Histol Histopathol (2000) 15: 85-94

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

Expression of galectin-l and -3 and of accessible binding sites during murine hair cycle

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Summary. Although protein-carbohydrate interactions are supposed to play key roles in cell adhesion, signalling and growth control. Their exact role in skin physiology has only recently been investigated. The endogenous lectins galectin-l and galectin-3 have been identified in skin including hair follicles. Here, we analyzed the expression and distribution of these galectins and their binding sites in C57BL/6 mice during hair cycle. The expression of galectin-l and galectin-3 binding sites was found to be predominantly hair cycle-dependent showing some overlapping to the expression of galectin-l and -3. The outer root sheath (ORS) expressed galectin-l binding sites during anagen IV to VI and in early catagen, whereas galectin-l was expressed from early anagen to late catagen. The ORS expressed galectin-3 binding sites during catagen transition corresponding to a galectin-3 expression during anagen V and catagen. The innermost layer of the ORS expressed galectin-3 binding sites during anagen VI until catagen VIII, but galectin-3 during anagen III to IV and catagen. The inner root sheath (IRS) expressed galectin-3 binding sites only in anagen IV but missed expression of any of the two galectins. The matrix cells expressed galectin-3 binding sites in catagen II-III as well as galectin-3 during anagen V to catagen IV. The present study provides the first evidence for a cycle-related expression of both galectin-l and -3 and their binding sites during murine hair cycle.

Key words: Galectins, Galectin-binding sites, Hair cycle

Introduction

Cellular glycoconjugates are potential ligand sites for endogenous receptors, thereby being involved in morphogenesis and other cellular activities (Gabius and Gabius, 1997). As tools for glycoconjugate mapping plant lectins have proven their suitability. The expression of plant lectin binding sites in anagen hair follicles has been investigated in different species including humans (Tezuka et al., 1991; Tsubura et al., 1991; Wollina et al., 1992; Wollina, 1997). These findings and additional studies have suggested a differentiation-related expression of complex carbohydrates in hair follicle epithelium and their modification in pathological conditions like androgenetic alopecia (Ohno et al., 1990; Heng et al., 1992). Assigning a potential role for the glycan chains of complex carbohydrates in molecular recognition, it is essential to document the presence of endogenous receptors, i.e. animal lectins (Gabius, 1997b, 1998; Zanetta, 1997; Kaltner and Stierstorfer, 1998). Terminal, spatially readily accessible positioned ß-galactosides and their derivatives are of special interest as docking points for endogenous receptors. Binding cross-linking of ligands on the cell surface can elicit signaling regulating proliferation or secretion of mediators (Brewer, 1996; Gabius, 1997b; Kopitz et al., 1998; Villalobo and Gabius, 1998). Among the families of animal lectins, for example the galectins recognize such epitopes (Gabius, 1997b; André et al., 1997; Hirabayashi, 1997; Hughes, 1997; von der Lieth et al., 1998).

One member of this family, i.e. galectin-l or its avian equivalent, has been identified in skin including the connective tissue around hair follicles (Oda and Kasai, 1984; Catt et al., 1987). In human skin, dermal extracellular matrix and hair follicles have been found to contain abundant galectin-l. It appeared to be absent in basal cell carcinomas and their adjacent stroma (Gabius et al., 1990; Santa Lucia et al., 1991; Akimoto et al., 1995). Galectin-l has been localized in the cell membranes of keratinocytes of the epidermal basal and spinous layers. In epidermal Langerhans cells, galectin-l presence was observed in both the cytoplasm and the nucleus. In *situ* hybridization showed gene expression in basal and spinous cell layer keratinocytes and dermal fibroblasts (Akimoto et al., 1995). These results are in

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line with the suggestion that galectin-1 is important for cell-cell contact and/or adhesion in the epidermis and for cell-extracellular matrix interactions in the dermis.

In addition to galectin-1 the chimeric-type galectin-3 is of particular interest for skin biology. Galectin-3 (syn.: εBP, low affinity IgE-binding protein, Mac-2 or CBP-35) is a 29-35 kDa protein expressed by human keratinocytes, sebaceous and sweat glands, hair follicle epithelium and basal cell carcinomas. The amount of expression seems to be related to the degree of epithelial differentiation, as also reflected by loss of occurrence in certain skin tumors (Crittenden et al., 1984; Gabius et al., 1990; Wollenberg et al., 1993; Konstantinov et al., 1994).

The hair follicle is not a constant organ but permanently undergoes rhythmic changes known as the hair cycle, which is characterized by anagen growth, catagen regression and telogen resting phases (Paus, 1996; Stenn et al., 1996). There are no data available on differential galectin expression during these hair cycle phases. In order to clarify this question, in the present study we have investigated the hair cycle of C57BL/6 mice. In this animal model, deliberately-induced anagen allows a detailed investigation of hair cycling (Paus et al., 1990; Slominski et al., 1991; Paus, 1996). In addition to the immunohistochemical monitoring of galectin-1 and -3 expression during the murine hair cycle, we also determined accessible ligand sites for the two galectins, employing the mammalian receptors as physiological probes (Gabius et al., 1986a,b; Gabius, 1997a). We have revealed the first evidence available so far that murine hair follicles are a potent source of endogenous lectins that are known to bind a limited set of ligands *in vitro*.

