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PhD THESIS

MOLECULAR AND THERAPEUTIC APPROACH TO CORNEAL ANGIOGENESIS

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Introduction

The cornea forms the anterior part of the eyeball, representing the most important component of the refractive system, with a power of about 40 diopters. Transparency status of this organ is possible due to corneal avascularization, a process achieved by balancing pro- and anti-angiogenic factors. Also the cornea offers an unique possibility to follow both the angiogenic phenomenon and the therapeutic action of antiangiogenic therapy. Current doctoral research aims to establish a useful corneal experimental model, capable of providing support for the comparative testing and validation of antiangiogenic therapies on rabbit cornea and also to monitor the expression of VEGF and PDGF genes in the normal pig cornea. Two distinct methods, which are represented by chemical burn induced in the cornea and implantation of human tumor cells in the anterior chamber of the eyeball, both experiments will be performed on the rabbit eye model in order to induce corneal neovascularization. This paper also aims to study therapeutic methods used in treatment of corneal neovascularization. In case of corneal burns we will use an artisanal compound based on fresh Aloe Vera extract. Talking about superficial lesions we will adopt a gentle, cheap therapy, having therapeutic potential in various diseases of the ocular surface taking into account the multiple beneficial effects of the fresh Aloe Vera extract. In case of tumor implant, an accentuated inflammatory response is expected accompanied by denser and deeper corneal neovascularization compared to mild neovascular reaction following corneal burn method, in this case a treatment based on anti-CLIC1 antibodies will be used.

The elements of originality of this work are represented in the first place by the little data available on the genetic profile of angiogenic growth factors and corresponding receptors in the normal cornea. Also corneal wound healing studies have used only Aloe Vera extract, which is not combined with other substances. We aimed to study the effects of a gel made of Aloe Vera extract mixed with hyalruonic acid on corneal lesions induced by sodium hypochlorite.

The element of originality is also found in the tumor experiment, due to the fact that tumor fragments from human kidney and breast tissue implanted in the anterior chamber of the eyeball in rabbits are rarely found in the literature. Also anti-CLIC 1 antibodies represent a promising therapeutic target in malignant tumors, especially those with increased risk of invasion, due to the fact that CLIC1 is expressed not only in tumor cells but also in blood vessels acquired by the tumor.

I. The General Part

I.1 Vasculogenesis and angiogenesis, physiological mechanisms. The formation of the vascular system (vasculogenesis) is one of the first processes of embryogenesis. From the first stages of embryonic development, mesodermal cells differentiate into hemangioblasts, representing the progenitors of both hematopoietic cells and endothelial cells, giving rise through this complex process to blood vessels. Following this process, hemangioblasts differentiate into angioblasts, which leads to the formation of so-called "capillary islands", so that the fusion of these islands leads to the formation of the first vascular plexuses. In adults, this process of formation and development of new vessels is under strict control, the processes being activated under certain specific conditions such as the healing of damaged tissues. The human cornea is avascular only in the case of normal homeostasis, this privilege of corneal avascularization is not absolute. The appearance of corneal neovasculature is an immune response in conditions of local inflammation, limbic stem cell deficiency, hypoxia or trauma .

I.2 Pathological angiogenesis. Inflammation and hypoxia contribute to the production of VEGF-A through several cell types such as pericytes, smooth muscle cells, macrophages and T cells. There are two distinct elements in the process of angiogenesis, namely: endothelial cell proliferation and the survival of new vessels. Angiogenesis is a well-coordinated process, based on balance between pro- and antiangiogenic factors, so that under pathological conditions there is an imbalance in favor of proangiogenic factors. There are four essential stages of angiogenesis : Vasodilation and vascular permeability, Endothelial proliferation and cell migration, Degradation of the basement membrane, Supportive cell recruitment and Vascular fusion.

I.3 Implications of angiogenesis in ophthalmology. Avascularization and corneal accessibility make this organ an ideal model for studying the process of angiogenesis. Corneal neovascularization is formed when the blood vessels of the limbus penetrate the avascularized corneal tissue. The new blood vessels follow the path of collagen fibers and develop in the form of brushes. The development of corneal neovascularization essentially presents three clinical models, the first model involves the formation of the vascular panel, the second model involves stromal neovascularization, a process resulting from stromal keratitis or alkaline burn, the third clinical model is summarized in neovascularization occurring in the corneal Descemet layer.

