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Expression of endogenic lectins and their glycoligands in the tear fluid, human corneal and conjunctival epithelium under physiological and disease conditions

Doctoral thesis

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ABSTRACT

Purpose: Lectins play an important role in many biological processes. The aim of this work was to analyse mainly the expression of endogenic lectins, such as galectins and plant lectin, e.g. *Dolichos biflorus* agglutinin (DBA), and their glycoligands in the tear fluid, human corneal and conjunctival epithelium in physiological and disease conditions. Further, we studied the human natural antibody against Galα1,3Gal–R, which is mainly responsible for hyperacute rejection of xenografts transplants. We tried to investigate its localization in human corneal epithelium, lacrimal gland and tears.

Material and Methods: Human tissue (lacrimal gland, tear fluid, conjunctiva, cornea, epidermis, keratinocyte and cultured corneal epithelium), as well as porcine tissue (cornea, liver and epidermis) were examined. Endogenous galectins (galectins–1, –3 and –7) were detected using immunohistochemistry methods. Binding sites for galectins, as well as binding sites for plant lectin *Dolichos biflorus* agglutinin, were localized by lectin histochemistry. Reverse lectin histochemistry was used for the study of binding reactivity of endogenous lectins using labelled (neo)glycoligands. Employing biotinylated natural human IgG anti α–galactosides, as well as anti β–galactosides, we detected reactive epitopes in human cornea, lacrimal gland, tear fluid, skin, muscle capillaries and in porcine cornea, skin, vein and liver. The expression of galectin–1 and –3, laktoferrin and α, β galactosides in tear fluid was confirmed by using western blot.

Results: Galectin–1 was markedly present in tear fluid, corneal and limbal epithelium, and was absent in conjunctival epithelium. Galectin–3 was found in tears from patients with ocular surface disorders, in normal conjunctival and corneal epithelium, but not in the lacrimal gland. Inflammatory leucocytes and goblet cells found in galectin–3-containing tear fluid also expressed galectin–3. Galectin–3–binding sites were detected on the surface of conjunctival and corneal epithelium colocalizing with desmoglein. All cell layers of the corneal epithelium were positive for galectin–7. The binding of *Dolichos biflorus* agglutinin was typical for postmitotic early differentiated epithelial cells. Concerning cellular reactivity, the porcine corneal epithelium was negative for Galα1,3Gal structures, which are known to be abundantly expressed on cells of non–primate grafts, consequently causing an immunological barrier between humans or other Old World primates and non–primate mammals.

Conclusions: The monitoring of the presence of galectin–3 and its binding sites prompts the elucidation of the functional role of galectin in the eye under both normal and pathological condition. The results show potential participation of galectin–3 in mediation of intercellular contacts of corneal epithelium, namely in suprabasal cells. The specific binding of *Dolichos biflorus* agglutinin for postmitotic early differentiated epithelial cells lends strong support for using glycohistochemical methods in the study of differentiation of cells of the squamous epithelium. The absence of Galα1,3Gal structures in the porcine corneal epithelium raise the question whether it might be possible to use porcine cornea and the epithelial cell layer in clinical medicine as viewed from the perspective of α Gal.

ABSTRAKT

Cíl: Lektiny hrají důležitou roli v mnoha biologických procesech. Cílem této práce bylo analyzovat expresi endogenních lektinů a jejich glykoligandů v slzách a také v epitelu lidské rohovky, případně spojivky, a to jak u fyziologických, tak i u patologických stavů. Dále jsme se zabývali lidskou protilátkou Galα1,3Gal-R, která je zodpovědná zejména za hyperakutní rejekci xenotransplantátu. Snažili jsme se prozkoumat její lokalizaci v epitelu lidské rohovky, slzné žláze a slzném filmu.

Materiál a metodika: Zkoumali jsme lidské tkáně (slzné žlázy, slznou tekutinu, spojivku, rohovku, epidermis, keratinocyty a kultivovaný epitel rohovky), i prasečí tkáně (rohovka, játra a epidermis). Endogenní galektiny (galektin-1, -3 a -7) byly detekovány pomocí imunohistochemických metod. Vazebná místa pro galektiny, stejně jako vazebná místa pro rostlinný lektin aglutinin extrahovaný z rostliny *Dolichos biflorus*, byla lokalizována lektinovou histochemií. Reverzní lektinová histochemie byla použita při studiu vazebné reaktivity endogenních lektinů označené pomocí (neo)glykoligandů. Pomocí přírodních lidských biotinylovaných IgG anti α-galaktosidů a β-galaktosidů jsme zjistili reaktivní epitopy v lidské rohovce, slzných žlázách, slzné tekutině, kůži, svalových kapilárách a v prasečí rohovce, kůži a játrech. Exprese galektinu-1 a -3, laktoferinu a α, β galaktosidů v slzách byla potvrzena pomocí western blot analýzy.

Výsledky: Galektin-1 byl přítomen v slzném filmu, rohovce a v limbálním epitelu, ale nevyskytoval se v konjunktiválním epitelu. Galektin-3 byl nalezen v slzách pacientů s poruchami povrchu oka, v normálním spojivkovém a rohovkovém epitelu, ale nebyl přítomen v slzné žláze. Zánětlivé leukocyty a pohárkové buňky nacházející se v slzách obsahujících galektin-3 také exprimovaly galektin-3. Vazebná místa pro galektin-3 byla detekována na povrchu spojivkového a rohovkového epitelu, spolu s desmogleinem. Všechny buněčné vrstvy epitelu rohovky vykazovaly přítomnost galektinu-7. Vazba aglutininu z rostliny *Dolichos biflorus* byla typická pro postmitotické časně diferencované buňky epitelu. Pokud jde o buněčnou reaktivitu, epitel prasečí rohovky nevykazoval přítomnost Galα1,3Gal struktur, o kterých víme, že jsou hojně exprimovány v buňkách štěpů u savců s výjimkou primátů a v důsledku toho způsobuje imunologickou bariéru mezi člověkem či jinými primáty Starého světa a ostatními savci, nepatřícími mezi primáty.

Závěry: Sledování přítomnosti galektinu-3 a jeho vazebných míst pomocí imunohistochemie může přispět k objasnění funkčních rolí galektinu v oku za normálních a patologických podmínek. Tyto výsledky ukazují na možnou účast galektinu-3 při zprostředkování mezibuněčných kontaktů epitelu rohovky, a to v suprabazálních vrstvách. Specifická vazba *Dolichos biflorus* aglutininu na postmitotické časně diferencované buňky epitelu ukazuje význam využití glykohistochemických metod při studiu diferenciace buněk dlaždicového epitelu. Nepřítomnost Galα1,3Gal struktur v epitelu prasečí rohovky nám pokládá otázku, zda by bylo možné použít prasečí rohovku a vrstvu epiteliálních buněk v klinické medicíně z pohledu α-Gal.

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ABBREVIATIONS

BMZ	Basement Membrane Zone
BSA	Bovine Serum Albumin
CESC	Corneal Epithelial Stem Cells
CRD	Carbohydrate Recognition Domain
DBA	*Dolichos Biflorus* Agglutinin
ECM	Extracellular Matrix
ELISA	Enzyme–Linked Immunosorbent Assay
HAA	*Helix Aspersa* Agglutinin
HGF	Hepatocyte Growth Factor
IEF	Isoelectric Focusing
PAA	Poly–2–hydroxyethyl–Acrylamide
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate–Buffered Saline
PMN	Polymorfonuclear
PNA	Peanut Agglutinin
PRK	Phototerapeutic Refractive Keratectomy
TAC	Transient Amplifying Cells
WGA	Wheat Germ Agglutinin

1. GENERAL INTRODUCTION

A. THE EYE

1.1. Tear film

The tear film is a complex composite whose components have multiple sources, which include the lacrimal gland, meibomian glands, goblets cells, and the accessory glands of the ocular surface (Lamp et al., 2011). The base of the tear film is the outer surface membrane of the corneal and conjunctival epithelial cells.

The function of the tear film includes lubrication, protection from disease, nutrition of the cornea, and a critical role in the optical properties of the eye (Klyce et al., 1988). Normal tear volume is around 6 µl and the production is 1.2 µl / minute with a turnover rate of about 16 % per minute.

Normal tears contain 6–10 mg / ml total proteins and almost 500 proteins have been reported (De Souza et al., 2006). Major tear proteins include lysozyme, lactoferrin, secretory immunoglobulin A (IgA), serum albumin, lipocalin (Redl, 2000), and lipophilin (Lehrer et al., 1998). Tears are a dilute protein solution and both the electrolytes and protein content of tears varies from that of serum.

Tear film has a trilaminar structure, consisting of a thin anterior lipid layer, an intermediate aqueous layer, and an innermost mucous layer whose component particles are loosely bound to the glycocalyx of the corneal and conjunctival epithelial surface (Michael et al., 2011). Most tear samples are collected using either a glass capillary tube or a filter paper strip. The entire volume of minimally stimulated tears is around 7 µl. Despite sampling problems, a number of analytical methods that couple microliter samples size with high sensitivity and resolution have prompted more detailed studies in tear composition. Qualitative and quantitative techniques include one- and two-dimensional polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF), crossed immunoelectrophoresis, enzyme–linked immunosorbent assay (ELISA).

