Aus dem Zentrum für Augenheilkunde der Universität zu Köln
Klinik und Poliklinik für Allgemeine Augenheilkunde
Direktor: Universitätsprofessor Dr. med. C. Cursiefen

The Lymphogenic and Hemangiogenic factors affecting The Human Sclera

Inaugural-Dissertation zur Erlangung der Doktorwürde
der Medizinischen Fakultät
der Universität zu Köln

vorgelegt von
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promoviert am 25. November 2022

Gedruckt mit Genehmigung der Medizinischen Fakultät der Universität zu Köln,
2022

Dekan: Universitätsprofessor Dr.med. G. R. Fink

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Die in dieser Arbeit angegebenen Experimente sind nach entsprechender Anleitung durch Herrn Univ.-Prof. Dr.med. Ludwig. M. Heindl von mir von mir selbst ausgeführt worden.

ACKNOWLEDGEMENTS

First and foremost, I would like to cordially thank my respectful supervisor, Univ.-Prof. Dr. med. Ludwig M. Heindl, to whom I am so grateful for his constant and valuable support on this project. To his continuous encouraging me and support throughout the whole process. I want to express my gratitude to him for his not only academic guidance but also for his trust and kindness which encourages me to overcome all the challenges in my scientific and personal way.

I am also thankful to Univ.-Prof. Dr. med. Claus Cursiefen, Director of the Center for Ophthalmology of the University of Cologne, for the opportunity to carry out this project through the hospital.

I also would like to greet especially Dr.med Herbert Tempfer for his constant, very friendly support throughout each phase of my work as well as his suggestions that have contributed to the completion of this work. I am grateful to him for his energetic support in carrying out the project.

I would like to express my deepest thanks to my colleagues and friends at the Paracelsus Medical University in Salzburg, Austria for their consistent scientific support, generous help, and sincere friendship.

I really appreciate all the support, encouragement, and joy from my brothers, Ahmed, and Islam in Germany

Furthermore, I want to express my special thanks to my beloved parents who gave me the wonderful life and always support me without any requests.

Dedication

To your sole Dad …

to you Mom …

and….

to you my Karma

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ABBREVIATION

APCs	Antigen-Presenting Cells
α-SMA	Alpha Smooth Muscle Actin
BSA	Bovine serum albumin
CO_2	Carbon Dioxide
CHP	Collagen Hybridizing Peptide
COX2	Cyclooxygenase-2
C57BL/6	Congenic strain 57 black laboratory mouse 6
CTGF	Connective Tissue Growth Factor
CQ	Quantification Cycle
DM	Diabetes Mellitus
DAPI	4′,6-diamidino-2-phenylindol dihydrochloride
DNA	Deoxyribonucleic Acid
ECM	Extra Cellular Matrix
E17	Embryonic Day 17
E 0.5	Embryonic Day 0.5
GFP	Green Fluorescent Protein
HINT1	Histidine Triad Nucleotide-Binding Protein 1
HPRT1	Hypoxanthine Phosphoribosyl Transferase 1
iNOS	Inducible Nitric Oxide Synthase
IL1-ß	Interleukin 1-ß
IL6	Interleukin 6
LYVE1	Lymphatic Vessel Endothelial Hyaluronan Receptor 1
mRNA	Messenger Ribonucleic Acid
MMP2	Matrix metalloproteinase 2
MMP3	Matrix Metalloproteinase 3
MMP13	Matrix Metallopeptidase 13
Mkx	Mohawk
NF-κB	Nuclear Factor kappa light chain enhancer of activated B cells
PBS	Phosphate-Buffered Saline
qRT-PCR	Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
RT	Room Temperature
RNA	Ribonucleic Acid
RQI	Ribonucleic Acid Quality Indicator
RA	Rheumatoid Arthritis
SLRPs	Small Leucine-Rich Proteoglycans
SLE	Systemic Lupus Erythromatousis

SCX_GFP	Scleraxis Green Fluorescent Protein
Tnmd	Tenomodulin
TSP-4	Thrombospondin 4
TNC	Tenascin C
Tg	Transgenic
TBP	TATA-Box Binding Protein
BECs	Blood endothelial cells
CD11b	Cluster of Differentiation 11b
CD11c	Cluster of Differentiation 11c
CD 31	Cluster of Differentiation 31
CD36	Cluster of Differentiation 36
CD45	Cluster of Differentiation 45
CD45b	Cluster of Differentiation 45b
CD 68	Cluster of Differentiation 68
CD117	Cluster of differentiation 117
CD163	Cluster of Differentiation 163
CCR7	C-C chemokine receptor type 7
CXCL12	C-X-C motif chemokine 12
CCR2	C-C chemokine receptor type 2
CXCR4	C-X-C chemokine receptor type 4
c- kit	transmembrane tyrosine kinase receptor
FGF-2	Fibroblast growth factors 2
FBLN5	Fibulin-5
FLT4	Fms-related tyrosine kinase 4
GFAP	Glial Fibrillary Acidic Protein
GFs	Growth factors
HGF	Hepatocyte growth factor
IGF-1	Insulin like growth factor 1
IGF-2	Insulin like growth factor 2
LECs	Lymphatic endothelial cells
MHCII	Major histocompatibility complex class II
M1	M1 macrophages
M2	M2 macrophages
NK-cell	Natural killer cell
PDGF	Platelet-derived growth factor
PEDF	Pigment epithelium-derived factor
PDT	Photodynamic therapy

PGF	Placental growth factor
PROX1	Prospero homeobox protein 1
SER- PINF1	Serpin Family F Member 1
SEMA3F	Semaphorin-3F
TNM	Tumor (T), Nodes (N), and Metastases (M)
TGFA	Transforming growth factor alpha
TSP-1	Thrombospondin 1
TSP2	Thrombospondin 2
TIMP2	Tissue inhibitor of metalloproteinases 2
TIMP3	Tissue inhibitor of metalloproteinases 3
VEGF	Vascular Endothelial Growth Factor
VEGFR-1	Vascular endothelial growth factor receptor 1
VEGFR-2	Vascular endothelial growth factor receptor 2
VEGFR-3	Vascular endothelial growth factor receptor 3
VEGF-A	Vascular endothelial growth factor A
VEGF-B	Vascular endothelial growth factor B
VEGF-C	Vascular endothelial growth factor C
VEGF-D	Vascular endothelial growth factor D
VAP-1	Vascular adhesion protein 1
VPF	Vascular Permeability Factor

1. DEUTSCHE ZUSAMMENFASSUNG

Titel der Inaugural dissertation:
The Lymphogenic and Hemangiogenic factors affecting The Human Sclera
Von Ghada Atta
Aus dem Zentrum für Augenheilkunde der Universität zu Köln

Unser Projekt " Die lymphogenen und hämangiogenen Faktoren, die sich auf die menschliche Sklera auswirken", ist eine neuartige Studie, die sich auf die vaskulären und zellulären Veränderungen der menschlichen Sklera im gesunden Zustand und unter pathologischen Bedingungen konzentriert Unsere Forschungsarbeit basiert auf Beobachtungen und klinischen Erfahrungen, zusammen mit einer Literaturübersicht auf der Grundlage einer PubMed-Suche. Wir fanden heraus, dass das vaskuläre Privileg der gesunden menschlichen Sklera durch ein dichtes vaskuläres Netzwerk gekennzeichnet ist, das ein breites Spektrum an anti-angiogenen und anti-lymphangiogenen Faktoren aufweist, die stark exprimiert werden, während die Expression von pro-angiogenen Faktoren reduziert ist. In den gesunden menschlichen Skleral-schichten gibt es keine Lymphgefäße, die zum okulären Immunprivileg des inneren Auges beitragen. Unter pathologischen Bedingungen sind die vaskulären und zellulären Privilegien der Sklera beeinträchtigt, da sich die Fibrozyten in aktive Fibroblasten verwandeln, die die Sklera Matrix umbauen und proliferieren. Auch die zelluläre Aktivierung und das sekundäre Einwachsen von intraokularen Lymphgefäßen, die mit LYVE-1+ Makrophagen assoziiert sind, dringen in die Sklera und die intraokularen Strukturen ein und beeinträchtigen die Heilungsergebnisse. immunologische Verteidigung gegen eindringende Mikroorganismen und Autoimmun-Reaktion gegen intraokulare Antigene [21].

In unserer zweiten Publikation geben wir einen neuen Einblick in die Charakterisierung von SCX-GFP, einem Sehnenmarker, der Sklera Zellen exprimiert, und in die Reaktion von Sklera Fibroblasten auf Entzündungsreize, indem wir explantiertes Sklera Gewebe von erwachsenen Mäusen in einem neuartigen Ex-vivo-Modell der Skleritis untersuchen, das die Kennzeichen dieser Krankheit in Form einer geschädigten Kollagenmatrix, einer Hochregulierung von Entzündungsfaktoren und der Expression von Matrix abbauenden Enzymen darstellt. Mittels qRT-PCR und Immunhistochemie wurde das explantierte Sklera Gewebe von SCX-GFP-Mäusen mit 10 ng/ml IL1-ß bzw. IL1-ß in Verbindung mit Dexamethason behandelt. Ein Kollagen-Hybridisierungs-Peptid-Bindungstest analysiert den Abbau von Kollagenfasern in den durch Immunfluoreszenz gefärbten Geweben der entzündungs- und Fibrose bezogenen Proteine. Dabei wurde SCX-GFP im Sklera Gewebe der Maus stark exprimiert. Nach Stimulation durch IL1-ß erhöhten SCX-GFP+-Zellen die Expression aller untersuchten Proteine stark. Die Zugabe von Dexamethason führte zu einer signifikanten Verringerung der

Reaktion auf IL1-ß-Stimulation. Außerdem nahm der Kollagenabbau in der IL1-ß-Gruppe stark zu. Das Kortikosteroid Dexamethason zeigte dagegen eine starke Heilwirkung [20].

Unseres Wissens bieten diese beiden Studien einen neuartigen Einblick in zelluläre und vaskuläre Mechanismen im gesunden und pathologischen Zustand und stellen ein neuartiges organotypischen Ex-vivo-Modell der Skleritis zur Verfügung. Außerdem liefern sie eine erste Charakterisierung von Sehnenmarker exprimierenden Skleralzellen SCX_GFP als Reaktion auf entzündliche Stimulationen, und Die Ergebnisse könnten eine Grundlage für die Wundheilung nach chirurgischen Eingriffen bilden und den Weg für die Entwicklung neuer wirksamer Therapien für unheilbare Augenerkrankungen ebnen, die eine neue Verbesserung in der klinischen ophthalmologischen Praxis darstellen werden.

2. INTRODUCTION

Sclera word comes from a Greek word Skleros which means “hard”. The sclera maintains the eyeball shape by providing a firm and stable substrate. Mechanically, it protects intraocular tissues and serves as a site of attachment for extraocular muscles which are responsible for the movement of the eye and prevention of high dynamic loading conditions of the muscular eye movements [1].

2.1. Anatomical structure of the Human Sclera

2.1.1 Macroscopic Anatomy

The three main layers of the sclera are: the first layer is the superficial vascularized episclera which found beneath the conjunctiva and Tenon’s capsule and facing the orbit., the second layer is main avascular scleral stroma [2], and the third layer is the thin pigmented lamina fusca that lies right above the uvea [3].

2.1.2 Microscopic Anatomy

The sclera is made up of dense, various-diameters fibrils that combine forming interlacing fibre bundles [4]. These fibrils are heterotypic and made up of collagen bundles, primarily type I collagen, with minor amounts of other collagen types (such as type III, IV, V VI, VII) and elastin [5,6]. Appropriate hydration and solute diffusion in the scleral tissue are facilitated by the abundance of both the elastic fibres in the lamina fusca and the small leucine-rich proteoglycans (SLRPs) in the inter-fibrillary matrix [7–9]. Finally, the large proteoglycan aggrecan-rich keratan sulphate side chains found in the scleral extracellular matrix (ECM) confer compressive resistance [10].

2.2. The Scleral Vascular Privilege

Antigen-presenting macrophages, anti and pro-angiogenic factors, and other lymphangiogenesis-related factors are parts of the healthy human sclera’s cellular and molecular mechanisms [11].

2.2.1. Macrophages

Within a human’s healthy sclera, macrophages act as antigen-presenting cells (APCs), regulate blood vessel homeostasis and allow maintenance of the eye’s physiological functions. Macrophages are present with highest density in the episclera [12], and to a lesser extent around blood vessels of scleral stroma. During inflammatory processes, macrophages respond to cellular damage by migrating inflammation from the periphery towards the site of inflammation and accumulating around blood vessels [12].

2.2.2. Anti-angiogenic and Pro-angiogenic factors

A wide range of anti-angiogenic and anti-lymphangiogenic factors are highly expressed within a healthy human sclera [13], while pro-angiogenic factors expression is reduced [11]. The healthy human scleral layers lack lymphatic vessels which contribute to ocular immune privilege in the inner eye [14]. Lymphatic Vessel Endothelial hyaluronan receptor 1 (LYVE1) / Cluster of Differentiation 68 (CD68) expressing macrophages surround scleral blood vessels. However, LYVE1+ podoplanin+ lymphatic vessel is absent in all the layers of the sclera (lamina fusca, stroma, and episclera). Cluster of Differentiation 31 (CD31+) blood vessels which are found mostly in the episclera's anterior section, form a tight network that decreases towards the back of the eye [14,15].

2.3. Pathological Conditions affecting the scleral vascular privilege

The scleral vascular privilege is completely impaired under pathological conditions like in ciliary body melanoma with extraocular extension and in open globe injuries, sympathetic ophthalmia. In our publication we studied the scleral vascular and cellular changes and [13,14] the disturbance of, the balance between the anti-angiogenic and anti-lymphangiogenic factors and pro-angiogenic factors which leads to breakdown of the tight vascular network of the sclera we also studied different mechanisms of lymphatic vessels proliferation [16].

