ORIGINAL ARTICLES—Clinical science

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

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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.[1] 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.[2] 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.[3 4] The sugar receptors in these tissues have been demonstrated by employing labelled neoglycoligands.[5] 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 β-galactosides, including histo-blood A and B group saccharides and poly-N-acetyl-lactosamines.[2 5] 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,[6–8] regulation of cell growth,[9 10] and programmed cell death.[11] In the immune system they modulate different steps of the inflammatory cascade,[12 13] 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.[2 6 14 15] It is a mitogen capable of stimulating fibroblast cell proliferation in a paracrine fashion through interaction with cell surface glycoconjugates.[10] Also, this protein can exert an anti-apoptotic activity underscoring its strong effect on cell growth.[16]

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

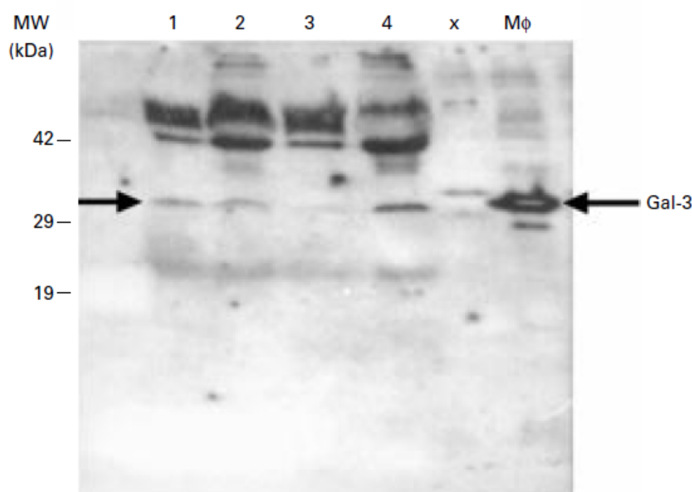

Figure 1 Western blotting for detection of galectin-3 using polyclonal antibody. MW: molecular weight (demonstrated according to position in acrylamide gel, because it is not visible in western analysis). Lanes 1, 2: tear fluid samples from patients with sarcoidosis, lane 3: tear fluid sample from patient 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,[14] 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 µl) 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.[17]

WESTERN BLOT ANALYSIS OF GALECTIN-3 PRESENT IN TEAR FLUID

The cells (if any) were separated from tear fluid by low rate centrifugation (minicentrifuge Qualitron, 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°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 samples) were then resolved by 14% SDS-PAGE.[18] After electrophoresis, the resolved proteins were transferred to a nitrocellulose membrane, and a western blot analysis was performed as previously described.[19] Galectin-3 was detected with a rabbit polyclonal antibody[7] (dilution 1:500) and 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 was used as a positive control. In addition, a monoclonal mouse anti-galectin-3 antibody, A1D6[20] (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.

IMMUNOFLUORESCENCE 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°C, then frozen in liquid nitrogen and stored in –70 °C. Cryostat sections (10 µm; 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.[20] 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

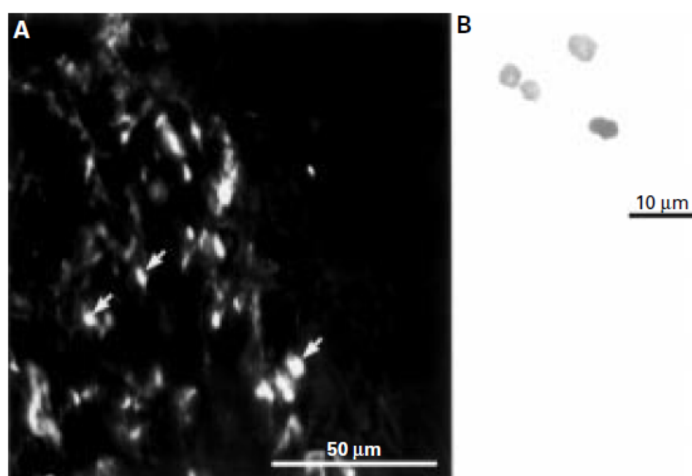

Figure 2 Positivity of inflammatory and goblet cells for galectin-3 detected by A1D6 antibody. Inflammatory cells in conjunctival stroma from a patient with Stevens-Johnson syndrome (A) expressed galectin-3 similarly to leucocytes (B) harvested from another patient with galectin-3 in tear fluid. The 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).

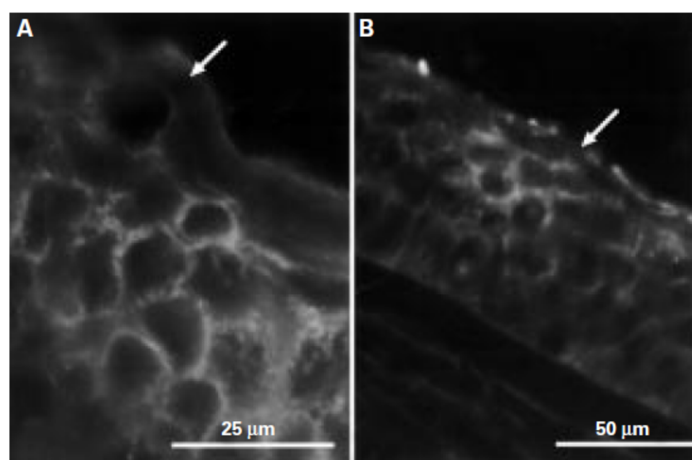

Figure 3 Galectin-3 expression in conjunctiva and cornea. Conjunctival (A) and corneal (B) areas are positive in the immunohistochemical analysis. The galectin-3 was expressed predominantly on the cell surface. However, the expression of galectin-3 in the superficial part of conjunctiva was non-uniform; 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.[21–24] The ExtrAvidin-TRITC was employed as a second step reagent. The double labelling technology at one cell level[24] was used to visualise desmoglein and galectin-3 binding sites simultaneously in one specimen.

The omission of the first step antibody or preincubation 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

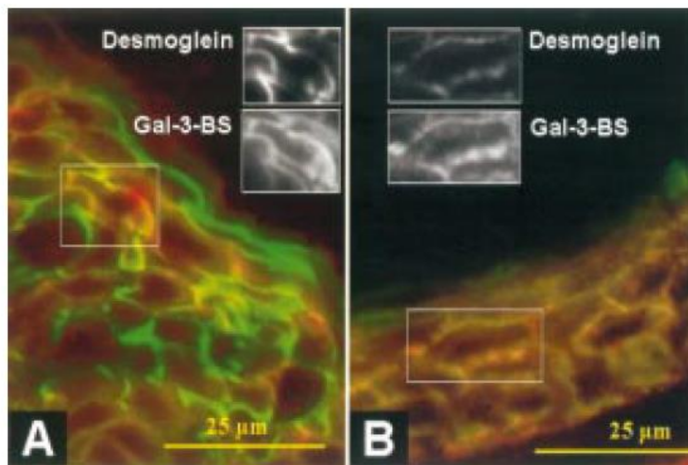

Figure 4 Co-localisation of galectin-3 binding sites with desmoglein in conjunctiva (A) and cornea (B). The binding sites for galectin-3 (red signal) co-localised with expression of the desmosomal protein desmoglein (green signal). Desmoglein was detected by immunohistochemistry and galectin-3 reactive glycloligands 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.[25] 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.[21 24] 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[14] 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.[26 27] 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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Cells of Porcine Epidermis and Corneal Epithelium Are Not Recognized by Human Natural Anti-α-galactoside IgG

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Abstract. **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 biotinylated 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. No reactivity of porcine and human epidermis as well as anterior corneal epithelium was observed for human anti-α-Gal IgG. Serving as positive control, porcine capillaries gave the expected signal with the anti-α-Gal antibody. The anti-β-Gal subfraction recognized cell nuclei in the epidermis of both these species. The pig liver cells interacted with antibodies against α- and β-galactosides like cells of the human lacrimal gland. α-galactoside-reactive glycoproteins were also detected in the human tear fluid. The carbohydrate specificity of the reaction was ascertained by using melibiose as competitive sugar for α-galactoside-mediated binding. These results reveal the presentation of Galα1,3Gal in epithelial cells of human lacrimal gland, its biosynthetic origin being unclear. With respect to a potential clinical perspective, the given results facilitate consideration of the use of porcine epidermal cells in engineering of non-permanent wound covers to improve treatment.**

It is well known that approximately one percent of the circulating human IgG is directed against α-galactosyl epitopes of general structure Galα1,3Gal-R, the so-called Galili antigen, which occurs in mammals except Old World monkeys, apes and humans (Galili et al., 1988a). Compared to natural antibodies against carbohydrate epitopes of A or B histo-blood group antigens these antibodies are not present in neonates and can be detected after the colonization of the intestine with bacterial flora (Wiener, 1951). Interestingly, the titre of these natural antibodies significantly increases with bacterial/parasitic antigenic challenge, a proven target of the antibodies (Springer and Horton, 1969; Galili et al., 1988b; Avila et al., 1989). The abundance of the polyclonal antibody against α-Gal autoreactivity to human tissues was postulated to contribute to autoimmune diseases such as thyroiditis. In this case, α-Gal epitopes were found on normal as well as autoimmune human thyroid cells, rendering this explanation rather unlikely (Thall et al., 1991). In a different context, the presence of this carbohydrate antigen is unquestionably 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; Cooper and Oriol, 1997). The porcine vascular endothelium is damaged by exposure

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Abbreviations: Gal – galactoside; PBS – phosphate-buffered saline.

to the antibody and the organ is eventually destroyed during hyperacute rejection (Cooper 1996; Cooper and Oriol, 1997).

Trophic wounds such as pressure and venous ulcers or diabetic foot represent a serious medical problem. Using human allogenic cells, tissue engineering led to production of bioactive matrices as protective covers, also contributing to re-epithelization of the wound bed by eliciting production of cytokines favourable for epidermal stem cell division (comercially available products such as Apligraf and Dermagraft). As a substitute for human cells in the preparation of such biocompatible non-permanent covers, porcine epidermal cells might find a place to improve the treatment of chronic wounds. Taking stock of applying pig dermoepidermal grafts in provisional therapy of burn injuries without obvious negative side effects gives reason to test porcine epidermal cells also in this context. At any rate the expression of α-galactosides in these cells is to be evaluated concerning their potential to be a target of natural antibodies limiting applicability. The first step is visualization of the reactivity of cells with the damage-conferring human antibodies obtained by affinity chromatography of the serum. Employing the human natural anti-α- or β-Gal antibody fractions of the IgG class, we studied the presence of reactive carbohydrate epitopes in porcine and human epidermis and anterior epithelium of cornea of the human and porcine nature. Moreover, the occurrence of antibody-reactive Gal determinants in a panel of human and porcine tissues and human tear fluid was studied.

Material and Methods

Tissue and tear sample processing

The porcine epidermis was harvested using the punch-biopsy procedure from highly keratinized (foot) and poorly keratinized (snout) areas of miniature pigs (crosses of the Minnesota and Gottingen strains) after local anaesthesia. The samples of porcine liver and cornea were received post mortem. The specimens of human skin were obtained from the Department of Aesthetic Surgery (Charles University, 3rd Faculty of Medicine, Prague, Czech Republic) with the informed consent of donors. The human cornea, lacrimal gland samples and *musculus levator palpebrae* samples were received post mortem. Non-stimulated tear fluid samples were collected from healthy volunteers (N = 10), from another patient with idiopathic chronic conjunctivitis and one patient with metaherpetic keratitis. The pieces of tissue were embedded with Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), incubated for 1 h at 4°C and frozen in liquid nitrogen. The tear fluid samples were also deeply frozen. All samples (tissue and tear fluid samples) were stored frozen up to further processing at –20°C.

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

Immunohistochemical analysis of tissues

The 5–10 μm thick cryostat sections (Cryocut-E. Reichert-Jung, Wien, Austria) were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2–7.4) and washed carefully with PBS. A PBS solution with 0.1% (w/v) human serum albumin (fraction V, Sigma-Aldrich, Prague, Czech Republic) was employed to block a non-specific protein binding-site solution. The α- and β-Gal-containing glycoepitopes were visualized with biotinylated human natural antibodies of the IgG class at a dilution of 10 μg/ml of PBS for 1 h at room temperature (Dong et al., 1997; Smetana et al., 1998). Isolation, subfraction and activity assays of the IgG preparations have been described in detail previously (Dong et al., 1997; Siebert et al., 2000). After careful washing with PBS, TRITC-labelled ExtrAvidin (Sigma-Aldrich, Prague, Czech Republic) was used for visualization of the immunocytochemical procedure. To assess the masking effect of N-acetylneuraminic acid at the terminal position of oligosaccharide chains on antibody binding, a part of sections were pretreated with neuraminidase applied at a dilution 1:100 recommended by the supplier for 12 h at 37°C (Institute of Epidemiology and Microbiology, Gorkij, Russia). One of the control experiments (see also section on competitive inhibition) was performed by omitting the first-step antibody or its replacement with human albumin to exclude any binding of the kit reagents to the section.

Because the Galili antigen shares the core structure with the histo-blood B group epitope (the branching with α1,2-linked L-fucose is not present) which is known to be a glycoligand for Gal-3, we tested the possibility that Gal-3 is reactive with the core structure. Sections of the human lacrimal gland containing this glycoepitope were preincubated with label-free Gal-3 prepared and tested for activity as described previously (André et al., 1999; Plzák et al., 2000), and after extensive washing the specimens were incubated with labelled anti-α-Gal antibody as described.

The sections were mounted using Vectashield (Vector Laboratories, Burlingame, CA). An Optiphot-2 Nikon fluorescence microscope equipped with a CCD integrating camera (Cohu) and computer-assisted image analysis system LUCIA (Laboratory Imaging, Prague, Czech Republic) was used for photodocumentation.

