

## Detection of galectin-3 in tear fluid at disease states and immunohistochemical and lectin histochemical analysis in human corneal and conjunctival epithelium

Enkela Hrdličková-Cela, Jan Plzák, Karel Smetana Jr, Zora Mělková, Herbert Kaltner, Martin Filipec, Fu-Tong Liu, Hans-Joachim Gabius

Charles University, 1st Faculty of Medicine, Department of Ophthalmology, Prague, Czech Republic  
E Hrdličková-Cela  
M Filipec

1st Faculty of Medicine, Institute of Anatomy  
E Hrdličková-Cela  
J Plzák  
K Smetana Jr

1st Faculty of Medicine, Department of Otorhinolaryngology, Head and Neck Surgery  
J Plzák

Centre for Cell Therapy and Tissue Repair  
K Smetana Jr

1st Faculty of Medicine, Department of Pathological Physiology  
Z Mělková

Ludwig-Maximilians-University, Faculty of Veterinary Medicine, Institute of Physiological Chemistry, Munich, Germany  
H Kaltner  
H-J Gabius

La Jolla Institute for Allergy and Immunology, San Diego, CA, USA  
F-T Liu

Correspondence to: Karel Smetana, Charles University, 1st Faculty of Medicine, Institute of Anatomy, U nemocnice 3, 128 00 Prague 2, Czech Republic  
ksmet@lf1.cuni.cz

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### Abstract

**Background/aim**—Components of the tear fluid contribute to the biochemical defence system of the eye. To reveal whether the immune mediator and lipopolysaccharide binding galectin-3 is present in tears, tear samples were collected from eyes in healthy and pathological states. Investigation of expression of galectin-3 and galectin-3 reactive glycoligands in normal human conjunctival and corneal epithelia was also initiated as a step to understand the role of galectin-3 in ocular surface pathology.

**Methods**—Immunoblot analysis using either a rabbit polyclonal or a mouse monoclonal antibody against galectin-3 was employed to detect galectin-3 in tear fluid. Galectin-3 expression in tissue specimens was detected by immunocytochemistry employing A1D6 mouse monoclonal antibody, and galectin-3 reactive glycoligands were visualised by lectin histochemistry using labelled galectin-3.

**Results**—Galectin-3 was found only in tears from patients with ocular surface disorders. It was expressed in normal corneal and conjunctival epithelia but not in lacrimal glands. 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 epithelial cells co-localising with desmoglein.

**Conclusions**—This study revealed expression of galectin-3 in tear fluid obtained from patients with eye diseases. The role of this endogenous lectin (produced by inflammatory as well as epithelial cells) in antimicrobial action and inflammation modulation could be expected.

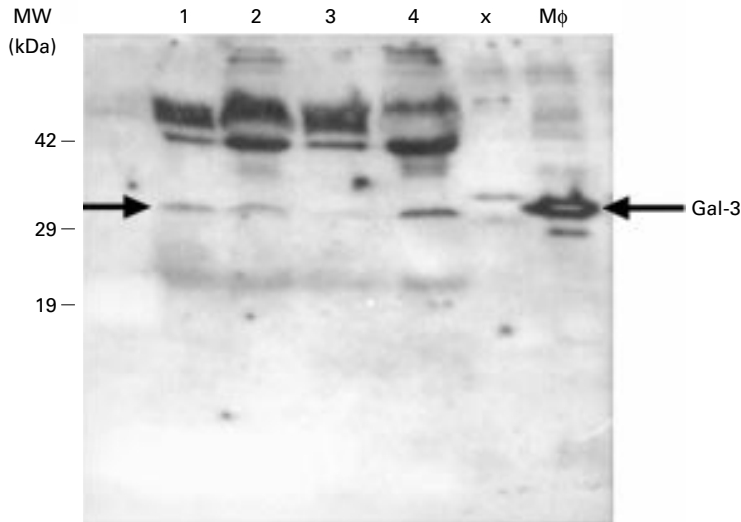
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Progress in glycosciences has documented that biological information transfer not only exploits protein-protein and nucleic acid-protein