2.4. Scleraxis

Scleraxis (SCX) is a transcription factor with a basic helix-loop-helix structure. It is a marker present in tendon cells where and damage of SCX gene causes disruption in tendon development, resulting in matrix disorder and motion impairments [17]. SCX expression was detected in the tendon cells attached to the ocular muscles [18]. Our research uses Scleraxis immunopositively cells in the sclera of e17 embryos as well as adults to investigate how these cells respond to inflammations in an organotypic way. Therefore, our study examines the properties of sclera and establishes an ex vivo scleral inflammation model. Using Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) and immunohistochemistry. SCX Positive cells are in accordance with studies of scleral tenomodulin expression, an antiangiogenic tissue that was also expressed using tendon cells [19] (Scleraxis- Green Fluorescent Protein) SCX-GFP expression is higher in Interleukin 1-ß (IL1-ß) treated tissue and lower in IL1-ß+ dexamethasone-treated samples [20]. This finding is consistent with the observation that dexamethasone, by targeting the SCX gene, inhibits the differentiation of tendon stem cells into mature tenocytes.

2.5. Aims of work

The study of The Lymphogenic and Hemangiogenic factors affecting The Human Sclera aimed to focus on the vascular and molecular changes of human sclera in healthy status this vascular privilege compromised under pathological conditions. Therefore, we explain the cellular and molecular mechanisms [11] related to pro- and anti- angiogenic factors during the process of wound healing after surgical treatments, as well as various ocular diseases [12].

As a continuation to the first publication, the aim of the second publication studying the response mechanism of scleral cells to an inflammatory environment [17]. We detect Scleraxis (SCX) expression which is a transcription factor a tendon marker cells in tendon cells attached to the ocular muscles and scleral tissues [18] where damage of SCX gene leading to matrix disorder which causes disruption in tendon development and motion impairments [17]. Also, we define the expression of markers associated with tendons in adult and embryonic mice eyes using a novel ex-vivo organotypic technique. As noted, tendon and sclera tissues have a lot of characteristics in common. As both are considered collagen-rich matrix with low cell density, sparse vascular tissues, as well as high mechanical stress.

The results of the two studies will provide a novel study focused on cellular and vascular privilege of human sclera which compromised under pathological conditions which affecting healing outcomes Some landmark reviews have been lately published, paving the path for the development of new therapy options for incurable ocular disorders. Therefore, the first publication examines the human sclera's lymphangiogenic and hemangiogenic privileges. And the second publication provides insights by characterization of scleral cells and establishes a novel ex vivo model of scleral inflammation. therefore, this study will help in studying the healing outcomes in the clinical practice in ophthalmology and will open a new field to devise more effective therapeutic strategies.

3. THE LYMPHANGIOGENIC AND HEMANGIOGENIC PRIVILEGE OF THE HUMAN SCLERA

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Financial Disclosure
The authors have no financial interest to disclose.

Funding
Supported by the German Research Foundation FOR 2240 “Lymph Angiogenesis and Cellular Immunity in Inflammatory Diseases of the Eye” to LMH; HE 6743/2-1, HE 6743/3-1, and HE 6743/3-2, GEROK-Programme University Hospital of Cologne to Y.G., and PMU-FFF R-15/02/067-KAS and ÖNB 17617 to AKE and FS

Short Running Head

Privilege of the Human Sclera

Pre-Release:

Atta G, Tempfer H, Kaser-Eichberger A, Guo Y, Schroedl F, Traweger A, Heindl LM., The lymphangiogenic and hemangiogenic privilege of the human sclera. Ann Anat. 2020 Jul; 230:151485. doi: 10.1016/j.aanat.2020.151485. E pub 2020 Feb 28. PMID: 32120002.

In this publication methods and results of my work are described and explained.

3.1. ABSTRACT

Purpose: Most organs of the human body are supplied with a dense network of blood and lymphatic vessels. However, some tissues are either hypo vascular or completely devoid of vessels for proper function, such as the ocular tissues sclera and cornea, cartilage, and tendons. Since many pathological conditions are affecting the human sclera, this review is focusing on the lymphangiogenic and hemangiogenic privilege in the human sclera.

Methods: This article gives an overview of the current literature based on a PubMed search as well as observations and experience from clinical practice.

Results: The healthy human sclera is the outer covering layer of the eye globe consisting mainly of collagenous extracellular matrix and fibroblasts. Physiologically, the sclera shows only a superficial network of blood vessels and a lack of lymphatic vessels. This vascular privilege is actively regulated by balancing anti- and proangiogenic factors expressed by cells within the sclera. In pathological situations, such as open globe injuries or ciliary body melanomas with extraocular extension, lymphatic vessels can secondarily invade the sclera and the inner eye. This mechanism most likely is important for tumor cell metastasis, wound healing, immunologic defense against intruding microorganism, and autoimmune reactions against intraocular antigens.

Conclusions: The human sclera is characterized by a tightly regulated vascular network that can be compromised in pathological situations, such as injuries or intraocular tumors affecting healing out- comes Therefore, the molecular and cellular mechanisms underlying wound healing following surgical interventions deserve further attention, in order to devise more effective therapeutic strategies

Keywords: Human, sclera, Angiogenesis, Lymphangiogenesis, VEGF, Macrophages.

3.2. MACRO- AND MICROSCOPIC ANATOMY OF THE HUMAN SCLERA

The sclera is the rigid outer layer of the eye (Fig. 1) protecting intraocular structures and maintaining intraocular pressure[1]. It affects the visual process by distortion of the retina and the lens-iris-diaphragm. Its structural integrity is a consequence of a dense connective tissue providing stability. It provides attachment sites for extraocular muscles, sharing anatomical and cellular similarities with tendons [1]. It is separated into three main anatomical layers: (1) the superficial vascularized episclera lies underneath the conjunctiva and Tenon's capsule and faces towards the lateral and back of the eye facing the orbit [2]; (2) the scleral stroma is the main part of the sclera which is mainly avascular [3]; and (3) the lamina fusca as a thin pigmented layer forming the sclera-choroid border [4].

The trabecular meshwork (lined by trabeculocytes) is located around the base of the cornea, near the ciliary body, and is responsible for draining the aqueous humor from the eye via the anterior chamber and filters into Schlemm's canal eventually draining into the blood system. It is divided into three parts with characteristic ultra-structures [5]: (1) the inner uveal meshwork is located closest to the anterior chamber angle, contains thin cord- like trabeculae, and is predominantly orientated in a radial fashion, enclosing trabeculae spaces larger than the corneoscleral mesh- work [6] (2) the corneoscleral meshwork contains a large amount of elastin, arranged as a series of thin, flat, perforated sheets arranged in a laminar pattern, and is considered the ciliary muscle tendon [7], and (3) the juxtacanalicular tissue (the cribriform meshwork) lies immediately adjacent to the Schlemm's canal, composed of connective tissue ground substance rich in glycosaminoglycans and glycoproteins, and is covered by a monolayer of endothelial cells [8–10]. The uveo-scleral pathway also assists in the aqueous drainage [11] and this mechanism is increased with the use of anti-glaucoma drugs such as prostaglandins (e.g., latanoprost, travoprost, tafluprost) [12,13].

The lamina cribrosa is a hole in the sclera in the back of the eye where the nerve fibers of the optic nerve exit the eye [1]. It is formed by a multilayered network of collagen fibers inserting into the scleral canal wall. The nerve fibers of the optic nerve run through pores formed by these collagen beams [14,15]. The lamina cribrosa helps to maintain the pressure gradient between the inside of the eye and the surrounding tissue. Being structurally weaker than the much thicker and denser sclera [16], the lamina cribrosa is more sensitive to changes in the intraocular pressure and tends to react to increased pressure through posterior displacement. This is thought to be one of the causes of nerve dam- age in glaucoma, as the displacement of the lamina cribrosa causes the pores to deform and pinch the traversing nerve fibers and blood vessels [1,17].

Microscopically, the sclera consists of dense fibrils with various diameters combining into interlacing fibre bundles [18]. The fibrils are heterotypic structures made up of collagen bundles, mainly of type I collagen and in smaller amounts of collagen type III, IV, V, VI, VII and elastin [19,20]. Scleral elastic fibres are abundant in the lamina fusca and trabecular meshwork and the inter-fibrillary matrix is rich in small leucine-rich proteoglycans (SLRPs) allowing hydration and solute diffusion through the scleral tissue, such as decorin which is closely associated with collagen fibrils at specific binding sites situated to the C-terminus of the collagen molecules and the small SLRP biglycan, which contains dermatan and dermatan/chondroitin sulphate [14,21,22]. Finally, the scleral extracellular matrix (ECM) is also rich in the large proteoglycan aggrecan-rich keratan sulphate side chains, conferring resistance to compression [23].

3.3. THE VASCULAR PRIVILEGE OF THE HUMAN SCLERA

Arterial supply (Fig. 2) is derived from the ophthalmic artery originating from the internal carotid artery. The following branches can be differentiated: central retinal artery, short and long posterior ciliary arteries, and anterior ciliary arteries [24]. The iris and ciliary body are supplied by the anterior ciliary arteries, long posterior ciliary arteries, and anastomotic connections from the anterior choroid [25]. The anterior ciliary arteries travel with extraocular muscles and pierce the sclera near the limbus to join the major arterial circle of the iris. The two long posterior ciliary arteries pierce the sclera near the posterior pole, and then travel anteriorly between the sclera and choroid to join the major arterial circle of the iris. The major arterial circle of the iris gives off branches to the iris and ciliary body [26]. The superficial vascularized episclera is supplied by the anterior as well as short and long posterior ciliary arteries, with the exception of some perforating vessels from which nutrients penetrate from the choroid and the vascular plexus in the Tenon capsule as well as episclera, where arterio-arterial anastomosis is located in which blood oscillates rather than flows rapidly [27,28].

Venous outflow primarily occurs via the vortex veins and the central retinal vein which merges with the superior and inferior ophthalmic veins draining into cavernous sinus, pterygoid venous plexus, and facial vein. Most of the venous drainage from the anterior segment is directed posteriorly into the choroid and then into the vortex veins [24].

The lymphatic system is a prerequisite for maintenance of tis- sue fluid balance and immunity in the body, enabling removal of interstitial fluid and macromolecules, proteins or immune cells and directing transport to the lymph nodes before entering the blood circulation [29]. The adult human sclera is devoid of lymphatic vessels. While the conjunctiva, corneal limbus, lacrimal gland, orbital meninges, and extraocular muscles contain lymphatic vessels, the inner part of the eye is completely devoid of classic lymphatic vessels contributing to the ocular immune privilege [30–32].

It was shown that the scleral blood vessels are surrounded by lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1)/CD68-expressing macrophages, but LYVE1+/podoplanin + lymphatic vessels were not observed in any of the three scleral layers (episclera, stroma, and lamina fusca). CD31+ blood vessels are mainly found in the anterior part of the episclera which build a tight net decreasing toward the posterior part of eye [33–37].

During development the fetal human sclera is primarily devoid of lymphatic vessels while the fetal scleral stroma contains blood vessels [38]. In analogy to the collagen-rich cartilage tissue, the lymphangiogenic potential of vessel associated LYVE1+ cells are inhibited in the scleral microenvironment. CD31+ blood vessels spread in the episclera and anterior stroma, but not through the lamina fusca where they are absent at any develop- mental stage so far investigated [39]. Episcleral blood vessels are present as early as gestational week 13 and their amount increases during pregnancy, whereas stromal CD31+ blood vessels are elevated in early pregnancy and regress with ongoing pregnancy. The function of extra-luminal macrophages within the perivascular region is clearance of perivascular fluid or debris by phagocytosis and a protective role by facilitating influx of inflammatory leukocytes [38].

3.4. ACTIVE REGULATION OF THE SCLERAL VASCULAR PRIVILEGE

Cellular and molecular mechanisms of the healthy human sclera include antigen-presenting macrophages, other lymphangiogenesis-related factors and pro- and anti-angiogenic factors [40]. Macrophages located in the healthy human sclera function as antigen-presenting cells (APCs) and allow for maintenance of physiological function by regulating blood vessel homeostasis within the eye, with the highest density being present in the episcleral [41]. These tissue resident macrophages express a plethora of markers, including CD68, CD163 and CD11b, CD45 (a general leukocyte marker), MHCII (expressed by APCs), CD11c (dendritic cell marker), lymphatic endothelium hyaluronan receptor-1 (LYVE1; expressed on lymphatic endothelium and macrophage subsets), chemokine receptor 7 (CCR7, a homing receptor for leukocytes and regulates the homing to lymph nodes), CXCL12 (expressed by activated leukocytes), CCR2 (a marker for inflammatory monocytes), and glial fibrillary acidic protein (GFAP; astrocytic marker; [41]).

Further, populations co-expressing CCR7 and CD68, which is generally assigned to the “pro-inflammatory”M1 subset, and CD68+/CD163+ macrophages, belonging to the “anti-inflammatory”M2 subset have been described. In the episclera, a high number of cells (>40 cells/mm2) were immunoreactive for CD68, CD45+ leukocytes and cells of a potential bone marrow–derived origin was demonstrated, expressing MHCII, CCR7, LYVE1, and CD11b. Lower numbers (<20 cells/mm2) were positive for the C-X-C-motif chemokine ligand 12 (CXCL12) known to regulate cell migration, proliferation, and angiogenesis through its receptor CXCR4 and both are expressed by endothelial progenitor cells. Further, these cells were positive for CCR2 and GFAP [26,30,41,42]. MHCII+ cells were either double positive for CCR7, CD45, CD11c (used in combination with MHCII to detect dendritic cells), or CD11b and rarely CXCL12. Macrophages were most likely from the M1 subtype (CD68 b, CD163; [41]).

Characterization of the immunophenotype of scleral cells indicated that the episclera and to a lesser extent the scleral stroma contains numerous APCs such as CD11c+/MHCII + dendritic cells and CD11+/MHCII + macrophages, which are either yolk sac or bone marrow derived (CD45b). Finally, CCR7+/MHCII + macrophages were observed in healthy episclera. Interestingly, there are similarities between scleral and cerebral perivascular macrophages: they are located around blood vessels and are both CD45+ and have antigen-presenting functions. In cases of inflammation MHC II+/CD45+ macrophages migrate towards the site from the periphery accumulating around blood vessels and responding to cell damage [41,43].