Table 1.1: Properties of Human Tear Fluid (from American Academy of Ophthalmology, Basic and clinical science course 1998)

Composition (%)	Water	98
	Solid	1.8
Thickness (μm)	Total	6.5
	Lipid layer	0.1–0.2
Volume (μl)	Unanesthesized	7.4
	Anesthesized	2.6
Secretory rate (μl)	Unanesthesized	
	Shirmer	3.8
	Fluorophotometry	0.9
	Anesthesized	
	Shirmer	1.8
	Fluorophotometry	0.3
Turnover rate (% / min)	Normal	12–16
	Stimulated	300
Evaporation rate (μl / cm^2 / min)		0.06
Osmolarity (mOsm)		296–308
pH		6.5–7.6
Electrolytes (mmol / l)	Na	134–170
	K	26–42
	Ca	0.5
	Mg	0.3–0.6
	Cl	120–135
	HCO_3	26

Table 1.2: Functional peptides of tears (*Reference: Michael A. Lemp, Roger W. Beuerman Basic Science, Chapter Tear film, 2013 33—39)*

Growth factors	Association
Epidermal growth factor (EGF)	Epithelial wound healing Tear concentration higher than saliva or serum
Transforming growth factor alpha (TGF–α)	Wound response
Transforming growth factor beta–1 (TGF–β1	Wound response
Transforming growth factor beta–2 (TGF–β2)	Found in normal tears, increases after wounding
Hepatocyte growth factor (HGF)	Wound response
Basic fibroblast growth factor (FGF–2)	Wound response
Vascular endothelial growth factor (VEGF)	Wound response, increases after wounding
Platelet derived growth factor–BB	Did not change after PRK
Neuropeptides	
Substance P	Wound healing, neurogenic inflammation
Calcitonin gene related peptide	Wound healing, neurogenic inflammation
Interleukins	
IL–4	Increases in vernal conjunctivitis
IL–1α, IL–1β	Elevation of IL–1 in dry eye patients
IL–2, IL–4, IL–6, IL–8, IL–10	Increases with contact lens wear, ocular allergy
Immunoglobulins	
IgA, IgE, IgG(1–4) and complement	Ocular allergy
Proteases	
MMP–1, MMP–3, MMP–9, TIMP–1, capthepsin, alpha2–macroglobulin	Role in pterygium migration and vernal keratoconjunctivitis, protection of the ocular surface
Antimicrobial peptides	
Lysozyme, lactoferrin, αand β defensins, phospholipase A2	Increases in infections, wound healing, may decrease in dry eye

1.2. Conjunctiva

The conjunctiva arises from surface ectoderm and neural crest tissue (Spencer et al., 1985). It is a mucous membrane that protects the soft tissues, critical in maintaining the integrity of the eye. The epithelium varies in thickness and appearance from the eyelid margin to the limbus. The location of the stem cell populations for the conjunctival epithelium is believed to be located in the fornix (Pellegrini et al., 1999). At the limbus, the conjunctiva converts to

stratified squamous epithelium of the cornea. The flattened surface cells of the conjunctival epithelium demonstrate many microvilli and are coated by a glycogalyx and mucin (Dilli et al., 1998). Keratinization of the conjunctival epithelium is always pathologic and can occur in conditions such as vitamin A deficiency, pemphigoid, Steven–Johnson syndrome, and keratoconjunctivitis sicca.

The conjunctiva contains nonepithelial cells similar to those in the skin. Melanocytes are present basally and Langerhans cells are scattered throughout.

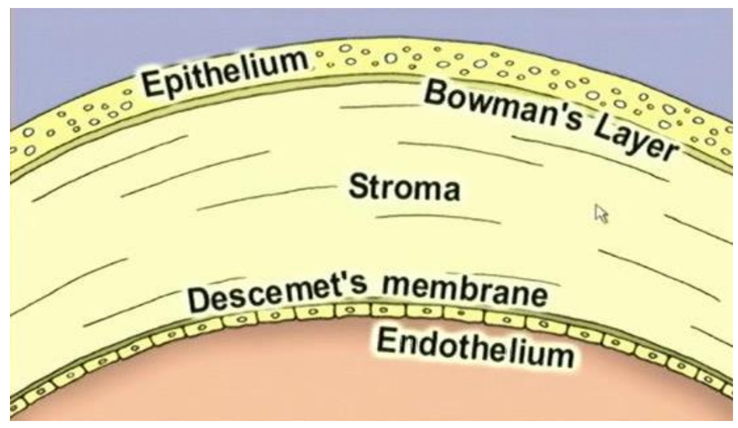
The bulbar conjunctival epithelium consists of six to nine layers of stratified squamous epithelial cells piled up in an irregular fashion in contrast to the more orderly corneal epithelium. Cytoplasmic organelles are similar to the cornea but more abundant. The basal and intermediate epithelial cells contain more and larger mitochondria than the corneal epithelium, suggesting a higher level of oxidative metabolism (Daniel et al., 2011). The epithelial cellular membranes show marked infoldings, with incomplete interdigitation with adjacent cells. This configuration causes wide intracellular spaces in which antibodies and other plasma constituents and inflammatory cells from underlying vessels can accumulate.

The conjunctival basement membrane zone (BMZ) does not normally show immunochemical reactivity to any immunoglobulins, complement components, or albumin. The superficial cells in normal subjects do show variable amounts of IgA and IgG reactivity. BMZ immunoreactivity to IgM, IgD, and IgE may be seen in patients with ocular cicatricial pemphigoid but is not found in normal conjunctiva. Fibrinogen is normally found at the BMZ and can serve as a positive control when processing conjunctival specimens for immunoreactivity (Foster et al., 1994).

1.3. Cornea

The World Health Organization estimates that 45 million people suffer from bilateral blindness worldwide, 10 million of whom are affected by corneal disease.

The cornea consists of three different cellular layers and two interfaces: the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium. The cell types that constitute the cornea include epithelial cells, keratocytes (corneal fibroblast), and endothelial cells (Terua et al., 2011).



Picture 1.1: Corneal layers *(from Pulmonary & Ophtalmic Drug Absorption, Ismail Mortada Medical Blog, August 26, 2009)*

Epithelium

The anterior surface of the cornea is derived from surface ectoderm and is made up of nonkeratinized, stratified squamous epithelium. The corneal epithelium consists of five or six layers of three different types of epithelial cells: superficial cells, wing cells, and columnar basal cells, the latter of which adhere to Bowman layer. Only the basal cell of the corneal epithelium proliferates. The daughter cells differentiate into wing cells and subsequently into superficial cells. The differentiation process requires about 7 to 14 days (Hana et al., 1968). An important physiological role of the cornea epithelium is to provide a barrier to external stimuli. The presence of junctional complexes between adjacent corneal epithelial cells prevents the passage of agents into the deeper layers of the cornea. Tight junctions (zonula occludens) are present mostly between cells of superficial cell layers. Hemidesmosomes (zonula adherence) and desmosomes are present in all layers of corneal epithelium (Terua et al., 2011).

In corneal epithelial cells, intermediate filaments of the cytoskeleton are formed by specific type of acidic (type I) and basic (type II) keratin molecules. Basal cells of the corneal epithelium express keratin 5/14, like basal epidermal cell of the skin. However, keratin 3/1 (64–kDa keratin) is specifically expressed in the epithelium of the cornea, not being found in that of the conjunctiva or in the epidermis (Kurpakus et al., 1990, Schermer et al., 1987).

Nonepithelial cells

Nonepithelial cells may appear within the corneal epithelial layer. Histocytes, macrophages, lymphocytes, and pigmented melanocytes are frequent components of the peripheral cornea. Langerhans cells have also been described (Terua et al., 2011).

Bowman's layer

The Bowman's membrane is beneath the basal layer. It consists of randomly dispersed collagen fibrils and is 8–14 µm thick (Terua et al., 2011). Its posterior border merges with the corneal stroma. Unlike Descemet's membrane, it cannot be regenerated by itself and may become opacified by scar tissue following injury.

Stroma

The stroma constitutes about 90 % of the total corneal thickness in humans. It is composed of collagen–producing fibroblast cells (keratocytes), collagen lamellae, and proteoglycan.

– *Cells*. Keratocytes are the predominant cellular components of the corneal stroma and are thought to turn over about every 2 to 3 years. Keratocytes are quiescent in the normal cornea but they are readily activated and undergo transformation into myofibroblast that express α–smooth muscle actin in response to various types of injury to the stroma (Ueda et al., 1987).

– *Collagen.* Collagen constitutes more than 70 % of the dry weight of the cornea. Collagen in the corneal stroma is mostly type I, with small amount of type III, V and VI (Linsenmayer et al., 1984). The turnover of collagen molecules in the cornea is slow, requiring 2 to 3 years. Fibrils form obliquely oriented lamellae in the anterior third of the stroma and parallel lamellae in the posterior two thirds of the cornea.

– *Proteoglycans.* The major matrix components located in the spaces among major collagen fibres, are composed of a core protein and glycosaminoglycan chains and are thought to modulate collagen fibrillogenesis (Iozzo et al., 1998). Glycosaminoglycans (Tab. 1.3) themselves also play important role regardless of the core protein to which they are attached. The functions of proteoglycans can thus be considered from the points of views of both the core protein and glycosaminoglycans.

Table 1.3: Glycosaminoglycans in the cornea *(from Terua Nishida, Shizuya: Basic Science, Chapter I, Cornea and Sclera 2013: 3–22)*

Glycosaminoglycan	Size (kDa)	Constituent disaccharide
Heparan sulfate	5–12	*N*–acetylgalactosamine, glucuronic acid
Heparin	6–25	*N*–acetylgalactosamine, glucuronic acid
Dermatan sulfate	15–49	*N*–acetylgalactosamine, iduronic acid
Chondroitin 4,6–sulfate	5–50	*N*–acetylgalactosamine, glucuronic acid
Keratan sulfate	4–19	*N*–acetylgalactosamine, galactose
Hyaluronan	4–8000	*N*–acetylgalactosamine, glucuronic acid

Descemet's Membrane

The basement membrane of the corneal endothelium, Descemet's membrane, is periodic acid Schiff (PAS)–positive. At birth it is 3–4 µm thick and its thickness increases throughout life to the adult level of 10–12 µm. Descemet's membrane is composed of an anterior banded zone that begins to develop in utero and a posterior nonbanded zone that is laid down by the corneal endothelium throughout life. Descemet's membrane is composed primarily of collagen types IV and VIII (Fitch et al., 1990) and laminin but also contains fibronectin.

Endothelium

The corneal endothelium is derived from the neural crest and is therefore neuroectodermal, not mesodermal. A single layer of mostly hexagonal cells forms the corneal endothelium. Typically, the young endothelial cells have a large nucleus and abundant mitochondria (Terua et al., 2011). These organelles play an important role in active transport and maintenance of deturgescence of the normal corneal stroma. Mitosis of the endothelium seldom occurs in humans, and the overall number of endothelial cells decreases with age (Laule et al., 1978). Adjacent endothelial cells interlock and form a variety of tight junctions, including zonula

occludens, macula occludens and macula adherence (Terua et al., 2011). Injury to the endothelium from surgery, from raised intraocular pressure, or from other disease processes may destroy endothelial cells. In the absence of mitosis, destruction of cells results in decreasing density of cells and ultimately endothelial decompensation, edema, and clouding of the cornea.