Materials and methods

Animals

Six- to nine-week-old, syngenic female C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). The animals were housed in community cages wih 12h light periods at the Humboldt University, Charité Berlin, Virchow Campus, and were fed water and mouse chow *ad libitum*. Anagen was induced in the back skin of mice in the telogen phase of the hair cycle by depilation, as described, which induces reliably predictable and homogeneous development of anagen follicles that are morphologically indistinguishable from spontaneous anagen follicles (Paus et al., 1990; Slominski et al., 1991).

At defined periods (days 0, 1, 3, 5, 8, 10, 12, 17, 18, 19, and 25) after depilation, mice were sacrificed (n= 3-5) and their back skin was harvested perpendicular to the paravertebral line to obtain longitudinal hair follicle sections. Back skin samples from the neck and the tail region were formalin-fixed, paraffin-embedded, cut at 8 μm and deparaffinated. Sections were stored at -20 °C until histochemical analysis.

Galectins and antigalectins

Galectin-1 and -3 were purified to homogeneity as described recently (Gabius et al., 1991; Siebert et al., 1997). They were biotinylated with biotinyl-N-hydroxysuccinimide ester in the presence of lactose to protect the active site (Bardosi et al., 1990). Galectin-1- and -3-specific polyclonal IgG antisera were raised in rabbits and controlled rigorously for the lack of cross-reactivity (Gabius et al., 1986b, Kopitz et al., 1998).

Histochemistry

Lectin- and immunohistochemical staining was performed on 8 μm-thick sections, which had been dewaxed, rehydrated and treated subsequently with 1% methanolic H_2O_2 for 30 min to block endogenous peroxidase activity. The sections were then rinsed in phosphate-buffered saline (20mM PBS buffer; pH 7.4) and incubated with 0.1% bovine serum albumin in PBS buffer for 30 min to block nonspecific protein-binding sites. Biotinylated galectins-1 and -3 were used at a final concentration of 1 μg/ml, and the solutions were applied overnight at 4 °C. Thorough washes, application of commercial avidin-peroxidase kit reagents (MaxiTags®, Quartett, Berlin/Germany), repetitive rinses and development of the chromogenic product from 3-amino-9-ethylcarbazole as substrate concluded the processing.

Control reactions included competitive inhibition with 0.2M lactose and 0.5 mg asialofetuin (ASF)/ml PBS buffer and omission of the incubation step with the labeled marker to exclude any nonspecific staining by binding of the kit reagents. Positive controls were performed with galactoside specific *Viscum album* agglutinin (Gabius et al., 1991). In addition, antisera against galectin-1 and -3 were used at a final dilution of 1:200 and an avidin-peroxidase kit as described above.

Results

The specificity of the probes was verified by the standard controls. Hapten inhibition by glycosubstances proved the specificity of galectin-carbohydrate recognition. The expression of galectin-1 and galectin-3 binding sites was found to be predominantly hair cycle-dependent (Table 1, Fig. 1).

The outer root sheath (ORS) expressed galectin-1 binding sites only during a short period between day 5 to day 18 (anagen IV to catagen V). Other parts of the follicle epithelium remained nonreactive (Fig. 1a). In epidermal keratinocytes we found a weak to moderate perinuclear and/or nuclear staining at days 3 to 5 (anagen II-V), and 17 (catagen I-IV) (Fig. 1a, 4).

Hair matrix and the innermost layer of the ORS disclosed a moderate or strong expression of galectin-3 binding sites, respectively, from day 12 (anagen VI) to 19 (catagen VIII) (Fig. 1b, 6). The ORS itself was reactive from day 17 (catagen I-IV) and the epithelial strand of catagen VIII was decorated (Fig. 1b). The inner

root sheath (IRS) was reactive on day 12 (anagen VI). Matrix cells were reactive from day 12 (anagen VI) to day 17 (catagen IV). The dermal papilla was stained from day 3 (anagen II-III) onwards.

Epidermal keratinocytes were found to react in a nuclear pattern at days 3-5 (anagen II-V), and 12-17 (anagen VI and catagen I-IV). Since nuclear presence of galectins has been described with relevance for pre-mRNA splicing 40 (Patterson et al., 1997), this staining should not be dismissed as non-specific. On day 8 (anagen V) the staining changed to intercellular (Fig. 1b).

Antibodies against galectins have been employed to immunolocalize endogenous galectin-1 and galectin-3. A weak epidermal staining was seen for galectin-1 between day 1 (anagen I) and day 17 (catagen I-IV). The ORS was labeled weakly to moderately in the period between day 8 and 19 (anagen V to catagen VIII) (Figs. 2a, 3).

Table 1. Time-course of expression of endogenous lectins, galectin-1 and -3, and their accessible ligand sites in murine skin.