3.5. OTHER LYMPHANGIOGENESIS-RELATED FACTORS

Several growth factors (GFs) have been described: insulin like growth factors (IGF-1 and IGF-2) which stimulate proliferation and migration of lymphatic endothelial cells; hepatocyte growth factor (HGF) which induces corneal lymphangiogenesis and can be blocked by VEGFR-3 inhibition and platelet-derived growth factor (PDGF), which directly stimulates the lymphatic endothelium [44,45]. Finally, using a corneal micro-pocket assay it was shown that a low dose of fibroblast growth factor 2 (FGF-2) induces lymphangiogenesis, demonstrating that lymphatic growth is possible without concur- rent angiogenesis [46]. These growth factors and cytokines are angiogenic [47] and also promote lymphangiogenesis either by driving proliferation or migration of lymphatic endothelial cells directly or by up-regulating VEGF-C/-D to activate VEGFR-3 [48,49].

3.6. PRO- AND ANTI-ANGIOGENIC FACTORS IN THE HUMAN SCLERA

Vascular endothelial growth factor (VEGF) originally known as vascular permeability factor (VPF) is a signal protein that stimulates the formation of blood vessels involved in both vasculogenesis (the de novo formation of the embryonic circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature) to restore the oxygen supply to tissues when blood circulation is inadequate. Overexpression of VEGF can cause vascular disease in the retina and other parts of the body. Drugs such as aflibercept, bevacizumab, ranibizumab, and pegaptanib can inhibit VEGF and control or slow these diseases. Five members of VEGF family are known: VEGF-A, placenta growth factor (PGF), VEGF-B, VEGF-C and VEGF-D and the cognate VEGF receptors are VEGFR-1 (Flt- 1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) [50,51].

VEGF-A is involved in angiogenesis and lymphangiogenesis via a VEGFR-3-dependent and VEGFR-3-independent manner [52]. The temporal and spatial association of angio- and lymphangiogenesis indicates interdependencies between blood and lymphatic vessels. VEGF-A binds and activates two tyrosine kinase receptors: VEGFR-1 and VEGFR-2 while it does not show any binding affinity to VEGFR-3 [46].

VEGF-C and VEGF-D are the main lymphangiogenic factors in both physiological and pathological conditions [53]. VEGF-C is a potent lymphangiogenic factor by direct effect on the lymphatic endothelium as well as by macrophage recruitment [54,55]. VEGFR-3 is the receptor for both VEGF-C and VEGF-D and the mature form of VEGF-C and human VEGF-D is known to bind and activate VEGFR-2 which selectively suppresses the physiological growth of lymphatic vessels (the first specific lymphangiogenesis inhibitor) [56–58]. Constitutively expressed VEGFR-2 in angiogenic tips acts as a scavenger receptor for VEGF-C, thereby

spatially inhibiting lymphangiogenesis and thus balancing angiogenesis and lymphangiogenesis [59]. Importantly, delayed lymphangiogenesis gives immune cells originating from angio- genic vessels additional time at the inflammatory sites before being drained through lymphangiogenic vessels [60].

Within the healthy human sclera, a broad spectrum of anti- angiogenic and anti-lymphangiogenic factors are expressed [61]. Together with a reduced expression of pro-angiogenic factors this milieu maintains an anti-angiogenic and a pro- lymphangiogenic niche. The expression of a panel of 96 known pro- and antiangiogenic factors has been quantified comparing mRNA quantities in 12 scleral or conjunctival control samples from healthy human donors. Several antiangiogenic factors were demonstrated to be higher expressed in the human sclera com- pared to the conjunctiva, such as TIMP2 or FBLN5. Further, several proangiogenic factors were down-regulated in the sclera com- pared to the conjunctiva, e.g., FLT4, HGF or PROX1. Functionally, scleral homogenate was co-cultured with blood- and lymphatic endothelial cells (BECs and LECs) in vitro and immunohistochemistry performed on scleral fibroblasts and BECs [40]. Three anti-angiogenic factors were up-regulated in human sclera compared to conjunctiva, including FBLN5 (fibulin 5), SER- PINF1 (serpin peptidase inhibitor, clade F, member 1 = pigment epithelium derived factor) and TIMP2 (Tissue inhibitor of metalloproteinase 2; [40]). Immunohistochemistry of three major anti-angiogenic factors confirmed TSP1, TSP2, TIMP3 and PEDF expression both in scleral fibroblasts & in blood endothelial cells, whereas TIMP2 was not detectable [40]. This confirms that scleral fibroblasts maintain the vascular privilege of the sclera and con- tribute to limitation of blood vessels in the episcleral [40]. Prior research has shown that PEDF expression can also be detected in healthy human cardiac fibroblasts, in human retina and in photodynamic therapy (PDT)-treated choroid endothelial cells [62,63]. Also, the anti-angiogenic factor thrombospondin-1 (TSP-1) is an endogenous anti-lymphangiogenic factor by suppressing macrophage-derived VEGF-C and VEGF-D via ligation of CD36 on the cell surface [64].

Finally, six pro-angiogenic factors were found to be down-regulated in sclera, including FLT4 (Fms-related tyrosine kinase 4 = VEGF-R3), HGF (hepatocyte growth factor), KIT (CD117/c-kit), PROX1 (Prospero homeobox 1), SEMA3F (semaphorin-3F) and TGFA (transforming growth factor alpha; [40]).

3.7. BREAKDOWN OF THE SCLERAL VASCULAR PRIVILEGE UNDER PATHOLOGICAL CONDITIONS

Under pathological conditions (Fig. 3), the vascular privilege of the sclera can be impaired. Ciliary body melanoma with extraocular extension compromises approximately 4% of uveal melanomas and are associated with poor survival prognosis [49]. Metastasis occurs by haematogenous spread or by lymphogenous spread via proliferating lymphatic vessels [49,65]: (1) Conjunctiva lymphatics proliferate and invade the melanoma by formation of newly dividing nuclei in lymphatic endothelial cells through outgrowth of lymphatic capillaries [66,67]. (2) Melanoma lymphatics proliferate and invade the lymphatic system by expansion and invasion of per-tumor lymphatic by cancer cells [66,68,69]. (3) Combination of mechanisms (1) and (2) [49]. Intraocular lym- phatic vessels were found in 60% of melanomas with extraocular extension. Overall, the intraocular lymphatic vessels had a reticular architecture with numerous tiny lumina that differed from the larger and more dilated architecture of lymphatic vessels found in the periphery of the extraocular tumor component [68].

Sympathetic ophthalmia is a bilateral diffuse granulomatous T-cell mediated uveitis that occurs as early as five days after penetrating ocular injury [70]. Lymphatic vessel growth is multifactorial influenced by the expression of various cytokines, driving local inflammation after trauma and various external factors, e.g., the interval between trauma and treatment, anatomic site of rupture and mechanism of trauma. Overall, persisting lymphatic vessels act as an afferent pathway for immunologic response [71]. Even years after open globe injury lymphatic vessels persist and localize within the eye wall or inside the eye in retro corneal membranes, underneath the sclera and adjacent to uveal tissue ciliary body and iris [72–74].

Podoplanin is a widely accepted lymphatic marker and expressed in lymphatic endothelium and on almost all cells of the trabecular meshwork, endothelial cells of Schlemm’s canal and cells of anterior ciliary muscle tips despite the lack of lymphatic vessels [68]. Intraocular LYVE-1 and podoplanin-positive lymphatic vessels were only observed at the tumor periphery directly adjacent to the sclera. The central aspects of the tumor showed no LYVE-1 or podoplanin-positive lymphatic vessels [68]. Further, the intraocular and extraocular ratios of proliferating lymphatic ratios were shown to be associated with age, sex, tumor size, and distance to optic disc, tumor pigmentation, tumor cell type, mitotic rate, micro vascular patterns, and TNM classification. (i.e., large tumor size, epithelioid tumor cell type, high mitotic rate, and closed connective tissue loops; [68])

The concept of attracting conjunctival lymphatic vessels (Fig. 4) into the eye (secondary intraocular lymphangiogenesis), also referred to as “lymphangiotaxis”, may be the result of increased levels of pro-lymphangiogenic growth factors such as VEGF-C and decreased levels of anti-lymphangiogenic inhibitors owing to the scleral weakness [65]. Based on these facts, anti-hemangiogenic therapies such as ranibizumab, an antibody fragment that binds and inhibits all identified VEGF isoforms, and bevacizumab were developed. Bevacizumab is thought to remain in the eye longer than ranibizumab and therefore possibly allows for less frequent injections. Further, more specific anti-lymphangiogenic therapies, such as anti-VEGF receptor 3 anti- bodies or integrin α5 blocking peptides in uveal melanomas with extraocular extension (Fig. 5) to prevent lymphatic spread of the tumor to regional lymph nodes are being employed [75–77]. If an additional lymphvasculogenesis, as detected in other systems [78], is also present in scleral needs further proof.

The scleral vascular privilege can also be comprised through open globe injuries. After open globe injury two histopathological findings are typically observed: (1) Podoplanin+/laminin + lymphatic vessels are detected within the sclera and the intraocular space of globes enucleated after trauma indicating secondary lymphangiogenesis has occurred. If no signs of post traumatic diffuse or cystic epithelial ingrowth are evident, it can be excluded that lym- phatic vessels originate from displaced conjunctiva [71]. (2) The secondary ingrowth of intraocular lymphatic vessels with a significant number of associated LYVE-1+ macrophages (de novo formation of lymphatic vessels), supporting wound healing, defense against invading microorganisms and autoimmune reactions against intraocular antigens [71].

Under pathologic conditions, invading lymphatic vessels serve as the “afferent arm of the immune reflex arc” by facilitating pas- sage of antigen-presenting cells with foreign antigens to regional lymph nodes (Fig. 6). Blood vessels represent the “efferent arm of the immune reflex arc” by allowing invasion of immunologic effector cells and thus, enhancing an immune rejection [72]. The density of lymphatic vessels was observed to be diminished in the deeper parts of the sclera. This kind of active “lymphangiotaxis” may be the result of increased levels of pro-lymphangiogenic factors, such as interleukin-7, VEGF-C or - D, and decreased levels of anti-lymphangiogenic inhibitors as a consequence of the corneal or scleral weakness ultimately driving lymphangiogenesis [71,79].

Vascular adhesion protein-1 (VAP-1) is an endothelial trans-membrane glycoprotein that regulates leukocyte transmigration and adhesion at the inflamed environment. Its soluble form released into the circulation from vascular endothelial cells has been utilized as a prognostic biomarker in human pathologies. Thus, VAP-1 could be a therapeutic target for lymphangiogenesis related ocular diseases [80–82].

On a cellular level, during pathological conditions macrophages contribute to lymphangiogenesis. CD11b+ macrophages infiltrate and trans-differentiate into lymphatic endothelium and provide lymphangiogenic factors by activating NF-nB signaling pathways, resulting in secretion of VEGF-A, -C, and -D. Macrophage polarization is a physiological process by which existing macrophages acquire different functional programs in response to input from their microenvironment. They are powerful effector cells of the innate immune system, and they have a role e.g. in removal of cellular debris, embryonic development, and tissue repair. By simplified classification, the macrophage phenotype can be divided into 2 groups: M1 (classically activated macrophages) and M2 (alternatively activated macrophages). M1 macrophages were described as the pro-inflammatory type, important in direct host-defense against pathogens, such as phagocytosis and secretion of pro-inflammatory cytokines and microbicidal molecules. M2 macrophages were described to have quite the opposite function: regulation of the resolution phase of inflammation and the repair of damaged tissues [83]. If other putative cell populations, such as e.g., pericytes, telocytes, or other quiescent cells of any kind, also are involved in these scleral processes remains to be established [84–86].

3.8. SUMMARY

The sclera is the rigid outer coat of the eye which is made mainly of collagen type I and smaller amounts of collagen types III, IV, V, VI, VII and elastin. It consists of three anatomical layers the superficial vascularized episclera which contains a tight network of blood vessels, and the scleral stroma is the main part which is mainly avascular and the lamina fusca is a thin pigmented layer which forming the sclera-choroid border. The sclera is not an acellular tis- sue but it is rich in perivascular extra-luminal macrophages, their highest density being present in the episclera where they maintain blood vessel homeostasis. It expresses a broad spectrum of anti hemangiogenic and lymphangiogenic factors, while the expression of pro-angiogenic factors is generally reduced. The balance of both factors maintains the scleral anti (lymph-) angiogenic status, ensuring that the adult scleral layers are devoid of lymphatic vessels and creating a lymphatic-free border to the inner eye, ultimately contributing to the ocular immune privilege.

The balance of pro-and antiangiogenic factors is involved in maintaining the scleral vascular status and potentially the vascular privilege of the inner eye. Under pathologic conditions like choroidal melanoma, the vascular privilege of the sclera is impaired. Metastasis occurs by haematogenous spread or by lymphogenous spread via proliferating lymphatic vessels. After open globe injury, the scleral vascular privilege is comprised and secondary ingrowth of intraocular lymphatic vessels with a significant number of associated LYVE-1+ macrophages, supporting wound healing, defense against invading microorganisms and autoimmune reactions against intraocular antigens.

Taken together, future work should focus on pro- and anti- (lymph-) angiogenic factors, further delineating their functions and their therapeutic effects in different ocular diseases, paving the way for the development of novel therapeutic strategies for incurable ocular diseases.

3.9. ACKNOWLEDGEMENTS

This research was supported by the German Research Foundation FOR 2240 “Lymph Angiogenesis and Cellular Immunity in Inflammatory Diseases of the Eye” to LMH; HE 6743/2-1, HE 6743/3-1, and HE 6743/3-2, GEROK-Programme University Hospital of Cologne to Y.G., and PMU-FFF R-15/02/067-KAS and ÖNB 17617 to AKE and FS

3.10. FIGURES

Figure Legends

Fig. 1:

The anatomy and structures of the eye (modified with permission from “Sobotta, Atlas der Anatomie des Menschen”, 2017, [87]).