1.4. Limbal stem cells

Corneal epithelial cells renew continuously to maintain the normal layered structure of the epithelium. The centripetal movement of corneal epithelial cells has been well demonstrated, as has the fact that only the basal epithelial cells are capable of proliferation. Thoft and Friend proposed that an equilibrium exists between the centripetal movement of epithelial cells, the differentiation of basal cells into superficial cells, and the desquamation of epithelial cells from the corneal surface (X, Y, Z hypothesis) (Thoft et al., 1993). The existence of corneal epithelial stem cells at the limbus has also been postulated (Cotsareils et al., 1989). Indeed, the limbal epithelium exhibits a higher proliferative activity and a lower differentiation capability than those of the corneal epithelium, and basal limbal epithelial cells are thought to be a type of undifferentiated stem cells because they do not express corneal epithelium-specific keratin (keratin 3/12) (Daniels et al., 2006).

Basal epithelial cells of the human limbus express various possible stem cell markers (O'Sullivan et al., 2007). The expression of p63, α–enolase, keratin 5/14, and the hepatocyte growth factor (HGF) receptor have also been shown to be higher in the limbal epithelium than in the corneal epithelium.

LECTINS

1.5. Introduction to the lectins

The ability of plant agglutinins to distinguish between erythrocytes of different blood types led Boyd and Shapleigh (1954) to propose for them the name lectins, from the Latin legere, to pick out or choose. This term was generalized to embrace all sugar–specific agglutinins of nonimmune origin, irrespective of source and blood type specificity (Sharon and Lis, 1972).
The primary criterion for subdivision into categories was based on their monosacharide specificity. Lectin has been found in plants, animals and microorganisms. Naturally, these initial investigations have only been a prelude to the search for evolutionary relationship, reflected in the sequence and the structure of the carbohydrate recognition domain (CRD). Lectins are proteins that bind to specific carbohydrate structures and can thus recognize particular glycoconjugates among the vast array expressed in animal tissues (Himansha et al., 2012).
Functions of animal lectins: The outlined diversity of CRD appearance in multimodular proteins and the exceptional complexity of the sugar code make it difficult to comprehensively describe the spectrum of proven and likely functions. Some of the functions of animal lectins are as follows: cell growth control and apoptosis, cell–cell interaction (Lasky, 1991), cell–matrix interactions (Viquier et al., 2014) ligand–selective molecular chaperones in endoplasmic reticulum (Karaveq et al., 2005), intracellular routing of glycoproteins and vesicles, intracellular transport and extracellular assembly (Yamamoto 2014), recognition of foreign glycans. In the field of medicine, lectins are likely to play some role in infection processes, immune reaction, oncology.
All lectins contain a specific structure called carbohydrate recognition domain (CRD), which is specific for each lectin and it is the basic unit for classification of a protein as a lectin. Based on this structure, lectins are classified in five distinct families: (1) C–type lectins (including the selectins), (2) I–type, (3) Galectins—formerly S–type, (4) Penetraxins, (5) P-type (Himansha et al., 2012).
The doctoral thesis is focused mainly on the expression of galectins, plant lectin *Dolichos biflorus* agglutinin, their glycoligands in ocular tissue.

1.6. Lectins and the eye

In recent years lectins were used to detect carbohydrate–related abnormalities in the dystrophic cornea and species–specific differences in the carbohydrates of normal corneas. There are studies which demonstrate specific alteration in glycoconjugates occurring in the corneal matrix of patients with macular corneal dystrophies, namely the presence of oligosaccharides with terminal alpha–fucase, beta–galactose, N–acetylglocosamine and N-acetylgalactosamine residues (Panjwani et al., 1986).

Plant lectins contributed to a better understanding of the mechanisms that modulate corneal epithelial sheet migration and wound healing by detecting the expression of membrane glycoproteins (Gipson et al., 1980).

Fuchs' endothelial dystrophy is commonly regarded as an endothelial cell disorder. In a recent study glycoconjugates of Fuchs' and normal cornea using FITC conjugated lectins were compared. It came out that Fuchs' corneas contained stromal collagens with altered biochemical properties (Calandra et al., 1089). They postulate that the characteristic deterioration of endothelial function in Fuchs' endothelial dystrophy may compromise the microenvironment of the stroma and its keratocytes, and thereby lead to an altered collagenous extracellular matrix.

Keratoconus is characterized by central thinning and an increased curvature of cornea. Scarring of the cornea accompanies the disease as it proceeds, and keratoplasty is the ultimate treatment. The pathogenesis of keratoconus is not yet completely known. Panel of conjugated lectins were used in these corneal disorders as well, in an attempt to better understand the mechanisms of scarring and thinning of the cornea (Tuori et al., 1998). The results indicate that the blood group status influences lectin binding in the cornea. Furthermore, the difference in lectin binding to defects in keratoconus corneas (HAA and DBA conjugates) and to scar regions in scarred corneas (PNA and WGA conjugates) suggests that the defects in keratoconus are not solely the results of scarring (Tuori et al., 1998).

Another field in which lectins were used as probes is limbal stem cells. The initial differentiation event for the corneal epithelial cell lineage occurs as the limbally localized stem cells yield, through mitosis, the highly proliferative, transiently amplifying corneal peripheral cells. This differentiation is characterized by the expression of tissue specific cytokeratins, as well as by the loss of alpha enolase and pigmentation. Lectin PNA can be used as another marker for limbal stem cells. Limbal stem cells express on the cell surface unsialylated galactose residues that are recognized by PNA and lack of sialic acid bound

through alpha–2,3 bonds. The initial differentiation involves sialylation of these residues and the concurrent appearance of alpha–2,3 sialic acid residues, suggesting expression or activation of alpha–2,3 sialytransferase. Changes in the basement membrane composition, its charge, or both may underpin this expression (Wolosin et al., 2005).

1.7. **Galectins:** an evolutionarily conserved family of animal lectins with multifunctional properties

Galectins constitute a family of evolutionarily conserved animal lectins, which are defined by their affinity for poly–N-acetyllactosamine–enriched glycoconjugates and sequence similarities in the carbohydrate recognition domain.

1.7.1. Biochemical and molecular overview

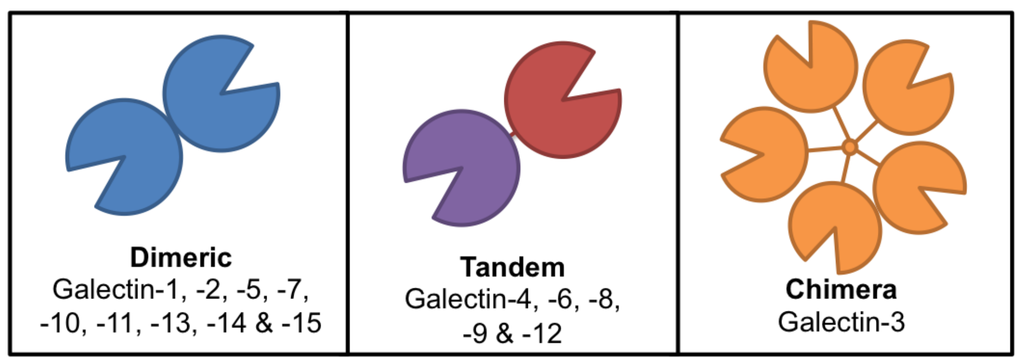
Galectins are an enigmatic family of evolutionarily conserved proteins widely distributed in nature from lower invertebrates to mammals. They share sequence similarities in the carbohydrate recognition domain (CRD) in addition to specificity for N-acetyllactosamine-enriched glycoconjugates (Barondes et al., 1994). The typical carbohydrate–binding domain consists of 135 amino acids tightly folded into a sandwich structure of 5 ± 6–stranded β-sheets and recognizes the basic structure of LacNAc; (Galβ14GlcNAc) (Kasai et al., 1997).

Mammalian galectins have so far been identified in a wide variety of tissues of several species. According to their architecture, Hirabayashi and Kasai classified this protein family into dimeric–type, chimera type, and tandem–repeat type (Rabinovich, 1999).

- **dimeric–type galectins**, include galectins (–1, –2, –5, –7, –10, –11, –13, –14, –15). Dimeric–type galectins are non–covalent homodimers composed of two identical CRDs. The best studied among them, galectin–1 (formerly referred to as L–14, galaptin and BHL), has been localized in skeletal, smooth and cardiac muscle, motor and sensory neurons, thymus, kidney and placenta.
- On the other hand, **chimera–type galectins** show a combined structure composed of a carboxy terminal CRD linked to a proline–, glycine–, and tyrosine–rich N–terminal domain. The only member of this family, galectin–3 (previously known as Mac–2, L–29, CBP–35 or IgEBP), has been predominantly found in mammals, although it has

been also identified in chickens. It has been shown to be expressed by activated macrophages, basophils, mast cells and certain epithelial and tumour cells.

- The third family of **tandem–repeat galectins** are proteins with two distinct CRDs and includes galectins–4, –6, –8, –9, and –12.



Picture 1.2: Basic structures of galectins *(from Glycobiology: 2013Feb, 23(2): 241-58)*

1.7.2. Regulation of galectin expression, subcellular distribution and secretion

Galectins have been proposed to play crucial roles by recognizing carbohydrate moieties on intracellular ligands, cell signaling receptors and extracellular glycoproteins, according to subcellular compartamentalization, developmentally regulated expression and cell activation status (Cooper et al., 1997). Expression of galectins was found to be up– or downregulated during embryogenesis, being a typical hallmark of specific developmental stages (Poirier et al., 1992). Moreover, galectin expression and subcellular distribution have been reported to be highly susceptible to modulation by diverse stimuli such as sodium butyrate (Gillenwater et al**.,** 1998), viral infections (Hsu et al., 1996), tumour suppressor genes or inflammatory agents (Sato et al., 1990). Galectins have been implicated in several physiopathological processes requiring carbohydrate recognition, such as cell adhesion, cell growth regulation, immunomodulation, apoptosis, inflammation, embryogenesis, reproduction, tumour spreading and premRNA splicing (Rabinovich, 1999). The precise knowledge of the mechanism of action of galectins is lacking, although their effects are believed to relate to interactions with specific carbohydrate ligands. It should be emphasized that the vast majority of galectin functions have been assigned to galectins–1 and –3. In contrast, functions corresponding to other members of this protein family are still a virgin land, and remain to be elucidated in future work.

