Review Article

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Corneal Neovascularization: A Translational Perspective

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Abstract

Corneal neovascularization (CNV) results from the growth of new vessels from the limbal vasculature into the normally avascular cornea. As the second cause of severe visual impairment worldwide, it represents a major clinical problem. This occurs because CNV often leads to corneal opacification, thus reducing visual acuity. CNV is a well characterized and complex process, which includes different aspects of the corneal tissue: degradation of the epithelial basement membrane, remodeling of the extracellular matrix (ECM), and proliferation of endothelial cells.

A number of studies have investigated the molecular mechanisms regulating corneal angiogenesis, and various mechanisms have been identified. This paper will review corneal neovascularization and its relevance to human disorders. Additionally, we will describe experimental models commonly used to study CNV, the role of innate immune cells in inflammatory-induced angiogenesis, current therapies, and future directions.

Keywords: Corneal neovascularization; Angiogenic privilegeanti-angiogenic treatments

Angiogenic Privilege

The cornea is one of the few tissues of the body, together with cartilage, completely devoid of both blood and lymphatic vessels. This particular condition is termed "angiogenic privilege" and is necessary for optimal visual acuity.

The avascularity of the cornea is evolutionarily highly conserved [1] and it is regulated by anti- and pro-angiogenic factors. In normal conditions, anti-angiogenic molecules are highly expressed and act as inhibitors by using different mechanisms. Soluble VEGFR-1 -2 and -3 expressed in the corneal epithelium may act as decoy receptors [1-3], hence inhibiting VEGF activity. In addition, it has been shown that the cornea expresses natural inhibitors of angiogenesis such as thrombospondins (TSPs), Pigment epithelium-derived factor (PEDF), angiostatin, endostatin and metalloproteinases (MMPs).

TSP-1 and TSP-2 belong to a family of proteins found in the ECM and act by inducing vascular endothelial cell apoptosis as well as by blocking growth factors mobilization through interactions with the ECM [4].

PEDF belongs to the superfamily of the serine protease inhibitors and can inhibit CNV by suppressing VEGF expression via its anti-oxidative properties [5].

Angiostatin is a 38 kDa proteolytic fragment of plasminogen found in the corneal epithelium. It has been shown to inhibit both bFGF-induced and injury-induced CNV in mice [6,7].

Endostatin is a 20 kDa proteolytic fragment of collagen XVIII found in the cornea, retina, and lens. Endostatin is able to inhibit both FGF-induced and VEGF-induced CNV [8]. Both angiostatin and endostatin act as inhibitors by inducing vascular endothelial cell apoptosis.

Finally, MMPs are a group of zinc-dependent enzymes involved in ECM remodeling and angiogenesis [9]. MMPs are able to be pro- or anti-angiogenic under different conditions. In fact, MMPs activate proteolytic cleavage of proenzymes, which results in the production of both pro- and anti-angiogenic fragments.

Pathological Neovascularization

Highly prevalent ocular aliments such as infection-related inflammation, trauma, hypoxia or limbal stem cell deficiency can induce loss of the "angiogenic privilege". As a consequence, blood and lymphatic vessels grow into the cornea, resulting in CNV. CNV can induce tissue scarring, lipid deposition, stromal hemorrhage and corneal edema, further reducing visual acuity.

Although CNV may increase corneal resistance to perforation in the acute phase [10] and its real impact on best corrected visual acuity is still under debate [11], development of neovessels in the cornea is generally regarded as a sign of disease. In fact, the neovascularized cornea loses its "immunologic privilege". As a consequence, the risk of immune-mediated allograft rejection after keratoplasty is increased [12,13]. While literature originally focused on corneal hemangiogensis, it soon became clear that lymphangiogenesis is also highly relevant during CNV. This was achieved through employment of specific markers such as vascular endothelial growth factor receptor-3 (VEGFR-3) and lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1) [14]. It is now clear that lymphatic vessels play a pivotal role in the pathophysiology of corneal transplant rejection by allowing the antigen-presenting cells (APCs) to reach the regional lymph nodes and lymphoid organs [15]. Moreover, lymphangiogenesis facilitates tumor (e.g: ocular melanoma) metastasis by spreading the tumor cells via lymphatic vessels [16]. Thus, blocking lymphangiogenesis or removing the draining lymph nodes could inhibit the immune response and promote graft survival, as well as block tumor cells spread [3,17,18].