Detection of α-galactosides in human tear fluid

The proteins of human tear fluid were separated on a gradient of 5–20% SDS polyacryalmide gel (Laemmli, 1971). Following electrophoretic transfer of proteins to nitrocellulose (Amersham, Freiburg, Germany) at 0.9 mA/cm^2 in a semi-dry blotting apparatus in transfer buffer (48 mM Tris, 39 mM glycine, and 20% methanol (Bjerrum and Schafer-Nielsen, 1986)), the binding sites on the membrane were blocked with 0.1% Tween 20 (Sigma, Prague, Czech Republic) in PBS or with low-fat bovine milk. The membrane was then incubated with a solution containing biotinylated 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 (Galα1,6Glc; ICN, StarLab, Prague, Czech Republic) at a concentration of 10–40 mM added to a diluted antibody as mentioned above was employed as a competitive inhibitor in immunohistochemical as well in Western blot experiments.

Detection of lactoferrin in tears

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

Results

Immunohistochemical analysis of α-Gal- and β-Gal-containing epitopes

The purified and labelled immunoglobulin G fractions were tested in solid-phase assays for carbohydrate-dependent activity and then tested as glycohistochemical markers. Epithelial and endothelial cell layers were monitored for reactivity to the human natural anti-carbohydrate immunoglobulin G fraction. Under the conditions used, no signals of probe binding against α-Gal to porcine and human epidermal cells and cells of anterior epithelium of cornea were observed (Figs. 1, 2). The antibody against β-Gal recognized nuclei in porcine and in a lesser extent in human epidermis (Figs. 1, 2). A very low signal intensity only for the presence of β-galactosides was observed in the nuclei of porcine anterior epithelium in contrast to the human corneal epithelium, which was negative (Fig. 2). Pretreatment of epithelium with neuraminidase had no effect on the binding of anti-α-Gal antibodies (not shown).

Cultured foetal porcine cells showed no signal for the presence of α-Gal in contrast to murine 3T3 cells with rather a low, but significant positivity of this glycoepitope (Fig. 3). The endothelium of porcine dermal capillaries expressed epitopes definitely recognized by the antibody fraction against α-Gal (Fig. 4). The endothelial layer of porcine veins was reactive with antibodies against β-galactosides after the neuraminidase treatment (not shown). Human capillaries were highly reactive for the antibody against β-Gal (Fig. 4).

Both porcine liver and human lacrimal gland were highly reactive for the human natural antibody fractions, i.e. against α- or β-Gal (Fig. 5). Preincubation of sections from human lacrimal gland with label-free Gal-3 had no influence on the binding of anti-α-Gal to the lacrimal gland cells (Fig. 6).

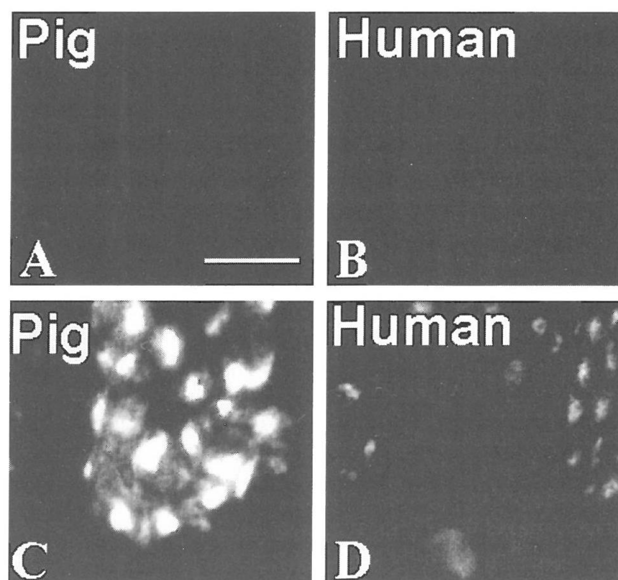

Fig. 1. Detection of glycoepitopes containing probe-reactive α-Gal (A, B) and β-Gal (C, D) moieties in pig (A, C) and human (B, D) epidermis. Scale is 20 μm.

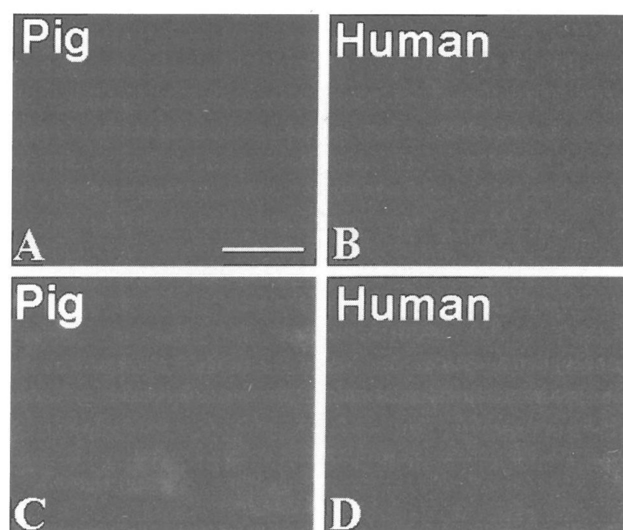

Fig. 2. Detection of glycoepitopes containing probe-reactive α-Gal (A, B) and β-Gal (C, D) moieties in pig (A, C) and human (B, D) anterior corneal epithelium. Scale is 20 μm.

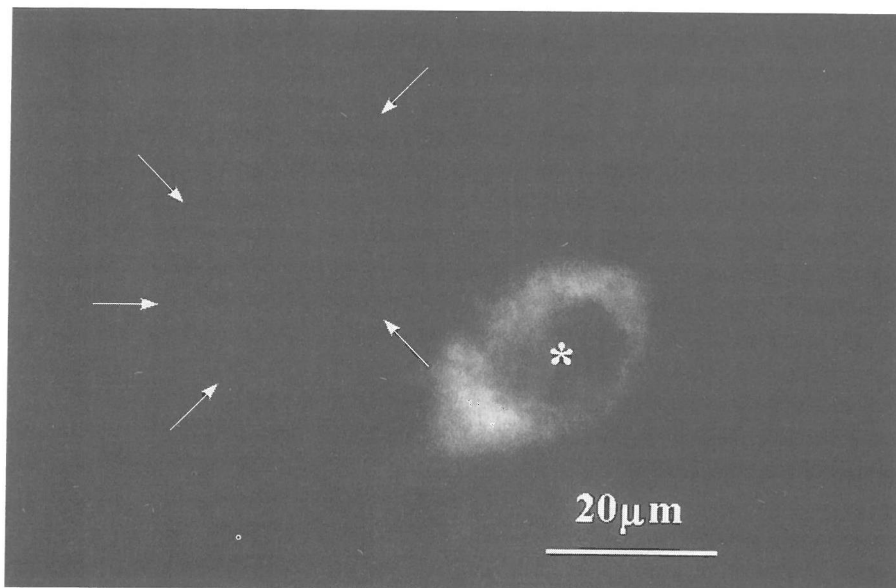

Fig. 3. Detection of glycoepitopes containing probe-reactive α-Gal in cultured pig foetal epidermal cells (position of one cell is indicated by arrows). A murine 3T3 fibroblast is marked with an asterisk. Scale is 20 μm.

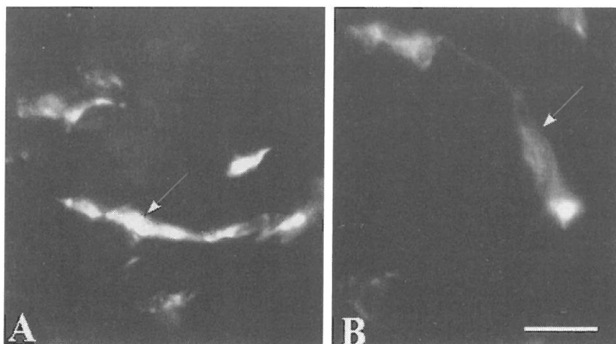

Fig. 4. Detection of glycoepitopes containing probe-reactive α-Gal (A) and β-Gal (B) moieties in pig (A) and human (B) capillaries (arrows). Bar is 20 μm.

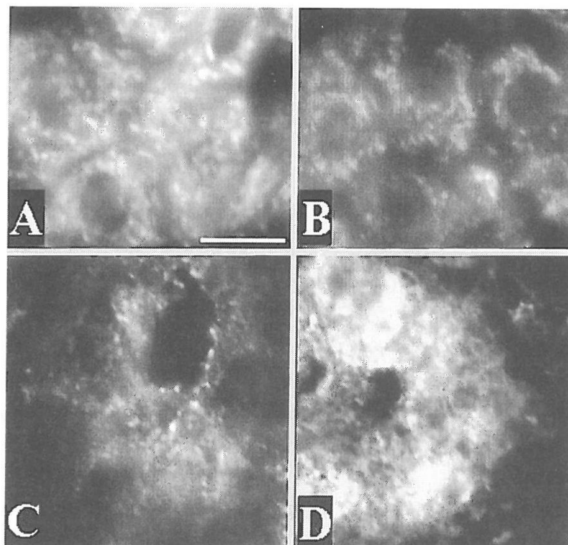

Fig. 5. Detection of glycoepitopes containing probe-reactive α-Gal (A, C) and β-Gal (B, D) moieties in pig liver (A, B) and human lacrimal gland (C, D). Bar is 20 μm.

Detection of α-galactosides by immunoblotting

Distinct bands were detected in blots of the (glyco)protein mixture of tear fluid of healthy persons and the proband with the postherpetic lesion. No bands were detected when bovine milk was used for blocking. 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 reactivity, proving the sugar-dependent antibody binding (Fig. 7).

The Western analysis of tear fluid showed that the human lactoferrin bands had identical mobility at the α-Gal-reactive glycoantigen (Fig. 8).

Discussion

Fixed cells of porcine epidermis, including cultured epidermal cells, and of anterior corneal epithelium were not reactive for anti-α-Gal using labelled natural human IgG without and after neuraminidase pretreatment. The possibility for 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 inhibitor 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 surfaces 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

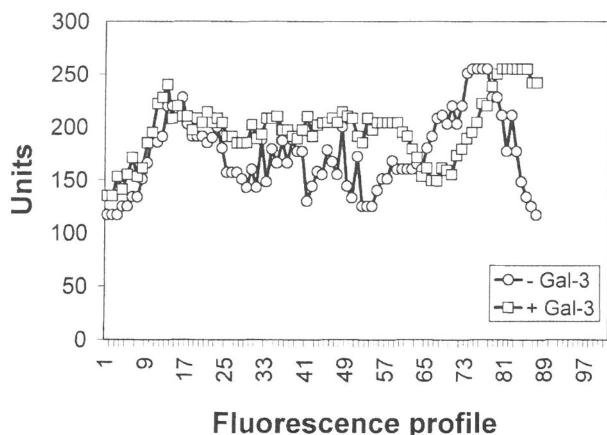

Fig. 6. Fluorescence profile for the presence of α-Gal-containing epitopes measured in cells of the lacrimal gland without and with preincubation using label-free Gal-3.

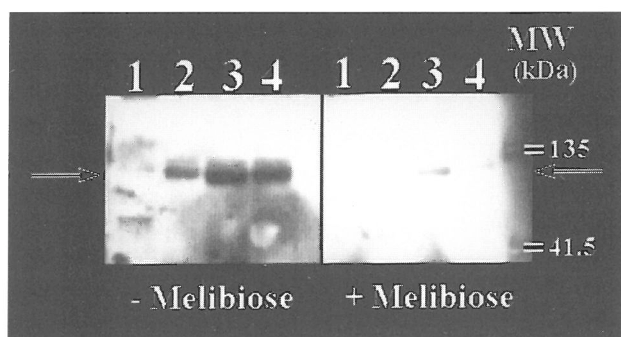

Fig. 7. Presence of α-Gal-containing glycoepitopes in tear fluid collected from donors with idiopathic chronic conjunctivitis (1), postherpetic lesion (2) and from two healthy persons (3, 4) without or with competitive inhibition by melibiose (Galα1,6Glc).

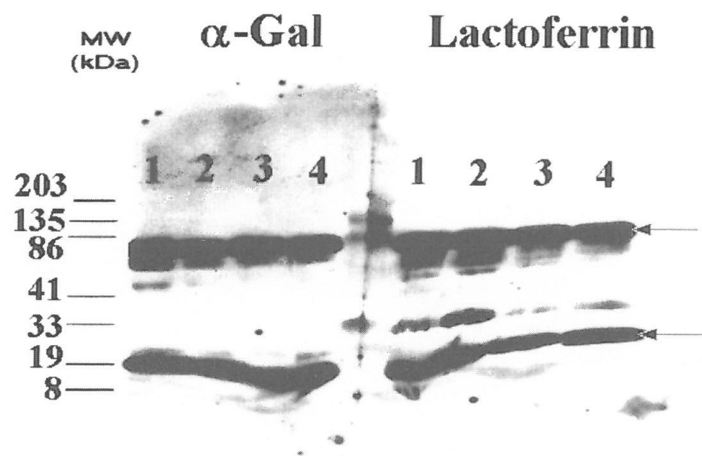

Fig. 8. Presence of α-Gal-containing glycoproteins and lactoferrin in tear fluid collected from four healthy persons (1–4). The arrows indicate position of bands positive for both antibodies, i.e. anti-α-Gal and anti-lactoferrin.

presence of IgG-reactive α-Gal in tear fluid of healthy volunteers indicates the physiological occurrence of this glycoepitope 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 Rudolff, 1993; Nascimento de Araujo and Giogliano, 2000). Lactoferrin as well as lactalbumin also exert direct bactericidal activity (Ellison et al., 1988; Hakansson et al., 2000). Moreover, material from a patient with a chronically inflamed eye surface contained no band recognized by human natural anti-α-Gal antibody in the same position as that from healthy donors. Our preliminary studies revealed the absence of Gal-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. An α-Gal-containing glycoprotein(s) was detected in tear fluid from normal eyes and not in the tear samples from inflamed eyes. This result points to the possibility of an interaction of α-Gal with Gal-3. However, the preincubation of lacrimal gland sections with label-free Gal-3 for epitope masking had no inhibitory effect on anti-α-Gal binding to lacrimal gland cells. Further explanation could be the absence of α-Gal in these individual donors or a 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.