1.7.3. Galectins in cell–cell, cell–matrix interactions

Adhesion and migration of cells through basement membranes and the extracellular matrix (ECM), is a multistep procedure. Consistently, a variety of extracellular and intracellular candidate ligands have been reported to bind galectins, such as laminin, fibronectin (Do et al., 1990), lysosome–associated membrane proteins 1 and 2 (LAMP–1 and 2). In view of this specific recognition, galectins have been postulated as powerful modulators of cell–cell and cell–ECM interactions.

1.7.4. Galectins in cell growth regulation

In multicellular organisms, homeostasis is maintained through a delicate balance between cell proliferation and cell death (Osborne et al., 1996). In addition to promoting or inhibiting cell attachment in the context of the ECM, galectins have been also reported to exert critical but contradictory effects on cell growth. Thus, the effects of galectin–1 seem to be double-edged, since it may trigger either proliferation or cell growth arrest, depending on the presence of concomitant environmental signals, dose, cell cycle stage, or the expression of carbohydrate receptors on the cell surface (Adams et al., 1996). It should be highlighted that the concept of a bifunctional protein with both growth–stimulatory and growth–inhibitory activities is not limited to galectins. Other signaling molecules such as TGF–β and FasL, have been shown to trigger different effects depending on the type and functional state of the cell.

1.7.5. Galectins in the immune system

Although the precise mechanisms involved in these properties in vivo still remain to be elucidated, it has been proposed that galectin–1 may affect processes in T–cell suppressor commitment and in sensitization or deletion of antigen specific T cells. The conceptual idea that a carbohydrate binding protein could affect the apoptotic threshold of T cells not only provided a breakthrough in galectin research, but also revolutionized the field of glycoimmunology (Rabinovich, 1999). Moreover, galectin–1 expression in the context of the eye would protect this sensory organ from the devastating effects of an inflammatory response. In addition to the immunosuppressive and inhibitory functions reported for galectin–1, recent studies have shown that galectin–3, formerly defined as an IgE–binding protein, was able to inhibit IL–5 gene transcription and protein release from human eosinophils and allergen specific T-cell lines (Cortegano et al., 1998).

1.7.6. Galectins: a novel paradigm in the regulation of programmed cell death

The first clue indicating that a β–galactoside–binding protein could be associated with apoptosis was provided by Goldstone and Lavin (1991), who reported that the galectin–1 gene was overexpressed during glucocorticoid–induced cell death. Apoptosis occurs in a wide variety of specific physiological situations and plays a crucial role in normal tissue homeostasis (Osborne et al., 1996). Regarding cell death regulation, overall opposite functions from galectin–1 have been assigned to galectin–3, providing clues to a novel paradigm. While galectin–1 has been shown to induce T–cell apoptosis, galectin–3 has been conversely shown to prevent cell death. Thus, galectins–1 and –3 may represent an additional family of proteins similar to the Bcl–2 family, where different members exhibit sequence similarity, yet opposite effects on cell survival. Moreover, other members of the galectin family may show redundancy in these pro– and anti–apoptotic effects. Indeed, it has been recently demonstrated that galectin–9, a novel tandem repeat lectin, is able to trigger apoptosis of murine thymocytes.

1.7.7. Galectins modulate different steps of the inflammatory cascade

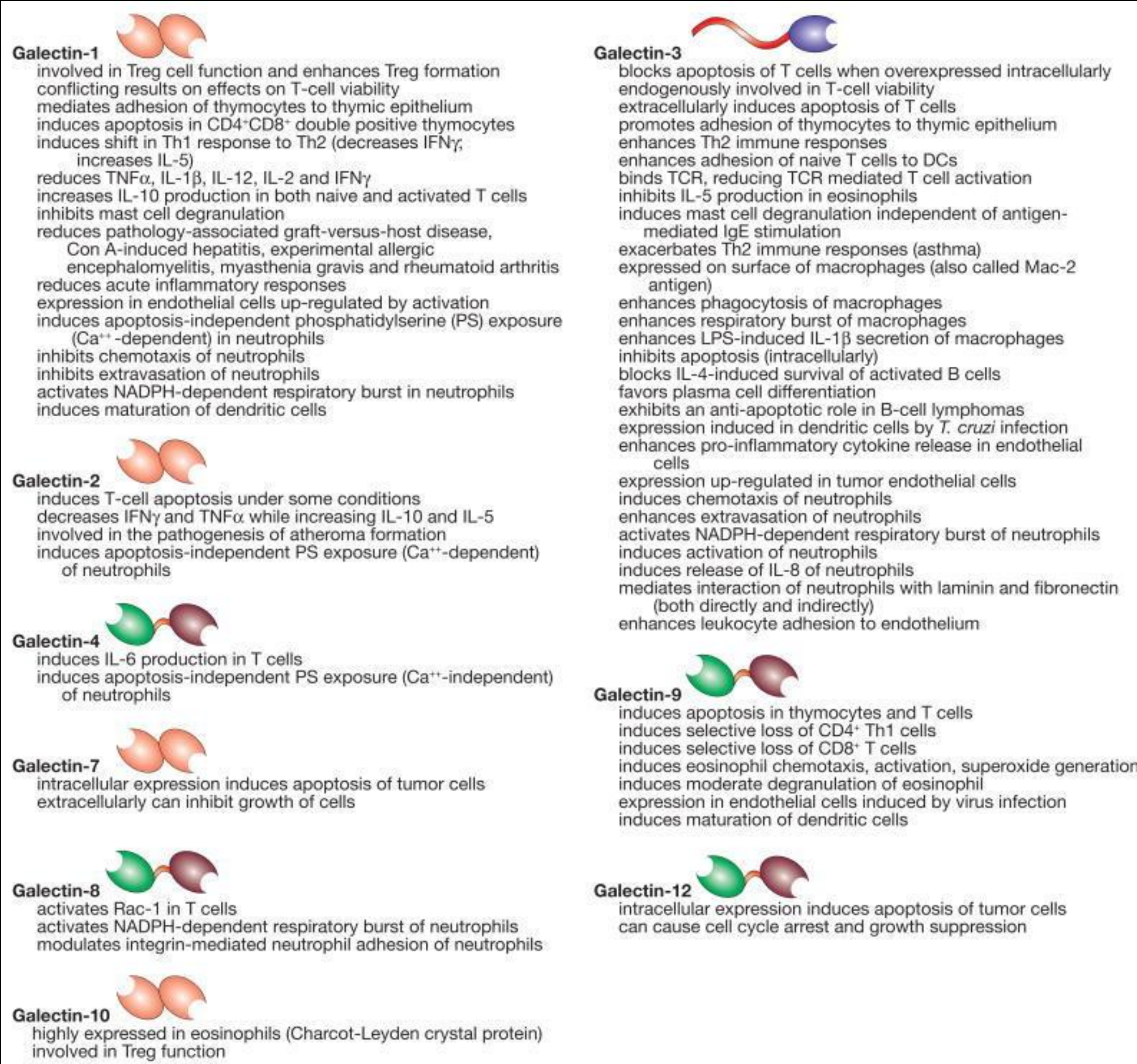
The first suggestions that galectins could play important roles in inflammatory processes were made for galectin–3, which was described as an antigen (Mac–2) expressed on the surface of thioglycollate–elicited peritoneal macrophages. Whatever their mechanisms of action at the molecular level, galectins are supposed to modulate inflammation acting in an autocrine or paracrine fashion.

Participation of galectins in inflammation is not limited to adhesion and migration and could be extended to the regulation of the respiratory burst and leukocyte chemotaxis.

Galectin–3 has been reported to activate the NADPH oxidase and stimulate superoxide production from peripheral blood neutrophils, but only if they were pre–treated with cytochalasin B, which facilitated degranulation by disrupting the microfilament system. (Yamacka et al., 1995). Galectin–3 knock-out mice were also evaluated to test the effect of gene targeting toward an inflammatory challenge in a model of acute peritonitis. Four days after thioglycollate injection, galectin–3 mutant mice exhibited a reduced number of granulocytes compared to wild–type mice, suggesting that this protein could be involved in the control of acute inflammation in vivo. In the context of newly discovered galectins, ecalectin, a variant of human galectin–9, has been recently proposed as a novel eosinophil

chemoattractant produced by T lymphocytes. This is particularly important in terms of inflammatory diseases mediated by eosinophil accumulation, such as bronchial asthma, rhinitis and atopic dermatitis.

A list of known and putative functions and biological activities of galectins. From Cummings and Liu Essentials of Glycobiology. 2nd edition.2009



1.8. Lectins studied in this thesis

1.8.1. Galectin–1

Galectin–1 is a non–covalent homodimeric galectin, with a 14 kDa monomer which contains one CRD (dimeric type galectin). Galectin–1 preferentially recognizes Galb1,4GlcNAc (LacNAc) sequences that can be present on N– or O–linked glycans. Galectin–1 is present both extracellularly and intracellularly (André et al., 1999). It is also found at the cell surface because secreted galectin–1 binds to lactosamines on glycoconjugates expressed by the same cell or by neighbouring cells. Galectin–1 can be characterised as a truly matricellular protein which serves as an adapter between ECM modulating cell–cell and cell–ECM adhesion, migration, proliferation, and apoptosis. Sometimes, 'pro– or anti–effects' of galectin–1 (i.e. adhesive/anti–adhesive; proliferative/antiproliferative) are observed and this depends on the cell type, cell activation status, relative cell surface expression and glycosilation of particular receptors.