Angiogenic factors

Several factors are known to play a role in CNV. Among these, some have been extensively studied. These include Vascular Endothelial Growth Factors (VEGFs), Basic Fibroblast Growth Factor (bFGF) and Platelet-derived Growth Factors (PDGFs). Understanding the role of these factors in hem- and lymphangiogenesis is essential for the development of new therapies for CNV.

VEGFs and their receptors (VEGFRs) are the principal mediators of angiogenesis. VEGF-A is one of the better extensively characterized pro-angiogenic molecule and plays a crucial role in the induction of both hem- and lymphangiogenesis via its interaction with VEGFR-1 and VEGFR2, with VEGFR2 identified as the main receptor [19-21]. The soluble form of VEGFR-1 acts as decoy receptor. Recently, it has been demonstrated that injection of VEGFR1-specific morpholino was able to decrease the membrane-bound VEGFR-1 levels and increase the production of soluble VEGFR1, thereby limiting corneal angiogenesis in animal models [22]. VEGF-A has been demonstrated to be upregulated in inflamed and vascularized corneas of various animal models [23]. This factor promotes angiogenesis by inducing endothelial cells proliferation, migration, tubulogenesis and vascular permeability [24,25]. VEGF-A is mainly produced by corneal epithelial and endothelial cells, vascular endothelial cells and macrophages, in response to inflammatory stimuli [25]. VEGF signaling is also modulated by Angiopoietins and their receptors Tie1 and Tie2 [26]. Additional members of the VEGF family include VEGF-B VEGF-C and VEGF-D [27]. They bind to different VEGF receptors and control hem- and lymphangiogenesis. In particular, it has been shown that VEGF-C and VEGF-D play a central role in lymphangiogenesis through activation of VEGFR-328, but they also participate in hemangiogenesis by binding VEGFR-2 [29] or VEGFR-3 [30]. Furthermore, it has been demonstrated that molecular mechanisms involving VEGF-C and VEGFR-2 can regulate the temporal and spatial association of hem- and lymphangiogenis [31].

b-FGF (FGF-2) is a member of the fibroblast growth factor family that has been shown to be over expressed in the corneal epithelium after injury [32]. FGF-2 has a pro-angiogenic effect by binding the FGF receptors on the endothelial cells and stimulating their proliferation and migration together with the induction of integrins, cadherins and proteases, thus causing ECM degradation [33]. It has been shown that lymphatic vessels are significantly more sensitive to FGF-2 than blood vessels [34]. VEGFs and bFGF have distinct biological roles but their cross-talk is important to control angiogenesis [35].

Other important angiogenic factors in the cornea are PDGFs. They have important functions on endothelial cells and can induce new blood vessel growth. The protein family is composed by different isoforms. The PDGFs are produced by vascular endothelial cells and interact with their receptor expressed in the surrounding pericytes and vascular smooth muscle cells. PDGF-AA is able to display only a weak angiogenic response in the mouse cornea, even if it is used in combination with bFGF [36]. PDGF-BB is involved in the recruitment of mural cells during angiogenesis [37]. Thus, blocking PDGF-BB signaling interfere with mural cell recruitment to corneal vessels and results in vessel regression [38]. PDGF-CC is able to produce a robust angiogenic response and it is the only member that has structural similarities with the VEGF family and could probably coadiuvate the vascular function of VEGFs [39].

Animal Models of CNV

Because of its optical clarity and the possibility to easily visualize and quantify vessel growth, the cornea is considered the ideal tissue to study the molecular basis of hem- and lymphangiogenesis. Several rodent models have been developed; these also allow testing pro- or anti-angiogenic drugs. Indeed, results obtained from CNV studies can be easily applied to other research fields, such as tumor angiogenesis.

Suture placement model

The suture placement model is commonly used to study inflammatory angiogenesis. In this model, nylon sutures are placed into the corneal stroma without penetrating into the anterior chamber [40]. The time course and distribution pattern of suture-induced corneal hem- and lymphangiogenesis have been thoroughly characterized, making this model highly reproducible [41,42]. Moreover, angiogenesis induced by sutures is easily measurable by standard image analysis programs [43].