I.4 Experimental models in ophthalmology.

Rabbit intrastromal pocket model. This method is based on a pro-angiogenic implant placed in a stromal pocket in the cornea created with the scalpel. The sterile pellet of Endotoxin Copolymer was implanted, and the evolution of neovascularization was followed under a biomicroscope. The endotoxin-free implant was shown to be avascular. Extraction of endotoxin pellets resulted in a regression of neovascularization over two weeks. It should be noted that the method based on the implantation of tumor cells has developed the most intense process of neo-vascularization, but this technique is limited by the much too rapid development of tumors, thus obstructing close monitoring of the development of new blood vessels.

Corneal suturing model. The short time, simplicity and prompt response of the corneal lymph-angiogenetic following the suture are the features that make this experiment a reliable model, especially since the growth rate of new corneal vessels is measurable.

Alkane burn model. This is done with a NaOH-soaked filter paper that touches the center of the cornea without affecting the corneal limbus, conjunctiva or sclera.

Corneal transplant model. This experiment was used to study the mechanism by which graft rejection occurs. Transplant surgery can be performed both on a surface that has an inflammatory reaction and on a clear cornea without an inflammatory reaction, to study the effect in both situations.

Fibroblast grow factor model. The implantation of a micropellet containing FGF2 in an intrastromal pocket is a good model for inducing corneal neovascularization in animals.

I.5 Lymphangiogenesis Direct arguments and practical applications on the anterior pole of the eyeball in experimental model. Various studies have shown the involvement of VEGF-C and VEGF-D together with the VEGFR-3 receptor in the lymphatic invasion of various cancers and in metastases located in the lymph nodes. The lymphatic characteristic of the cornea was unknown until recently when a study showed that a soluble form of VEG-R2 secreted by corneal epithelial cells selectively suppresses the development of the lymphatic system at this level. Major involvement of IGF-1 and IGF-2 factors in stimulating lymph endothelial cell proliferation and migration, processes that support corneal lymphangiogenesis, has been demonstrated. It follows that VEGFR-A can induce the proliferation of lymph endothelial cells directly without the involvement of the VEGF-R3 receptor. PDGF is involved in lymphangiogenesis through direct stimulation of the lymphatic endothelium. HGF can also cause corneal lymphangiogenesis that can be blocked by partial inhibition of the VEGF-R3 receptor.

I.6 Corneal repair mechanisms without angiogenesis. The corneal epithelium benefits from an autonomous healing mechanism based on migration and differentiation of stem cells from the limbic level, but nevertheless does not undergo mutations to transform into another type of cell, unlike stromal keratocytes which at this level it is differentiated into fibroblasts and myofibroblasts. The healing process of the corneal endothelium is based on cell migration and differentiation, while being able to undergo a process of transformation of mesenchymal epithelial cells.

I.7 Current therapies in pathological lymphangiogenesis and angiogenesis. The method based on subconjunctival injection of Bevacizumab was found to be superior to topical treatment with the same active substance in terms of significantly reducing corneal neovascularization and graft life in experimental corneal transplant models in laboratory mice and also in human corneal transplantation. Steroid anti-inflammatory drugs are considered to be the main anti-angiogenic therapy, however they have a limited indirect anti-angiogenic effect due to the association of this therapy with the occurrence of cataracts. At the same time, the reduction of CLIC1 expression has the effect of reducing endothelial migration, cell development, reducing the formation of neoformation vascular networks.

I.8 Morphological and molecular architecture of corneal endothelial cells. The corneal endothelium consists of a single layer of polygonal cells with a diameter of 20 microns and a thickness of 4 microns. Their main role is to maintain an optimal corneal transparency, by the pump effect. These cells actively participate in enzymatic transport. The average density of endothelial cells in adult is around 3000 cells per square mm, which decreases by 0.6% per year, without the ability to regenerate, neighboring cells undergo a process of hyperplasia. At a value of less than 500 cells per square mm, corneal edema and decreased visual acuity may appear. The central portion of the cornea, including the endothelium, derives from neural crest cells. The corneal endothelium, composed mainly of structurally modified cells, has been shown to be more susceptible to intraoperative microtraumas than an endothelium consisting largely of polygonal cells. Also the cells in the central area have a more compact distribution than those located in the periphery.