Fig. 2:

The vascular supply of the eye is provided by two sources: the inner layers of the retina (facing the vitreous) receive blood from the central retinal artery. The posterior uvea is supplied by the long and short posterior ciliary arteries, and with its choroidal innermost layer, the choriocapillaris, supplying exclusively the retinal photoreceptors. These vessels represent together with the vorticose veins, also the vascular supply of the sclera (modified with permission from “Sobotta, Atlas der Anatomie des Menschen”, 2017, [87]).

Fig. 3:

Schematic overview of the processes involved in tumor lymphangiogenesis via secreted lymphangiogenic growth factors and lymphatic metastasis (Langheinrich et al., 2012, with permission, [88]).

Fig. 4:

The multiple functions of VEGF-C in tumor progression where VEGF-C binds to the receptors expressed on natural killer cells (NK-cell), tumor cells and endothelial cells (from Wang and Tsai, 2015, with permission, [89]).

Fig. 5:

Ciliary body and choroid melanoma: uveal melanomas may affect all parts of the uvea, i.e., the iris, ciliary body, and choroid. Tumors arise from the melanocytes that reside within the uvea, and large tumors often encompass multiple parts of the uvea and are distinct in their etiology and prognosis (graphic used with permission of Mayo Foundation for Medical Education and Research, all rights reserved).

Fig. 6:

The pathway of chemokines mediating metastasis via the lymphatic system (from (Christiansen and Detmar, 2011, with permission, [90]).

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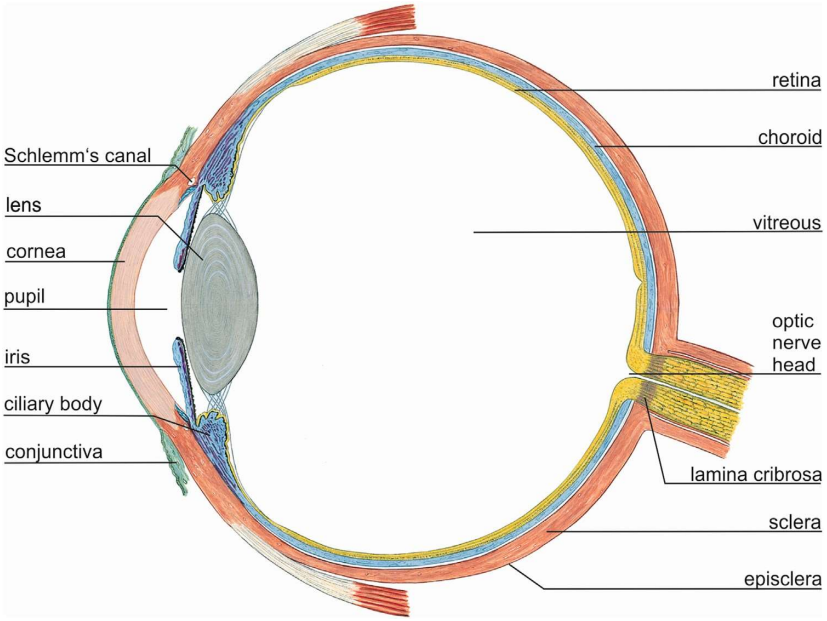

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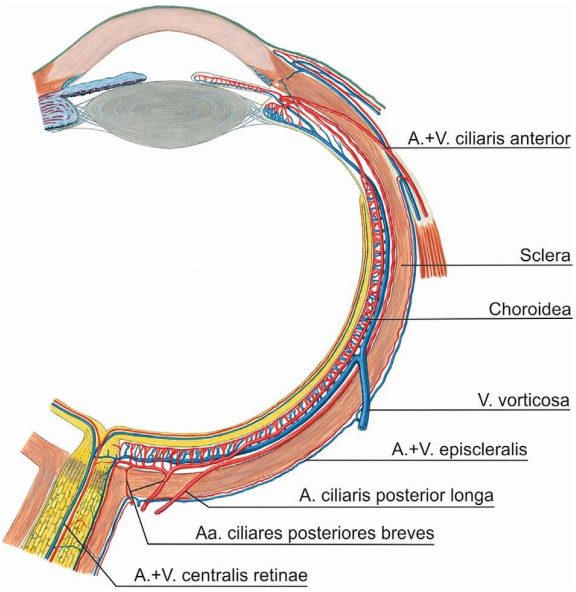

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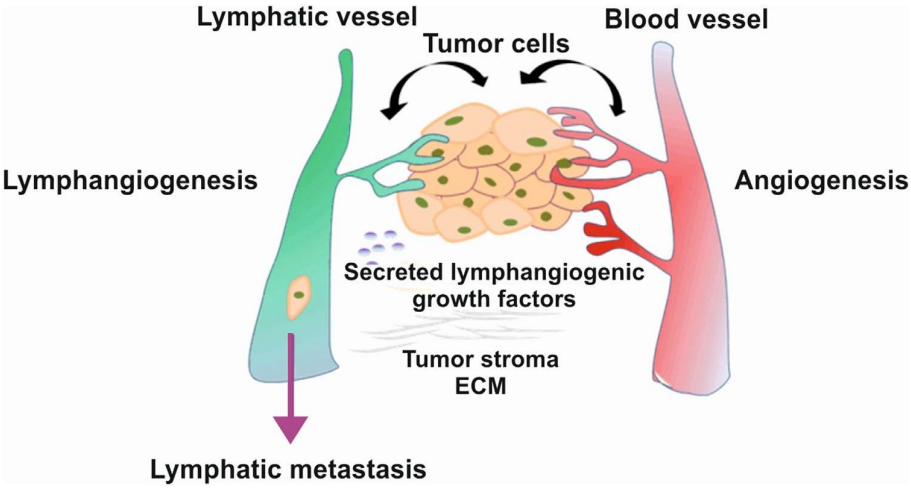

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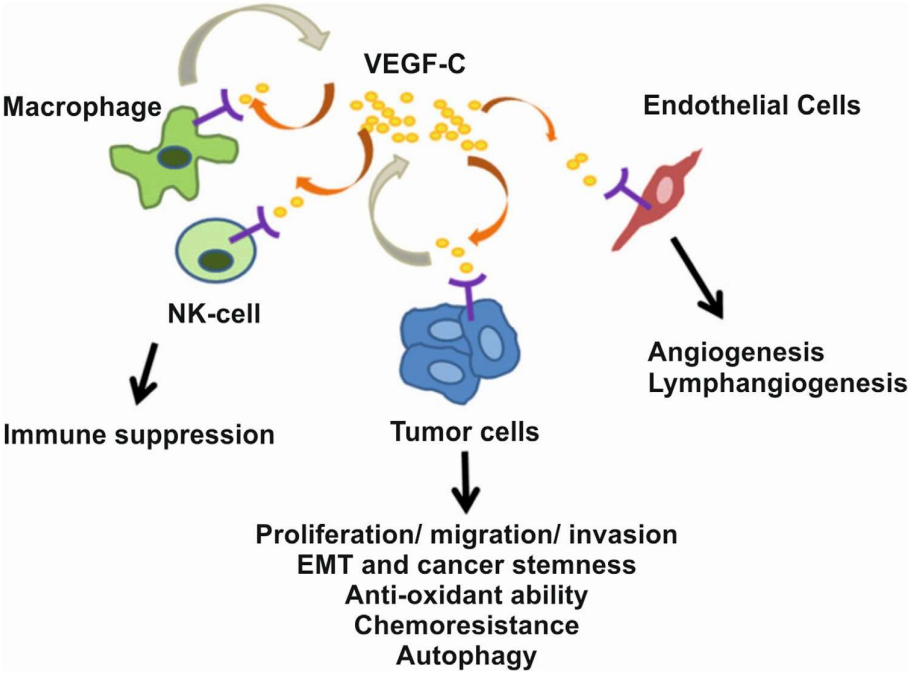

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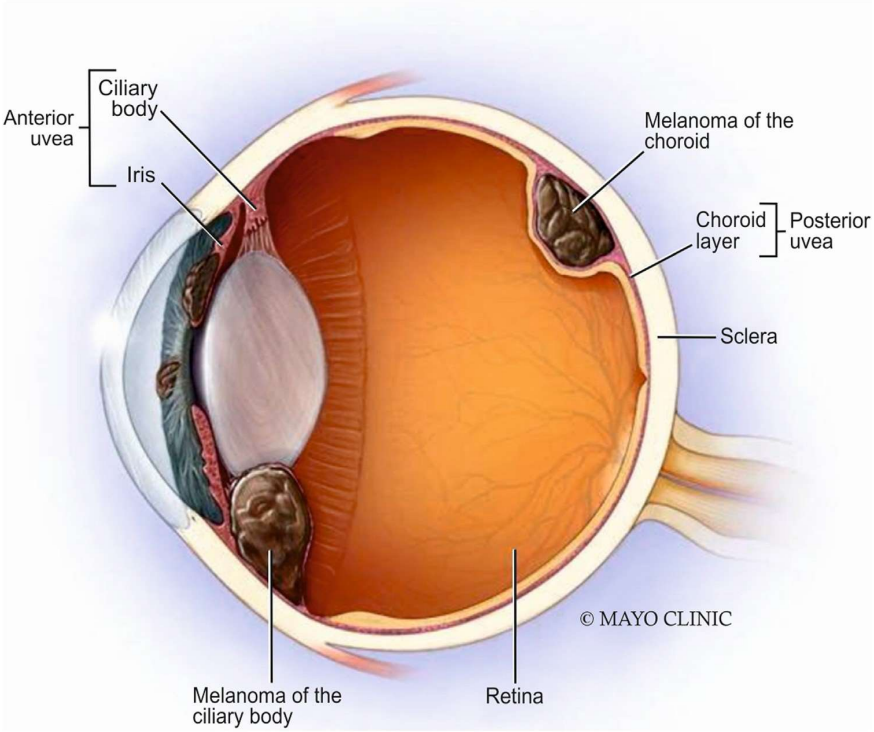

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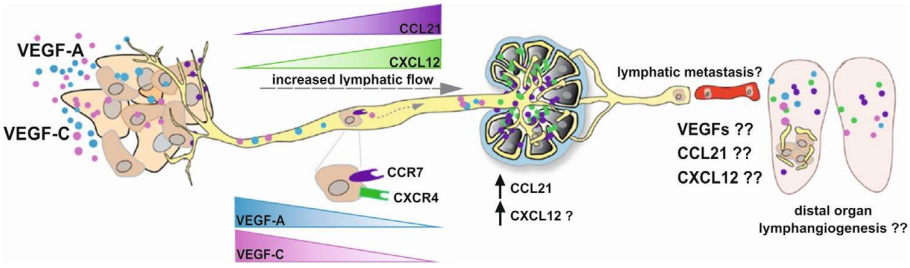

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4. SCLERAXIS EXPRESSING SCLERAL CELLS RESPOND TO INFLAMMATORY STIMULATION

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Financial Disclosure
The authors have no financial interest to disclose.

Funding
Open access funding provided by Paracelsus Medical University. German Research Foundation (FOR2240; HE 6743/3-1, HE 6743/3-2, and HE 6743/5-1 to LMH), Dr. Gabriele Lederle Foundation, Taufkirchen (to LMH), Brigitte and Dr. Konstanze Wegener foundation (to LMH), Marie-Louise Geissler foundation (to LMH).

Short Running Head

Scleraxis expressing scleral cells

Pre-Release:

Atta G, Schroedl F, Kaser-Eichberger A, Spitzer G, Traweger A, Heindl LM, Tempfer H., Scleraxis expressing scleral cells respond to inflammatory stimulation. Histochem Cell Biol. 2021 Aug;156(2):123-132. doi: 10.1007/s00418-021-01985-y. Epub 2021 May 8. PMID: 33966129

In this publication methods and results of my work are described and explained.

4.1. ABSTRACT

The sclera is an ocular tissue rich of collagenous extracellular matrix, which is built up and maintained by relatively few, still poorly characterized fibroblast-like cells. The aims of this study are to add to the characterization of scleral fibroblasts and to examine the reaction of these fibroblasts to inflammatory stimulation in an ex vivo organotypic model. Scleras of Scleraxis-GFP (SCX-GFP) mice were analyzed using immunohistochemistry and qRT-PCR for the expression of the tendon cell associated marker genes Scleraxis (SCX), mohawk and tenomodulin. In organotypic tissue culture, explanted Scleras ofadult Scleraxis GFP reporter mice were exposed to 10 ng/ml recombinant interleukin 1-ß (IL1-ß) and IL1-ß in combination with dexamethasone. The tissue was then analyzed by immunofluorescence staining of the inflammation- and fibrosis- associated proteins IL6, COX-2, iNOS, connective tissue growth factor, MMP2, MMP3, and MMP13 as well as for collagenfibre degradation using a Collagen Hybridizing Peptide (CHP) binding assay. The mouse sclera displayed a strong expression of Scleraxis promoter-driven GFP, indicating a tendon cell-like phenotype, as well as expression of Scleraxis, tenomodulin and mohawk mRNA. Upon IL1-ß stimulation, SCX-GFP+ cells significantly upregulated the expression of all proteins ana- lysed. Moreover, IL1-ß stimulation resulted in significant collagen degradation. Adding the corticosteroid dexamethasone significantly reduced the response to IL1-ß stimulation. Collagen degradation was significantly enhanced in the IL1-ß group. Dexamethasone demonstrated a significant rescue effect. This work provides insights into the characteristics of scleral cells and establishes an ex vivo model of scleral inflammation.