In conclusion, this study demonstrated the absence of α-Gal epitopes (so-called Galili antigen) in porcine epidermal cells and corneal epithelium *in situ* or cultured *in vitro*. 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., 1998; Kayser et al., 2000), probably as a product of aberrant galactosylation or glycolytic degradation.

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Postmitotic basal cells in squamous cell epithelia are identified with *Dolichos biflorus* agglutinin – functional consequences

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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;109:714–20.

Dolichos biflorus agglutinin (DBA) is a plant lectin specifically recognizing α-N-acetylgalactosamine. Controversial reports regarding the binding of DBA to the epidermis have been published. Using a double labeling procedure at the single-cell level, we studied the expression of DBA-reactive binding sites in conjunction with markers of cell proliferation and differentiation in normal human epidermis, cornea, and malignant tumors as well as in cultured keratinocytes. The results characterize the cells recognized by DBA as postmitotic early differentiating cells, identifiable by their lack of expression of the proliferation marker (Ki-67). The Golgi complex of a limited number of cultured keratinocytes was recognized by DBA and some of these cells show the accumulation of β_1 integrin chain in the Golgi complex. This process seems to be important for the migration of postmitotic cells from the basal to the suprabasal layers.

Key words: Squamous cell epithelia; carcinoma; lectin; *Dolichos biflorus* agglutinin; integrin; differentiation.

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The squamous cell multilayered epithelia represent morphologically and functionally stratified tissue (1). The basal cell layer contains a pool of mitotically active (epidermal stem and transit amplifying) as well as inactive (postmitotic differentiating) cells. The epidermal stem cells can be characterized as slowly cycling elements with an unlimited number of mitoses and the transit amplifying cells as rapidly cycling cells with a restricted number of divisions (2, 3). In special epidermal structures such as the hair follicle, the epidermal stem cells are located in the so-called bulge region (4). The stem cells of corneal anterior epithelium are present in the basal layer in the periphery of the limbal region. They are characterized by the absence of cytokeratin type 3, which is normally expressed suprabasally in

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limbal epithelium and uniformly in central corneal epithelium.(5–7). The epidermal stem cells in the bulge region of hair follicles express cytokeratin type 19, which is specific for monolayered epithelia (8). The cell populations located in the basal cell layer of the epidermis or mucosa are characterized mainly according to functional criteria, position, and quantitative markers. For example, the epidermal stem cells express β_1 chain of integrins more extensively than other molecules in the basal layer. On the other hand, other receptor molecules, such as E-cadherin and β-catenin, are less well represented in epidermal stem cells in comparison with other basal cell types (9). The disorders of epidermal (or mucosal) stem cell function, differentiation and maturation represent a crucial element in the pathology of multilayered squamous epithelia as well as cancer. From this point of view, markers enabling us to distinguish different cell types in the compartment of the basal cell layer are very important for basic research and for diagnostic purposes.

The cell glycophenotype sensitively reflects the cell differentiation pattern. This can be used in diagnostics (10). According to some authors, basal layer cells of the epidermis are highly reactive for the plant lectin *Dolichos biflorus* agglutinin (DBA) (11–13). Others observed no binding of DBA to the epidermis (14, 15). These observations suggest a possible developmental control of DBA-reactive glycoligand expression in squamous cell epithelia. In this study we try to resolve this problem and characterize the DBA-binding cells by a procedure of double labeling where the biotinylated DBA combined with antibodies for proliferation (Ki-67) and differentiation (intermediate filaments, β_1 chain of integrin) markers was used to stain human epidermis, basal and squamous cell carcinomas, and cultured epidermal cells.

MATERIAL AND METHODS

Skin samples

The samples of human skin harvested from three consenting donors were received from the Department of Aesthetic Surgery (Charles University, 3rd Faculty of Medicine, Prague). The samples of three basal cell carcinomas were received from the Department of Dermatovenerology (Charles University, 2nd Faculty of Medicine, Prague) and samples of four squamous cell carcinomas (tongue) from the Department of Otorhinolaryngology, Head and Neck Surgery (Charles University, 1st Faculty of Medicine, Prague). The samples of cornea from two cadaverous donors were received from the Department of Ophthalmology (Charles University, 1st Faculty of Medicine, Prague). The tissue samples were frozen in liquid nitrogen using Tissue-Tek (Sakura, Zoeterwoude, Netherlands) as cryoprotective medium and stored until further processing.

Epidermal cell culture

The epidermal cells were cultured on the surface of 10 coverslips. The modified method of Green and coworkers using 3T3 cells with arrested mitosis as feeders was used (16, 17). Epidermal cells were cultured up to the stage of confluency (approx 9 days), then the cells from the center of five coverslips were gently removed (wounded culture) and the remaining cells were cultured for an additional 48 h. This procedure was used to increase the number of mitotic cells in culture.

Immunohistochemistry and lectin histochemistry

The 7 μm thin frozen sections (Cryocut-E Reichert-Jung, Vienna, Austria) from normal epidermis, cornea and tumors were washed in PBS, fixed in 4% paraformaldehyde (w/v) in PBS, and permeabilized with cooled acetone. After that, simultaneous detection of biotinylated lectin-reactive binding sites and antibody-reactive epitopes (18, 19) was used for visualization of DBA-reactive glycoligands (Vector Laboratories, Burlingame, CA), Ki-67 antigen (IgG1, Immunotech, Prague, Czech Republic), cytokeratins 3 (IgG1, Progen, Heidelberg, Germany) and 10 (IgG1, Dako, Glostrup, Denmark), and β_1 chain of integrin (IgG1) (19). Collagen type IV (IgG1, Sigma, Prague, Czech Republic) was also visualized in combination with the DBA-binding sites. Similar technology was used for detection of the above-mentioned molecules in cultured cells. The FITC-labeled swine anti-mouse antibody (TEMDA, Prague, Czech Republic) and TRITC-labeled ExtrAvidin (Sigma, Prague, Czech Republic) were used for visualization of the immuno- and lectin histochemical reaction.

Control experiments were performed by replacing the antibodies with the ED1 monoclonal antibody against rat macrophages (IgG1) and anti-CD1a (IgG2), which recognizes only the dendritic cells of Langerhans type in the epidermis. The specificity of the lectin histochemical reaction was verified by blocking the DBA binding of histo-blood A group trisaccharide with polyhydroxyethyl acrylamide polymer chain.

The specimens were mounted in Vectashield medium (Vector Laboratories, Burlingame, CA, USA). The Nikon Optiphot-2 (Nikon, Prague, Czech Republic) fluorescence microscope equipped with FITC and TRITC filter blocks, CCD camera and com-

puter-assisted image analysis system LUCIA (Laboratory Imaging, Prague, Czech Republic) was employed for the detection of signals and their analysis. This arrangement enables us to observe the antibody binding as a green signal, lectin binding as a red signal, and their co-localization as a yellow signal.

RESULTS

Strong expression of DBA-binding sites (DBA-BS) was observed in more than 50% of the basal layer cells in normal human epidermis. The DBA-BS$^+$ basal cells were significantly longer (17.94±3.36 μm) than the DBA-BS$^-$ basal cells (9.22±2.06 μm) ($p<0.05$). These basal epidermal cells strongly positive for the DBA-BS were in intimate contact with the basement membrane and they were very occasionally observed in the lower spinous layer (Figs. 1, 2). Both DBA-BS-positive as well as -negative basal cells expressed β_1 integrin. However, no association between DBA-BS and Ki-67 nuclear positivity was observed (Figs. 2a, 3, 4). Also, no relationship between the presence of DBA-BS and expression of cytokeratin 10 was observed in the basal cell layer (not shown). In contrast to in the basal cells, the signal of the DBA binding to the majority of suprabasal cells was significantly reduced or completely absent (Figs. 1, 2a, b, 5).

Both types of malignant tumors (i.e. basal and squamous cell carcinomas) expressed DBA-BS in tumor cells with Ki-67-negative cell nuclei. In contrast, cells with Ki-67 nuclear expression completely lacked DBA-BS (Fig. 2c, d).

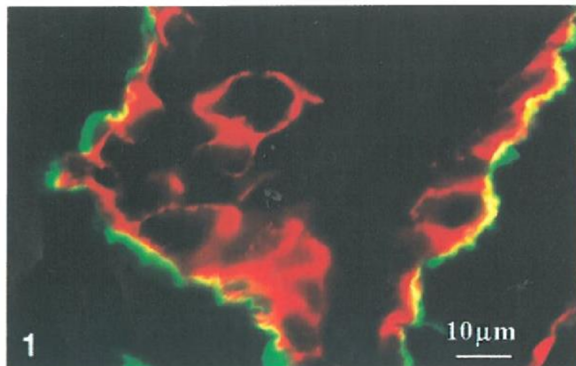

Fig. 1. Collagen type IV (green signal) and DBA-reactive glycoligands (red signal) in human epidermis. Intimate contact of DBA-reactive glycoligands and collagen (yellow signal) and Superficial migration of DBA-BS$^+$ were observed. Scale=10 μm.

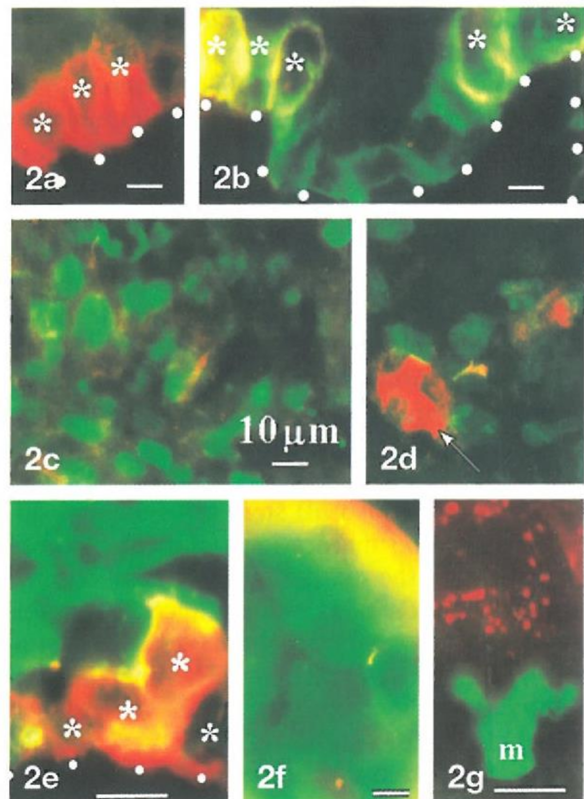

Fig. 2. DBA-reactive glycoligands (A-G; red signal), Ki-67 (A, C, D, G; green signal), β_1 subunit of integrin receptor (B; green signal), cytokeratin 3 (E; green signal) and cytokeratin 10 (F; green signal) in normal human epidermis (A, B), basal cell carcinoma of the skin (C), squamous cell carcinoma of the tongue (D), limbus of the cornea (E), epidermal cell culture (F), and "wounded" epidermal cell culture (G). The position of the basement membrane is indicated with white spots. Asterisks indicate DBA-binding site$^+$ cells in human epidermis (A, B) and limbus of the human cornea (E). Arrow indicates DBA-binding site$^+$ cell in squamous cell carcinoma (D). Cultured mitotic Ki-67$^+$ is indicated by m (G). The figures show that DBA-binding site positive cells never express Ki-67 as a marker of proliferation in vivo as well as in vitro (A, C, D, E). These cells express β_1 integrin (B; yellow signal) in epidermis and cytokeratin 3 in limbus of the cornea (E; yellow signal). Co-localization of cytokeratin 10 and DBA-binding sites in cultured cells (F; yellow signal) is also demonstrated. Scale=10 μm.

The highly specialized squamous cell epithelium, such as the anterior epithelium of the cornea, showed a remarkable co-localization of DBA-BS and cytokeratin 3 in the limbus (Fig. 2e).

The cultured confluent keratinocytes were

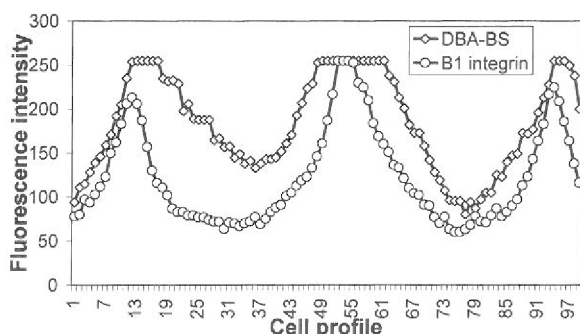

Fig. 3. Measurements of DBA-binding sites and β_1 integrin fluorescence intensity through three intercellular contacts in DBA-binding site positive area of basal layer. Co-localization of the markers is demonstrated.

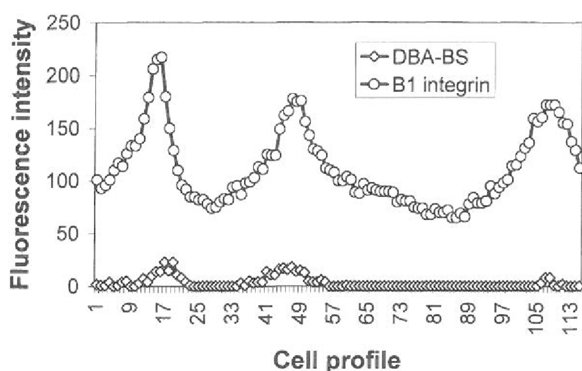

Fig. 4. Measurements of DBA-binding sites and β_1 integrin fluorescence intensity through three intercellular contacts in DBA-binding site negative area of basal layer. Only a negligible signal of DBA binding was observed.