Micropocket assay model

The corneal micropocket assay consists in the placement of an angiogenic factor, such as cytokines or cell suspensions, into a small intrastromal space created by a surgical knife [44]. This assay was first introduced by Folkman in 1971 [45] to study tumoral angiogenesis and then it was partially modified and adopted from several research groups. The major advantage of this model is that it can isolate the angiogenic effect of the factor of interest. Among the angiogenic factors VEGF or bFGF [34,46] are commonly used. This model is relatively simple and has a high reproducibility.

Alkali burn model

A third effective, but less "clean" model to study CNV is the alkali burn model [47]. This is generated by applying a filter paper disc soaked in NaOH in the central cornea without affecting the limbus, conjunctiva and episclera.

Transplantation model

The corneal transplantation model is achieved by replacing the central cornea of the recipient with a donor cornea [48]. Donor and recipient can be matched differently to achieve low or high rejection risk grafts. This model has been widely used to dissect the mechanisms of graft rejection. The transplantation surgery can be performed either on inflamed or non-inflamed beds with different outcomes [20,49]. Thus, these two models are ideal to study the angiogenic response in settings mimicking the cornea.

In addition to the corneal angiogenic models, a variety of transgenic and knockout mice have been generated to further study the mechanisms of corneal angiogenesis and a number of studies have been conducted so far. For example, it has been shown that transgenic mice expressing only one of the three VEGF-A isoforms develop reduced corneal hem- and lymphangiogenesis [46]; and that TSP-1 and CD-36 deficient mice develop increased spontaneous corneal lymphangiogenesis [50]. Moreover, it has been recently demonstrated that corneal lymphangiogenesis is completely inhibited in Ang2 knockout mice [51]. Thus, all these mice models are of great importance to understand the different roles of angiogenic molecules in the cornea.

Strain-dependency

Another important consideration is that the extent of CNV varies among different mouse strains [52]. The amount of preexisting lymphatics as well as the VEGF-C-induced lymphangiogenesis were demonstrated to be significantly higher in C57BL/6 mice compared with BALB/c mice [53]. Lymphangiogenesis is also more pronounced in FVB mice as compared with C57BL/6 mice in the suture placement model [42]. Furthermore, it has been shown that C57BL/6 mice have a higher risk of corneal graft rejection because they contain more activated macrophages and lymphatic vessels as opposed to BALB/c mice, thus resulting in more effective alloantigen detection in C57BL/6 than BALB/c [54].

Innate Immune Cells

Inflammation plays a central role in the development of CNV. In fact, inflammation-induced angiogenesis is a key component in both pathogen defense and repair mechanisms. Specifically, immune cells are particularly relevant. Indeed, vessel growth is always preceded by leukocyte infiltration into the corneal stroma. It has long been known that angiogenesis does not develop in an alkali burn model after leukocyte ablation, thus suggesting a crucial role for immune cells in CNV [55].

Macrophages

Macrophage infiltration into inflamed corneas has a key role in CNV physiopathology. In fact, macrophages secrete pro-angiogenic factors such as VEGFs, thus stimulating vascular endothelial cells proliferation and amplifying the angiogenic response [46,56]. Moreover, it has been shown that macrophages express VEGF receptors, including constitutive expression of VEGFR-1,-3 and inducible expression of VEGFR-2, which allow them to be recruited to the site of inflammation [46,57].

It has also been demonstrated that CNV is significantly inhibited after macrophages depletion through administration of clodronate liposomes or bone marrow depletion by irradiation [46], and that macrophage depletion has the greatest inhibitory effect on VEGFR-3-mediated angiogenesis [30]. Moreover, a study in C57BL/6 mice showed that both intravenous and subconjunctival clodronate liposome injection reduced the number of lymphatic vessels and CD11b+ cells in the corneal stroma, suggesting that CD11b+ cells may contribute to the maintenance of lymphatic vessels in physiological settings [54]. There are also evidences that the formation of corneal lymphatic vessels correlates with the number of CD11b+ cells and that macrophages are able to form vessel-like tubes and to integrate into preexisting lymphatic vessels [56]. Additionally, macrophages can inhibit lymphangiogenesis by binding TSP-1 and consequently down regulate their VEGF-C expression [50].