Keywords: Sclera, Tendon, Scleraxis, Scleritis model

4.2. INTRODUCTION

The sclera forms around 85% of the rigid outer tunic coat of the human eyeball, which consists of three anatomical layers: (1) the superficial vascularized episclera which contains a tight network of blood vessels, (2) the scleral stroma resembling the main part, which is mainly avascular, and (3) the lamina fusca, a thin pigmented layer which is located above the uvea, an also strongly pigmented layer below the sclera. The sclera is a remarkably resistant, stable connective tissue that performs various functions critical to vision: its primary role includes providing a firm and stable substrate for the retina to protect retinal and other internal structures of the eye from damage due to their mechanical vulnerability [1]. Physiologically, the adult sclera shows only a superficial network of blood vessels but lacks lym- phatic vessels, thereby creating a lymphatic-free border to the inner eye [2], ultimately contributing to the ocular immune privilege. This vascular privilege is actively regulated by balancing anti- and proangiogenic factors expressed by cells within the sclera [3]. Despite sclera being considered a quiescent tissue in healthy state, pathologic conditions such as injuries or tumours trigger tissue responses leading to matrix disruption and cellular activation [4]. Consequently, the scleral vascular privilege is compromised and secondary ingrowth of intraocular lymphatic vessels with a significant number of associated LYVE-1 + macrophages take place, invading the sclera and the inner eye [5,6]. This mechanism supports wound healing, defense against invading microorganisms and autoimmune reactions against intraocular antigens [5,6].

Regarding the scleral cellular phenotype(s), there is still a lack of characterization of scleral resident cells. Except for the innermost layer of the sclera connected to the choroid, the lamina fusca, most regions of the sclera are sparsely populated by cells. The vast majority of resident cells of the scleral stroma are defined as “fibrocytes”, transforming to an active fibroblast upon insult. While fibroblasts are responsible for synthesis of all scleral ECM components, such as collagen, proteoglycans, and elastic fibres, they also respond to mechanical stimuli from their surrounding ECM, potentially inducing matrix remodeling and undergoing proliferation. Under mechanical stimulation, the expression of thrombos-pondin-1, HINT1, vimentin, actinin, and α-smooth muscle actin was shown to be increased in scleral fibroblasts [1,7]. In an RNA seq approach, cultured scleral fibroblast cells derived from healthy human eyes were demonstrated to express fibronectin, collagen I, III, and VI and further also MMP2 under non- pathologic culture conditions [8].

Under inflammatory conditions, the scleral matrix is severely affected, resulting in structural changes and tissue disintegration with necrosis of the outer tunic, as it is the case in scleritis [9]. While this dis- ease is associated with severe pain and possibly permanent loss of vision, the mechanisms of this disease remain unclear [10]. In most of the cases, autoimmune

mechanisms are likely the underlying cause, while post-traumatic and iatrogenic cases have also been reported. However, generally other aetiologias remain enigmatic and the underlying mechanisms are poorly understood. The few existing animal models are not quite satisfying [10,11], as they partly use non-physiologic types of scleritis induction such as ovalbumin application. Most of the human data derive from Histo-pathological investigations.

Interestingly, tendon tissue shares various similarities with the sclera; they are mainly composed of collagen- rich, extracellular matrix, built up and maintained by relatively few, spindle shaped fibroblasts. They are sparsely vascularized in a healthy state, whereas a hallmark of tendon disease includes hypervascularity [12]. Similar to scleritis, degenerative tendon pathologies are often associated with systemic inflammatory diseases such as psoriatic arthritis [13]. Similar to the sclera, tendon cells are still rather poorly characterized, and progress is in part hampered by the lack of truly specific marker proteins. Generally, tenomodulin (Tnmd), thrombospondin 4 (TSP-4), tenascin C (TNC) and collagens type 1 and 3 are commonly used for identification and characterization of tendon fibroblasts. Tenascin C, tenomodulin and thrombospondin 4 were also found to be expressed in various tissues, the latter two also in the sclera [14–16].

The most accepted marker for tendon cells, however, is the basic helix loop helix transcription fac- tor Scleraxis. The loss of the Scleraxis gene leads to disrupted tendon development with matrix disorganization and motion deficits [17]. Despite not being a master regulator of tendon development, fate map- ping and Scleraxis-GFP reporter mouse models underline the importance of Scleraxis as a marker for tendon cells [18]. In the eye, expression of Scleraxis has been shown in the tendons attaching to the ocular muscles [19]; however, examination of its expression in the sclera is still lacking. As tendon and sclera share a large variety of properties, such as collagen rich matrix, low cell density, sparse vascularization, and high mechanical stress under physiological conditions, it is the aim of the present study to further characterize scleral cells regarding their expression of tendon associated markers in adult and embryonic mouse eyes and to examine the response of these cells to an inflammatory environment in an organotypic approach.

4.3. MATERIALS AND METHODS

4.3.1. Animals

All procedures involving animals were carried out in an approved animal facility by authorized staff and were in accordance with all relevant Austrian laws. As only tis- sue from euthanized animals was used, no further ethics approval was required. Scleraxis-GFP (Scx_GFP) reporter mice [18] were kindly shared by Prof. Denitsa Docheva (University of Regensburg, Germany). All animals were acclimatized to standard laboratory conditions (12-h light, 12-h dark cycle) and given free access to rodent chow and water. Tissue was harvested from 12-week-old mice. For generation of E17 mouse embryos, embryonic day 0.5 (E0.5) was specified as the day when the experimenter confirmed the presence of a vaginal plug. Embryos were obtained from the pregnant mice, which were sacrificed by cervical dislocation. For qRT-PCR analyses, 12-week-old male C57Bl/6 mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France).

4.3.2. Organotypic sclera inflammation model

For the experiment, three female Tg (Scx-GFP) 1 Stzr mice (3 months of age) were sacrificed by cervical dislocation. Subsequently, the eyes were dissected, and all non-scleral tissue was removed under sterile conditions. Sclera has been divided into three segments with the optic nerve in the centre and three cuts reaching from the centre to 12, 4 and 8 o'clock direction. Obtained segments/triangles were further subdivided in tangential cuts from centre to midline periphery and sagittal cross sections (i.e., at 2, 6 and 10 o'clock) obtained and immediately transferred to 6 well plates containing 10% fetal bovine serum in minimum essential medium. One group was further supplemented for 3 days with 10 ng/ml IL1-ß (PeproTech, Vienna, Austria), or 10 ng/ml IL1-ß and 100 nM dexamethasone (Sigma-Aldrich, Vienna, Austria), respectively. These concentrations were chosen according to several published protocols on tendon cells [20,21]. The tissue pieces were incubated for 3 days at 37 °C and at 5% CO_2, with daily media change. The tissues were then fixed in 4% paraformaldehyde overnight, washed three times in PBS and processed for further cryosectioning (Supplementary Fig. 1).

4.3.3. Preparation of tissue sections

Mouse eyes were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 12 h at 4 °C. Following several rinses in PBS and cryo-preservation in PBS containing 30% sucrose, tissues were embedded in cryomedium (Surgipath Cryogel®, Leica Microsystems, Vienna, Austria) and 15 µm cryosections were prepared (CM1950, Leica, Vienna, Austria).

4.3.4. Detection of denatured collagen

To detect damaged or denatured collagen, collagen hybridizing peptide (CHP, 3helix Inc, Salt Lake City, Utah, USA) was applied onto cryo-sections of organotypic cultured mouse sclera, according to the manufacturers' protocol. This peptide specifically binds to unfolded collagen chains, thus detecting degraded collagen only. Briefly, the sections were incubated with 5 µM CY3- labelled CHP and 1 µg/ml 4′,6-diamidino-2-phenylindol dihydrochloride (DAPI) at 4 °C in a humified chamber for 18 h. Confocal images were acquired on a confocal laser scanning microscope (LSM710, Zeiss, Vienna, Austria) using a 20× objective. For semi-quantitative analysis of expression intensity, the images were analyzed by ImageJ software, calculating % area with CHP positive signal (Fig. 2).

4.3.5. qRT-PCR analysis

Total RNA was isolated from sclera, hind limb digital flexor tendons and optic nerve from 12 months old male C57Bl/6 mice (*n* = 4 animals, tissues pooled from left and right side) using TRI® Reagent (Sigma-Aldrich) according to the manufacturer's protocol. RNA yield was quantified by Nanodrop 2000C (Thermo Fisher Scientific, Vienna, Austria), RNA integrity was verified using an Experion Automated Electrophoresis system (Bio-Rad, Munich, Germany). A mini- mum requirement of RNA quality indicator (RQI) > 7.5 was chosen. qRT-PCR was performed as described by Gehwolf et al. (2019) using TaqMan® assays from Integrated DNA Technologies (Coralville, IA, USA). Amplification conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were run in duplicate. CQ values were analyzed using qBasePlus v. 2.4 (Biogazelle NV, Zwijnaarde, Belgium) and normalized relative quantities were calculated by normalizing the data to the expression of previously validated endogenous control genes as described by Vandesompele et al. [22]. As housekeeping genes, TATA-Box Binding Protein (TBP) and hypoxanthine phosphoribosyl transferase 1 (HPRT1) were used. The normalized quantities were then determined for the candidate genes scaled against the expression values determined for the controls to generate fold changes in expression.

4.3.6. Immunofluorescence

Immunofluorescence detection of inflammation- and fibro- sis-associated markers was performed on cryosections of mouse sclera. After a 5 min rinse in PBS, slides were incubated for 1 h at room temperature (RT) in PBS containing 1% bovine serum albumin (Sigma-Aldrich, Vienna, Austria). Slides were subsequently incubated for double or triple immunohistochemistry (overnight at 4 °C) with antibodies directed against CD68 (NB100-683, Novus Biological, Colorado, USA, 1:50), COX2 (12282, Cell Signaling, Massachusetts,

USA,1:200), IL6 (ab6672, Abcam, Cam- bridge, UK, 1:100), connective tissue growth factor (CTGF) (ab6992, Abcam, Cambridge, UK, 1:200), Matrix Metalloproteinase 2 (MMP2) (66366-1-Ig, Proteintech, Manchester, UK, 1:200), Matrix Metalloproteinase 3 (MMP3) (66338-1-Ig, Proteintech, Manchester, UK, 1:200), Matrix Metallopeptidase 13 (MMP13) (18165-1-AP, Proteintech, Manchester, UK, 1:200), Cleaved Caspase 3 (Asp175, #9661, Cell Signaling, Massachusetts, USA, 1:100). For all antibodies used, the manufacturers provided proof of validation in the antibody data sheets.

After a rinse in PBS (three times 5 min) binding sites of primary antibodies were visualized by corresponding Alexa 568- or Alexa 647-tagged antisera (1:500; Invitro- gen, Karlsruhe, Germany) in PBS, containing 1% BSA (1 h at RT) followed by another rinse in PBS (three times 5 min). The GFP signal of the transgenic animals was enhanced using a goat anti-GFP antibody (GFP, #600- 101-215S, Rockland, Limerick, USA; 1:500). The slides were counterstained for nuclei using DAPI. For that, slides were incubated for 10 min (1:4000, stock 1 mg/ml, VWR, Vienna, Austria) followed by a brief wash in PBS (three times 5 min). All slides were embedded in Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich, Vienna, Austria). Negative controls for background correction prior to semi-quantitative evaluation were performed by omission of the primary antibodies during incubation and resulted in absence of immunoreactivity. For semi-quantitative analysis, six sections of each animal were analyzed, the mean values were used for statistical analysis (n = 3).

4.3.7. Confocal imaging

Confocal images were acquired using a confocal laser scanning microscope (LSM710, Zeiss) equipped with 405, 488, 555, and 639 nm diode lasers, a 10 × EC Plan-Neofluar (10 × /0.3) or a 20 × Plan-Apochromat (20×/0.8) objective (Zeiss, Munich, Germany). Image acquisition was per- formed with the ZEN 2010 software (Zeiss), with image dimensions of 1024 × 1024 pixels and image depth of 16 bit. During image acquisition, two times averaging was applied, and laser power and gain were adjusted to avoid saturation of single pixels. All images were acquired using identical microscope settings based on the control labelling of the secondary antibodies.

4.3.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism v.5.04 (La Jolla, CA, USA). Numerical data are presented as means ± standard deviation. For analysis of qPCR data, Mann–Whitney tests were performed, for semi-quantitative analysis of immunofluorescence stains Kruskal–Wallis tests for multiple comparisons were carried out. Statistical significance was set at $\alpha = 0.05$.

4.4. RESULTS

Analysis of Scx-GFP reporter mouse eyes revealed a Scleraxis immunopositively cell population in the sclera in both E17 embryos and adult animals (Fig. 1). The cells appeared spindle shaped with scarce cytoplasm, and cell diameters in the longitudinal axis of about 45 µm, and transverse axis of 15 µm, and with ovoid nucleus located in the center of the cell (Fig. 1d, inset). These GFP-positive cells located in all areas of the sclera with no obvious accumulation in a specific area. Double labelling with the macrophage marker CD68 revealed the presence of CD68+ cells in the sclera; however, these cells displayed no overlap with the Scx-GFP positive cell population (Fig. 1d). In addition, qRT-PCR analysis revealed expression of Scleraxis (Fig. 1e), tenomodulin (Fig. 1f) and mohawk-mRNA (Fig. 1g) in tendon tissue as well as in the sclera. Also, in the optic nerve these markers are detectable, however to a significantly lesser extent.

In the organotypic tissue culture protocol, the addition of IL1-ß elicited a significant damage to scleral collagen, as seen by analysis of CHP binding. Quantification of the CHP positive area revealed an increase from 0.38% ± 0.25 in the control group compared to 8.89% ± 5.70 in the inflammatory-primed group (Fig. 2 a-f, j). To inhibit this effect, co-incubation experiments with dexamethasone were per- formed. As expected, CHP-positive areas were reduced to control levels (0.38% ± 0.14; Fig. 2g-i, j).

To verify the inflammatory response of Scx-GFP expressing scleral cells upon stimulation with IL1-ß, the expression of COX2 and IL6 was investigated. These experiments revealed an about 30-fold increase in expression in the experimental group (13.33% ± 11.01 vs. 0.31% ± 0.24 in the control samples), and again this effect was inhibited by the addition of dexamethasone (1.12% ± 0.24) (Fig. 3a–d) and IL6 (Fig. 3e–g) upon IL1-ß stimulation.

