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

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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 their localization in human corneal epithelium, lacrimal gland and tears.

Material and Methods: Human tissue (lacrimal gland, tear fluid, conjuctiva, 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 labeled (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 galaktosides 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 conjuntival epithelium. Galectin–3 was found in tears from patients with ocular surface disorders, in normal conjunctival and corneal epithelial, 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 conjuntival and corneal epithelium colocalizing with desmoglein. All cell layers of the corneal epitelium 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.

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 tohoto lektinu 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.

1. GENERAL INTRODUCTION

1.1. Ocular surface anatomy

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. 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 lubrification, protection from the 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 production is 1.2 µl / minute with a turnover rate of about 16 % per minute. Normal tear film contains 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).

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 to maintain 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. 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 cornea consists of three different cellular layers and two interfaces: the epithelium, Bowman's layer, the stroma, Descement's membrane, and the endothelium. The cell types that constitute the cornea include epithelial cells, keratocytes (corneal fibrobast), and endothelial cells. 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. Only the basal cell of the corneal epithelium proliferates. The differention 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 complexis between adjacent corneal

epithelial cells prevents the passage of agents into the deeper layers of the cornea. Tight junctions (zonula occludence) are present mostly between cells of superficial cell layers. Hemidesmosomes (zonula adherence) and desmosomes are present in all layers of corneal epithelium.Keratin 3/12 (64 –kDa keratin) is specifically expressed in the epithelium of the cornea, not being found in that of the conjunctiva or in the limbus (Kurpakus et al., 1990, Schermer et al., 1987). The stroma constitues about 90 % of the total corneal thickness in humans. It is composed of collagen-producing fibroblast cells (keratocytes), collagen lamellae. The corneal endothelium is derived from the neural crest and is therefore neuroectodermal. A single layer of mostly hexagonal cells forms the corneal endothelium. Mitosis of the endothelium seldom occurs in humans, and the overall number of endothelial cells decreases with age. In the abscence of mitosis, destruction of cells results in decresing density of cells and ultimately endothelial decompensation, edema, and clouding of the cornea.

Corneal epithelial cells renew continuously to maintain the normal layered structure of the epithelium.The existence of corneal epithelial stem cells at the limbus has 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 cell because they do not express corneal epithelium-specific keratin (keratin 3/12) (Daniels et al., 2006).

1.2. 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 non-immune origin, irrespective of source and blood type specificity (Sharon and Lis, 1972).

The nomenclature termin “lectin” was established 25 years ago. However the knowledge of lectins is far older.

The primary criterion for subdivision into categories was based on their monosaccharide specifity. 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.

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: ligand-selective molecular chaperones in endoplasmic reticulum, intracellular ruting of glycoproteins and vesicles, intracellular transport and extracellular assembly, recognition of foreign glycans, cell growth control and apoptosis, cell-cell interaction, cell-matrix interactions.

In the field of medicine, participation of lectins is shown in infections 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 the 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.

The doctoral thesis is focused mainly in the expression of the galectins and their glycoligands, and plant lectin such as *Dolichos biflorus* agglutinin binding site in the ocular surface tissue.

1.2. Galili antigen

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. This antigen occurs in mammals except Old World monkeys, apes and humans (Galili et al., 1988). The titer of this natural antibodies significantly increases in the present of bacterial/parasites antigen (Spinger et al., 1969). The abudance 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 presentation on surfaces of animal endothelial cells represents the main barrier for the use of animal, mainly porcine, organs in xenotransplantation (Bach et al., 1995). Employing the human natural anti α- or-β-Gal antibody fractions of the IgG class, we studied the presence of reactive

carbohydrate epitopes in human tear fluid and in porcin and human epidermis and the corneal epithelium.

1.4. 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 is one of the components of the immune system of the body, it has antimicrobial activity (bacteriocide, fungicide) and is part of the innate defense, mainly at mucoses. In particular, lactoferrin provides antibacterial activity to human infants.

1.5. 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. Desmoglein is one of the glycoproteins found in the core of desmosomes, responsible for adhesive recognition between cells.