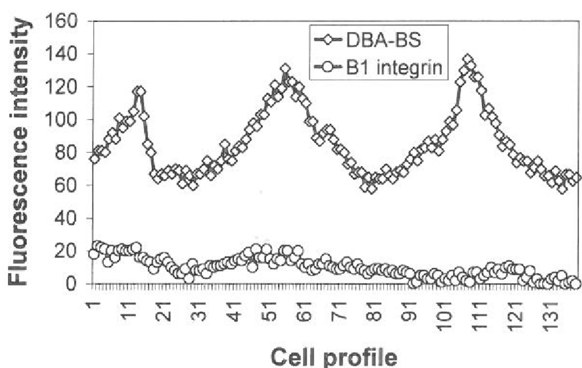

Fig. 5. Measurements of DBA-binding sites and β_1 integrin fluorescence intensity through three intercellular contacts in DBA-binding site positive area of spinous layer. The signal for DBA binding was reduced by approximately 50% and no β_1 integrin was expressed in the cells of the measured zone.

positive for β_1 integrin. A limited number of small keratinocyte colonies exhibited co-localized expression of DBA-BS and cytokeratin 10 (Fig. 2f). However, the number of cells exhibiting DBA-BS was very limited (maximally 10%). The mitotic cells and interphasic cells highly positive for Ki-67 did not express DBA-BS (Fig. 2 g). The most usual pattern of DBA-BS in cultured cells was in the Golgi complex (perinuclear with a polarized appearance), and granular in the cytoplasm (Fig. 2g, 6). DBA-BS frequently (but not always) co-localized with β_1 integrin. This phenomenon was easily visible in the Golgi complex and in the overlapping margins of epidermal cells at the stage of beginning cell multilayer formation (Figs. 7, 8).

DISCUSSION

The results presented in this paper, such as the absence of a signal for Ki-67 in DBA-BS$^+$ cells (including cells in malignant tumors) and co-localization of cytokeratin 3 with DBA-BS in the limbus of human cornea, clearly support the hypothesis that the DBA-BS$^+$ elements can be characterized as early postmitotic differentiating cells. This hypothesis is in agreement with the observation in vitro of a negative correlation between Ki-67 and DBA-BS expression. The basal expression of DBA-BS was also observed by others (see Introduction), but the procedure of multiple labeling combined with computer-assisted analysis characterizes this cell population more precisely.

The high expression of DBA-BS in this well-defined population of epidermal basal cells can be interpreted by differentiation-dependent glycosylation. This is in very good harmony with our previous findings (19) that demonstrated the absence of galectin-3-reactive binding site expression in the basal cell layer, although the suprabasal cells were well recognized by this animal lectin. The precise cellular localization of DBA-BS is not possible at the resolution level of fluorescence microscopy, but the cell membrane signal in the epidermis in situ can be distinguished. The perinuclear pattern observed in vitro points to the involvement of the Golgi complex where glycosylation takes place in eukaryotic cells. The binding reactivity of integrin receptors is clearly influenced by the glycosyl-

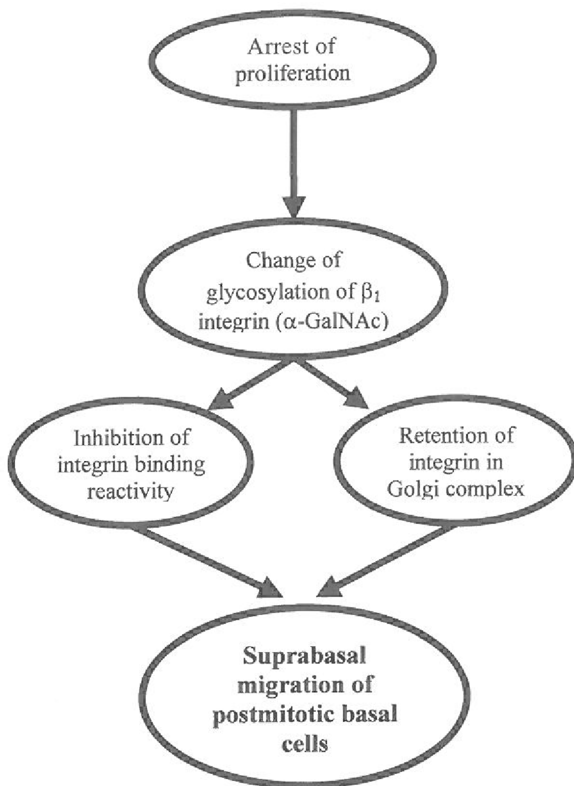

Fig. 9. A proposed model explaining the physiological role of DBA-reactive binding sites in the course of superficial migration of postmitotic basal cells.

From a practical point of view, the DBA as a probe recognizes a distinct population of postmitotic cells which are at the beginning of their differentiation route. Because this probe is a lectin, it can be used in a simple procedure for double labeling together with an antibody. These properties support the employment of DBA in cutaneous diagnostics also of malignancies.

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Detection of New Diagnostic Markers in Pathology by Focus on Growth-regulatory Endogenous Lectins. The Case Study of Galectin-7 in Squamous Epithelia

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Abstract: 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, no matter of origin of epithelium. Its expression is significantly reduced in malignant cells, thus galectin-7 might be a differentiation marker of epithelial malignancies.

Key words: Carcinoma – Basal cell carcinoma – Galectin-7 – Lectin – Squamous cell – Sugar code

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signal recognition [5, 9]. Of note, they can even read changes in glycan conformation introduced by common substitutions [10]. Reflecting their functionality, they have already become targets of drug design [11]. In addition to their specificity to glycans they also home in on peptide/lipids motifs in intracellular sites, hereby contributing to growth regulation or pre-mRNA splicing [8, 12, 13]. In fact, galectins are known to harbor pro- and anti-apoptotic activities linked to growth and malignancy [8, 14]. In this report, we focus on a homodimeric galectin present in the tissue compartment mentioned above, i.e. galectin-7 (Gal-7). This galectin is characteristic for squamous stratified epithelia, where it appears to be connected with the program of tissue stratification [15]. It figured prominently as p53-induced gene-1 in a colon cancer line, and initial reports indicate that expression of Gal-7 is actually downregulated in basal and squamous cell carcinomas of the skin [16, 17].

To illustrate our activity in the delineation of the functionality of the sugar code the histochemical monitoring of Gal-7 in normal and cancer squamous cell epithelia of different histogenetic origin and the comparison of these *in situ* results with observations performed under *in vitro* conditions are the objective of the presented study.

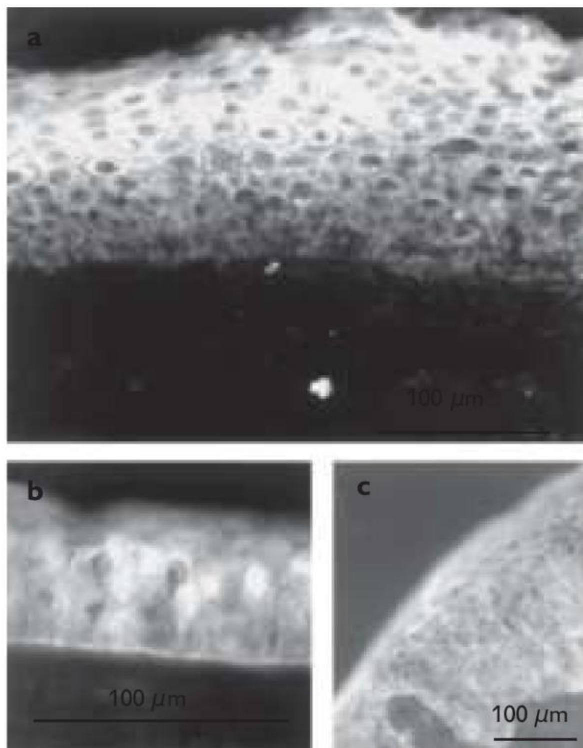

Figure 1 – Detection of galectin-7 in human epidermis (a), cornea (b) and vocal cord epithelium of larynx (c).

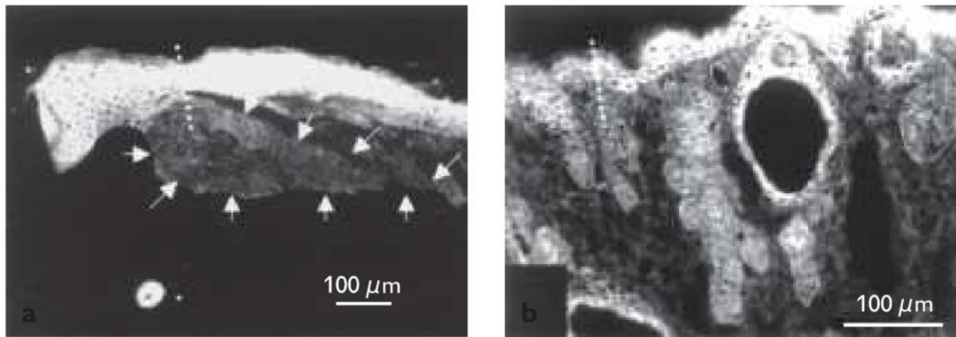

Figure 2 – Detection of galectin-7 in basal cell carcinoma (surrounded by arrows) (a) and follicular trichoepithelioma (b) dashed line marks the site of the measurements of fluorescence profile.

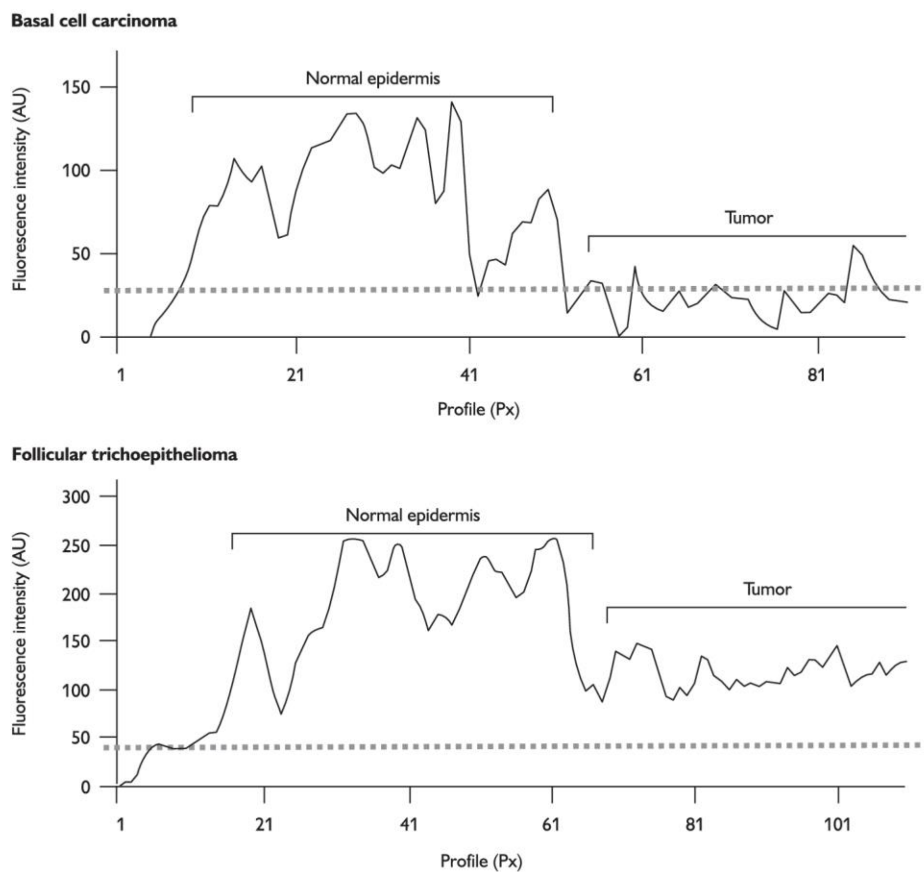

Figure 3 – Fluorescence profile of galectin-7 expression in basal cell carcinoma and follicular trichoepithelioma. See the significant difference of expression on non-affected surface epidermis and tumor site.

Material and methods

The specimens of human tissue (Table 1) were obtained with the explicit informed consent of patients according to the Helsinki Declaration. The tissue samples were frozen in liquid nitrogen using Tissue-Tek (Sakkura, Zoeterwoude, The Netherlands) and stored deeply frozen until further processing started. 7μm thin frozen sections were prepared using Cryocut E (Reichert, Vienna, Austria). Tissue-Tek was removed by PBS immediately before immunohistochemical processing. Interfollicular epidermis and epithelium from the periphery of cornea containing limbus was used for cultivation experiments as described [18].

Gal-7 was detected by a routine immunohistochemical procedure using rabbit polyclonal antibody free of cross-reactivity against any other galectin diluted 1:50. FITC-labeled swine-anti rabbit antibody (AlSeVa, Prague Czech Republic) diluted 1:50 was used as the second-step reagent. Substitution of anti Gal-7 antibody by

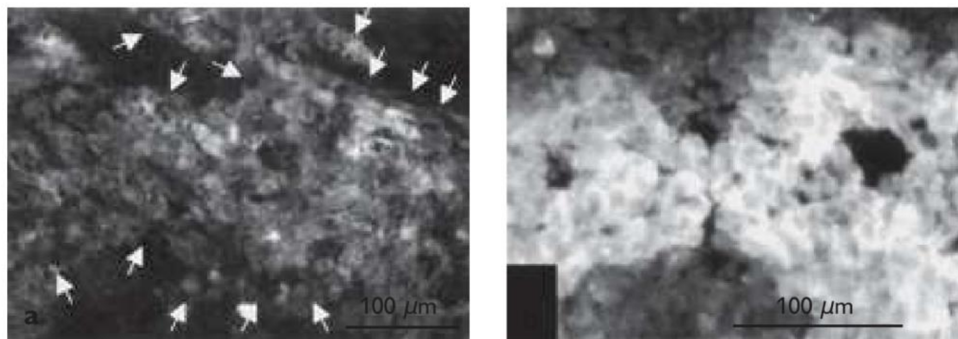

Figure 4 – Expression of galectin-7 in squamous cell carcinoma of larynx (a) and of lymphatic metastasis of the carcinoma of the same origin (b).