interactions but also protein-carbohydrate recognition.<sup>1</sup> Proteins involved in interaction with carbohydrates are known as lectins. Based on structural analysis of the carbohydrate recognition domains, animal lectins are currently classified into five categories: C type, I type, P type, galectins, and pentraxins.<sup>2</sup> Cells as well as extracellular matrix molecules of normal and pathological corneas and conjunctivas in mammals are already known to contain glycans recognised by numerous plant lectins.<sup>3,4</sup> The sugar receptors in these tissues have been demonstrated by employing labelled neoglycoligands.<sup>5</sup> This experimental basis encourages us to further investigate expression of endogenous lectins on the eye surface. In this report, we focus on a member of the animal lectin family of the galectins. Mammalian galectins at present comprise nine proteins sharing the property of secretion via a non-classic pathway and cation independent binding capacity to  $\beta$ -galactosides, including holo-blood A and B group saccharides and poly-N-acetyl-lactosamines.<sup>2,5</sup> Functionally, galectins have been proposed to have crucial biological roles by recognising carbohydrate ligands on intracellular and extracellular compartments and glycoproteins of the extracellular matrix, thus contributing to cell-cell and cell-matrix interaction,<sup>6–8</sup> regulation of cell growth,<sup>9,10</sup> and programmed cell death.<sup>11</sup> In the immune system they modulate different steps of the inflammatory cascade,<sup>12,13</sup> The only chimera-type galectin, galectin-3, deserves special attention in this context.

Galectin-3 is a protein of  $M_r$  of 29 000–35 000 depending on the animal species, which is expressed and secreted by various types of cells, especially monocytes, macrophages, mast cells, and epithelial cells including corneal epithelium.<sup>2,6,14,15</sup> It is a mitogen capable of stimulating fibroblast cell proliferation in a paracrine fashion through interaction with cell surface glycoconjugates.<sup>10</sup> Also, this protein can exert an anti-apoptotic activity underscoring its strong effect on cell growth.<sup>16</sup>

The cornea is a transparent, avascular tissue that is exposed to the external environment. The anterior corneal surface is covered by the



**Figure 1** Western blotting for detection of galectin-3 using polyclonal antibody. MW: molecular weight (demonstrated according to position in acrylamide gel, because it is not visible in western analysis). Lanes 1, 2: tear fluid samples from patients with sarcoidosis, lane 3: tear fluid sample from patient with corneal degeneration, lane 4: tear sample from patient with adenovirus conjunctivitis, lane M: extract from cultured human macrophage cell line producing galectin-3 used as a positive control. Only the tear samples from a few of the investigated patients are shown.

tear film, which has a protective, lubricative, and nutritive function. Both the corneal and conjunctival epithelia form the biodefence system of the anterior surface of the eye. The epithelium, together with the tears, has a pivotal role in maintaining the corneal integrity and its constituents affect eye surface immunology and responses to inflammation. Since galectin-3 is expressed by the human corneal epithelium and binds lipopolysaccharides purified from *Pseudomonas aeruginosa* as was demonstrated by the multiple inhibition assay,<sup>14</sup> 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. Moreover, we employed labelled galectin to analyse the binding sites for an endogenous lectin in this system. The employment of galectin-3 as a probe represents an important step to infer the presence of potential binding sites for this molecule, which can be important in elucidating its biological function in the eye.

## Materials and methods

### MATERIALS

Samples of normal corneas (n = 3), conjunctivas (n = 4), and lacrimal gland (n = 1) were obtained post mortem from donors without eye problems. The conjunctiva of a patient suffering with Stevens-Johnson syndrome (n = 1) was taken by biopsy. All samples were obtained

after receiving the consent forms from the donors. The tear fluid samples (volume 5–12  $\mu$ l) were collected from normal, healthy people (n = 4) without applying an irritant. 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.<sup>17</sup>