II. The special part

II.1 Objectives of doctoral project. The objectives of this doctoral project were structured in for main points such as : Characterization of angiogenic factors in the normal cornea on experimental model ; Macroscopic and microscopic characterization of lesions induced by non-tumor agents (which induce lesions with corneal angiogenic potential) ; Characterization of the behavior of two types of human malignancies (breast and kidney) implanted in the anterior chamber of the eyeball in rabbits ; Effects of anti-CLIC1 antibody therapy on tumor implant-induced angiogenesis.

II.2 Materials needed to perform the experiments. Five pig corneas harvested from pigs sacrificed for commercial purposes. Laboratory animals (9 male rabbits of the Albino breed), dry food, ensuring the microenvironment necessary for the optimal development of the experimental model. Fresh specimens of renal cell carcinoma, clear cell type and breast cancer, ductal invasive type. 1fl Sodium chloride 0.9%, 1fl Xylazin Bio 2%, 1fl Ketamidor 100mg/ml, face mask for the anesthetic gas. Surgical instruments, 45 Scalpel, slitknife, surgical scissors, surgical pens, sutures. Therapeutic agents such as anti CLIC1antibodies.

II.3 Gene expression profile of VEGF and PDGF pathways in the normal cornea. Angiogenic growth factors expression is not known in the normal cornea. The aim was to study corneal gene expression profile of VEGF and PDGF pathways influencing the avascular state of cornea. **Materials and Methods**: cDNA synthesis was performed from mRNA extracted from five fresh pig corneas followed by cDNA synthesis and analysis of VEGF and PDGF pathways by TaqMan Array gene expression profile.

Results: Normal pig cornea lacks VEGFR2 and VEGFR3 gene expression. MK2 and AKT1 genes were significantly overexpressed ($p=0.000684$, $p=0.050995$, respectively). Six PDGF pathway genes were overexpressed: TIAM1 ($p=0.047$), PIK3CA ($p=0.00005$), IKBKG ($p=0.000006$), PAK4 ($p=0.034$), RAC1 ($p=0.000006$ and PTGS2, $p=0.00375$). PDGF A was up- regulated, but not with a statistical significance ($p=0.79911$), while PDGFRα was down-regulated and PDGFRβ was not expressed.

Conclusion: Normal cornea avascularity is given by growth factor receptors down-regulation. Rapid corneal neovascularisation is induced by activation of the main angiogenic growth factors that induce angiogenic cascade and vessel recruitment.

II.4 Hypochlorite-induced lesions treated with fresh Aloe Vera extract. The effects of corneal burns induced by sodium hypochlorite, a substance commonly used in dental practice, which have not been sufficiently studied so far. The aim was to study the therapeutic effects of fresh aloe vera extract and hyalruonic acid on corneal lesions induced by sodium hypochlorite.

Materials and Methods. two healthy male Albino rabbits were used for the experiment, Fresh Aloe Vera leaves were used, harvested from plants older than 3 years. The leaves were prepared using usual aseptic methods and cut into 2 cm fragments which were then removed from the hard shell with a scalpel. The gelatinous contents of the leaf was collected and stored in 2 ml syringe, which was applied 1 drop per day for 5 days to the right eye, the lesions of the left eye remainied untreated. The chemical burn was obtained by daily instillations of 40 microliters, for 3 consecutive days of sodium hypochlorite.

Results. The first macroscopic changes in the eyeballs surface appeared 3 days after the application of sodium hypochlorite, the effect was generalized conjunctival hyperemia and ephitelium destruction. Lesions induced by sodium hypochlorite were observed microscopically at both epithelial and stromal levels. Accelerated reepithelialization was observed after 5 days of treatment, the most important changes took place on the corneal stroma wich was regenerated for the most part by restoring collagen fibers and densifying the stromal structure.

Conclusion. Extract of fresh Aloe Vera in combination with hyalruonic acid can be effective on mild or medium corneal burns, both in epitelium restauration and stromal regeneration.