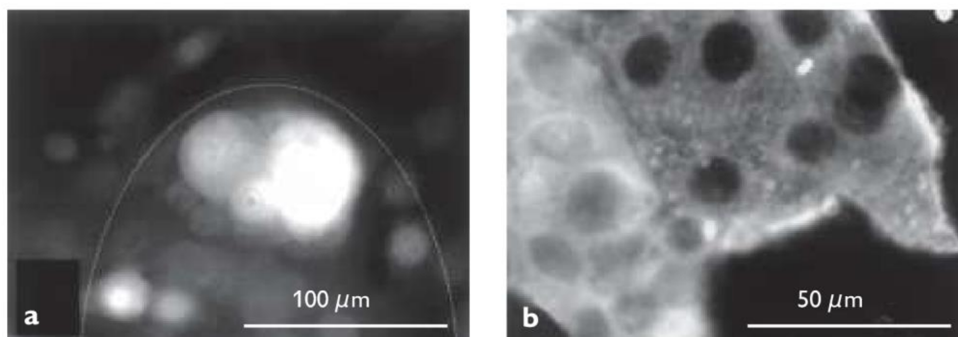

Figure 5 – Expression of galectin-7 in cultured cells of corneal epithelium at the 5th day of cultivation (the group of cells is surrounded by dashed circle) (a) and of cultured epidermis at the 10th day of cultivation (b). The second layer of differentiated cells is positive only.

non-immune rabbit serum was used as a control of the specificity of reaction, as was saturation of antibody by Gal-7. The specimens were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and inspected employing an Optiphot-2 (Nikon, Praha, Czech Republic) fluorescence microscope equipped with a CCD camera Cohu and a computer-assisted image analysis system LUCIA (Laboratory Imaging, Prague, Czech Republic).

Results

Cells of the layers of the different types of studied epithelia, i.e. of epidermis (Figure 1a), cornea (Figure 1b) and larynx (Figure 1c), expressed Gal-7 (Table 1). While the cells of basal cell carcinomas were devoid of Gal-7 expression (Figure 2a), cells of follicular trichoepithelioma were positive (Figure 2b), although the extent of signal was lower than in the non-affected epidermis (Figure 3) as is visible from the measurements of fluorescence profiles. The samples of squamous cell carcinoma (SCC) of epidermis and a majority of samples of the same type of tumor but from the laryngeal epithelium exhibited significant regulation of galectin expression (Figure 4a). A similar phenomenon was also observed in lymph node metastases of the laryngeal SCC (not shown). Interestingly, cells of one sample of laryngeal SCC and its lymphatic metastasis presented Gal-7 expression in tumor cells (Figure 4b).

The initial step of the cultivation of epidermal cells and corneal epithelium is associated with absence of Gal-7 expression. At the beginning of the formation of multilayered colonies Gal-7 expression is apparently initiated (Figure 5a and b). Two-weeks-old confluent culture of human keratinocytes constitutes a mosaic of monolayer and multilayered regions. Expression of Gal-7 reflects this growth of culture, where the cell multilayer is only positive for the studied lectin (Figure 6).

Discussion

Our results support the notion that Gal-7 is involved in the negative growth regulation of epithelial tumor cells. Fittingly, the follicular trichoepithelioma,

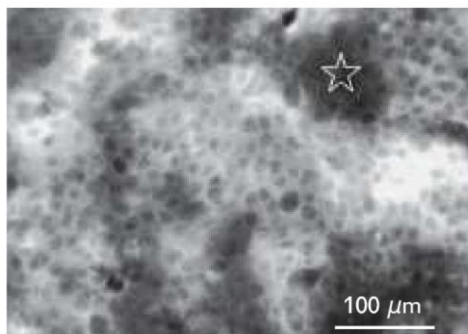

Figure 6 - Expression of galectin-7 in cultured epidermal cells at the 2nd week of cultivation. Cells of the monolayer (asterisk) are weekly positive in comparison with multilayered formation.

a rare benign tumor of the skin with high degree of tumor cell differentiation, showed only small reduction of Gal-7 expression in comparison with the epidermis. However, recent results by proteomics demonstrate an increase of Gal-7 expression in buccal squamous cell carcinoma in comparison with normal epithelium [19]. Evidently, cell biological studies are required to clarify the role of Gal-7 in each cell type under study. Looking at the colon cancer (DLD-1) cells, galectin-7 offers the potential for application as suppressor [16, 20], as was similarly reported for neuroblastoma cells by virtue of cell surface binding to ganglioside GM_1 [21]. In epithelia it is reasonable to assume that Gal-7 is linked to the program of stratification in squamous type of epithelia arising from both the ectoderm and entoderm. This process is under the control of transcription factor $\Delta Np63\alpha$. Decrease of Gal-7 expression in epithelial tumors can thus serve to indicate the lack to develop an organized multilayered tissue. These tumors present a high level of expression of $\Delta Np63\alpha$ and decrease or absence of adhesion molecules responsible for interacellular contacts typical for suprabasal cells in squamous cell carcinomas. At this stage, further study of Gal-7 might be instrumental to define a diagnostic marker for this type of malignancy. Having developed a panel of antibodies for galectins, it will be a next step to comparatively map the network and relate its status to the level of differentiation and tumor progression. A new perspective is opened by the introduction of labeled galectins to localize endogenous binding sites, recently reported [22]. Especially, galectins-1 and -3 and their reactive ligands have role in biology and spreading of squamous cell carcinomas [23]. The comparison of expression of these galectins and their ligands with Gal-7 could be suitable for better characterization of malignant epithelial tumors.

Conclusion

Galectin-7 expression represents the specific feature of squamous epithelia of different nature. Monitoring of this endogenous lectin can be employed in the histopathology of tumors originating from this type of epithelium.

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Defining the glycophenotype of squamous epithelia using plant and mammalian lectins. Differentiation-dependent expression of α2,6- and α2,3-linked N-acetylneuraminic acid in squamous epithelia and carcinomas, and its differential effect on binding of the endogenous lectins galectins-1 and -3

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Holíková Z, Hrdličková-Cela E, Plzák J, Smetana K Jr, Betka J, Dvořánková B, Esner M, Wasano K, André S, Kaltner H, Motlík J, Hercogová J, Kodet R & Gabius H. Defining the glycophenotype of squamous epithelia using plant and mammalian lectins. Differentiation-dependent expression of α2,6- and α2,3-linked N-acetylneuraminic acid in squamous epithelia and carcinomas, and its differential effect on binding of the endogenous lectins galectins-1 and -3. APMIS 2002;110:000–0.

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 glycan 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 (α2,3- or α2,6-linked) by plant lectins. Based on the hypothesis that this factor regulates accessibility of ligands for endogenous lectins we introduced two labeled 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 α2,6-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.

Key words: Epidermis; galectin; glycohistochemistry; sialic acids; stem cells; T-antigen.

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Squamous epithelia (epidermis, oropharyngeal mucosa, vocal cord epithelium, anterior corneal epithelium) represent morphologically and functionally stratified tissue types. The pool of proliferatively active cells, including the stem cells, is present in the basal layer (1, 2). Specialized squamous epithelia such as corneal epithelium and epithelial derivatives such as hair follicles are characterized by distinct occurrence of stem cells in the limbus and bulge region of the hair sheet, respectively (3). In contrast, terminally differentiated postmitotic cells are present basally and mainly suprabasally (1–3). The basal and suprabasal cells can be distinguished not only by their morphological features but also by a set of characteristic markers. For example, basal cells express a typical pattern of intermediate filaments and integrin receptors, which is helpful in cell biology and clinical monitoring (1, 4). Besides protein markers, the products of the cells' capacity for glycosylation, i.e. the glycan chains of cellular glycoconjugates, harbor the potential to reveal distinctive features. Hence, on examining their glycosylation with plant and mammalian lectins, these cells have been characterized by an absence of galectin-3 (Gal-3)-reactive glycoligands, and postmitotic basal cells express *Dolichos biflorus*-agglutinin-reactive glycoepitopes (5–7). Considering glycans as information-bearing code units, the mapping especially of those sugar epitopes which are spatially accessible for interactions is of interest (8)

In this respect, branch termini with typical modifications by α2,3(6)-sialylation deserve special attention. This reasoning is underscored by observations in mice deficient for either α2,3- or α2,6- sialyltransferase activities (9, 10). Besides a local effect on protein structure by the different sugar configuration, the display of docking sites for endogenous lectins (for example galectins, siglecs or selectins) is altered, disturbing certain aspects of the translation of the sugar code (8). Underscoring the information obtained with KO mice, cell transfectants with α2,6-sialyltransferase expression in glioma (U373) cells or its downregulation in colon cancer (HT29) cells revealed impaired invasiveness, linking changes in the pattern of sialylation state with cell biological properties (11, 12). Exploiting this approach, colon cancer cell adhesion to endothelial cells was enhanced by raising the level of cell surface α2,3-sialylation (13). Fittingly, α2,6-sialylation together with sialyl-Tn expression was correlated with survival in colon cancer patients and associated with malignant transformation of colon enterocytes (14, 15). The developmental course of expression of α2,6-linked sialic-acid-bearing epitopes is consistent with the suggestion of oncofetal regulation (16, 17). These results prompt the question as to whether these epitopes might also exert notable functions in epidermal cell layers, but information on their presence at this site is rather limited (18). This lack of relevant data on the glycophenotype of cells in this region was one reason for initiating this study. In order to correlate presence of glycan epitopes with functional implications the ligand properties for endogenous lectins were then assessed. In particular, the presence of endogenous lectins such as Gal-3 and the switching between inert and galectin-reactive sialylated structures with shifting from α2,6- to α2,3-linked sialylation provided the incentive to extend classical monitoring with plant lectins (*Maackia amurensis* lectin, MAL; *Sambucus nigra* lectin, SNL) using mammalian lectins as probes (19, 20).

In this study, we report on the presence and topological expression profile of α2,6/α2,3-linked Neu5Ac, and T-antigen in adult human squamous epithelia at different anatomic sites (epidermis, larynx, cornea, oropharyngeal mucosa), in embryonic epidermis of the chick and in fetal epidermis of the pig before and after the neuraminidase pretreatment. These expression profiles were correlated with the binding patterns of mammalian galectins. Due to fine-specificity differences between the individual members of the family of galectins, we employed galectins-1, -3, and -4, deliberately selecting one member of each subfamily, i.e. Gal-1 representing proto-type galectins, Gal-3 as chimera-type family member, and Gal-4 as tandem-repeat-type protein (19, 20). These results are set in relation to those obtained with carcinomas and also cultured human epidermal and mouse embryonic stem cells representing non-differentiated pluripotent elements. A differential role of α2,6/α2,3-linked Neu5Ac in masking galectin binding is indicated.

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MATERIAL AND METHODS

Tissue samples

Specimens of human epithelia and carcinomas (type and number specified in Tables 1 & 2) were obtained with the explicit consent of the patients at surgery, or postmortem (corneas). Porcine fetal skin samples (85th day of pregnancy) of Minnesota×Göttingen strain (Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic) and those of chick embryonic skin (White Leghorn, 14th day of incubation) (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic) were also included. Samples of human and porcine origin were routinely processed and embedded in paraplast. Alternatively, they were frozen with liquid nitrogen using Tissue-Tek (Sakura-Finetek Europe B. V., Zoeterwoude, The Netherlands) as a cryoprotective agent and stored in the frozen state until histochemical processing started.

Cell culture

Human epidermal cells were harvested from skin samples obtained from the Department of Aesthetic Surgery (Charles University, 3rd Faculty of Medicine) and cultured according to a modified version of a published procedure (21–23). The embryonic stem cells were isolated from blastocysts of C57BL/6xBALB/c mice. ES cells were maintained on mouse embryonic fibroblasts in DMEM medium supplemented with 20% fetal calf serum, MEM non-essential amino acids, 100 mM nucleosides, penicillin/streptomycin, 0.1 mM β-mercaptoethanol and 1000 U/ml leukemia inhibitory factor (24, 25). The cells were fixed with a mixture of ethanol (100%) and acetic acid (98%) at a 4:1 ratio (v/v) and also used for lectin cytochemistry.

Immunohistochemistry and lectin histochemistry

The tissue preparation protocols, including our double-labeling protocol, were described in detail in a previous report (26). The 7-µm-thin frozen or rehydrated paraplast sections were used for lectin histochemistry or immunohistochemistry. Frozen sections or cultured cells were washed with phosphate-buffered saline (PBS, pH 7.4) and fixed with either 2% (w/v) paraformaldehyde in PBS (pH 7.4), methanol, or methanol with acetic acid (4:1, v/v) prior to further processing. Because paraplast sections were fixed prior to embedding, no further fixation was performed.