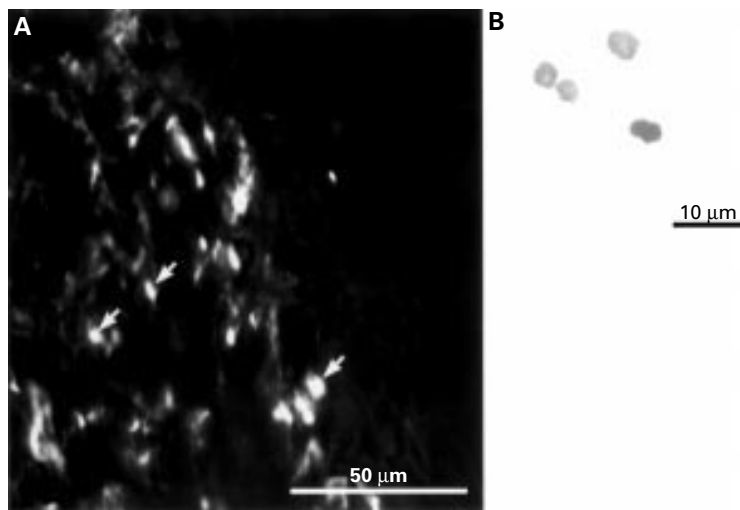
### WESTERN BLOT ANALYSIS OF GALECTIN-3 PRESENT IN TEAR FLUID

The cells (if any) were separated from tear fluid by low rate centrifugation (minicentrifuge Quatriton, Sigma, Prague, Czech Republic) preventing cell damage and contamination of samples by galectin-3 content of these cells. The samples were then stored at  $-20^{\circ}\text{C}$ . Upon analysis, samples were combined with a sample buffer, denatured for 3 minutes at  $100^{\circ}\text{C}$ , and centrifuged for 10 minutes at 16 000 g. Supernatants (12  $\mu$ l of each samples) were then resolved by 14% SDS-PAGE.<sup>18</sup> After electrophoresis, the resolved proteins were transferred to a nitrocellulose membrane, and a western blot analysis was performed as previously described.<sup>19</sup> Galectin-3 was detected with a rabbit polyclonal antibody<sup>7</sup> (dilution 1:500) and peroxidase conjugated goat anti-rabbit IgG (Cappel Research Products, USA; dilution 1:5000) using enhanced chemiluminescence (ECL, Amersham, Pharmacia, Biotech, Freiburg, Germany). Extract of cells from mouse macrophage line J774.GB producing galectin-3 was used as a positive control. In addition, a monoclonal mouse anti-galectin-3 antibody, A1D6<sup>20</sup> (dilution 1:150), a secondary antibody SwAM-Px (Temda, Prague, Czech Republic; dilution 1:200 000), and enhanced chemiluminescence (SuperSignal Wets Femto, Pierce) were used in a separate western blot.