In a further set of experiments, the reaction of the fibrosis- associated marker connective tissue growth factor (CTGF) was investigated upon IL1-ß stimulation. Here, an increase in regions positive for expression due to IL1-ß treatment from 3.65% ± 0.10 to 8.73% ± 2.89 was observed, while this increase was reduced with addition of dexamethasone to 2.29% ± 1.39% (Fig. 4a–d). A similar response pattern was observed when investigating the change in expression of various matrix metalloproteases: MMP2 (control 0.29% ± 0.23, IL1-ß 2.24% ± 0.84, IL1-ß + Dexamethasone 0.41 ± 0.28, Fig. 4e–h), MMP3 (control 0.96% ± 0.01,IL1-ß 5.51% ± 4.87, IL1-ß + Dexamethasone 0.27% ± 0.04, Fig. 4i–l) and with MMP13 (control 1.99% ± 1.33, IL1-ß 5.87% ± 3.38, IL1-ß + Dexamethasone 0.23% ± 0.11, Fig. 4m–p). Treatment of scleral samples with 10 ng/ml IL1-ß induced apoptosis to a significant extent (control 0.50% ± 0.71, IL1-ß 7.11% ± 4.48, IL1-ß + Dexamethasone 6.86 ± 1.71), with no rescue effect caused by addition of dexamethasone (Fig. 5).

4.5. DISCUSSION

We here describe a population of cells in the sclera expressing tendon-related markers responding to inflammatory stimulation. By size and shape, the Scleraxis-positive scleral cells rather resemble fibroblasts. Since they do not express CD68, they do not seem to belong to macrophage-like cells. The observed presence of CD68 + scleral macrophages (Fig. 1d) is in line with previous reports showing macrophages in human and mouse sclera [23,24]. The observed expression of tenomodulin and mohawk-mRNA further substantiates the presence of cells expressing tendon-related markers in scleral tissue.

Regarding the expression of tendon related markers in the sclera, our observation of Scleraxis-positive cells is in line with reports on tenomodulin expression in the sclera, a presumably antiangiogenic factor also expressed by tendon cells [15]. There are relatively few studies that have systematically studied the histopathology of scleritis, as a scleral biopsy is not commonly performed for diagnostic purposes due to the risks of worsening the inflammatory process or structurally weakening an already compromised organ. Most information on structural and histological alterations in eyes affected by scleritis stem from eyes removed surgically because they were blind and painful, or from eyes obtained at autopsy [10]. Therefore, particularly early events in this disease and immunohistochemical aspects are not well studied.

In tendon, we could show that cells respond to stimulation with IL1-ß by upregulation of inflammatory and fibrotic marker genes in vitro [25]. Similarly, the Scx-GFP positive scleral cells upregulate COX2 and IL6.The observed upregulation of CTFG indicates a fibro- sis-like response to stimulation with IL1-ß, as it has been described in fibrotic tendon [26]. In the sclera, CTGF was shown to be regulated in recovering experimental myopia, with a downregulation during a hyperopic refractive error causing GO signals, whereas it was found to be upregulated in the remodeling process during recovery [27].

Regarding the involvement of matrix metalloproteinases in scleritis, relatively little is known so far. Both MMP2 and MMP3 were shown to be expressed by resident scleral fibroblasts as well as inflammatory cells such as macrophages and T lymphocytes in necrotizing scleritis [28]. MMP2 was found to be expressed in samples of human melanoma-associated spongiform scleropathy, whereas MMP13 could not be detected in this study [29]. In tendon, MMPs are well described to be involved in matrix remodeling and degeneration in tendinopathy, causing collagen fibre disruption and tissue weakening [30].

The observation of apoptosis induction in scleral cells by IL1-ß is in line with our previous findings of tendon cells undergoing apoptosis following stimulation by 10 ng IL1-ß/ ml [20]. The fact that dexamethasone does not significantly rescue scleral cells from IL1-ß induced apoptosis in our hands may be due to ambivalent influence of dexamethasone on apoptosis. In proliferative chondrocytes, dexamethasone has been shown to induce apoptosis itself via activation of caspases [31]. Interestingly, several other cell types, such as neutrophils or keratinocytes have an anti-apoptotic response to glucocorticoids that is cytoprotective [32].
A limitation of this study is the indirect proof of Scleraxis expression in the sclera by a transgenic animal model. In our hands, no antibody was sufficiently specific to credibly label Scleraxis by immunohistochemistry. Therefore, also the intensity of the Scleraxis-GFP signal should not be over-interpreted.

However, with this work, we introduce a novel organo- typic in vitro model of scleritis, using a Scleraxis-GFP reporter mouse model, and provide a first characterization of these tendon marker expressing scleral cells in response to inflammatory stimulation.
Summarizing, with this study, we show that Scleraxis- positive scleral fibroblasts respond to inflammatory stimulation in a similar fashion as it has been demonstrated for tendon. Moreover, we establish a novel ex vivo model of scleritis, leading to hallmarks of this disease like damaged collagen matrix, upregulation of inflammatory factors as well as to the expression of matrix degrading enzymes.

4.6. ACKNOWLEDGMENTS

Open access funding provided by Paracelsus Medical University. German Research Foundation (FOR2240; HE 6743/3-1, HE 6743/3-2, and HE 6743/5-1 to LMH), Dr. Gabriele Lederle Foundation, Taufkirchen (to LMH), Brigitte and Dr. Konstanze Wegener foundation (to LMH), Marie-Louise Geissler foundation (to LMH).

4.7. DECLARATIONS

Conflict of interest: The authors have no financial/conflicting interests to disclose.

Ethics approval Not applicable.

Consent to participate Not applicable.

Informed Consent for publication: All authors give consent for publication of the manuscript in the present form.

4.8. FIGURES

Figure Legends

Fig. 1:

Scleral cells express tendon-associated markers. **a**, **b** Fluorescence microscopy revealed numerous Scleraxis-GFP (Scx-GFP, *green*) positive cells in the sclera of embryonic mice at E17. **b** represents a magnification of the boxed area in a, highlighting the Scx- GFP positive cells (*arrows*). *Blue* = DAPI. **c**, **d** A similar situation was found in adult mice: spindle shaped Scx-GFP positive cells (*green*) were present throughout the entire sclera. Immunohistochemistry with CD68 revealed absence of co-localization with the Scx- GFP signal (**c**), while CD68- immunoreactivity was detected in few cells within the sclera (**d**, *arrows*). *Blue* = DAPI Scale bars: **a** = 100 µm, **b**–**d** = 20 µm. qRT-PCR analysis shows the expression of mRNA of the tendon-associated markers *Scleraxis* (**e**), *tenomodulin* (Tnmd, **f**) and *mohawk* (Mkx, **g**), in both tendon and sclera. To a significantly lesser extent, expression is also detectable in the optic nerve.

Fig. 2:

Interleukin treated scleral tissue undergoes collagen degradation. **a**–**i** Immunohistochemistry of Scx-GFP (*green*) and collagen hybridizing peptide (CHP, *red*, indicating collagen degradation). Compared to controls (**a**–**c**), a clear increase in signal intensity was evident in tissue incubated with IL1-. (**d**–**f**) for both Scx-GFP and CHP that was abolished when treated with IL1-. and dexamethasone (**g**–**h**). **j** Quantitative analysis of the signal increase of CHP **j** revealed statistical significance for IL-. compared to IL1-. and dexamethasone and control. Scale bars: 50 µm.

Fig. 3:

Interleukin treatment enhances the expression of COX2 and IL6 in scleral tissue. The inflammation-associated marker proteins COX2 (**a**–**d**) and IL6 (**e**–**h**) were increased when Scx-GFP mouse scleral tissue was incubated with IL1-. compared to controls (**a**, **e**), and the effect is abrogated by addition of dexamethasone (**c**, **g**). Signal analysis revealed statistical significance for both COX2 (**d**) and IL6 (**h**) when incubated with IL-1. compared to control. Scale bars: 50 µm.

Fig. 4:

Interleukin treatment enhances the expression of fibrosis associated proteins in scleral tissue. The fibrosis-associated marker protein Connective Tissue Growth Factor (CTGF, **a–d**), and matrix metalloproteinases MMP2 (**e**–**h**), MMP3 (**i**–**l**), and MMP13 (**m**–**p**) were increased in Scx-GFP mouse scleral tissue when stimulated with IL1-. compared to controls, and the effects were abrogated by addition of dexamethasone (**c**, **g**, **k**, **o**). Signal analysis revealed statistical significance for all markers when incubated with IL-1. compared to controls. Insets in the IL1-. group images show the Scx-GFP channel and reveal co-expression of the respective proteins with Scx-GFP (insets in IL1-. group). Scale bars: 50 µm.

Fig. 5:

Interleukin treatment induces apoptosis in scleral cells. The apoptosis-associated marker protein Cleaved caspase 3 was increased in Scx- GFP mouse scleral tissue when stimulated with IL1-. compared to controls, dexamethasone did not abrogate this effect (**a**–**d**). Scale bar: 50 µm.

Fig. 1 Scleral cells express tendon-associated markers. **a**, **b** Fluorescence microscopy revealed numerous Scleraxis- GFP (Scx-GFP, *green*) positive cells in the sclera of embryonic mice at E17. **b** represents a magnification of the boxed area in a, highlighting the Scx-GFP positive cells (*arrows*). *Blue* = DAPI. **c**, **d** A similar situation was found in adult mice: spindle shaped Scx-GFP positive cells (*green*) were present throughout the entire sclera. Immunohistochemistry with CD68 revealed absence of co-localization with the Scx- GFP signal (**c**), while CD68- immunoreactivity was detected in few cells within the sclera (**d**, *arrows*). *Blue* = DAPI Scale bars: **a** = 100 µm, **b**–**d** = 20 µm. qRT-PCR analysis shows the expression of mRNA of the tendon-associated markers *Scleraxis* (**e**), *tenomodulin* (Tnmd, **f**) and *mohawk* (Mkx, **g**), in both tendon and sclera. To a significantly lesser extent, expression is also detectable in the optic nerve.

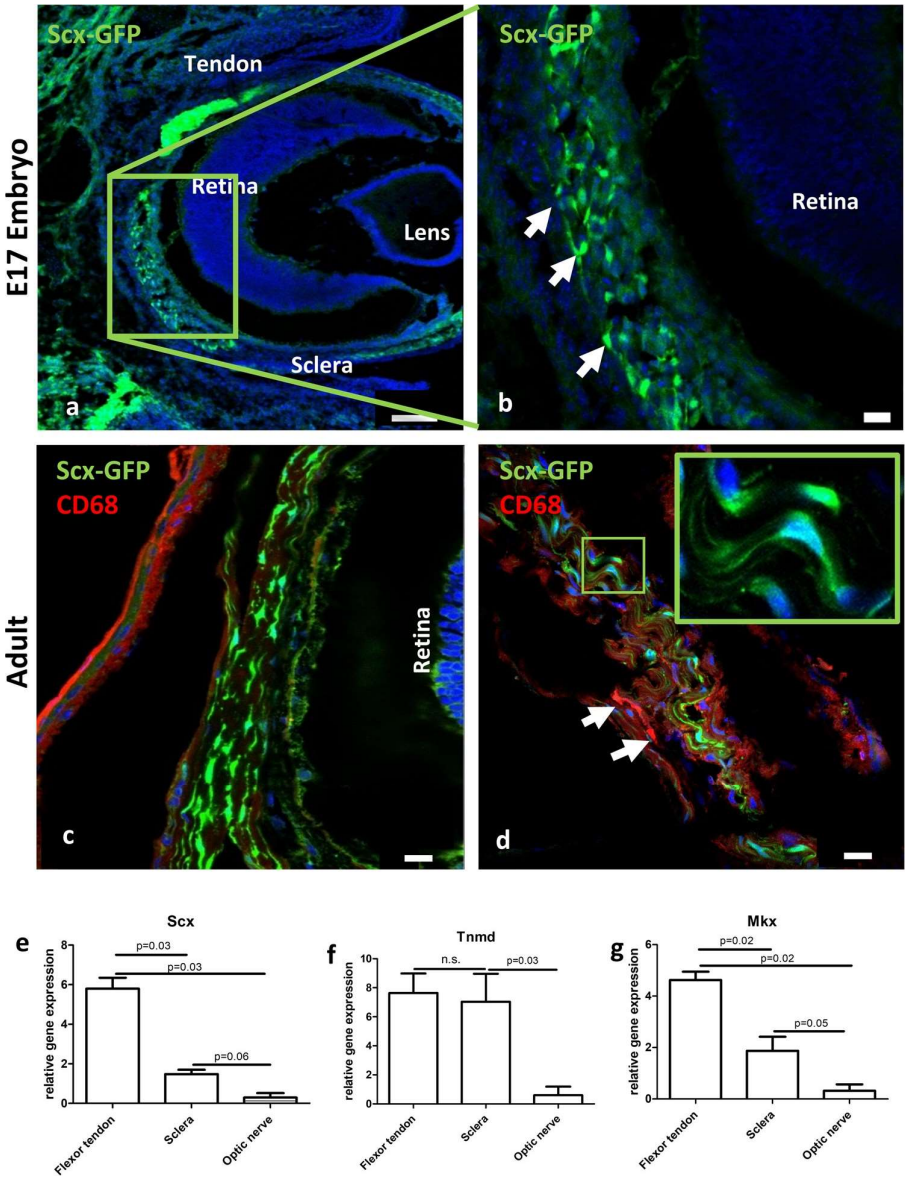


Fig. 2 Interleukin treated scleral tissue undergoes collagen degradation. **a**–**i** Immunohistochemistry of Scx-GFP (*green*) and collagen hybridizing pep- tide (CHP, *red*, indicating collagen degradation). Compared to controls (**a**–**c**), a clear increase in signal intensity was evident in tissue incubated with IL1-ß (**d**–**f**) for both Scx-GFP and CHP that was abolished when treated with IL1-ß and dexamethasone (**g**–**h**). **j** Quantitative analysis of the signal increase of CHP **j** revealed statistical significance for IL-ß compared to IL1-ß and dexamethasone and control. Scale bars: 50 µm