The level of carcinoma cell differentiation was evaluated by analysis with a monoclonal antibody against cytokeratin type 10 (IgG1 isotype) and LP34 (CK1) (IgG1) monoclonal antibody (DAKO, Glostrup, Denmark). The LP34-reactive cytokeratins are present in basal and suprabasal layers of normal squamous epithelia in contrast to the positivity with an antibody against cytokeratin-10 recognizing the suprabasal layers only (4). The â1 integrin was detected by a monoclonal antibody (IgG2a) from Immunotech (Prague, Czech Republic) as a marker of the basal cell layer (1). Biotinylated plant lectins were used to detect the α2,3-/ α2,6-linked sialic-acid-bearing epitopes and the T-antigen (Thomsen-Friedenreich antigen; Galβ1,3GalNAc). *Maackia amurensis* lectin type 2 (MAL) specific for α2,3-linked NeuNAc, *Sambucus nigra* lectin (SNL) specific for α2,6-linked Neu5Ac, and Jacalin (isolated from *Artocarpus integrifolia* seeds) specific for Galβ1,3GalNAc (Neu5Ac-free or sialylated) were purchased from Vector Laboratories (Burlingame, CA, USA) and applied as recommended by the supplier. Biotinylated galectins-1 and -3 were prepared as described (27, 28), while galectin-4 domains were directly used as fusion proteins containing the glutathione-S-transferase (GST) of *Schistosoma japonicum* origin (29). Gal-4 binding was therefore detected by a rabbit polyclonal antibody against GST (Santa Cruz Biotechnology, CA, USA). Specific binding of biotinylated neoglycoproteins presenting N-acetyllactosamine with α2,3- or α2,6-linked Neu5Ac was also studied by reverse lectin histochemistry (20, 30). Cell nuclei were counterstained with 4;6-diamidino-2-phenylindole dilactate (DAPI) (Sigma-Aldrich, Prague, Czech Republic) in selected specimens. Neuraminidase treatment for control removal of sialic acids from glycoconjugate chains of cells and in sections was performed with an enzyme preparation purchased from the Institute of Epidemiology and Microbiology (Gorkij, Russia) and diluted 1:100. To evaluate the possible effect of glycolipids on staining profile and intensity, an extraction procedure using a mixture of chloroform: methanol (2:1; v/v) was performed in certain sections. Lactose (Sigma-Aldrich, Prague, Czech Republic) was employed as competitive inhibitor of galectin binding. FITC-labeled swine anti-mouse or anti-rabbit immunoglobulins (Alseva, Prague, Czech Republic) and TRITC-labeled ExtrAvidin (Sigma-Aldrich, Prague, Czech Republic) were used as second-step reagents for visualization of specific marker binding. The specificity of the 1st step antibodies was tested by their omission or replacement by monoclonal anti-rat macrophages ED1 (IgG1 isotype) and ED3 (IgG2a isotype) (Serotec, Oxford, UK). The specimens were mounted on Vectashield (Vector Laboratories, Burlingame, CA, USA) and analyzed with an Optiphot-2 fluorescence microscope (Nikon, Prague, Czech Republic) equipped with specific filter blocks and a computer-assisted image analysis system (LUCIA, Laboratory Imaging, Prague, Czech Republic).

RESULTS

Normal adult epithelia

Analysis of the data for galectin-specific binding revealed that Gal-1 and Gal-3 have

TABLE 1. *Semiquantitative evaluation of staining intensity using labeled plant and mammalian lectins*

Epithelium	Marker							
	SNL α2,6 Neu5Ac		MAL II α2,3 Neu5Ac		Gal-1		Gal-3	
	BC	SBC	BC	SBC	BC	SBC	BC	SBC
Human epidermis (n=5)	++	−	++	++	++	++	−	++
Human cornea (limbus) (n=3)	++	−(n=1) +(n=2)	++	++	++	++	−	−(n=2) +(n=1)
Human oropharyngeal mucosa (n=5)	+	−	−	+	++	++	−	++
Human larynx (vocal cord) (n=2)	++	−/+ lower suprabasal	−	+	++	++	−	++
Porcine fetal epidermis (day 85) (n=4)	++	−/+ lower suprabasal	−	+	ND	ND	−	−
Chick embryonal epidermis (day 14) (n=3)	−	−	+	+	ND	ND	−	−

BC: basal cells; SBC: suprabasal cells; ND: not determined.
Scale: − (no signal), + (positive cells), ++ (strongly positive cells).

non-uniform profiles of ligand sites. While Gal-1-binding sites were observed in cells of basal and also suprabasal layers of all studied samples of epithelia, Gal-3-binding sites were detected only suprabasally, with the exception of human tonsillar epithelium showing no signal for Gal-3 binding (Table 1, Fig. 1). Because of the restricted expression profile of Gal-4 relative to the widely present Gal-1 and -3, expression of reactive binding sites for the tandem-repeat-type galectin was studied in tonsillar epithelium only. The results showed different binding reactivities of the C- and N-terminal carbohydrate recognition domains, reflecting their non-identical sequences (Fig. 1). Detection of T-antigen by Jacalin was performed in the epidermis only. The positive signal for the expression of this disaccharide was found basally and suprabasally (Table 1, Fig. 1). The cells of epidermis, oropharyngeal epithelium and vocal cord of larynx expressed α2,6-linked Neu5Ac recognized by SNL in the basal layer and in the lower spinous suprabasal layer (Table 1, Figs. 1 & 2). The limbal corneal epithelium presented a heterogeneous staining pattern, and SNL binding was observed either restricted to the basal layer or in all layers of the epithelium (in each case strong signal intensity was expressed by the basal cells) (Table 1, Fig. 3). The binding of MAL, which specifically recognizes α2,3-linked Neu5Ac, was observed in cells throughout the epidermis and the anterior epithelium of the limbal region of the cornea (Table 1, Figs. 1 & 3). The vocal cord and oropharyngeal epithelium expressed α2,3-linked NeuNAc strictly suprabasally (Table 1, Fig. 2). To control the requirement of binding for carbohydrate ligands, neuraminidase treatment to remove sialic acids was performed. Binding of both plant lectins was completely inhibited by neuraminidase pretreatment, as expected on the basis of their sugar specificities (Fig. 4). Gal-3-reactive glycoligands were detected also basally in these neuraminidase-treated sections (Fig. 4). Interestingly, this basal signal was sensitive to lipid extraction by a mixture of chloroform and methanol (Fig. 4). No accessible binding sites for the neoglycoprotein with α2,6-linked Neu5Ac were detected in the studied epithelia, in contrast to when testing the probe with α2,3-linked Neu5Ac, which was bound to dermal leukocytes (Fig. 4). The visualization of cytokeratins, β1 integrin and collagen type IV (green signal) was employed as an independent marker to assign the signal of Gal-1 and -3 binding to epithelial cells.

Chick embryonic and porcine fetal epidermis

No binding of SNL and Gal-3 to native chick embryonic epidermis was detected, in contrast to the neuraminidase-sensitive signal with MAL which was observed in cells of all layers of the epidermis (Fig. 5). Treatment of the epidermis

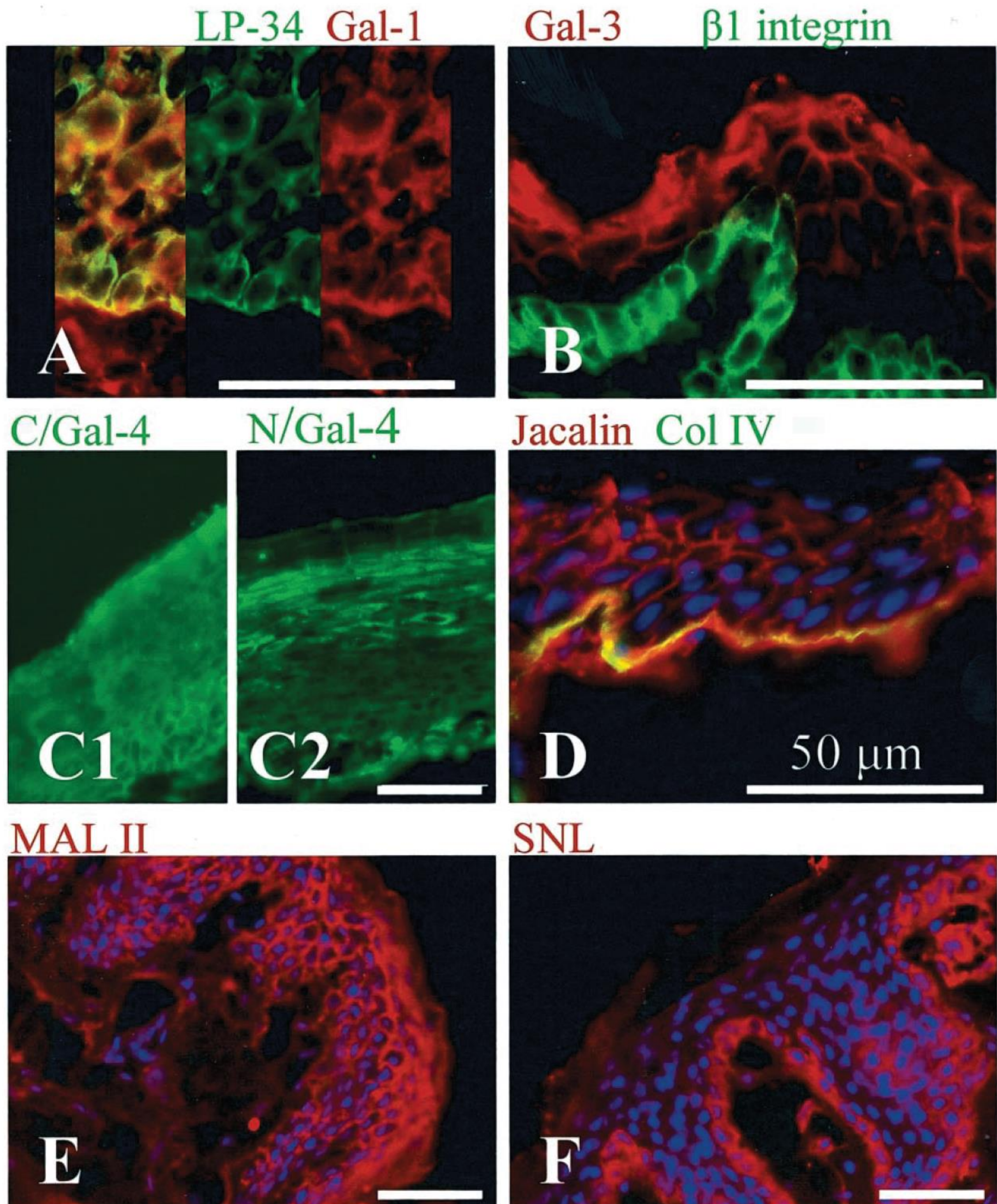

Fig. 1. Detection of binding sites for Gal-1 (A, red signal), Gal-3 (B, red signal), and the C-terminal (C1, green signal) or N-terminal (C2, green signal) carbohydrate recognition domains of tandem-repeat-type Gal-4, and also of Jacalin (D, red signal)-, MAL II (E, red signal)- and SNL (F, red signal)-reactive glycoligands in the adult human tonsillar epithelium (A, C1, C2) and epidermis (B, D, E, F). Presence of LP-34-positive cytokeratins (A), β1 integrin (B) and collagen type IV (D) is visualized with a green signal, and nuclei were counterstained by DAPI (D, E, F). A yellow signal represents colocalization of cytokeratins with Gal-1-reactive glycoligands (A) or of T-antigen with collagen type IV (D). Bar = 50 μm.

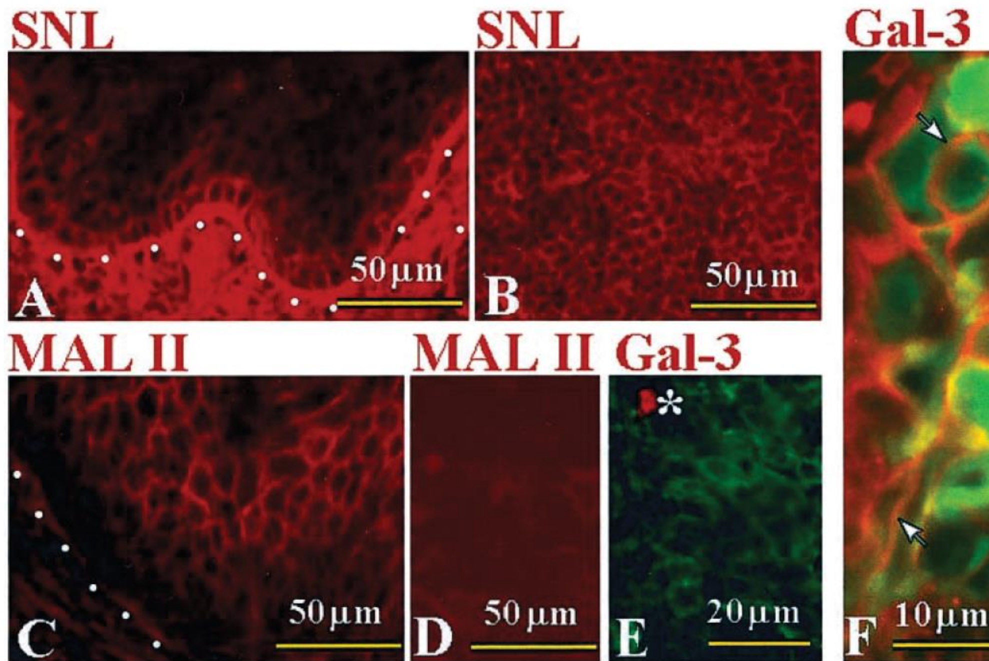

Fig. 2. Detection of SNL (A, B, red signal)-, MAL II (C, D, red signal)- and Gal-3 (E, F, red signal)-reactive glycoligands in representative sections of a tumor-free area of a case with differentiated squamous cell carcinoma of the vocal cord (A, C) as well as of poorly (B, D, E) and highly (F) differentiated squamous cell carcinomas of the vocal cord. Presence of LP-34-positive cytokeratins yields the green signal (E, F). The position of the basement membrane is indicated by the dashed line (A, C). The bars represent 10, 20 and 50 ìm, respectively.

with neuraminidase had no influence on Gal-3 binding.

The basal layer of porcine fetal epidermis and the developing hair follicle were recognized by SNL. MAL-reactive glycoligands were observed in suprabasal cells, but not in any tested specimens of developing hair follicles (Fig. 5). The staining pattern for the two plant lectins reactive with either α2,3- or α2,6-linked Neu5Ac was sensitive to removal of sialic acids by neuraminidase digestion. No Gal-3-binding sites were detected in the epithelium irrespective of another or not neuraminidase was applied.