II.5 Behavior of intracameral malignant implants and effects of anti-CLIC1 antibodies. The rabbit cornea model offers the opportunity to notice with the help of accessible optical instruments the effects of the anti-CLIC1 antibody therapy on the human tumor fragments implanted in the anterior chamber of the eyeball. Two of the most aggressive malignancies found in humans, breast carcinoma and kidney carcinoma, were used, because of the poor information about this subject in the literature.

Material and methods. Human tumor fragments were inserted into the anterior chamber of the rabbit eyeball. Two rabbits were used for the experiment, one of which had a breast tumor inserted and the other the kidney tumor. A Zeiss operating microscope was used to monitor weekly changes in both the anterior chamber of the eyeball and the cornea. The

stereomicroscoic changes were certified by the histological examination performed with the help of Axio Zoom 2 Observer microscope.
Results. The two tumor fragments implanted in the anterior chamber evolved differently, the growth rate of the renal tumor being higher compared to the growth rate of the breast tumor. At the same time, the acquisition rate of new blood vessels at the corneal level was different between the two experimental models. At week 5 for renal tumor and at week 8 for breast tumor, anti-CLIC1 antibody therapy was applied for 5 consecutive days, 20 microliters, in a single administration. Following the application of anti-CLIC 1 antibody treatment, a reduction in vascular density at the corneal level was observed, from a macroscopic point of view. Treatment with anti-CLIC1 antibodies to the RCC implant resulted in the destruction of tumor cells that had suffered an intense process of necrosis. Also, the density of the blood vessels from breast tumor was significantly reduced in the treated specimen.
Discussion. Intracellular chlorine channels type 1 are cellular structures present in many tissues and involved in various processes such as oxidative stress, endothelial destruction, inflammation, and tumor development. Our study tested for the first time the effects of anti-CLCI1 antibodies on intraocular implants of human kidney tumors known to be extremely aggressive.

III Conclusions and personal contributions

Regarding the absence of corneal vascularization in physiological conditions, it can be concluded that this is possible due to the balance between proangiogenic and antiangiogenic factors. At the same time, the dual role of VEGF and PDGF pathways was demonstrated, on the one hand, and the antiangiogenic pathway, on the other hand, the role in maintaining corneal integrity. Low values of VEGF A and VEGF C were detected in the normal cornea and also the absence of adjacent VEGFR2 and VEGFR3 receptors. PDGF α and β receptors are also absent in the normal cornea. The experiment performed on normal pig cornea highlights the overexpression of genes with proangiogenic role only in conditions of corneal suffering. Thus, synthesizing the results of the experiment performed on the normal pig cornea, it can be concluded that the proangiogenic status exists in the normal cornea, but it is counteracted by the absence of VEGF2, PDGFα and PDGFβ effectors, which are absent in the normal cornea.

Also, it can be concluded that the lesions produced by sodium hypochlorite burn affected both the corneal epithelium and the underlying layers, a fact demonstrated by the destruction of the local architecture of the collagen fibers at the stromal level. Fresh Aloe Vera extract and

Hialuronic Acid has been shown to be effective both in accelerating the process of corneal reepithelialization and in restoring the integrity of collagen fibers at the stromal level, thus having a beneficial effect at both the superficial and deep levels.

The results of the third experiment demonstrated both macroscopically and microscopically the double effect of anti-CLIC1 antibodies, which act both on the neovascularization process, inducing a limiting and even regression effect of neoformation vessels and also necrosis process on tumor cells.

Regarding the element of originality of this paper work it can be said that the literature does not present concrete data regarding the expression of VEGF and PDGF in the healthy cornea on experimental models, all the data published so far refer to the study of these proangiogenic factors in different pathological conditions of the cornea. Second, the effects induced by sodium hypochlorite on the cornea have not been studied so far, data from the literature show results of skin lesions induced by this substance. At the same time, the data present in the literature regarding the effects of local treatment with a substance composed of fresh aloe vera extract and hyaluronic acid on the corneal epithelium and corneal stroma are insufficient and incomplete, the studies focusing especially on the effects of treatment on the cornean epithelium. Last but not least, the use of tissue from aggressive human tumors such as: renal carcinoma and breast carcinoma, as proangiogenic material inserted in the anterior chamber of the rabbit eye is also a subject little studied in the literature. Anti-CLIC1 antibody therapy in such a situation is again a model little studied so far.