1.8.2. Galectin–3

In contrast to galectin–1, galectin–3 is found in solution as a monomer with two functional domains. C–terminus is the carbohydrate-binding domain which defines the molecule as a galectin. N–terminus contains tandem repeats of nine amino acids. Human galectin–3 is 35-kDa protein coded by a single gene, LGALS3, located on chromosome 14. Because of the presence of an N–terminal domain adjacent to the CRD, galectin–3 is classified as a chimera-type galectin. Galectin–3, like most members of the galectin family, binds glycoconjugates containing N–acetyllactosamine, but its affinity toward ligands is modulated by the presence of additional saccharides in proximity to the galactose residue. Differential recognition of cell surface glycans by different galectins correlates well with their distinct biological and signalling activities. Although galectin–3 exists as a monomer in solution, it can self–associate through intermolecular interactions involving the N–terminal domain when bound to a multivalent ligand and, therefore, can mediate crosslinking of glycoproteins. The effects of galectin–3 are complex; intracellular forms typically protect cells against apoptosis through carbohydrate–independent mechanisms. Extracellularly, the lectin mediates cell–cell and cell–matrix interactions and promotes apoptosis by binding to lactosamine–containing cell surface glycoconjugates via the CRD. In recent years, several studies have revealed that galectin–3, by the binding and cross–linking glycans on cell surface receptors, modulates signal transduction by novel carbohydrate–based recognition systems (Hsu et al., 2000). For

example, galectin–3 promotes corneal epithelial cell migration by cross–linking complex N-glycans on α3β1 integrin, and inducing lamellipodia formation by activating the α3β1 integrin–Rac1 signaling pathway, modulates VEGF– and bFGF–mediated angiogenesis by binding, via its CRD, to N–glycans on integrin αvβ3. Additionally, galectin–3, through interactions with mucin O–glycans at the apical membrane of corneal epithelial cells, forms a cell surface lattice important to the barrier function of the ocular surface (Jerome et al., 2012). In addition, galectin–3 has been detected in conjunctiva, trabecular meshwork, retina, and in the lens where it plays a role in cell differentiation and adhesion of fiber cells. Several studies have implicated galectin–3 in disease processes (Panjwani et al., 1986). It has been demonstrated that the rate of re–epithelialization of corneal wounds is significantly reduced in Gal3$^{-/-}$mice compared to Gal3$^{+/+}$mice, and that the lectin plays a key role in integrin signalling that modulates wound closure following injury (Yabuta et al., 2014). Interestingly, the glycosyltransferases involved in the biosynthesis of galectin–3 counter receptors are altered in dry eye and wound healing, supporting the concept that disruption of galectin-ligand interactions leads to ocular surface epithelial dysfunction.

Also, studies aimed at determining whether exogenous galectin–3 can be used to treat patients with nonhealing epithelial defects, dry eye, and chronic inflammation are likely to prove to be rewarding.

Since galectin–3 is expressed by the human corneal epithelium and binds lipopolysaccharides purified from *Pseudomonas aeruginosa,* as was demonstrated by the multiple inhibition assays (Gupta et al., 1997), the participation of galectin–3 in eye surface biology is likely and its role as a member of the multiple adhesion family can be expected. The glycoconjugates represent the important component of the cell surface and no data about galectin–3-reactive glycoligands on the ocular surface epithelia are available. This knowledge is important for a rational explanation of the role of this endogenous lectin in eye physiology and pathology. Thus, we investigated the occurrence of this protein in the cornea, conjunctiva, and tears using a specific antibody.

1.8.3. Galectin–7

Galectin–7 is a member of a family of proteins with affinity for β–galactosidase–containing oligosaccharides. Galactin–7 contains one carbohydrate recognition domain (CRD) in its biological structure and functions as a homodimer. In contrast to other galectins such as galectin–1 and –3, the function of galectin–7 is still largely unknown. It is known to interact

with a wide range of potential receptors, including non–reducing terminal LacNac residues and internal LacNac oligosaccharide residues, but its carbohydrate binding affinity is weaker than that of galectin–1 and –3. It has been suggested that the function of galectin–7 may vary according to its cellular localization because the protein is present in the nucleus and the cytoplasm, and also in the cell–to–cell contact region (Saussez et al., 2006).
Over the last two decades researches have shown that galectin–7 is associated with the differentiation and development of epithelia, including epithelial cell migration and epidermal and corneal wound healing (Cao et al., 2003). The regulation of apoptosis induction by galectin–7 also has been studied. In addition, there are several reports of a role of galectin–7 in cancer development.

1.8.4. Dolichos biflorus agglutinin

Dolichos biflorus agglutinin (DBA) is a plant lectin with a molecular weight of about 111 kDa and consists of 4 subunits of approximately equal size. This lectin has a carbohydrate specificity toward α–linked N–acetylgalactosamine. It has been used to establish secretor status in blood group A individuals by hemagglutination inhibition techniques and for blood typing (Hormia et al., 1988). This lectin has also been used as a general marker of developing renal collecting ducts.
The cell glycophenotype sensitively reflects during the cell differentiation process. This can be used in diagnostic procedures (Gabius et al., 1997). According to some authors, basal layer cells of the epidermis are highly reactive for the plant lectin DBA (Kariniemi et al., 1989). In this study we try to characterize the DBA–binding cells by a procedure of double labelling.

1.9. Galili antigen

The potential of corneal xenotransplantation

There are many similarities between pigs and humans in regard to anatomy and physiology (Doughty et al., 2000, Lee et al., 2006). Pigs represent an acceptable choice as an alternative source of organs for humans (Faber et al., 2008). Immunologically, however, the pig is less desirable than a nonhuman primate as a source because of the genetic distance between pigs and humans. However, because the cornea is an immune–privileged tissue and is not

immediately vascularized, its fate as a xenograft is likely to be better than that of organ xenografts.

Human natural anti–α–galaktoside IgG

Human natural antibodies against Galα1,3Gal–R are mainly responsible for hyperacute rejection of xenografts transplanted to the human host. It is well known that approximately one percent of the circulating human IgG is directed against galactosyl epitopes of general structures Galα1,3Gal–R, the so called Galili antigen, which occurs in mammals except Old World monkeys, apes and humans (Galili et al., 1988a). When comparing natural antibodies to carbohydrate epitopes of A and B histoblood group antigens, these antibodies are not present in neonates. They can be detected after colonization of intestine with bacterial flora (Wiener, 1951). The titer of these natural antibodies significantly increases with bacterial and parasitic antigenic infection (Spinger et al., 1969). The abundance of the polyclonal antibody against α–Gal autoreactivity to human tissue was postulated to contribute to autoimmune diseases such as tyroiditis. In a different context, the presence of this carbohydrate antigen is crucial. Its presence on surfaces of animal endothelial cells represents the main barrier for the use of animal's (mainly porcine) organs in xenotransplantation (Bach et al., 1995).
Employing the human natural anti–α–Gal or β–Gal antibody fractions of the IgG class, we studied the presence of reactive carbohydrate epitopes in porcine and human epidermis and epithelium of the cornea of the human and porcine tissues and human tear fluid.

1.10. Lactoferrin

Lactoferrin, also known as lactotransferrin, is a multifunctional protein of the transferrin family. Lactoferrin is a globular glycoprotein with a molecular mass of about 80 kDa that is widely represented in various secretory fluids, such as milk, saliva, tears, and nasal secretions. Lactoferrin is also present in secondary granules of PMN and is secreted by some acinar cells. Lactoferrin can be purified from milk or produced recombinantly. Lactoferrin is one of the components of the immune system of the body; it has antimicrobial activity (bacteriocide, fungicide) and is part of the innate defence, mainly at mucoses. In particular, lactoferrin provides antibacterial activity to human infants.

1.11. Desmoglein

Desmosomes are adhesive cell junctions found in great abundance in tissues that experience mechanical stress. The transmembrane desmosomal glycoproteins have been proposed to play a role in cell adhesion. Desmoglein is a major member of this class of desmosomal molecules (Eshkind et al., 2002). Desmoglein is one of the glycoproteins found in the core of desmosomes, responsible for adhesive recognition between cells. The cells of the corneal epithelium exhibit numerous elements in their cell surfaces to ensure firm adhesion to neighbouring cells and the extracellular matrix below. Both desmosomes along the lateral cell surface and hemidesmosomes of basal cell surface serve as sites of reinforcement of adhesion as well as anchorage points for the intermediate filaments (Jones et al., 1994).

2. PURPOSE OF THE STUDY

- To study the expression of lectins, mainly endogenous galectins, and their glycoligands in normal and pathological human cornea, conjunctiva and tear fluid.

- To analyze the expression of DBA–reactive binding sites in conjunction with markers of cell proliferation and differentiation in normal human cornea, and as well as in cultured keratinocytes.

- To explore the presence of human natural anti–α–galactoside IgG in cells of porcine corneal epithelium and epidermis as a main barrier for xenotransplantation.

3. METHODS AND MATERIALS

3.1. Preparation of lacrimal glands, tear film, conjunctiva, cornea

Samples of normal cornea, conjunctiva and lacrimal gland were obtained post mortem from donors without eye problems. The conjunctiva of patients suffering from chronic conjunctival inflammation like ocular cicatrical pemphygoid and Stevens–Johnson syndrome was taken by biopsy. All samples were obtained after receiving the consent forms from the donors. The tear fluid samples (volume 5–12 µl) were collected by pipetman from normal, healthy people without applying any irritant (Table 3.1.). The same volume of pathological tears (bullous keratopathy n = 1, ocular manifestation of sarcoidosis n = 3, chronic blepharitis n = 1, toxic conjunctivitis n = 1, adenoviral conjunctivitis n = 1, pellucid marginal corneal degeneration n = 1, alkali burn of cornea treated with corticosteroids n = 1) was collected from patients as described.

The pig epidermis was harvested employing the punch biopsies from the highly keratinized areas such as foot and areas with lower keratinization with, or without, hairs of miniature pigs (breeding colony of the Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic) after local anesthesia.

Table 3.1. Characteristic of samples

Tissue	Number of samples
Normal human	
Conjunctiva	8
Cornea	25
Tear fluid	8
Pathologic human	
Conjunctiva	6
Tear fluid	9
Porcine lacrimal gland	2
Human lacrimal gland	3

Cornea, including limbal rims, conjunctiva and lacrimal gland were dissected and sections were embedded in Tissue–Tek (Sakura–Finetek Europe BV Zoeterwoude, Netherlands) and frozen by immersion in liquid nitrogen–cooled 2–methyl butane. Ten–micrometer sections were then cut at –20 °C in cryostat and deposited on slides. The sections were either processed immediately or stored at –70 °C. Next steps differed according to chosen histochemical methods.

3.2. Tear sample cytology

The tear drops containing cells (received as described above) were smeared on the surface of supporting glass and processed for the detection of galectin–3. The cell types present in tear fluid were evaluated according to characteristic morphological features.

3.3. Histochemical methods

We used the following main methods during experimental work:

3.3.1. Lectin histochemical method for investigation of lectin binding sites

The specific property of lectins – their affinity to specific terminal sugars or oligomers in complex carbohydrates – makes them valuable probes for studying glycoconjugate in corneal and conjunctival epithelium. It is an immunohistochemic-like reaction, where the interaction between the lectin and specified terminal sugar is very similar to antibody–antigen reaction. During experimental work we used endogenous and plant lectins.