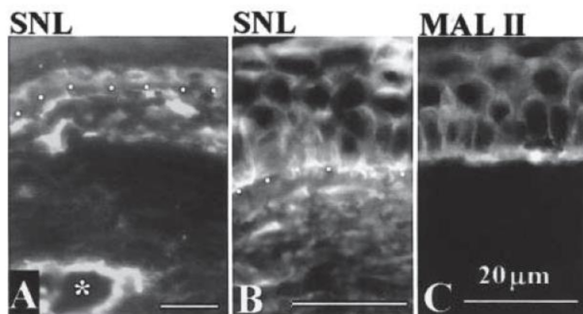

Fig. 3. Detection of SNL (A, B)- and MAL II (C)-reactive glycoepitopes in the corneal epithelium located at the limbar region. The asterisk denotes the position of the Schlemm canal, the dashed line the region of the basement membrane. Bar=20 µm.

Basal cell carcinomas of epidermis and squamous cell carcinomas of larynx and base of the tongue

The studied carcinomas expressed LP34-positive cytokeratins. Cytokeratin 10 was detected only in large polygonal cells of laryngeal or lingual carcinomas resembling the suprabasal differentiated cells of normal squamous epithelia. The basal cell carcinomas and small round cells (poorly differentiated elements from the morphological point of view) of laryngeal and tongue squamous cell carcinomas were recognized by SNL (Table 2, Figs. 2 & 6). MAL-reactive areas were observed in about one half of the studied basal cell carcinoma samples and in differentiated cell areas (large polygonal cells) of carcinomas of the larynx and base of the tongue (Table 2, Figs. 2 & 6). Whereas the

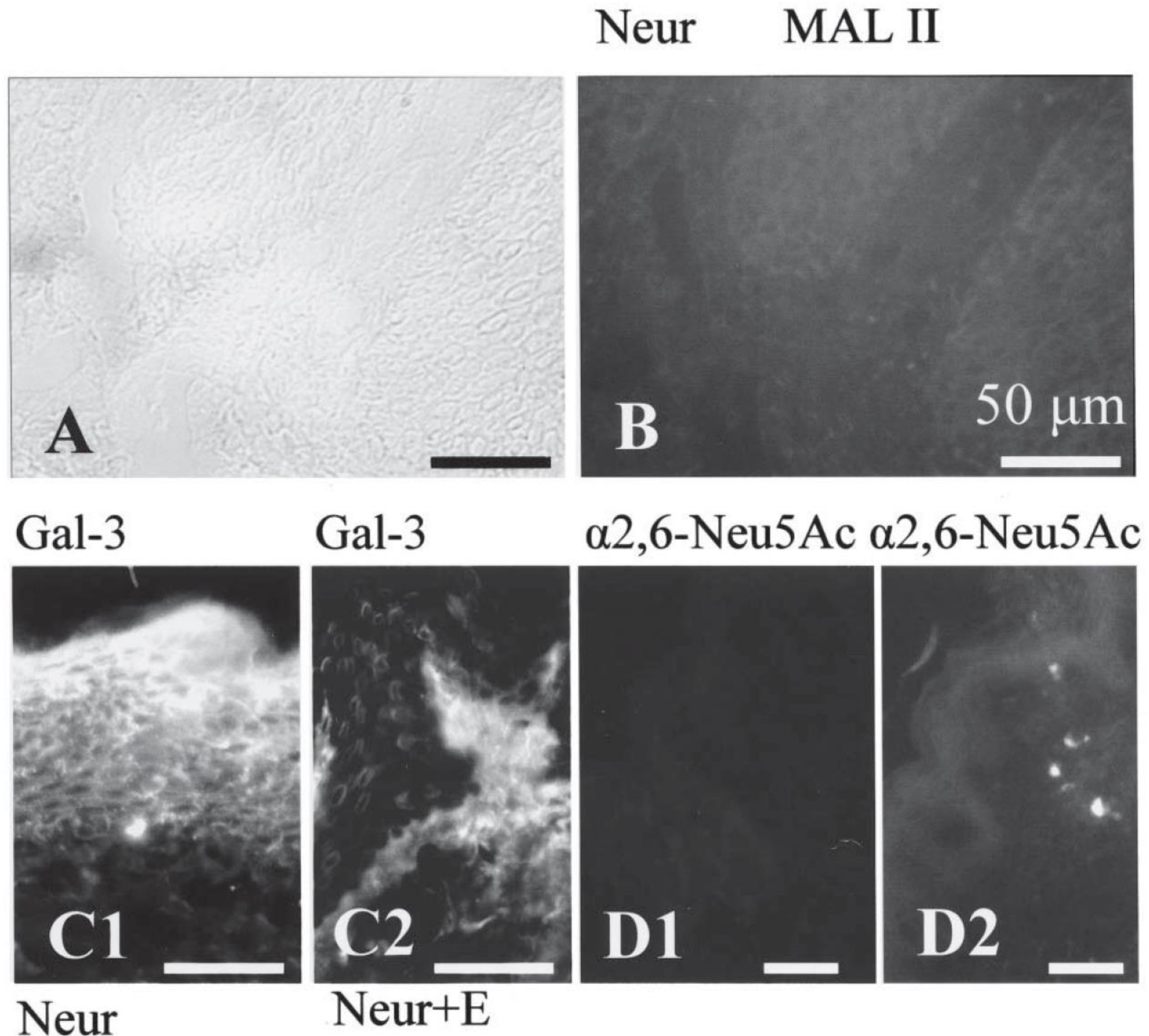

Fig. 4. Detection of MAL II (B)- and Gal-3-reactive glycoligands (C1, C2) after pretreatment with neuraminidase (B, C1) as well as pretreatment with neuraminidase and lipid extraction (C2) in sections of human epidermis (A-C2). The region shown in part B is also illustrated using interference contrast (A). Localization of α2,6- and α2,3-linked Neu5Ac-specific binding sites in human epidermis (D1, D2). Bar=50 μm.

poorly differentiated carcinoma cells reactive for SNL, negative for MAL II and without cytokeratin-10 expression were negative for Gal-3 binding, the differentiated cells (cytokeratin 10, MAL II-reactive and SNL-negative) were Gal-3-reactive (Table 2, Fig. 2). Gal-1-binding sites were observed in all cells of basal as well as squamous cell carcinomas (Table 2). The areas containing the large cells expressing cytokeratin 10 and binding sites for Gal-3 and MAL II were usually in the center of the tumor nodule, and they were surrounded by the smaller round cells negative for expression of cytokeratin 10 and binding sites for Gal-3 and MAL II. However, these cells were well recognized by the SNL. The removal of Neu5Ac from glycan chains by neuraminidase treatment rendered the majority of tumor cells accessible for Gal-3.

Cultured mouse embryonic stem cells and epidermal cell

While the colonies of mouse embryonic stem cells were not stained by labeled SNL or MAL, they were clearly reactive with Gal-3 without and also after the neuraminidase pretreatment (Fig. 6). The cultured epidermal cells were reac-

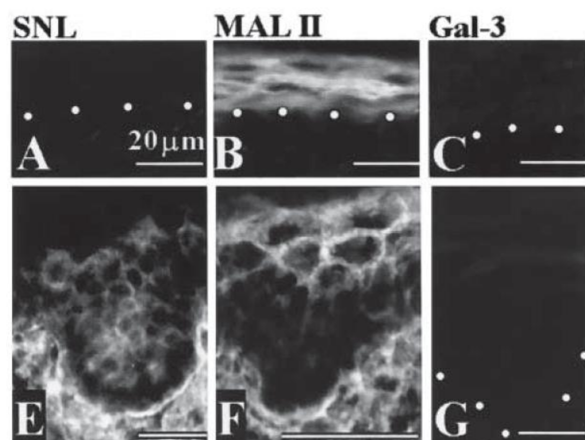

Fig. 5. Detection of SNL (A, E)-, MAL II (B, F)- and Gal-3 (C, G)-binding sites in sections of chick embryonic (A–C) and porcine fetal (E–G) epidermis. Bar=20 μm.

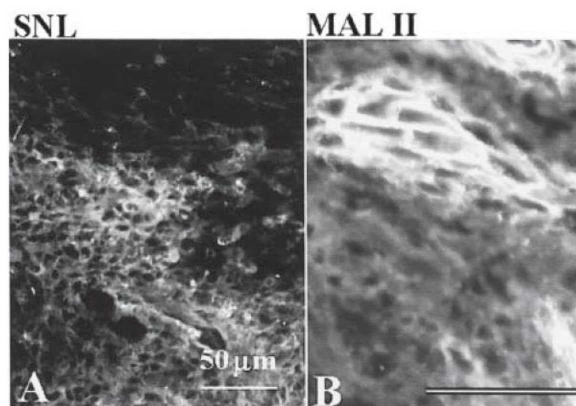

Fig. 6. Detection of SNL (A)- and MAL II (B)-reactive glycoligands in representative sections of a squamous cell carcinoma at the base of the tongue. SNL reactivity was found in round, poorly differentiated cells (A) in contrast to a strong positivity of large cells for MAL II (B). Bar=50 μm.

tive with SNL recognizing α2,6-linked Neu5Ac and very poorly so for MAL (α2,3-linked Neu5-Ac specificity) and for Gal-3. Neuraminidase treatment significantly increased the extent of Gal-3 binding to the epidermal cell (Fig. 7).

DISCUSSION

Application of plant lectins can be regarded as a classic technique for defining the glycophenotype (glycomic profiling) of cells by histochemistry. Differences in fine specificities between plant and endogenous lectins preclude any general conclusions as to the role of detected epitopes in information transfer. By introducing mammalian lectins to this technology, it is not only possible to detect distinct glycans but also to infer ligand presence and density for protein-carbohydrate interactions in situ (20). Thus, we performed a combined analysis with both types of lectins, characterizing the importance of sialylation of branch ends. To reveal whether members of the same family of animal lectins share target specificity or have developed divergence in this respect, analysis with more than one family member (here galectin) is essential. When a member of each subgroup of the family of galectins is examined, the individual localization profiles are not identical, indicating distinct fine-specificities. Gal-1-reactive epitopes

TABLE 2. *Semiquantitative evaluation of staining intensity using labeled plant and mammalian lectins and antibodies against LP34/CK10*

Carcinoma	Marker													
	SNL α2,6 Neu5Ac		MAL II α2,3 Neu5Ac		Gal-1		Gal-3		Gal-3 (neuraminidase)		LP34		CK10	
	UC	DC	UC	DC	UC	DC	UC	DC	UC	DC	UC	DC	UC	DC
Squamous cell: larynx (n=4)	+	−	−	+	+	+	−	+	+	++	++	++	−	++
Squamous cell: base of tongue, tonsils (n=6)	+	−	−	+	+	+	−	+	+	++	++	++	ND	ND
Basal cell: epidermis (n=6)	+	0	−(n=3) +(n=2)	0	+	0	−	0	+	0	++	0	−	0

DC: differentiated cells; UC: undifferentiated cells; ND: not determined; 0: cell type not present.
Scale: − (no signal), + (positive cells), ++ (strongly positive cells).

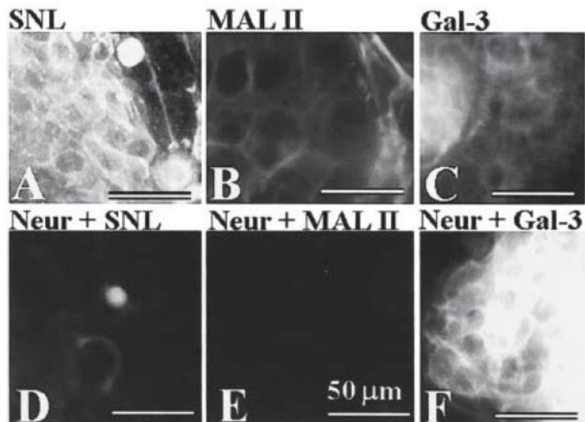

Fig. 7. Detection of SNL (A)-, MAL II (B)- and Gal-3 (C)-reactive glycoligands in cultured human keratinocytes without (A–C) and after neuraminidase pretreatment (D-F). Bar=50 µm.

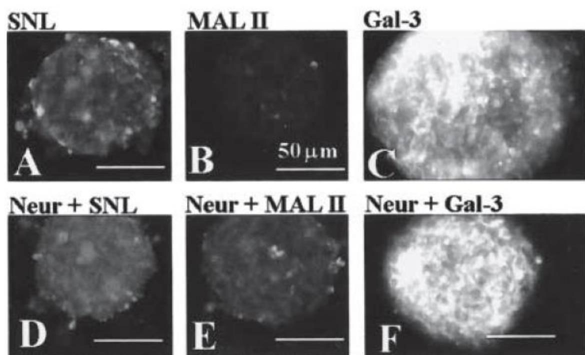

Fig. 8. Detection of SNL (A)-, MAL II (B)- and Gal-3 (C)-reactive glycoligands in cultured human epidermal stem cells without (A–C) and after neuraminidase pretreatment (D–F). Bar=50 µm.

were present in cells of all layers of the adult epithelia, in contrast to the suprabasal localization of Gal-3-specific glycoligands, confirming and extending previous reports on squamous cell and renal carcinomas (5, 6, 31). Gal-4 is expressed in epithelia of the digestive system, including carcinomas and gastrointestinal cell lines (32–34). As predicted from their sequence divergence, the two carbohydrate recognition domains of Gal-4 recognized distinct binding sites in the thickness of tonsillar epithelium with significant suprabasal binding of the N-terminal carbohydrate recognition domain. Notably, different binding patterns by these markers had been observed in intestinal epithelium (29).

