] 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 angiogenesis [7].

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 later [9]. These new tools helped in the discovery of the first angiostimulators: basic and acid fibroblast growth factor (bFGF, aFGF), and angiogenin [10]. Angiomodulators, most notably heparin, were added to the concept of angiogenesis [11]. Heparin antidote, protamine, was the first angioinhibitor with the known structure [12]. Then followed the discovery of a potent angioinhibitory effect of heparin in the presence of cortisone, [13–15] and of two endogenous angioihibitors, angiostatin [16] and endostatin [17].

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 hemoatracatant and endothlial cell mitogen. Its angiogenic action is regulated by hypoxia [20]. It is a dominant factor of ocular and general angiogenesis, being a perfect match for „Factor X" postulated by Michaelson [4] 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 VEGF [24]. Due to the lack of a signaling pathway for its release from cells and membranes, b FGF can act only upon their injury. Yet, its proangiogenic role is established by the findings of high levels of b FGF in the vitreous of the eyes with proliferative diabetic retinopathy, and large tumors, as well as the rodent corneal neovascularization after

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implantation of bFGF [25]. Finally, a simultaneous inhibition of both b FGF and VEGF activities in vitro is more efficient than inhibition of only VEGF [26].

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 angiogenesis [27]. This complex process is also influenced by angiopoietins, cyclooxygenases [28], platelet derived growth factor (PDF), hepatocyte growth factor (HGF), placenta growth factor (PlGF), transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α), and interleukines [29].

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 succeptible 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 angionhibitors are often bound is halted; soluble, decoy receptors, [42] like Fit-1, stop to bind angiostimulators, primarily VEGF; and notch signaling [43] starts navigating activated cells through a matrix prepared by proteinases [44] and bi-directional transmembrane receptors, integrins [45].

Corneal avascularity

One of the most useful ways towards understanding corneal angiogenesis is to study avascularity of the nomal cornea. The avascularity and easy accessability of cornea made it one of the most frequently used model of angiogenesis research [9, 46]. As Cogan [47] nicely put it: „Any theory which claims to explain corneal neovasculogenesis 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 vessels [48]. However, the shape of neovascularization induced by an isolated experimental corneal lesion led Campbell and Michaelson [49] to postulate the presence of a diffusible stimulator of blood vessel growth [49]. Ashton and Cook [50], 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 cells [52]. Consequently, antagonization of one of these soluble receptors by a tripeptide modulates angiogenesis [53]. 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 regeneration [57]. 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]. Thrombospodins induce apoptosis of vascular endothelial cells and shield them from the bFGF activity [60]. 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 receptor [61].

Matrix metaloproteinases (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 angiostatin [62]. It has recently been shown that these enzymes can also have an anti-inflammatory effect by changing a gene expression [63].

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 equilibrum of numerous pro- and anti-angiogenic activities.

Corneal neovascularization

Corneal neovacularization (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 growth [64]. 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.

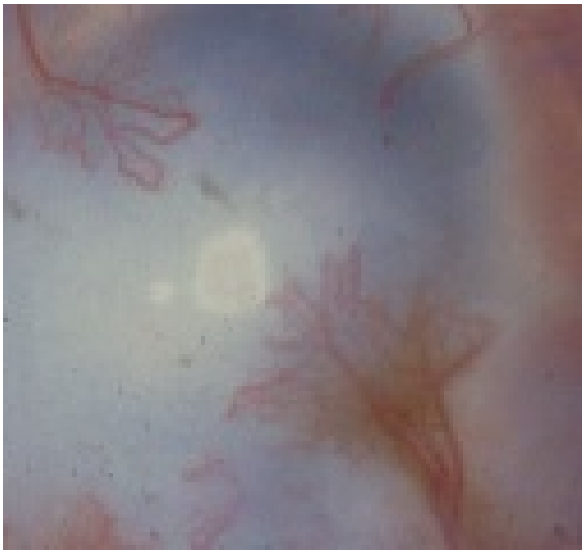

Fig. 1 – Corneal neovascularization.

Trachoma and onchocerciasis, both characterized by dramatic CONV, are among the world's most frequent causes of blindness. Their erradication needs measures that belong to economy rather that 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 year [66]. The world statistics reports fourty thousand new cases of a drastic monocular visual loss or blindness per year [67]. 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 negligeble, 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 fibroblasts [60]. VEGF expression is significantly upregulated in inflammed and vascularized corneas [68]. 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 angiopoietin [29]. 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 regeneration [73].

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 neutrophyl and macrophage accumulation (a hallmark of the late sensitivity reaction), their adhesion to the capillary endothelial cells, and formation of plasminogen activator [74]. 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 degradation [75]. and is enhanced in the presence of heparin or its pentasacharide 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 burn [76]. Prolonged topical corticosteroid therapy may cause cataract [77] and glaucoma [78].

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 harmful [79].

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 cornea [82]. 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 use [83–85].

Other medications

Well-known angioinhibitors, cyclosporine A, [86] methotrexate, [87] and tacrolimus [88] 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 competive 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 blocade 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 research [95] was USA Food and Drug Administartion (FDA) aproval 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 revolutionarized 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 partial [100]. It is possible that a humanized antibody cannot exert a full effect in experimental animals, while the inhbition of CONV in humans is significant [101–105]. Subconjunctival and deep stromal injections of bevacizumab have been given in hope for a better effect on the deep CONV [106]. 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 slighly better than the effect of bevacizumab [107]. The cost/effectiveness ratio of these two drugs calls for further investigations [69].

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]. Pegabtanib, which binds only one VEGF isoform, offers less probability of complications which is unfortunately associated with a lesser effect [111]. A novel therapeutic approach, VEGF trap, is the use of a soluble receptor molecule, aflibercept, that includes sequences from VEGFR 1 and 2, and posseses a high binding affinity for VEGF-A and B, as well as for PlGF. Its prevents VEGF fom 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-transcrptional processing [114] and signals from tyrosin-kinase receptors [115].

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 inhibitor [117]. It aseems to be one of the smart drugs, and has recently been used in oncology, [118] cardiology, [119] and neurology [120]. Its topical administration inhibits CONV in both experimental animals, [121] and in patients [122] by a MMP-independent mechanism [123]. Doxycycline acts in synergism with bevacizumab, and can additionally protect the corneal epithelium from untoward effects of the latter [124].

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.

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