For the detection of lectins binding site, the sections were washed with phosphate–buffered saline (PBS), fixed with 2 % paraformaldehyde in PBS for 5 min. After 10 minutes slides were washed with a large volume of PBS and for 5 minutes with absolute aceton. Nonspecific binding sites were blocked by incubation in 0.1 % bovine serum albumin for 60 min. Blocked sections were overlaid for 90 minutes with byotinilated lectin in 0.1 % bovine serum albumin at room temperature. The slides were washed three times every 5 minutes, the sections were overlaid with ExtrAvidin–TRITC (Sigma, St. Luis, USA), diluted 1:200 in PBS for 30 minutes.

3.3.2. Reverse lectin histochemistry for detection of neoglykoconjugates

The search for the probes suitable to study the expression of endogenous lectins and their involvement in different biological processes has led to the construction of neoglykoconjugates (Lee et al., 1997, Schmidt, 1997, Gabius, 2001). They are synthetically produced conjugate saccharide chains bound via tyrosine or lysine on the protein carrier, which is bovine serum albumin (BSA). This is generally labelled with biotin. Another variant is biotin connected directly to produced oligosaccharide or carbohydrate conjugation with biotinylated poly–2–hydroxyethyl–acrylamide (PAA). Neoglykoconjugates were synthesized by Prof. H.–J. Gabius (Institute of Physiological Biochemistry, Faculty of Veterinary Medicine, Ludwig–Maximilians University, Munich, Germany) and Prof. N.V. Bovin (Shemjakin and Ovcinikov Institute of Biochemistry and Organic Chemistry, Moscow, Russia).

3.3.3. Double labelling at one cell level to analyse two characters in one image and thus to assess their possible colocalization

The double labelling at one cell level technique is based on using two differently marked reagents. Using specific fluorescence filter makes it possible to analyse two characters in one image and thus to assess their possible colocalization (Lukas et al., 1997). Another frequently used procedure in this study was the examination of lectin binding site, mainly galectin, in correlation with other biological reagents. Lectin was labelled with biotyn and in the second step avidin–conjugated fluorophore TRITC (red signal) was applied. The second analysed reagent was in the first step detected by antibodies of animal origin, while in the second step avidin labelled with FITC (green signal) was applied. When both signals overlapped, yellow colour was observed.

3.3.4. Indirect immunohistochemical analysis of tissue for the detection of galectins and galili antigen in the cornea, conjunctiva, lacrimal gland

The specimens were stained for detection of galectin–1, –3, –7 with a porcine polyclonal antibodies produced by Prof. H.-J. Gabius. The desmosomal protein desmoglein was detected by using a commercial monoclonal antibody (Progen, Heidelberg, Germany). The cytokeratins in the lacrimal gland were detected by the monoclonal antibody LP–34 (Dako, Glostrup, Denmark) recognising a wide panel of cytokeratin types. The α– and β–

Gal-containing glycoepitopes were visualized with biotinylated human natural antibodies of the IgG class. FITC conjugated swine anti–mouse antibody (SwAM–FITC, Temda, Prague, Czech Republic) or swine anti–rabbit antibody (SwAR–FITC, Temda, Prague, Czech Republic) diluted 1:10 was employed as the second-step reagent. When the peroxidase labelled second step antibody was used, the Sigma Fast system (Sigma, Prague, Czech Republic) with the diaminobenzidine tetrahydrochloride as substrate was employed to visualise the localization of the bound antibody. To assess the masking effect of N-acetyl-neuraminic acid at the terminal position of oligosaccharide chains on antibody binding, parts of sections were pretreated with neoaminidase applied at a dilution 1:100 for 12 hours at 37 °C.
Antibodies in the second step were conjugated by FITC – fluorescein before examination under fluorescence microscope for detection of signals – green or labelled with peroxidase with the diaminobenzidine tetrahydrochloride as substrate to visualise the localization of the bound antibody.

3.3.5. Western blotting to analyse the presence of different proteins such as lactoferrine, galectins, galili antigen in tear fluid

- Detection of galectin–1, –3
- Detection of α–galactosides
- Detection of lactoferrin

The cells (if any) were separated from tear fluid by low rate centrifugation (minicentrifuge Qualitron, Sigma, Prague, Czech Republic). The samples were then stored at −20 °C. Upon analysis, samples were combined with a sample buffer, denatured for 3 minutes at 100 °C, and centrifuged for 10 minutes at 16 000 g. Supernatants (12 µl of each sample) were then resolved by 14 % SDS–PAGE (Laemmli, 1970). After electrophoresis, the resolved proteins were transferred to a nitrocellulose membrane, and a western blot analysis was performed (Harlow et al., 1988). Galectin–1 and –3 were detected with an IgG 14kDa rabbit polyclonal antibody and rabbit polyclonal antibody (dilution 1:500), respectively and with peroxidase conjugated goat anti–rabbit IgG (Cappel Research Products, USA; dilution 1:5000) using enhanced chemiluminescence (ECL, Amersham, Pharmacia, Bioteck, Freiburg, Germany). Extract of cells from mouse macrophage line J774 GB producing galectin –3 were used as a positive control. In addition, a monoclonal mouse anti–galectin–3 antibody, A1D6 (Liu et al.,

1996), (dilution 1:150), a secondary antibody SwAM–Px (Temda, Prague, Czech Republic; dilution 1:200 000), and enhanced chemiluminescence (SuperSignal West Femto) were used in a separate western blot.

For the detection of α–galactoside the membrane was incubated with a solution containing biotynilated human anti–α–Gal antibody (Dong et al., 1997) in a blocking buffer (1:500). After thorough washing with 0.05 % Tween–20 in PBS, the membrane was incubated with a solution containing peroxidase–labelled ExtrAvidin (Sigma, Prague, Czech Republic) diluted with blocking buffer (1:500), washed, and detected by chemiluminescence (ECL kit, Amersham, PPG Zlín, Czech Republic)

Competitive inhibition

Melibiose (Gal α1,6GLc:ICN, StarLab, Prague, Czech Republic) at a concentration of 10–40 mM added to a diluted antibody was employed as a competitive inhibitor in immunochistochemical as well western blot experiments.

Western blotting was performed as described above using rabbit polyclonal anti–human lactoferrin (Sigma, Prague, Czech Republic) diluted 1:50 to detect glycoproteins in the samples.

3.4. Cell culture of corneal human epithelium, porcine foetal keratinocytes and epidermal human cells

The culture of human epithelium was performed as described by BenEzra D. (1986)

The porcine foetal keratinocytes harvested from foetus on 90th day of pregnancy were cultured on the surface of histological coverslips using the feeder cells–murine 3T3 fibroblast with mitosis blocked by mitomycin C pretreatment (Sigma, Prague, Czech Republic) (Green et al., 1979, Dvořánková et al., 1996).

The adult epidermal human cells were cultured by the same procedure (Green et al., 1979, Dvořánková et al., 1996).

4. Results according to attached publications

4.1 ***Hrdličková–Cela E**, Plzák J, Smetana K Jr, Mělková Z, Kaltner H, Filipec M, Liu FT, Gabius HJ. Detection of galectin–3 in tear fluid at disease states and immunohistochemical and lectin histochemical analysis in human corneal and conjunctival epithelium. Br J Ophthalmol. 2001 Nov;85(11):1336–40 (**IF 2.902**)*

Although galectin–3 is known to be secreted by various cells *in vitro*, in contrast to patients with ocular surface inflammation, no galectin–3 was found in tears harvested from healthy volunteers. Concerning expression of galectin–3, it was found in corneal and conjunctival epithelium in normal eye, in goblet cells and leukocytes isolated from tears harvested from the galectin–3–positive eyes suffering from inflammation. Inflammatory cells infiltrating the conjunctival stroma from Steven–Johnson patient were also highly positive for galectin–3. The lacrimal gland expressed no galectin–3 and therefore this gland is not a likely source of this lectin in the tear film. The non–pathological corneal as well as conjunctival epithelium expressed galectin–3, but the tears harvested from healthy volunteers contained no galectin–3. To infer presence of binding sites for the endogenous lectins, we prepared biotinylated protein instead of a galactoside–binding plant agglutinin, because carbohydrate fine specificities of two lectins can differ. Hypothetically, galectin–3–binding sites expressed in both the corneal and conjunctival epithelium appear to immobilize produced galectin–3 exported from the cell to the cell surface. This phenomenon might be one of explanations of the absence of galectin–3 in tear film in healthy persons. This phenomenon could be of general relevance, because squamous epithelia, such as epidermis and oral mucosa, express galectin–3 and galectin–3–reactive glycoligands in a similar pattern (Holíková et al., 1999, Plzák et al., 2000). It should be noted that it is necessary to test the tissue endogenous lectin instead of a plant protein as a marker to reach this conclusion. The colocalization of galectin-3-reactive glycoligands with desmoglein suggests a participation of this endogenous lectin in intercellular contacts of the desmosomal type in the studied epithelia. However, this result must be verified. The irregular pattern of the accessible galectin–3–reactive glycoligands in conjunctiva can reflect the non–uniform distribution of these ligands in the conjunctiva or partial inaccessibility due to occupancy of some ligands by the endogenous lectin. This observation together with the ability of galectin–3 produced by corneal epithelial cells to immobilize bacterial lipopolysaccharides suggests a role for galectin–3 in the control mechanisms of the eye surface integrity and protection. In addition to epithelial cells, inflammatory cells such as polymorphonuclear leukocytes and macrophages are also known

as producers of galectin–3 (Truong et al., 1993, Liu et al., 1995). These cells may be a source of galectin–3 in the tear film under pathological conditions.

In conclusion, this study shows the presence of galectin–3 in the tear film in pathological eyes and reveals a difference from the normal condition. This, together with initial monitoring of the lectin and binding site by immunohistochemistry and lectin histochemistry respectively, should elucidate the functional role of galectin at this localization.