2. PURPOSE OF THE STUDY

- To study the expression of lectins, mainly endogenou galectins, and their glycoligand in normal and pathological human cornea, conjuntiva 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 corneas, conjunctivas, and lacrimal gland were obtained post mortem from donors without eye problems. The conjunctiva of a patient suffering with 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 from normal, healthy people without applying any irritant. (Tab 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 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	
Conjuctiva	8
Cornea	25
Tear fluid	8
Pathologic human	
Conjuctiva	6
Tear fluid	9
Pig lacrimal gland	2
Human lacrimal gland	3

Corneas, 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 section were either processed immediately or stored at -70 °C. Next steps were differently procced according to the choosed histochemical methods.

3.2. Tear sample cytology

The tear drops containing cells 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. Histochemistry methods

During the experimental work we used these main methods:

***Lectin histochemistry method for investigation of lectin binding sites*:** The specific property of lectins, the affinity of specific terminal sugars or oligomers in complex carbohydrates, makes them valuable probes for studing glycoconjugate in corneal and conjunctival epithelium. It is an immunochistochemic-like reaction, where the interaction between the lectin and specified terminal sugar is very similar to antibody-antigen reaction. During the experimental work we used endogenous lectins (galectins -1, -3, -7) and plant lectin (Dolichos biflorus agglutinin).

Reverse lectin histochemistry for detection of neoglykokonjugates: 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). It is a synthetically produced conjugate sacharides chains bounded via tyrosine or lysine on the protein carrier, which is bovine serum albumin (BSA). This is generally labelled with biotyn. Another variant is biotyn connected directly on produced oligosaccharide or carbohydrate conjugation with biotinylated poly-2-hydroxyethyl-acrylamide (PAA). Neoglykokonjugates were synthesized in the workplace Prof. H.-J. Gabius (Institute of Physiological Biochemistry, Faculty of Veterinary Medicine, Ludwig-Maximilians University, Munich, Germany) and Prof. NV Bovina (Semjakinuv and Ovcinikovuv Department of Biochemistry and Organic Chemistry, Moscow, Russia).

***Double labelling at one cell level*:** Double labelling at one cell level technic is based on using two differently marked reagents. Using specific flourescence filter is then possible to analyse two characters in one image and thus to assess their possible colocalization (Lukas et al., 1997). Another abundant use of this procedure in this study was the examination of lectin binding site, mainly galectins, in correlation with other biological reagent. Lectin was labelled with biotyn and in the second step was applicated avidin-conjugated fluroforem TRITC (red signal). The second analysed reagent was in the first step detected by antibodies of animal origin, in the second

step was applicated avidin labelled with FITC (green signal). When both signals overlapped, yellow colour was observed.

***Indirect immunohistochemistry analysis*:** The specimens were stained for detection of galectin-1,-3,-7 with a monoclonal mouse anti-galectin-1,-3,-7 antibody. 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. If 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 localisation of the bound antibody. To assess the masking effect of N-acetyl-neuraminic acid at the terminal position of ologosaccharide chains on antibody binding, a part of sections were pretreated with neoaminidase applied at a dilution 1: 100 for 12 hours at 37 °C. Antibody in the second step were conjugated by FITC - fluorescein before examination under fluorescence microscope for detection of signal (green) or labeled with peroxidase with the diaminobenzidine tetrahydrochloride as substrate to visualise the localisation of the bound antibody.

Western blotting: was used to analyse the presense of different proteins as lactoferrine, galectins, galili antigen in tear fluid.

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 immunochistochemistry as well as in Western blot experiments.

3.4. Cell culture of corneal human epithelium, the porcin foetal keratynocytes, the epidermal human cells.

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

The porcin foetal keratynocytes were harvested from foetus of 90th day of pregnancy were cultured on the surface of histological coverslips using the feeder cells-murin 3T3 fibroblast with mitosis blocked by mitomycin C pretreatment (Sigma,

Prague, Czech Republic)- was performed by Dr. Dvořanková B. as described by Green et al, 1979, Dvořanková at al 1996.