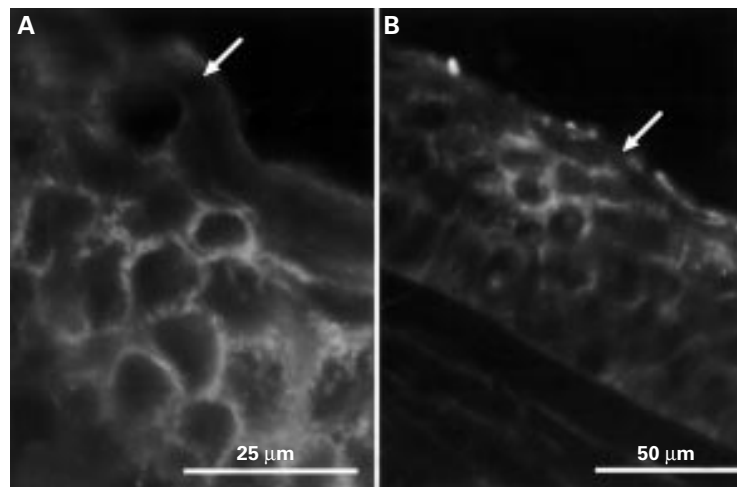
### IMMUNOFLOUORESCENCE ANALYSIS AND LECTIN HISTOCHEMISTRY

#### Tissue section analysis

Specimens for histochemical investigation were exposed to Tissue Tek (Sakura-Finetek Europe BV Zoeterwoude, Netherlands) as a cryoprotective agent for 2 hours at  $4^{\circ}\text{C}$ , then frozen in liquid nitrogen and stored in  $-70^{\circ}\text{C}$ . Cryostat sections (10  $\mu$ m; Cryocut-E, Reichert, Vienna, Austria) were fixed with 2% paraformaldehyde in PBS (pH 7.2) for 5 minutes; 0.1% bovine serum albumin in PBS was used as a blocking agent. After the extensive washing in TBS (pH 7.4), the specimens were stained for detection of galectin-3 with a monoclonal mouse anti-galectin-3 antibody, A1D6.<sup>20</sup> 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. FITC conjugated swine anti-mouse antibody (SwAM-FITC, Temda, Prague, Czech Republic) or swine anti-rabbit antibody



**Figure 2** Positivity of inflammatory and goblet cells for galectin-3 detected by A1D6 antibody. Inflammatory cells in conjunctival stroma from a patient with Stevens-Johnson syndrome (A) expressed galectin-3 similarly to leucocytes (B) harvested from another patient with galectin-3 in tear fluid. The inflammatory cells infiltrating the cornea express the galectin-3 in cytoplasm in contrast with absence of galectin-3 in conjunctival epithelium. Immunofluorescence and immunoperoxidase detection of galectin-3, scale 50 µm (A) and 10 µm (B).



**Figure 3** Galectin-3 expression in conjunctiva and cornea. Conjunctival (A) and corneal (B) areas are positive in the immunohistochemical analysis. The galectin-3 was expressed predominantly on the cell surface. However, the expression of galectin-3 in the superficial part of conjunctiva was non-uniform; some areas were galectin-3 negative. The surface of corneal and conjunctival epithelium is marked by arrows. Detection of galectin-3 using A1D6 antibody, scale 25 µm (A) and 50 µm (B).

(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.

Galectin-3 binding sites were visualised by the lectin histochemical procedure using the biotinylated recombinant lectin as a probe.<sup>21-24</sup> The ExtrAvidin-TRITC was employed as a second step reagent. The double labelling technology at one cell level<sup>24</sup> was used to visualise desmoglein and galectin-3 binding sites simultaneously in one specimen.

The omission of the first step antibody or pre-incubation of galectin-3 with lactose as a competitive inhibitor to block carbohydrate

dependent binding was used in control experiments to test the specificity of the immunohistochemical and lectin histochemical procedures. The specimens were mounted by Vectashield (Vector Laboratories, Burlingame, CA, USA). An Optiphot-2 (Nikon) fluorescence microscope and computer assisted image analysis system equipped with a CCD camera (Cohu) (Lucia, Laboratory Imaging, Prague, Czech Republic) was used for detection of signals.

#### Tear sample cytology

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

#### Results

The known secretion of galectins by other cells (macrophages, polymorphonuclear leucocytes, and epithelia) makes it likely that galectin-3 is present in tear fluid. Indeed, tears harvested from the eyes of patients with ocular inflammation contained galectin-3, although tears from healthy volunteers, the patient with corneal degeneration and alkali burned cornea (antibiotic and steroid treatment) did not (Fig 1). As detected by a rabbit polyclonal (or mouse monoclonal) antibody against gal-3, a band of apparent molecular weight around 30 kDa was found in pathological tears samples as well as in control macrophages. Additional bands of higher molecular weight are at the position of immunoglobulins that are recognised by the secondary antibody that was not pre-adsorbed with human immunoglobulins. In a control western blot with mouse monoclonal antibody against gal-3 and another secondary antibody, similar bands were found (not shown).

Inflammatory cells (granulocytes and macrophages) that express galectin-3 were found in tear fluid samples which were positive for galectin-3 (Fig 2).

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 (Fig 3). The results demonstrated no differences if the monoclonal and polyclonal antibodies were used. No expression of galectin-3 was observed in the cells of the lacrimal gland, although cytokeratin expression monitored by the monoclonal antibody LP34 was clearly visible, indicating that the glandular proteins were not autolysed post mortem (not shown).