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4.2 ***Hrdličková–Cela E**, Plzák J, Holíková Z, Dvořánková B, Smetana K Jr. Postmitotic basal cells in squamous cell epithelia are identified with Dolichos biflorus agglutinin – functional consequences. APMIS. 2001 Oct;109(10):714–20. (**IF 1.991**)*

Expression of DBA binding sites (DBA–BS) was observed partially in the basal cell layer of the corneal epithelium. DBA-binding cells were in intimate contact with the basement membrane. The most usual location of DBA–BS in cultured cells from the epithelium of the cornea was in the Golgi complex with a polarized appearance and granular in the cytoplasm. The cultured confluent keratocytes exhibited expression of DBA–BS at the stage of beginning cell multilayer formation and colocalization with galectin–3 and –7. The results,

such as the absence of a signal for Ki–67 in DBA–BS positive cells and colocalization of cytokeratin 3 with DBA–BS in the limbus of human cornea, clearly support the hypothesis that the DBA–BS-positive element can be characterized as early post mitotic differentiating cells. The basal expression of DBA–BS was also observed by others, but the procedure of multiple labelling characterizes this cell population more precisely. The high expression of DBA–BS in this well–defined population of corneal basal cells can be interpreted by the differentiation–dependent glycolisation. This corresponds to our previous findings (Plzák et al., 2000), which demonstrated the absence of galectin–3 reactive binding site expression in the basal cell layer, although the supra basal cells were well recognized by this animal lectin. From a practical point of view, the DBA as a probe recognizes a distinct population of post mitotic cells, which are at the beginning of their differential route. Because this probe is a lectin, it can be used in a simple procedure for double labelling together with an antibody.

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4.3 ***Hrdličková–Cela E**, Smetana K Jr, Plzák J, Holíková Z, André S, Hřebíček M, Hodanová K, Dvořánková B, Motík J, Gabius HJ. Cells of porcine epidermis and corneal epithelium are not recognized by human natural anti–alpha–galactoside IgG.. Folia Biol (Praha). 2001;47(6):200–5. (**IF 1.151**)*

Human natural antibodies against Galα1,3Gal-R are mainly responsible for hyperacute rejection of xenografts transplanted to the human host. In addition to the anti-α-Gal activity, human serum also contains anti-β-Gal IgG fractions. Employing biotynilated IgG

subfractions with anti-α and anti β-Gal activity purified from human natural IgG, we have studied expression of reactive epitopes in porcine and human skin, porcine cultured keratinocytes and porcine and human cornea, porcine liver and human lacrimal gland, tear fluid and capillaries. The cells of porcine epidermis including cultured epidermal cells and anterior epithelium of the cornea were not reactive for anti–α–Gal using labelled natural human IgG without and after neuraminidase pretreatment. The possibility of false negativity of this observation, e.g. due to a lack of probe activity, could be excluded with a positive signal of the marker binding to porcine endothelium and liver cells, which are known as carriers of the Galili antigen, the docking epitope for anti–α–Gal antibodies (Vaughan et al., 1994). Moreover, the reactivity of the anti–β–Gal antibody fraction in human and pig epidermal cells underscores the absence of anti–α–Gal reactivity in these cells. The accessibility of sugar epitopes for anti–β–Gal antibodies in the epithelium of porcine vessels was greatly improved by neuraminidase pretreatment, corroborating recent data published by Lucq et al., 2000.

The human lacrimal gland expressed both studied glycoepitopes, i.e. α– and β–Gal reactive with human natural antibodies. α–Gal–containing glycoproteins can evidently be secreted into tear fluid. Since the antibody reactivity was significantly inhibited with the competitive sugar melibiose, the carbohydrate specificity of the reaction within the immune recognition of α–Gal was clearly ascertained. This observation is supported by previous work noting α–Gal-containing deposits on contact lens surface by lectin histochemistry (Klotz et al., 1987). The molecular weight of band(s) positive for α–Gal presentation corresponded to that of tear lactoferrin or products of its enzymatic digestion (Kuizenga et al., 1991, Vorland, 1999). The presence of IgG–reactive α–Gal in tear fluid of healthy volunteers indicates the physiological occurrence of these glycoepitopes in healthy people with no signs of an autoimmune disorder. The functional consequences of the α–Gal presence in tear fluid are not yet clear but a protective role blocking bacterial adhesion to the eye surface could be of considerable significance. In line with this assumption, the complex mixture of milk oligosaccharides has been inferred to inhibit the docking of pathogenic bacteria to the susceptible cells (Kunz and Rudolf, 1993, Nascimento and Giogliano 2000). Lactoferrin as well as lactalbumin also exert bactericidal activity (Ellison et al., 1988, Hakansson et al., 2000). Moreover, material from a patient with chronically inflamed eye surface contained no band recognized by human natural anti–α–Gal antibody in the same position as that of healthy donors. Our preliminary studies revealed the absence of galectin–3 in the tear fluid

from people without eye problems and a high content of this lipopolysaccharide–binding lectin in tear fluid from inflamed eyes. α–Gal–containing glycoprotein was detected in tear fluid from normal eyes and not in tear samples from inflamed eyes. This result points to the possibility of an interaction of α–Gal with galectin–3. However, the preincubation of lacrimal gland secretion with label–free galectin–3 for epitope masking had no inhibitory effect on anti–α–Gal binding to lacrimal cells. Further explanation could be the absence of α–Gal in these individual donors or the breakdown of an anti–α–Gal reactive epitope by glycosidases produced by pathogens. Concerning cellular reactivity, the porcine corneal epithelium was negative for Galα1,3Gal structures, which are known to be abundantly expressed on cells of non–primate grafts, consequently causing an immunological barrier between humans or other Old World primates and non–primate mammals and preventing xenografting. These findings raise the question whether it might be possible to use pig cornea and the epithelial cell layer in clinical medicine, as viewed from the perspective of α–Gal.

Table 4.5. Expression of anti–α, β–Gal (Abbreviations: + positive, – negative.)

Tissue	anti–α–Gal	anti–β–Gal
Human Cornea epithelium	–	–
Porcine Cornea epithelium	–	+
Human lacrimal gland	+	+
Human tear fluid	+	
Human epidermis	–	+/–
Porcine epidermis	+	+
Porcine liver	+	+
Porcine foetal keratinocyte culture	–	
Porcine dermal capillaries	+	+

In conclusion, this study demonstrated the absence of α–Gal epitopes (so–called Galili antigen) in porcine epidermal cells and corneal epithelium. This result is an essential step to testing porcine epidermal cells in the development of non–permanent tissue–engineered devices improving the healing process of skin defects. The presence of α–Gal in human tear fluid adds the evidence that α–Gal could be present in human glycoproteins, as seen in

human tumour samples or inflammation (Bjerrun and Schafer–Nielsen, 1986, Tremont–Lukats et al., 1996, Kayser et al., 2000), probably as a product of aberrant galactosylation or glycolytic degradation.

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4.4 *Plzák J, Smetana K Jr, **Hrdlicková E**, Kodet R, Holíková Z, Liu FT, Dvoránkova B, Kaltner H, Betka J, Gabius HJ. Expression of galectin–3–reactive ligands in squamous cancer and normal epithelial cells as a marker of differentiation. Int J Oncol. 2001 Jul;19(1):59–64. (**IF 2.399**)*

The definition of biological markers for oropharynx and larynx cancer is essential to predict their clinical behaviour. Since cellular glycans play an important role in biological information transfer, we have employed an endogenous lectin, galectin-3, to examine in primary squamous carcinomas, lymph node metastases, and physiological squamous epithelia whether glycans recognized by this lectin are altered in relation to the state of differentiation. The expression of galectin-3 was concomitantly evaluated by immunohistochemistry using the A1D6 monoclonal antibody. In addition, other antibodies were used for the detection of cytokeratins and desmosomal proteins (desmoplakin-1 and desmoglein). The results show the expression of galectin-3-reactive ligands in moderately/highly differentiated carcinomas only in areas exhibiting a high level of keratinization. Except for one patient out of 14, metastatic cells in lymph nodes expressed no accessible binding sites for galectin-3. No galectin-3-reactivity was detected in the basal cell layer of all studied normal epithelia (which contain the proliferating cells). The suprabasal layers were positive in epidermis and epithelium of tongue and cornea and negative in epithelium of palatine tonsil. The tumour cells expressed galectin-3 with an intensity positively correlated with tumour differentiation. The position of galectin-3-reactive sites colocalized with the two tested desmosomal proteins. However, presence of

these proteins was also detected in areas of tumour and suprabasal layers of tonsil epithelium where no binding reactivity for galectin-3 was found. The present study showed that expression of galectin-3-reactive glycoligands is differentiation-dependent in normal as well as malignant squamous cells. Colocalization of galectin-3-reactive sites with desmosomal proteins (desmoplakin-1 and desmoglein) suggests an association of the galectin-3 ligand(s) with the cell surface, pointing to a potential participation of galectin-3 in mediation of intercellular contacts in these tumour types.

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A thorough characterization of the properties of squamous epithelial cells is necessary in order to improve our understanding of the functional aspects of normal development and malignant aberrations. Up to now, studies have focused almost exclusively on monitoring distinct protein markers. With our growing awareness of the coding function of glycan chains of cellular glycoconjugates and their interaction with receptors (lectins) in situ, defining the glycophenotype of these cells has become an important issue. Whereas the commonly applied plant lectins are tools used to map the presence and localization of biochemically defined saccharide epitopes, the introduction of endogenous (mammalian) lectins to this analysis enables us to take the step from monitoring the presence of glycans to understanding the functional implications by revealing ligand properties of the detected epitope for tissue lectin. Thus, in this study we investigated a distinct aspect of glycosylation using plant and mammalian lectins, i.e. the linkage type of sialylation. We first mapped the expression profile of the type of sialylation (alpha2,3- or alpha2,6-linked) by plant lectins. Based on the hypothesis that this factor regulates accessibility of ligands for endogenous lectins we introduced two labelled galectins to this study. Galectin-3 (but not galectin-1) binding was related to cell differentiation in normal adult and developing epithelia, cultured epidermal cells, and carcinomas derived from these epithelia. The presented data suggest that alpha 2,6-linked N-acetyl-D-neuraminic acid moieties could serve to mask galectin-3-reactive glycoepitopes. As a consequence, monitoring of the linkage type of sialic acid in glycans by plant lectins therefore has implications for the extent of glycan reactivity with endogenous lectins, pointing to a potential function of changes in sialylation type beyond these cell and lectin systems.