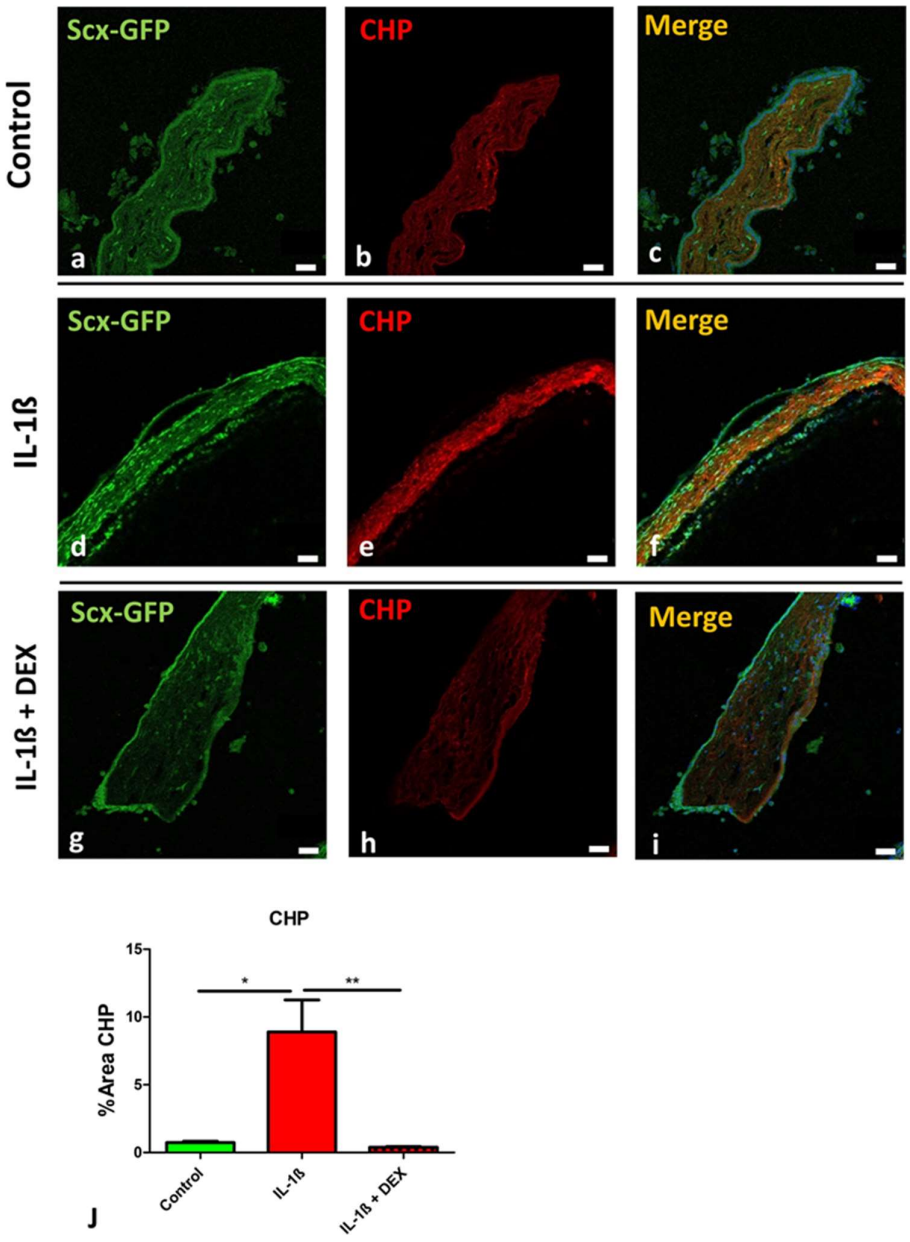


Fig. 3 Interleukin treatment enhances the expression of COX2 and IL6 in scleral tissue. The inflammation-associated marker proteins COX2 (a–d) and IL6 (e–h) were increased when Scx-GFP mouse scleral tissue was incubated with IL1-ß compared to controls (a, e), and the effect is abrogated by addition of dexamethasone (c, g). Sig- nal analysis revealed statistical significance for both COX2 (d) and IL6 (h) when incubated with IL-1ß compared to control. Scale bars: 50 µm

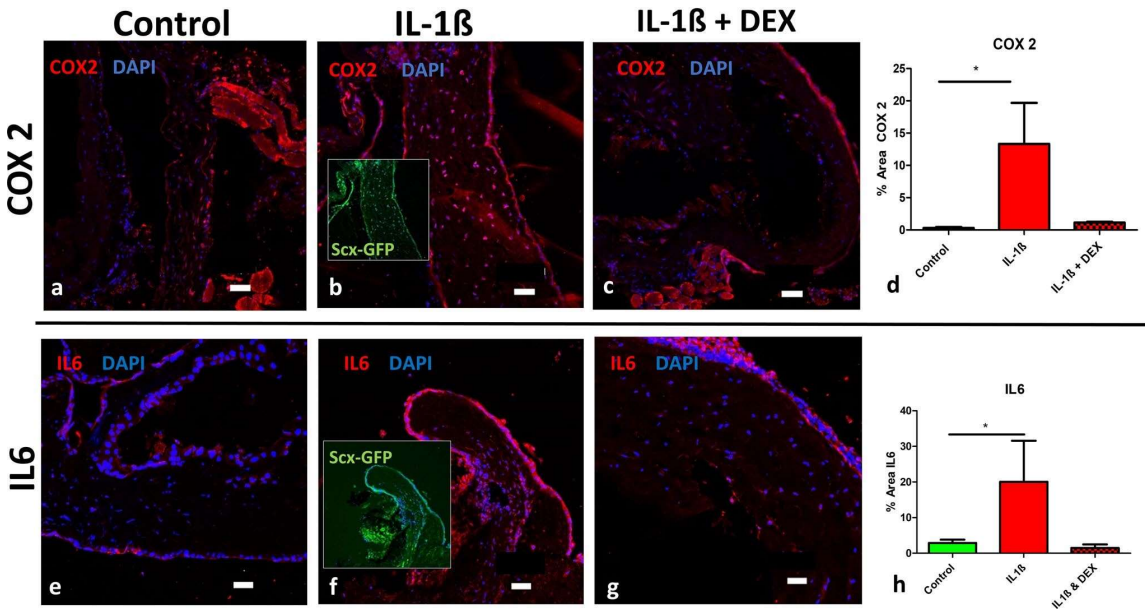


Fig. 4 Interleukin treatment enhances the expression of fibrosis- associated proteins in scleral tissue. The fibrosis-associated marker protein Connective Tissue Growth Factor (CTGF, **a–d**), and matrix metalloproteinases MMP2 (**e**–**h**), MMP3 (**i**–**l**), and MMP13 (**m**–**p**) were increased in Scx-GFP mouse scleral tissue when stimulated with IL1-ß compared to controls, and the effects were abrogated by addition of dexamethasone (**c**, **g**, **k**, **o**). Signal analysis revealed statistical significance for all markers when incubated with IL-1ß compared to controls. Insets in the IL1-ß group images show the Scx-GFP channel and reveal co-expression of the respective proteins with Scx-GFP (insets in IL1-ß group). Scale bars: 50 µm

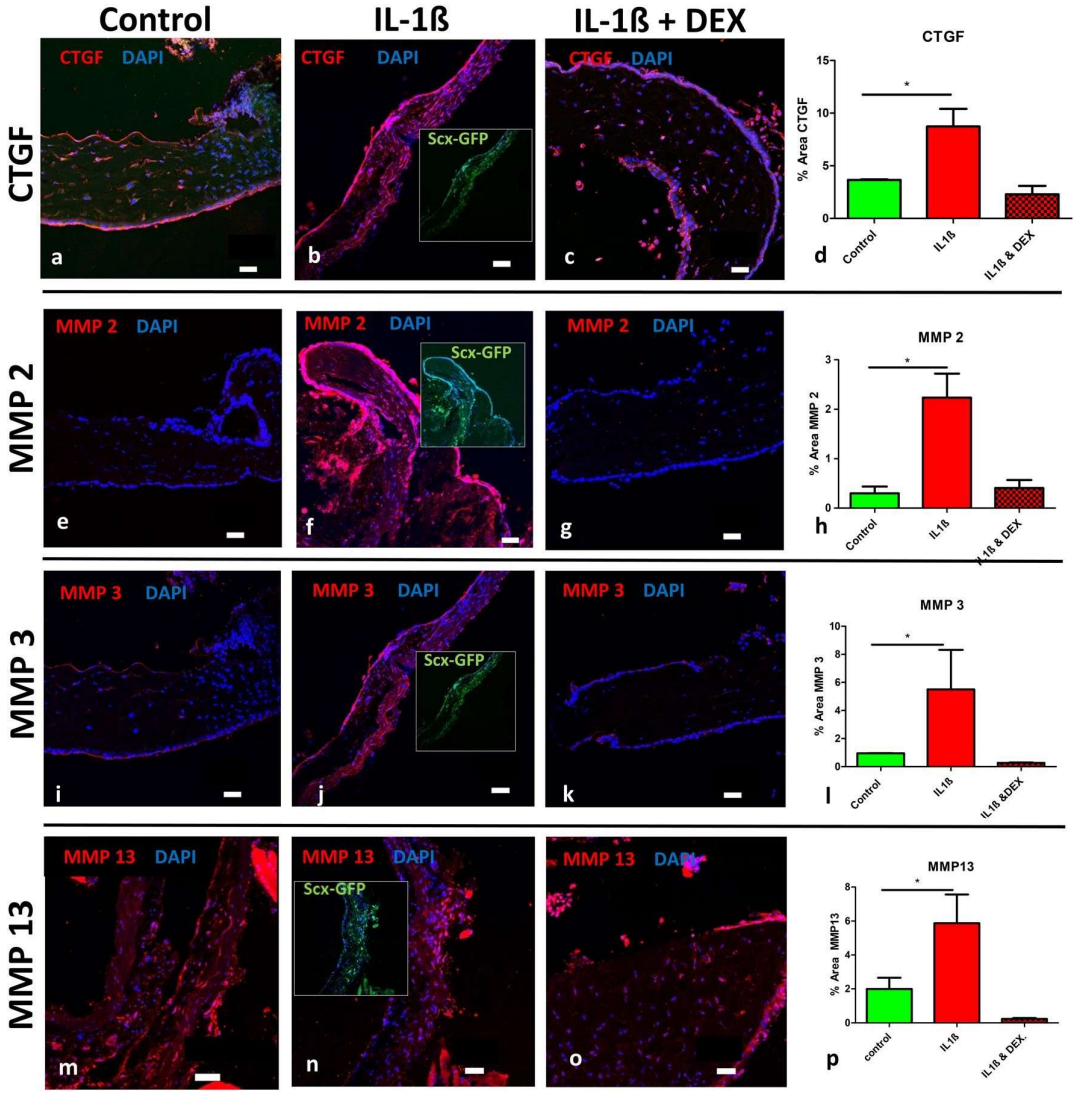
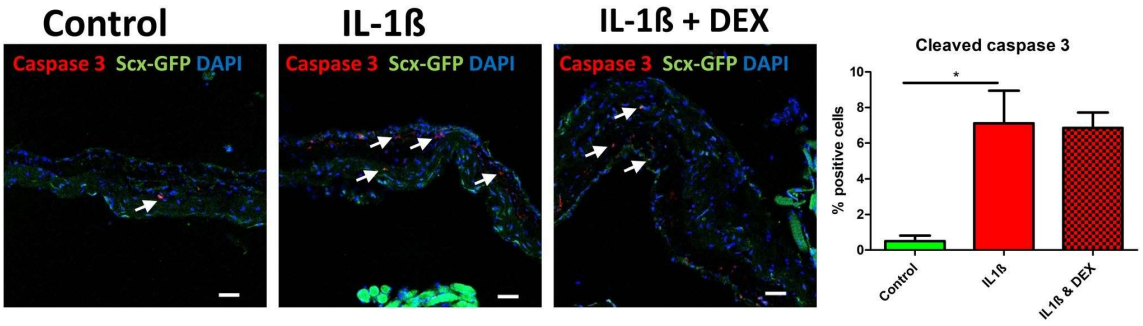


Fig. 5 Interleukin treatment induces apoptosis in scleral cells. The apoptosis-associated marker protein Cleaved caspase 3 was increased in Scx- GFP mouse scleral tissue when stimulated with IL1-ß compared to controls, dexamethasone did not abrogate this effect (**a**–**d**). Scale bar: 50 µm



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5. DISCUSSION

The publication of the Lymphogenic and Hemangiogenic factors affecting the Human Sclera is a novel study focused on the vascular and cellular privilege of human sclera in healthy status and under pathological conditions. Generally, the sclera is the eye globe's outer tunic covering coat. The sclera's opacity helps in prevention of off-axial light transmission that degrades the retinal image. The sclera affects the visual process through retinal and lens-iris-diaphragm distortion. Moreover, both scleral and corneal geometries play an important role in determining the eye's focal length and optical characteristics [1]. The superficial vascularized episclera, which includes a dense network of blood vessels the avascular scleral stroma which is the main layer, and the thin pigmented lamina fusca which forms the sclera-choroid border are the three anatomical layers forming the sclera [1]. The Sclera offers a stable firm surface for the globe. Thus, providing protection to the retina and other intraocular structures due to the high mechanical vulnerability of the eye. The scleral extracellular matrix is rich in collagen primarily type I collagen and elastin, which was formed by fibroblast-like cells that are still poorly characterized. The healthy sclera is characterized by a tight network of regulated vascularization which share many similarities with the tendon where both are hypovascularized collagen-rich extracellular matrix tissues where hypervascularity is the main feature of both tendon and scleral diseases [1].