Sialylation as monitored with two plant lectins was analyzed regarding linkage type and correlation with differentiation. The binding patterns of α2,3- and α2,6-Neu5Ac-specific plant lectins (i.e. MAL and SNL) to adult normal epithelia were clearly influenced by the degree of cell differentiation. α2,6-Neu5Ac-containing structures were preferentially expressed in cells of the basal layer, which harbors the pool of proliferatively active elements. Similar to the pronounced variation of SNL reactivity in colon tissue (15, 35), presentation of α2,6-linked Neu5Ac is thus subject to regulation with the differentiation status in these anatomic sites. As a consequence, galectin binding might be affected. To provide further indirect evidence for this suggestion, we included the plant lectin Jacalin in the marker panel. The binding of Jacalin, recognizing T-antigen (without or with sialic acid), to the surface of cells throughout all layers of the epithelium, including the basal layer, intimates a masking role of α2,6-linked Neu5Ac moieties for Gal-3 (and probably also for the N-terminal CRD of Gal-4), because the T-antigen belongs to the Gal-3-reactive glycoligands (36). Fittingly, our studies using enzymatic removal of sialic acids from glycan chains in the sections corroborated this reasoning. When further comparing SNL/Gal-3 binding profiles, the correlation can be interpreted as an indication that α2,6-sialylation can indeed be a switch for galectin binding in situ. The results of in vitro experiments with cultured keratinocytes lend this additional support. Further data sets presented in this report, however, add a cautionary note to the conclusion that such a correlation will necessarily always be present. Namely, observations on chicken embryonic and also on fetal porcine epidermis and embryonic stem cells demonstrated that the expression of α2,6-linked Neu5Ac and Gal-3-reactive glycoligands might not be directly connected in any of these cases.

In order to attribute staining to glycan chains of glycoproteins and glycolipids we exposed specimens to organic solvents. Extraction of glycolipids from the specimens treated with neuraminidase had a reducing effect on Gal-3 binding to the cells of the basal layer. This result indicated a contribution of glycolipids as glycoligands for Gal-3. In solid-phase binding assays, lactosylceramide was not recognized by Gal-1, in contrast to ganglioside GM1 (but not other sialylated gangliosides), which is Gal-1/3-

reactive (37, 38). Interestingly, ganglioside GM1 binding by galectins has implications for growth regulation of neuroblastoma cells in vitro (39) and therefore galectin binding to a glycolipid can be considered to trigger cell responses, as so far primarily assigned to glycoproteins as target sites, for example laminin, fibronectin and several integrin subunits (27). Lactosylceramide has been found in the basal layer of squamous epithelia (Hůlková & Elleder, unpublished), but a role of this molecule as a ligand of Gal-3 might be limited owing to the short sugar chain with a low level of spatial accessibility.

In conclusion, application of two plant lectins, and especially Gal-3, has produced evidence of masking of Gal-3-reactive epitopes in the basal layer by α2,6-sialylation. It is noteworthy in this context that suprabasal, not basal expression of this galectin has been documented immunohistochemically (40, 41). Suprabasally, Gal-3-reactive epitopes colocalize with desmosomal proteins (6), which are not expressed basally. In the light of this, the role of α2,6-linked Neu5Ac masking Gal-3-binding sites might be related to functional aspects of basal cells, which are able to migrate laterally in the course of epidermal self-renewal. Additionally, it is noteworthy that galectin-3 is a key factor in hunsin-dependent induction of terminal differentiation of epithelial cells (42). Equally interesting, the cell glycophenotype was dependent on the differentiation of epithelial cells under physiological conditions (normal epithelium) and in carcinomas in many of the studied markers. The differentiated areas of squamous cell carcinomas were similar to suprabasal cells of normal epithelium. A similarity between the lectin accessibility of basal cell carcinomas and the basal cell layer of epithelium was observed. Technically, these results demonstrate the merit of the double-labeling approach using endogenous and plant lectins to refine the glycophenotypic characterization in cell and tumor biology. It is suggested that this approach will find applications in other tissues and for other animal lectin families which function as cell adhesion molecules, such as C-type lectins and siglecs (19, 42–44).

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Galectins are proposed to mediate cell adhesion and cell-cell interaction, regulate cell growth and trigger or inhibit apoptosis (7,9,10,13-15). Due to their interaction with matrix glycoproteins a role in tumor spread has been proposed (16-18). In colon carcinoma, for example, laminin, carcinoembryonic antigen and lysosome-associated membrane glycoproteins are identified as reactive target molecules (19,20). Due to its anti-apoptotic activity (20-23) galectin-3, the only chimera-type member of this family, deserves special attention in immunohistochemical monitoring.

With respect to oral, pharyngeal and laryngeal cancers, decrease in the extent of expression of galectin-3-reactive correlates significantly with increased level of clinically detectable cancer aggressiveness and is related to the level of differentiation (24,25). Interestingly, galectin-3-reactive sites are subject to a similar regulatory mechanism (24). Further monitoring revealed that they are localized on the cell surface of suprabasal cells of normal oropharyngeal and laryngeal epithelium and in cornified areas of cancers (26). This observation points to an important issue in the analysis of expression of endogenous lectins. To exert their biological function, they will interact with complementary binding sites *in situ*. Thus, it is important to explore the localization and extent of expression of galectin-reactive binding sites by application of the lectin, extending classical plant/invertebrate lectin histochemistry. The value of applying labeled galectin-3 in histopathological analysis for differential diagnosis of uterine smooth muscle tumors and renal cell carcinomas and in evaluation of astrocytic, head and neck and metastatic colorectal tumors has already been documented (24,27-30). These studies encouraged to us extend this approach to analyze squamous cancer.

Therefore, we conducted the present study to: i) to compare the expression of galectin-3 in primary tumors of oropharynx and larynx with the expression in regional lymph node metastases and in distinct normal epithelium (root of tongue, tonsil, larynx, skin, cornea); and ii) determine the expression of galectin-3-reactive sites which could be compared to the immunohistochemical presence of desmoglein and desmoplakin-1. In this report, we present the pattern of expression of galectin-3-reactive sites and show that it is similar to that of desmosomal proteins desmoglein and desmoplakin-1, the expression of which is closely related to invasiveness of head and neck cancer (31).

Table I. Tissue and histopathological characteristics.

	No. of cases
Normal epithelium	
Keratinized	
Skin	1
Base of tongue	7
Larynx	3
Non-keratinized	
Tonsil	3
Cornea	2
Squamous cell carcinoma	
Poorly differentiated	
Base of tongue	8
Tonsil	2
Larynx	4
Moderately differentiated	
Base of tongue	2
Tonsil	4
Larynx	1
Well differentiated	
Base of tongue	2
Tonsil	3
Larynx	1
Regional lymph node metastasis	
Poorly differentiated	
Primary tumor:	
Base of tongue	1
Tonsil	1
Larynx	4
Moderately differentiated	
Base of tongue	2
Tonsil	2
Larynx	1
Well differentiated	
Base of tongue	0
Tonsil	2
Larynx	1

Materials and methods

The samples of cancer and normal epithelial tissue (Table I) were taken from the patients with their ascertained consent. The samples of cornea were obtained from cadavers. Parts of each sample from tumors and normal tissues were cut, exposed to Tissue-Tek (Sakura-Finetek Europe B.V. Zoeterwoude, The Netherlands) for 60 min at 4˚C and frozen in liquid nitrogen. The specimens were stored in the frozen state until further processing. The remaining materials were fixed in formaldehyde, embedded in paraffin, sectioned and stained by hematoxylin and eosin for routine histopathological examination and grading (32). Cryostat sections, 5-10 µm thin (Cryocut, Reichert, Wien, Austria), were fixed with 2% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2) for 5 min. This buffer containing 0.1% (w/v) bovine serum albumin was used as blocking solution for 30 min. The specimens after extensive washing in Tris-buffered saline (TBS, pH 7.2) were stained for simultaneous detection of galectin-3-reactive sites and cytokeratins immunohistochemically (26,33). Biotinylated galectin-3 and CK-1 (LP-34) monoclonal antibody (Dako, Glostrup, Denmark), which recognizes panel of cytokeratins (except cytokeratin 1, 8 and 19) present in basal and suprabasal layers of the epidermis and the mucous layer were employed for this purpose. The specimens were also processed for simultaneous detection of galectin-3-reactive sites and desmosomal proteins, i.e. desmoplakin-1 and desmoglein, using specific antibodies

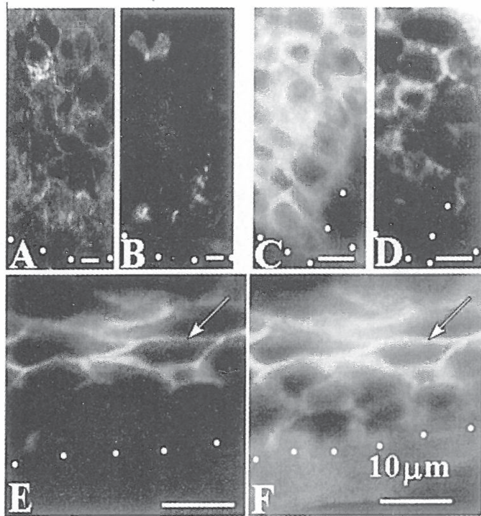

Figure 2. Detection of selected desmosomal proteins, i.e. desmoplakin-1 (A, C) and desmoglein (E), and galectin-3-reactive sites (B, D, F) in the same specimen of normal squamous epithelium of the tonsil (A, B), skin (C, D) and cornea (E, F). Both desmosomal proteins in the tonsillar and corneal epithelium and in the skin as well as binding of galectin-3 in the skin and cornea are localized suprabasally. There is no binding of galectin-3 to the tonsillar non-keratinized squamous epithelium. The position of arrows indicates the same cell. Small white spots mark the position of the basement membrane. Scale is 10 μm.

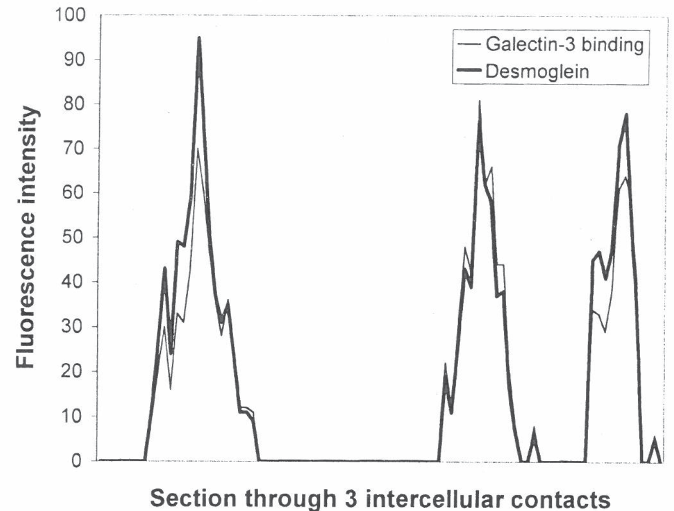

Figure 3. The measurements of the fluorescence profile of the expression of desmoglein and galectin-3-binding sites in human cornea revealed colocalization of both signals (see position of peaks).

were located at the periphery of tumor and normal epithelial cells in a pattern very similar to the position of those proteins (desmoplakin-1, desmoglein) participating in the formation of intercellular contacts such as desmosomes (Fig. 1). To elucidate the position of galectin-3-binding sites, double labeling with monoclonal antibodies against these desmosomal proteins and biotinylated galectin-3 was performed. The position of binding sites for galectin-3 colocalized with expression of the tested desmosomal proteins. However, presence of both desmosomal proteins was not restricted to this tissue area. They could also be detected in other parts of the tumors including the poorly differentiated regions that lacked galectin-3-reactive binding. The pattern of desmosomal protein expression in poorly differentiated parts of tumors was frequently atypical (cytoplasmic signal, small cell surface spots) (Fig. 1). The pattern of colocalization of galectin-3-reactive glycoligands and desmosomal proteins was also observed in one highly differentiated lymph node metastasis containing highly cornified cells (Fig. 1).

The position of galectin-3-reactive binding sites was congruent with the expression of both studied desmosomal proteins in physiological cornifying epithelia such as the base of the tongue and epidermis and non-cornifying epithelium of cornea. Desmosomal proteins in all studied epithelia, including tonsillar epithelium without reactivity for galectin-3, were expressed suprabasally (Fig. 2). The automatic computer-assisted analysis clearly revealed the same positions of expression of desmoglein and galectin-3 binding sites in these normal epithelia (Fig. 3).

Discussion

Galectin-3-reactive ligands were expressed in cells exhibiting the highest level of differentiation in moderately/highly differentiated squamous carcinomas of the head and neck. Lymph node metastases (except for one case with cornified metastatic tumor cells) and poorly differentiated primary carcinomas were negative. The cells of the basal layer of normal squamous epithelia (tongue, tonsil, epidermis, cornea) were negative concerning the expression of accessible galectin-3-reactive sites in contrast to suprabasal layers of lingual, epidermal and corneal epithelia, which were positive. The tonsillar epithelium was also negative suprabasally. Notably, highly differentiated carcinomas of tonsils displayed reactivity to galectin-3, whereas the normal epithelium was negative. The basal cell layer of normal squamous epithelia contains the mitotically active stem cells and transit-amplifying cells (35-37). In principle, an effect of cell keratinization on the binding of galectin-3 can be assumed, because the suprabasally located cells of cornifying epithelia such as epidermis and base of tongue were reactive to galectin-3, while non-keratinizing epithelium of tonsil was completely negative. Moreover, one case of cornified cancer cells in lymph node metastasis exhibited a high extent of galectin-3 binding to these cells. The corneal epithelium that is considered to be non-keratinizing exhibited the same pattern of galectin-3 binding as keratinizing epithelia. However, the corneal epithelium is able to cornify under non-physiological condition, such as vitamin A depletion (38). These observations point to a differentiation-dependent control of expression of galectin-3-reactive sites, corroborate previous observations on binding of galectin-3 to differentiated squamous carcinomas (24-26).

The galectin-3 expression appears to be also differentiation-dependent. It is expressed more highly by suprabasal keratinocytes of normal epidermis than basal cell and squamous carcinomas as well as keratoacanthomas and melanomas (39-42). *In vitro*, differentiation induces down-regulated expression of galectin-3 in mouse K-1735P melanoma cells (43). The findings are also consistant with the differential glycan display between basal and suprabasal cells which has been documented by classical plant lectin histochemistry (44).

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