To show whether the cells also express binding sites for this lectin, it is necessary to analyse the cells with the labelled galectin-3. Biotinylated galectin-3 was found to bind to the cell surface in normal corneal and conjunctival epithelium. The distribution of galectin-3 binding sites on corneal epithelium was uniform, whereas that on conjunctiva was rather irregular. The conjunctival epithelium from the patient with Stevens-Johnson syndrome expressed no galectin-3 reactive binding

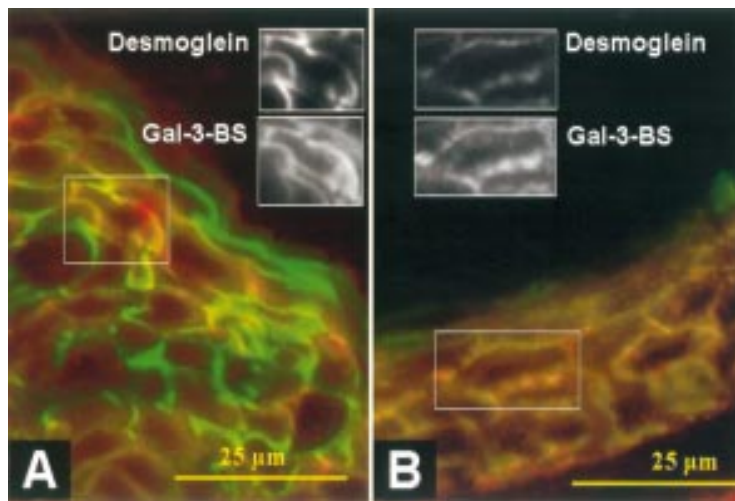


Figure 4 Co-localisation of galectin-3 binding sites with desmoglein in conjunctiva (A) and cornea (B). The binding sites for galectin-3 (red signal) co-localised with expression of the desmosomal protein desmoglein (green signal). Desmoglein was detected by immunohistochemistry and galectin-3 reactive glycoligands by lectin histochemistry, scale 25 µm (A, B).

sites (not shown) although the macrophages under the epithelium were intensely stained by antibody against galectin-3 (Fig 2A) and expressed the galectin-3 reactive binding sites. A remarkably strict co-localisation of galectin-3 reactive glycoligands with desmoglein was found in corneal and conjunctival epithelia (Fig 4). The employment of lactose as a competitive inhibitor completely blocked the binding of biotinylated galectin-3 to studied epithelia of the cornea and conjunctiva.

### Discussion

Although galectin-3 is known to be secreted by various cells in vitro, in contrast with patients with ocular surface inflammation, no galectin-3 was found in tears harvested from healthy volunteers. Expression of galectin-3 was found in corneal and conjunctival epithelium in the normal eye and in leucocytes isolated from tears harvested from the galectin-3 positive eyes suffering from inflammation. Inflammatory cells infiltrating the conjunctival stroma from the patient with Stevens-Johnson 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 expresses galectin-3, but the tears harvested from healthy volunteers contain no galectin-3. To infer presence of binding sites for the endogenous lectins, we prepared a biotinylated protein instead of a galactoside binding plant agglutinin, because carbohydrate fine specificities of two lectins can differ.<sup>25</sup> Hypothetically, galectin-3 binding sites expressed in both the corneal and conjunctival epithelium appear positioned to immobilise galectin-3 produced and exported from the cell to the cell surface. This phenomenon might explain the absence of galectin-3 in the tear film of healthy people, and 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.<sup>21-24</sup> The co-localisation 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 as a result of occupancy of some ligands by the endogenous lectin. This observation together with the ability of galectin-3 produced by corneal epithelial cells to immobilise bacterial lipopolysaccharides<sup>14</sup> suggest 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 leucocytes and macrophages are also known as producers of galectin-3.<sup>26-27</sup> 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 the initial monitoring of the lectin and binding site by immunohistochemistry and lectin histochemistry respectively, should prompt the elucidation of the functional role of the galectin at this location.

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