<u>The epidermal human cells</u> were harvested from cultured cells on the surface of histological coverslips using the feeder cells-murin 3T3 fibroblast with mitosis blocked by mitomycin C pretreatment (Sigma, Prague, Czech Republic)- was performed by Dr. Dvořanková B. as described by Green et al., 1979, Dvořanková et al., 1996.

Detailed description of the methods used are mentioned in the publications in the apendics.

4. RESULTS

4.1. Expression of galectins and their binding sites in human ocular surface

Galectin-1 : We performed immunohistochemistry and histochemistry method for detection of Gal-1 and his binding site in conjuntival and corneal epithelium. The normal, human corneal epithelium (suprabasal and basal cell layer) showed intracellularly immunopositivity for Gal-1, whereas the conjunctival epithelium was negative. We next performed analysis of galectin 1 in tear fluid by Wester blotting procedure and we detected a strong immunopositivity of galectin -1 in tear film harvested from normal, healthy people. These results informed us about the possibility of an influence of this endogenous galectin in physiologic homeostasis of the ocular surface. Other experimental work is required to clear up the specific biological function in ocular surface disease and physiological state, since the galectin -1 in general plays a number of crusional functions.

Galectine-3: Tears harvested from the eyes of patients with ocular inflammation contained galectin-3, although tears from healthy volunteers, the patients with corneal degeneration and alkali burned cornea (after antibiotic and steroid treatment) did not. Inflammatory cells (granulocytes and macrophages) that express galectin-3 were found in tear fluid samples which were positive for galectin-3. The immunofluorescence analysis with monoclonal and polyclonal antibodies against galectin-3 allowed the detection of galectin-3 in corneal and conjunctival epithelium. This protein was mainly present on the cell surface. No expression of galectin-3 was observed in the cells of the lacrimal gland. A remarkably strict co-localisation of

galectin-3 reactive glycoligands with desmoglein was found in corneal and conjunctival epithelia.

Galectin-7*:* Cells of the all layers of different types of studied epithelia of epidermis, cornea and larynx expressed Gal-7. While the cells of basal cell carcinomas were devoid of galectin -7. The initial step of the cultivation of epidermal cells and corneal cells is associated with an absence of Gal-7 expression. At the beginning of the formation of multilayered colonies Gal -7 expression is apparently iniciated. Two – weeks old confluent culture of human keratocyte were positive for galectin-7. **Galectins and limbus***:* The markers of limbal stem cells are divided in two major categories: negative and positive markers. Markers such as K3 and connexin 43 are regarded as negative markers of corneal differentiation since they are present in corneal epithelium but not in limbal region. In our study the immunohistochemical staining of corneal limbal stem cell by galectin -1, -3, -7 revealed the absence of galectin -3 and -7 in the limbal region and presence in the central cornea. Thus the absence of galectin-3 a -7 might serve as a negative marker for limbal corneal stem cells. The other galectins did not show any specific expression according to the type of cells.

4.2. Dolichos biflorus agglutinin **lectin**

Expression of DBA binding sites (DBA–BS) was observed parcialy in the basal cell layer of the cornea epithelium. These basal epithelial cells were in intimate contact with the basement membrane. The most usual pattern of DBA–BS in cultured cells from the epithelium of the cornea was in the Golgi complex with a polarized appearence and granular in the cytoplasm. The cultured confluent keratocytes exhibited expression of DBA–BS at the stage of beginning cell multilayer formation and co-localisation with galectin-3 and -7.