Also, we studied the scleral vascular privilege we found that the sclera is not essentially acellular, conversely, it is rich in perivascular extraluminal macrophages, with the episcleral layer having the highest density, where blood vessel homeostasis is being maintained. A wide spectrum of anti- angiogenic / lymphangiogenic factors is highly expressed within a healthy human sclera, while pro-angiogenic factors expression is reduced. This environment maintains an anti-angiogenic / lymphangiogenic balance, ensuring that the healthy human scleral layers are free of lymphatic vessels which contribute to the ocular immune privilege of the inner eye The stability of pro- and anti-angiogenic factors is important for preserving scleral vascular status and probably the vascular privilege of the internal eye [21].

Furthermore, we found that vascular scleral privilege compromised under in pathologic conditions Firstly, in ciliary body melanoma with extraocular extension, it represents about 4% of all uveal melanomas and is usually accompanied with high mortality rates [22]. Haemaogenous and lymphogenous metastasis can occur through different mechanisms of lymphatic vessels proliferation [16]. The first mechanism is the conjunctival lymphatics proliferation and melanoma invasion through the formation of newly dividing lymphatic epithelia cells nuclei and outgrowth of lymphatic capillaries[23]. The second mechanism is the proliferation and invasion of melanoma lymphatics to the lymphatic system when cancer cells

expand and penetrate per-tumour lymphatics [22,24]. Nevertheless, the third mechanism is a combination between both mechanisms[22]. Most extraocular-extension melanomas (almost 60%) have intraocular lymphatic vessels. Only the tumour periphery adjacent to the sclera has intraocular LYVE-1 and podoplanin-positive lymphatic vessels. Moreover, there are no LYVE-1 or podoplanin-positive lymphatic vessels in the central aspects of tumours [25].

Secondly, following an open eye injury, the vascular privilege of the sclera is impaired and many associated LYVE-1+ macrophages infiltrate intraocular lymphatic vessels. This mechanism is important for wound healing, autoimmune reactions against intraocular antigens, and defense towards intruding microorganisms[26].

Where two histopathological findings are spotted in enucleated globes following trauma:

1. Podoplanin+/Laminin+ lymphatic vessels, indicating secondary lymphangiogenesis occurrence, are observed in the sclera and intraocular space.
2. Secondary ingrowth of the intraocular lymphatic vessels with a large number of LYVE-1+ macrophages (de novo synthesis of lymphatic vessels), supports wound recovery, autoimmune reactions towards antigens, as well as defence against invading microorganisms [26].

Furthermore, In Sympathetic ophthalmia It is a rare bilateral, diffuse, granulomatous T-cell mediated inflammation that can happen soon following the penetration of ocular injury [27]. The growth of lymphatic vessel is influenced by a variety of factors, including the expression of different cytokines, local inflammations following trauma, as well as other external factors. Normally, persisting lymphatic vessels serve as an afferent pathway for the immune system [26]. Lymphatic vessels exist within the eye wall, in retro corneal membranes, beneath the sclera and close to the uveal tract, ciliary body and iris, even after years from open globe injury [28,29].

Under Pathological conditions, lymphangiotaxis is considered a secondary intraocular lymphangiogenesis in which the intruding lymphatic vessels act as ‘‘afferent arm of the immune reflex arc’’, allowing APCs carrying foreign antigens to reach regional lymph nodes. Also, it allows immunological effector cells to invade, and thereby, promoting immune rejection [30]. Due to scleral weakness, this process is characterized by elevated amounts of pro-lymphangiogenic growth factors and reduced amounts of anti-lymphangiogenic inhibitors [16].

Besides, macrophages play a role contributing to lymphangiogenesis at the cellular level. Secretion of Vascular Endothelial Growth Factor (VEGF-A, -C, and -D) is because of the infiltration and trans-differentiation of Cluster of Differentiation 11b(CD11b+) macrophages into lymphatic endothelium and the provision of lymphangiogenic components through activation

of Nuclear Factor kappa light chain enhancer of activated B cells (NF-κB) signalling paths. As noted, the physiological process by which the existing macrophages adopt various functional programmes responding to input from their milieu, is known as macrophage polarisation. They are innate immune system effector cells that help in cellular debris removal, embryonic development, and tissue repair [31].

Accordingly, to limit the lymphatic dissemination of the tumour to regional lymphatic nodes, nowadays we used anti-hemangiogenic treatments and an antibody fragment that bind and inhibit all identified VEGF isoforms, as well as more specialised anti-lymphangiogenic treatments [32].

Besides, our second publication discusses how expression of scleral tendon related markers and their response to an inflammatory stimulus. The SCX-positive scleral cells resemble fibroblasts in size and shape. They are not macrophage-like cells as they do not express CD68 [12,33]. Therefore, our study examines the properties of sclera and establishes an ex vivo scleral inflammation model. Using qRT-PCR and immunohistochemistry, we examined the sclera of SCX-GFP mice for expressing the tendons associated marker genes SCX, mohawk and tenomodulin. In a culture that is organotypic, explanted sclera of adult SCX-GFP mice were treated to 10 ng/ml recombinant interleukin 1-ß (IL1-ß) and IL1-ß in conjunction with dexamethasone respectively. A collagen hybridizing peptide (CHP) binding assay analyzes collagen fiber degradation in the tissue using immunofluorescence staining of the inflammation- and fibrosis related proteins Interleukin 6 (IL6), Cyclooxygenase-2 (COX-2), Inducible Nitric Oxide Synthase (iNOS), connective tissue growth factor (CTGF), Matrix Metalloproteinase 2(MMP2), Matrix Metalloproteinase 3 (MMP3), and Matrix Metallopeptidase 13 (MMP13). Scleraxis, tenomodulin, and mohawk mRNA, as well as Scleraxis promoter driven GFP were found to be expressed strongly in mouse scleral tissue, with an indication to a phenotype that is like a tendon cell. SCX-GFP+ cells highly increased the expression of all studied proteins, after being stimulated by IL1-ß [20].

In the present study of an ex vivo scleral inflammation model, tendon cells respond to IL1-ß stimulation by upregulating inflammatory and fibrosis-associated marker genes in vitro [34]. Correspondingly, COX2 and IL6 are upregulated by Scx-GFP positive scleral cells. The localized increase of CTFG suggests a fibrosis-like response to IL1-ß stimulation, like what described in the fibrotic tendon [35]. CTGF was regulated in the sclera throughout the recovery of experimental myopia, with downregulation at some point in a hyperopic refractive error inducing GO signal, while it was previously increased during the remodeling process through recovery [36]. In necrotizing scleritis, matrix metalloproteinases are involved, where MMP2 and MMP3 were both expressed by resident scleral fibroblasts and inflammatory cells such as T-

lymphocytes and macrophages. Moreover, MMP2 was shown in human melanoma-associated spongiform scleropathy samples. MMPs are involved in matrix remodeling and degeneration of tendons, leading to degradation of collagen fiber and weakness of tissues [37]. Furthermore, simulation of IL1-ß caused considerable degradation in collagen. The addition of dexamethasone led to a significant reduction in the reaction to IL1-ß stimulation. Moreover, degradation of collagen highly increased in the IL1-ß group. While dexamethasone corticosteroid showed a strong rescue effect.

Interestingly, Collagen is a fibrous protein that is considered the primary element that makes up the extracellular matrix. It provides multiple functions including tensile strength provision, cell adhesion regulation, chemotaxis, migration, and directing tissue development through providing stability and elasticity. It helps specialized connective tissues e.g., sclera and tendons to mechanically cope by enduring cyclic loads and deformations [38]. Generally, both tissues share many common structural and functional characteristics where both composed of collagen-rich matrix, and both characterized by low density, sparse vascular tissues, and high mechanical stress under normal physiologic conditions whereas both tissues characterized by sparse vascularity in healthy status and hypervascularity is a characteristic feature of both scleral and tendon disease [34]. The discussion of the similarities between sclera and tendon intended to provide new pathophysiological insights and mechanisms for orthopedic and ophthalmology research. In pathological conditions, as in degenerative processes in sclera and tendon, both are usually linked to Ageing, smoking, inflammatory diseases. Scleritis is the inflammatory state of sclera, that is usually linked to systemic diseases like Systemic Lupus Erythromatousis (SLE), Diabetes Mellitus (DM), Rheumatoid Arthritis (RA) [39]. Furthermore, these diseases usually associated with many tendinopathies which is a painful degenerative tendon disease [40].

As for Limitations for this study, that some of the existing animal models aren't quite satisfying, therefore the mechanism of most scleral diseases is obscure and poorly understood [39,41]. This lack of understanding is due to the unavailability of the human data, as it is derived from histopathological studies, because non-physiologic induction methods such as ovalbumin is partially used. Few studies have systematically examined the scleritis histopathology because if the scleral biopsy is not properly diagnosed, the inflammatory process will worsen, and the damaged sclera will become structurally weaker. As a result, most of the structural and histological data derived from scleritis comes from surgically removed eyes after they have become blind and painful or from postmortem eyes [41]. Therefore, early actions in this disease, as well as immunohistochemical components, remain improperly studied [42]. Moreover, the problem associated with the current study, is that using a transgenic animal model failed to directly prove SCX expression. Where no tested antibodies were precise

enough to reliably label SCX in immunohistochemistry. As a result, SCX- GFP sign should be interpreted in a reasonable way.

In summary, in our second publication, we are the first to propose a new organotypic ex-vivo model of scleritis was developed resulting in hallmarks of this disease, as well as a SCX-GFP reporter mouse model. It also provides the first characterization of these Scleraxis Positive fibroblast cells and tendon cell-like scleral cells in response to an inflammatory stimulus. Such as degraded collagen matrix upregulated inflammatory factors in addition to expression of matrix degrading enzymes [20].

Also, in our first publication, we studied the hemangiogenic and lymphangiogenic privilege in human sclera in healthy status which is characterized by tightly vascular network which is totally compromised under pathological conditions which leads to molecular and cellular changes which affects wound healing, metastasis of tumor cell, immunologic reactions against intruding microorganism, and autoimmune reactions against intraocular antigens [21].

In conclusion, more future research needs to emphasize on pro- and anti-lymph/angiogenic factors, and detailed definition of their function and therapeutic impact in various ocular disorders, along with paving the road for developing new effective therapeutic options for uncurable ocular disorders.

6. SUMMARY

Our project “The Lymphogenic and Hemangiogenic factors affecting the Human Sclera” is a novel study focused on the vascular and cellular changes of human sclera in healthy status and under pathological conditions Our research work based on observations and clinical experience, along with a literature review based on a PubMed search. We found that the vascular privilege of healthy human scleral characterized by tight vascular network showed by a wide spectrum of anti-angiogenic and anti-lymphangiogenic factors that is highly expressed, while pro-angiogenic factors expression is reduced. Where the healthy human scleral layers are devoid of lymphatic vessels which contribute to the ocular immune privilege of the inner eye. Under pathological conditions vascular and cellular privileges of the sclera are compromised where the fibrocytes change into active fibroblasts causing remodelling and proliferation of the scleral matrix. Also, cellular activation and secondary ingrowth of intraocular lymphatic vessels, associated LYVE-1+ macrophages invaded the sclera and intraocular structures affecting healing out-comes. immunologic defense against intruding microorganism, and autoimmune reactions against intraocular antigens [21].

Our second publication, we provide a novel insight on characterization of SCX-GFP which is a tendon marker expressing scleral cells and response of scleral fibroblasts to inflammatory stimuli by examining an explanted sclera tissue of adult mouse in a novel ex vivo model of scleritis which represent the hallmarks of this disease in damaged collagen matrix, upregulation of inflammatory factors and in matrix degrading enzymes expression. By using qRT-PCR and immunohistochemistry, the explanted scleral tissue of SCX-GFP mice was treated with 10 ng/ml IL1-ß and IL1-ß in conjunction with dexamethasone respectively. A collagen hybridizing peptide binding assay analyzes collagen fiber degradation in immunofluorescence staining tissues of the inflammation- and fibrosis related proteins. Where SCX-GFP expressed strongly in mouse scleral tissue. After stimulation by IL1-ß, SCX-GFP+ cells highly increased the expression of all studied proteins. the addition of dexamethasone led to a significant reduction in the reaction to IL1-ß stimulation. Moreover, degradation of collagen highly increased in the IL1-ß group. While dexamethasone corticosteroid showed a strong rescue effect [20].

To the best of our knowledge, these two studies provide a novel insight on cellular and vascular mechanisms in healthy and pathological status and provides a novel ex vivo organotypic model of scleritis and also provide a first characterization of tendon marker expressing scleral cells SCX_GFP in response to inflammatory stimulation and the results may provide a basis of wound healing following surgical interventions and paving the way for new effective therapeutic development for uncurable ocular disease which will make a new improvement in the clinical ophthalmology practice.

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8. PRE-PUBLICATION OF RESULTS

1. **Atta G**, Tempfer H, Kaser-Eichberger A, Guo Y, Schroedl F, Traweger A, Heindl LM., The lymphangiogenic and hemangiogenic privilege of the human sclera. Ann Anat. 2020 Jul; 230:151485. doi: 10.1016/j.aanat.2020.151485. E Pub 2020 Feb 28. PMID: 32120002
2. **Atta G**, Schroedl F, Kaser-Eichberger A, Spitzer G, Traweger A, Heindl LM, Tempfer H., Scleraxis expressing scleral cells respond to inflammatory Stimulation. Histochem Cell Biol. 2021 Aug;156(2):123-132. doi: 10.1007/s00418-021-01985-y. Epub 2021 May 8. PMID: 33966129
3. **Atta G**, Tempfer H, Kaser-Eichberger A, Traweger A, Heindl LM, Schroedl F, Is the human sclera a tendon-like tissue? A structural and functional comparison. Ann Anat. 2021 Nov 16:151858. doi: 10.1016/j.aanat.2021.151858. Online ahead of print. PMID: 34798297

This Cumulative thesis includes the papers of No. 1 and No. 2.