COX-2 and NF-kB pathways, which have a cardinal role in the control of stress response and inflammation, are also involved in CNV. Specifically, it has been shown that macrophages infiltrating the cornea express the COX-2 enzyme and, thus, play an important role in IL1β-induced CNV [58]. In fact, COX-2-induced production of prostanoids leads to the upregulation of several angiogenic factors such as VEGF-A, that in turn promotes angiogenesis. In this vein, treatment with COX-2 inhibitors decreases the VEGF-A expression level and inhibit CNV in mouse corneas. The NF-kB signaling pathway [59] modulates IL1β-induced activation of the COX-2. It has been demonstrated that blockade of NF-kB is able to reduce both hem- and lympangiogenesis together with the expression of VEGF-A, VEGF-B and VEGF-C [60].

Dendritic cells

The central cornea is also endowed with a population of Langherans cells (LCs) and anterior stromal dendritic cells (DCs) which can function as APCs. During inflammation and in particular after corneal keratoplasty, a majority of resident donor-derived DCs undergo activation, migrate to host cervical lymph nodes and activate T cells. Migration of DCs to cervical lymph nodes is, at least in part, regulated by the VEGFR-3 expressed on corneal DCs [61]. It has been demonstrated that VEGF-C blockade significantly inhibits hem- and lymphangiogenesis, suppresses corneal APCs trafficking to draining lymph nodes, inhibit APCs maturation, thus decreasing corneal transplants rejection [62]. Moreover, recent evidence suggests that sVEGFR-3 promotes corneal transplant survival also by suppressing T cell allosensitization [63].

Clinical Relevance

The growth of blood or lymphatic neovessels into the normally avascular cornea accounts for the second most common cause of blindness worldwide [64]. Data from World Health Organization (2002) suggest that corneal opacities-often associated with CNV-represent 5.1% of main causes of severe visual impairment worldwide, this adds to trachoma (3.6%) and onchocerchiasis (0.8%), both of which are often complicated with CNV. In the US 1.4 million people develop CNV every year, year and 12% of these cases are associated with decreased visual acuity [65]. Additionally, 20% of corneal specimens obtained during corneal transplantation –the standard procedure to treat end stage corneal disease-has pathologic evidence of vascularization, while 30% of vascularized corneas face the risk of graft rejection following transplantation, making the need for effective anti-angiogenic therapies real and urgent [66].

Corneal Neovascularization Treatments

Several treatments have been explored in order to reduce neovessels and to restore the angiogenic privilege of the cornea: these include medical and surgical options. Steroid eye drops are among the most common and effective medical treatment. Recently, topical application of VEGF inhibitors, such as Bevacizumab, Ranibizumab and Macugen, have been tested with promising results. More anti-VEGF factors are under development [67].

Surgical/parasurgical options include laser photocoagulation, fine needle diathermy, photodynamic therapy, and reconstruction of the ocular surface [68].

Finally, small anti-angiogenic molecules have been tested. Among these, Aganirsen, an antisense oligonucleotide against insulin receptor substrate (IRS-1) is able to inhibit corneal hem- and lymphangiogenesis [69,70]. Moreover, we previously showed that Infliximab, an anti-TNFα monoclonal antibody, is effective in reducing CNV [40]. Almost all therapies mentioned above show anti-CNV effects at some degree, but the most successful treatments are topical administration of corticosteroids and VEGF inhibitors for recent neovessels, while fine needle diathermy gives reasonable results in established CNV [71].

Corticosteroids

Topical corticosteroid administration represents the first-choice treatment for CNV and is often used after corneal transplantation [72,73]. Corticosteroids are potent anti-inflammatory molecules that are able to suppress the migration, activation, and recruitment of macrophages and other types of inflammatory cells, thereby blocking inflammatory cell recruitment into the cornea. It has been demonstrated that corticosteroids inhibit both hem-and lymphangiogenesis [74,75]. However, the use of corticosteroids is not always indicated because of their considerable side effects, which may include the development of cataract, glaucoma, delay wound healing and the increased risk of infection [76-78]. In addition, corticosteroid therapy is effective in inhibiting actively proliferating corneal vessels, but it is not useful for the treatment of established vessels. Thus, alternative therapeutic approaches are necessary.