4.3 Human natural anti-α-galactoside IgG

Galili antigen

No signal of binding of antibodies against α-Gal to porcine as well as human epidermal and cells of anterior corneal epithelium were observed. The antibody directed against β-Gal recognized nuclei of human and pig epidermal cells. A very low signal intensity only for the presence of β-Gal was observed in the nuclei of porcine corneal epitheliumin in contrast to human corneal epithelium, which was negative. The neuraminidase pretreatment had no influence on the binding of antibody against

α -Gal to human as well as porcin epithelial cells. The endothelium of porcine dermal capillaries express epitopes clearly recognized by antibody against α-Gal. The endothelial layer of porcine veins was reactive with antibodies against β-Gal. The pretreatment of samples with neuraminidase increased accessibility of epitopes recognized with anti-β-galactoside antibody in pig endothelium. Human capillaries were highly reactive for the antibody against β-Gal.

Both, porcine liver and human lacrimal gland were highly reactive to the human natural antibody fractions, i.e. against α- or β-Gal. No pathological findings such as the infiltration of lacrimal glands with inflammatory cells were observed. Preincubation of sections of human lacrimal gland with label-free galectin-3 had no influence on the binding of anti-αGal to the lacrimal gland cells.

Detection of α-galactosides by immunoblotting in tear fluid

Distinct bands were detected in blots of the (glyco) protein mixture of tear fluid from the healthy persons and the proband from patient with postherpetic lesion of the cornea. No bands were detected when bovine milk was used for blocking. Interestingly, no positivity was observed in the tear fluid sample from a patient with idiopathic chronic conjunctivitis. Preincubation of antibody with melibiose had a strong blocking effect on the antibody reaction, proving the sugar depended antibody binding. The Western bolotting analysis of tear fluid showed that the human lactoferrin bands had identical mobility at the α-Gal-reactive-glycoantigen.

5. DISCUSSION

The results presented in this thesis demonstrated an important role of glycobiological approach in ophthalmological research.

Glycosylation is an important and common form of posttranscriptional modification of proteins in cells and carbohydrates as medium for storage of secondary biological information were established (Pablo 2013, Solís et al., 2015). Biological functions has 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 carbohadrate synthesis are highly expressed at the human ocular surface include families of glycosyltransferases, proteoglycans, glycan

degradation proteins, as well as mucins and carbohydrate-binding proteins such as the 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 at 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 the literatures 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 co-localisation 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 process should not be ignored since large part of the picture is missing when proteins are being studied without its 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 ransformation. According to our results, the absence of a signal for Ki-67 in DBA-BS positive cells (including cells in malignant tumors) and colocalisation of cyrokeratin 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 investigated that galectin-3 binding was related to cell differentation in normal adult and developing epithelia, culutured epidermal cells, carcinomas derived from this epithelia (Holikova 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 -gal epitopes in tears harvested from patients with ocular inflammatory diseases and absence of galectin-3 together with presence of -gal epitopes in healthy eyes. Expression of lactoferrin did not depend on ocular surface inflammatory status Hrdličkova et al., 2002). Analysis of proteins in tear fluid therefore is valuable not only for diagnostic purposes but may also increase our knowledge in 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 (SDSPAGE) 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 quire 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 analyzed with great sensitivity.
It is important to mention three major differences between tissue-associated O-glycans in conjunctiva and those found in tears (Ana Guzman et al., 2002). Firstly, the conjunctival epithelium contains core 2-based structures, including galactosyl core 2 and di α2-3 sialyl galactosyl core 2. Secondly, 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 thirdly, 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 was expressed both in corneal and conjunctival epithelium but not in the tear fluid (Hrdličkova 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 issue 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 compare 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

- Galectin–1 can play its biological function in ocular tissue and can be useful as a histochemical marker for further experimental studies.
- 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.
- This study shows the presence of galectin–3 in the tear film in pathological eyes and reveals a difference from the normal condition. This finding should elucidate the functional role of the galectin at this localization.
- Abscence of galectin–3 and –7 binding site in basal cell layer in limbal region might be useful as a negative markers for corneal epithelial stem cells.
- The results characterize the cells recognized by 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 (so–called Galili antigen) are absented 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 payed 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 tumor samples or inflammation, probably as a product of aberrant galactosylation or glycolytic degradation.
- The new method of collection of tear film has the same efficacy as capillary tubes. It is a nontraumatic, easy to performe. There is no need for slit lamp. It 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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