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Lectins represent one of pivotal regulators of the cell proliferation. The potential of galectin-7 as a new prognostic marker was studied in normal and transformed squamous epithelia of both ectodermal (epidermis, cornea, vs. trichoepithelioma, basal and squamous cell carcinoma) and endodermal (vocal fold epithelium vs. carcinoma) origin. Studies on the cultured cells were also performed. Expression of galectin-7 seems to be connected to the process of stratification, notwithstanding the origin of epithelium. Its expression is

significantly reduced in malignant cells, thus galectin-7 might be a differentiation marker of epithelial malignancies.

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The study extends the previously reported profiling of Gal-1 and -7 presence of normal human cornea measuring the effect of a disease state (Acanthamoeba keratitis-AK) and a treatment modality (amnion membrane transplantation-AMT). Interestingly, in murine cornea infected by *Pseudomonas aeruginosa* or cauterized by silver nitrate stromal positivity of both galectins increased. Corneal stromal sheets contained significantly increased quantities of both proteins when detected by western blotting. In human specimen, the most prominent differences reaching statistical significance were a signal decrease for Gal-1 in corneal epithelium in AK+AMT specimen and for Gal-7 in AK specimen. Obviously, the levels of the two galectins were affected, and this in opposite directions. Because Gal-7 is known to favour re-epithelialization of corneal wounds, in contrast to Gal-1, the stimulation of re-expression by AMT can be considered as potentially advantageous. The noted Gal-1 decrease in the epithelium upon AMT may lead to consider external supplementation, in view of its effectivity to suppress bacterially induced corneal immunopathology. Gal-1, in different contexts, is present in granulation tissue of skin wounds and common to tumour stroma, e.g. of head and neck squamous cell carcinomas. Of note, its ability to generate cancer-associated fibroblasts with reprogrammed gene expression profiles and to favour wound healing but also tumour growth and spread qualifies Gal-1 to an important mediator of the local microenvironment. However, to trigger the effects, counterreceptors for the lectin must be presented locally. An orchestration of presentation of the receptor and its binding partners, even involving downregulation of an antagonist, will thus establish the prerequisites for bioactivity, as delineated in the case of tumour growth control by a suppressor. This

physiological interplay directs attention not only to extending galectin fingerprinting but also to monitoring availability of binding sites for galectin. In aggregate, our pilot study reveals an impact of AMT on galectin expression, obviously with opposite effects on Gal-1 and -7. Based on the known presence of other members of this family of adhesion/growth-regulatory galectins a detailed profiling including measurement of reactivity using labelled galectins as probes is warranted.

5. DISCUSSION

The results presented in this thesis demonstrate the important role of glycobiological approach in ophthalmological research.

Glycosylation is an important and common form of posttranscriptional modification of proteins in cells and carbohydrates were established as medium for storage of secondary biological information (Pablo 2013, Solís et al., 2015). Biological functions have been ascribed to glycans during the last decade thanks to a rapid evolution in glycomic technologies. Glycans on the cell surface glycocalyx and on secreted proteins modulate a wide variety of cell–cell, cell—pathogen, and cell–matrix events critical to the function of a multicellular organism and its interaction with the extracellular environment. Genes related to carbohydrate synthesis are highly expressed at the human ocular surface, and include families of glycosyltransferases, proteoglycans, glycan degradation proteins, as well as mucins and carbohydrate-binding proteins such as galectins (Pablo et al., 2013).

A critical finding in our research was the identification of galectin-3 and -7 as the most highly expressed carbohydrate-binding protein in human corneal and conjunctival epithelia. Oligomerization of galectin-3 occurs on cell surfaces in physiological concentrations of the lectin, resulting in galectin-3 lattices that are robust and resistant to lateral movement of membrane components on the glycocalyx (Nieminen et al., 2007).

The interaction of mucin O-glycans with galectin-3 would result in a highly organized and protective cell surface lattice barrier on the apical glycocalyx of ocular surface epithelial cells. Galactose is a major component of ocular surface mucins and, therefore, could potentially act as a ligand for the carbohydrate-binding domain of galectin-3. According to literature, the knockdown of core 1 β1,3-galactosyltransferase, a critical galactosyltransferase required for the synthesis of core 1 O-glycans, resulted in decreased cell surface O-glycosylation, reduced

cell surface galectin-3, and increased corneal epithelial cell permeability (Argueso et al., 2009).

Another interesting finding was the colocalization of galectin-3 reactive glycoligands with desmoglein. This suggests a participation of this endogenous lectin in intracellular contacts of the desmosomal type in the corneal epithelium.

Overall, these results indicate that two barriers contribute to the protection of the ocular surface epithelia against noxious molecules and pathogens: 1st - the traditional paracellular barrier containing the tight junctions that seal the space between adjacent cells, and, 2nd - the transcellular barrier formed by the association of transmembrane mucins and galectin-3 on the extensive apical glycocalyx of the ocular surface epithelia.

The role of glycans in biological processes should not be ignored since large part of the picture is missing when proteins are being studied without their glycans, or with wrong glycans attached during production of recombinant proteins in non-native organisms, cell types or cellular environment. Glycan structures actually change in association with variations in cellular metabolism. Such glycan structural diversification is highly regulated by signals that control cell differentiation, normal physiology, and even neoplastic transformation. According to our results, the absence of a signal for Ki-67 in DBA-BS positive cells (including cells in malignant tumours) and colocalization of cytokeratin 3 with DBA-BS positive in the limbus of human cornea, clearly support the hypothesis that DBA-BS positive elements can be characterised as early postmitotic differentiating cells. In this study we observed that galectin-3 binding was related to cell differentiation in normal adult and developing epithelia, cultured epidermal cells, carcinomas derived from this epithelia (Holíková et al., 2002) and in carcinoma from oropharynx and larynx tissues (Plzák et al., 2001).

Proteins and glycans in the ocular tear film play an important role in maintaining the surface integrity of the cornea and conjunctiva. They are involved not only in the defence against microbial invasion of the eye, but also in maintaining tear film stability and lubrication between the eye and the eyelids. Tear protein composition may be altered during eye disease. Our results showed expression of galectin -3 and absence of α-Gal epitopes in tears harvested from patients with ocular inflammatory diseases and absence of galectin-3 together with presence of α-Gal epitopes in healthy eyes. Expression of lactoferrin did not depend on ocular surface inflammatory status (Hrdličková et al., 2002). Analysis of proteins in tear fluid is therefore valuable not only for diagnostic purposes but may also increase our knowledge of the pathogenesis of certain external ocular diseases. Of the various techniques used to

investigate the protein composition of human tears, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an excellent technique that can be done rapidly on a microscale. These proteins can be stained with periodic-acid Schiff or alcian blue, but these dyes require a relatively large amount of tear sample and provide little information concerning individual carbohydrates in the glycoproteins. In recent years, lectins were found to be excellent probes for histochemical and biochemical analysis of glycoproteins in various ocular tissues and fluids, including human tears. Lectins are available as biotin-conjugated probes, and after coupling to avidin-horse radish peroxidase, tear glycoproteins can be analysed with great sensitivity.

It is important to mention three major differences between tissue-associated O-glycans in conjunctiva and those found in tears (Guzman et al., 2002). First, the conjunctival epithelium contains core 2-based structures, including galactosyl core 2 and di α2-3 sialyl galactosyl core 2. Second, α2-3 sialyl core 1 is the predominant O-glycan in human conjunctival tissue, whereas in tears the prominent O-glycan is α2-6 sialyl core 1. And third, disialyl core 1 is present in conjunctival mucin but not in tears. These discrepancies could be due to several factors. First, Royle et al. (2002) evaluated O-linked glycans in high-molecular-weight fractions of mucin isolates from conjunctival tissue. Second, differences in cellular trafficking may influence the O-glycosylation profiles in mucins. Third, it is also possible that specific mucin-type O-glycans in conjunctival tissue are associated with intracellular or cell surface glycoproteins and, therefore, are not secreted into the tear film. In our study galectin-3 binding sites were expressed both in corneal and conjunctival epithelium but not in the tear fluid (Hrdličková et al., 2002). And fourth, it is possible that the difference in O-glycan composition between conjunctival epithelium and tear film is due to degradation by glycosidase activity in tears (Matthews et al., 2001).

Expression of α-Gal epitopes was one of the main topics of the study. The α-Gal epitope is of major clinical significance. The α-Gal epitope (Galα1-3Galβ1-4GlcNAc-R, or Galα1-3Galβ1-3GlcNAc-R) is a unique carbohydrate structure that is absent in humans but is naturally produced on glycolipids and glycoproteins in non-primate mammals, prosimians and New World monkeys. Humans produce a natural antibody to this epitope. We observed a different pattern of α-Gal epitope expression compared to other works. We detected the distribution of α-Gal epitope in human lacrimal gland and in tear fluid in physiological condition, whereas (Galili et al., 2005) did not observe expression of α-Gal epitope in any human tissues or fluids.

6. SUMMARY

- The presence of galectin–3 in the tear film in pathological eyes reveals a difference to the normal condition. This, together with the initial monitoring of the lectin and binding site by immuno and lectin–histochemistry respectively, should prompt the elucidation of the functional role of this galectin at this location.
- Galectin–1 can play its biological function in ocular tissue and can be useful as a histochemical marker for further experimental studies.
- Absence of galectin–3 and –7 binding site in basal cell layer in limbal region might be useful as a negative marker for corneal epithelial stem cells.
- The results characterize the cells recognized by *Dolichos biflorus* agglutinin (DBA) as postmitotic early differentiating cells.
- The absence of α–Gal epitopes (so–called Galili antigen) in porcine epidermal cells is an essential step to testing porcine epidermal cells in the development of tissue-engineered devices improving the healing process of skin defects.
- α–Gal epitopes are absent in porcine corneal epithelium. These finding raise the question whether it might be possible to use pig cornea and the epithelial cell layer in clinical medicine. Among other barriers caution must be paid to retroviruses.
- The presence of α–Gal in human tear fluid adds the evidence that α–Gal could be present in human glycoproteins, as seen in human tumour samples or inflammation, probably as a product of aberrant galactosylation or glycolytic degradation.
- The new method of collecting tear film has the same efficacy as glass capillary tubes. It is nontraumatic and easy to perform. There is no need to use slit lamp. The method can be used for clinical and experimental purposes. The amount of tears that can be collected is around 70 µl and the samples of tears are not supposed to contain components from the conjunctiva.

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