Anti-VEGF therapy

Inhibition of angiogenesis by targeting VEGF seems like a logical approach to treat CNV. Indeed, several studies have confirmed inhibition of angiogenesis after blockade of the VEGF pathway, accompanied with fewer side effects [79]. High concentration treatment and treatment of active CNV, however, may be associated with corneal melting or neurotrophic keratopathy. It is important to keep in mind that both VEGF and VEGFR are expressed by vascular endothelial cells, macrophages and dendritic cells, thereby the beneficial effect of this kind of inhibitors could be also due to the modulation of immune cells.

Bevacizumab (Avastin) is a recombinant humanized monoclonal antibody directed against all isoforms of VEGF-A, thus inhibiting VEGF-mediated signaling pathways and angiogenesis [80]. It was approved originally for the treatment of metastatic colorectal cancer [81], but has been used off label for the treatment of exudative age-related degeneration, proliferative diabetic retinopathy and iris rubeosis, with promising results [82,83]. Although short-term use of topical and subconjunctival bevacizumab has been shown to give positive results, the effects of long-term anti-VEGF therapy have to be evaluated. Recently, topical and subconjuctival administration of bevacizumab has been reported to inhibit or regress CNV in both animal models and humans [84-86]. However, although CNV was reduced significantly, it was not completely eliminated. In addition, it is known that VEGF blockade alone cannot abolish established blood vessels. VEGF-A dependence of vessels may be influenced by vessel maturation and related to the presence of vascular mural cells [87]. Thereby, other regulators of angiogenesis may exist and work in conjunction with VEGF.

Ranibizumab (Lucentis) is a humanized monoclonal antibody Fab engineered to bind and inhibit all the isoforms of VEGF-A. It has been already approved for age-related macular degeneration, diabetic retinopathy, and retinal vein occlusions [88]. Recently, early subconjunctival administration of ranibizumab has been reported to inhibit CNV in an alkali burn model [89]. Clinical studies revealed that topical ranibizumab can be effective to treat CNV [90]; although no effect was found in pterygium-associated CNV [91]. Ranibizumab is also able to reduce VEGF levels in the aqueous humor and the iris, suggesting possible applications for treatment of angiogenesis in other structures of the anterior segment [89].

Pegaptanib (Macugen) is an aptamer that only binds the human VEGF-A isoform VEGF165 and its murine homologue VEGF164. It has been already approved for the treatment of age-related macular degeneration [92]. Recently, it has been shown that Pegaptanib is able to inhibit corneal hem- but not lymphangiogenesis, thus demonstrating that not all the VEGF-A isoforms play the same crucial role in angiogenesis [93].

Other possibilities include administration of soluble decoy receptors (VEGFTrap) that can bind to all the members of the VEGF family and block the activation of the VEGF-mediated angiogenic pathway, also with higher affinity compared to bevacizumab [94].

Finally, it has also been found that subconjunctival injection of synthetic siRNA targeting VEGF-A is able to inhibit angiogenesis in an alkali burn model [95]. This finding may represent an important therapeutic alternative in the treatment of CNV.

Topical and subconjunctival Bevacizumab and Ranibizumab administration has been tested in several trials and seems to be a safe treatment for CNV. However, some problems still remain unsolved. Bevacizumab is a large full-length immunoglobulin with a molecular weight of 149 kDa and topical application may be ineffective because the drug is not able to penetrate the intact corneal epithelium. Subconjunctival administration overcomes this problem but is more invasive. Ranibizumab has a molecular weight of 48 kDa, which allows for more effective corneal penetration and the establishment of therapeutic concentrations earlier in the course of treatment [96]. Additionally, ranibizumab lacks the Fc region of the antibody and is less likely to cause complement-mediated inflammation. Moreover, the binding affinity of ranibizumab is higher than bevacizumab, although bevacizumab has a longer half-life than ranibizumab [97].

Both bevacizumab and ranimizumab neutralize all isoforms of VEGF-A and have a similar clinical effect on CNV. In contrast, pegaptanib only binds the human VEGF-A isoform VEGF165 (mouse VEGF164). Even though it has been shown that topical inhibition of all VEGF-A isoforms has a stronger effect on CNV compared to the inhibition of VEGF164 alone, the specific action of pegaptanib may represent an advantage in terms of side-effects in comparison to bevacizumab and ranibizumab. In fact, it has been demonstrated that VEGF-A promotes corneal nerve regeneration and corneal epithelium wound healing, and its blockade may lead to some adverse effects such as superficial epitheliopathy and corneal thinning. In this context, the use of pegaptanib-which only inhibits VEGF165- could have a better safety profile.

Si-RNA administration represents a new technology which allows to target and silence the gene of interest with high efficiency. However, because of the difficulty of siRNA to penetrate cell membranes, this strategy is not always the best solution and further studies are needed to understand and prevent off-target effects [98].

In summary, although anti-VEGF therapies represent a good option to treat CNV, although their effectiveness in inducing regression of corneal neovascularization is still under scrutiny. In addition, only time will tell us whether long-term neutralization of VEGF may have unexpected local or systemic consequences.

Combination and surgical therapies

Although steroids and VEGF inhibitors represent the commonly used therapies, some combination approaches have been investigated with promising results.

For example, topically administered combinations of steroids have synergistic effects that contribute to efficient suppression of CNV [77].

Moreover, a study has reported that the inhibition of both VEGF-A and PDGF-B signaling is more effective in blocking angiogenesis than targeting VEGF-A system alone [99]. Another group has found that bevacizumab coupled with argon laser therapy is effective in reducing CNV. Indeed, argon laser-induced coagulation acts by closing pathological blood vessels while bevacizumab prevents new angiogenesis [100]. In addition, Su et al. have shown that the combination of doxycycline and bevacizumab enhanced inhibitory effects on CNV [101].

Other proposed treatments include combination of surgical and medical therapies. These include fine-needle vessel coagulation combined with anti-VEGFs [102] and verteporfin photodynamic treatment associated with subconjunctival administration of bevacizumab [103]. These treatments might represent new strategies to modulate the immune response and precondition vascularized high-risk corneas prior to keratoplasty.

Photodynamic therapy (PDT)

PDT, which uses verteporfin as a photosensitizer, has been reported to induce regression of CNV in both human and animal models, but several sessions are needed [104,105]. PDT destroys endothelial cells and vascular basement membrane by generating reactive oxygen species and leading to vessel thrombosis. It also has minimal local and systemic effects and offers a minimally invasive alternative treatment option.

Gene therapy

Gene therapy is another promising strategy for the treatment of CNV. Using recombinant viral vectors, specific angiogenic factors and their receptors can be easily targeted. Moreover, the isolation of the eye from the rest of the body makes the local administration of vectors easier and safe. The first attempts of corneal gene therapy date back to 1994 [106]. Potential applications for gene therapy have since then expanded to many corneal diseases such as allograft rejection, herpes simplex keratitis, CNV and corneal dystrophies.

Viral and non-viral vector systems are used to deliver genes in cells. Viral vectors show higher transgene expression efficiency but may develop immunogenicity and toxicity, thus limiting its use in clinic. On the other hand, non-viral vector systems are safer but have lower expression efficiency. Thus, several delivery methods and techniques have been investigated to increase gene delivery to corneal cells [107,108].

So far, gene therapy has been successful in inhibiting CNV in many cases [109-112].

Singh and colleagues have shown that the delivery of a plasmid expressing VEGFR-1 inhibits upregulation of VEGF and reduce CNV [111] and that intrastromal delivery of a plasmid expressing siRNA against VEGF-A suppresses VEGF expression, leukocyte infiltration and was able to induce CNV regression [112]. Other authors suggested that albumin nanoparticles are able to express VEGFR-1 for longer periods without toxicity to the cornea [113].

Future Directions

Although several treatments have been tested and the many molecules in the pipeline, an effective treatment for corneal neovascularization is still needed. In particular, an effective treatment to induce regression of established neovessels would represent a significant advantage, since available medical treatments generally fail to induce significant regression. A deeper understanding of the mechanisms driving neovascularization is also highly desirable. Specifically, it appears as if CNV may be helpful in promoting immune response and reduce the risk of perforations in some instances. Hence, it would be ideal to induce regression only in those cases and at those times where no beneficial effects from CNV are expected.

Finally, combined treatments targeting several molecules and modulating multiple signaling cascades may represent a valid approach for the future.

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