	Gal-1	Gal1LS	Gal-3	Gal-3LS
Epidermis	1-19*	3-5, 17	1-25	3-5, 12-17
Hair follicle				
ORS	8-19	5-18	8-19	17-19
IL (ORS)			8-19	12-19
IRS				12
Matrix		12	12-17	12-17
Dermal papilla	10-17	12	10-25**	3-25
Sebaceous gland	0-19	0-25	0-25	0-25
Dermal connective tissue	1-18	0-19	5-12	0-25
Panniculus carnosus	3-19	0-19	3-8	1, 17-19

gal-1: galectin-1; gal-3: galectin-3; BS: binding sites; ORS: outer root sheath; IRS: inner root sheath; IL: innermost layer; *: days of expression; **: expression starts between days 10-12 in most, but not all follicles.

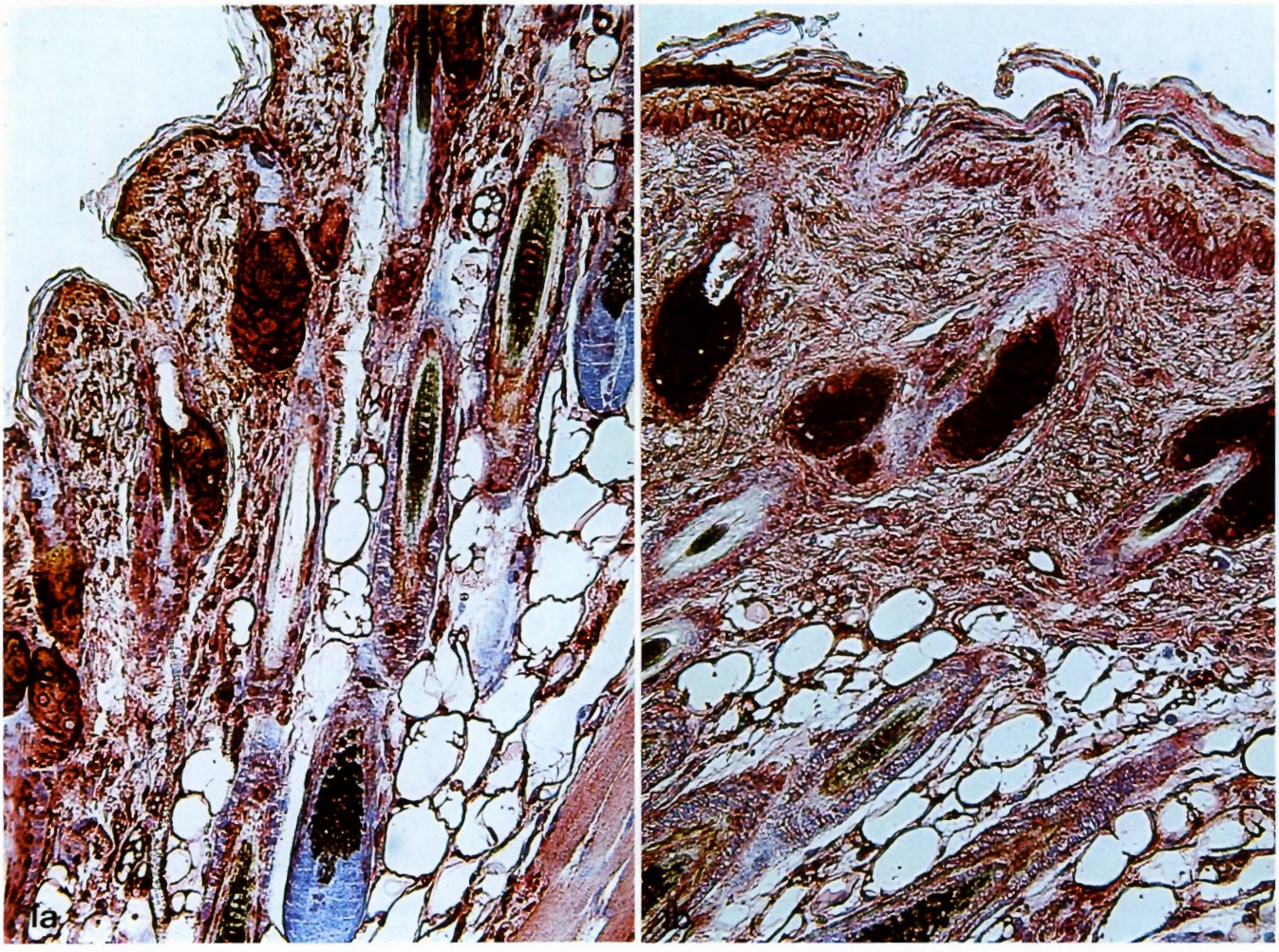


Fig. 1. Demonstration of accessible ligand sites for galectins in murine skin. **a.** Galectin-1 ligand sites, day 8 (anagen V): perinuclear staining of basal epidermal keratinocytes, nuclear staining of outer root sheath epithelium. Expression of galectin-1 binding sites in sebaceous glands, dermal connective tissue and panniculus carnosus. **b.** Galectin-3-accessible ligand sites, day 8 (anagen V): Intercellular staining of the epidermis, heavy staining of sebaceous glands, faint labeling of the dermal connective tissue and the outer root sheath. x 120

Other parts of the follicle apparatus remained negative during the whole hair cycle under the chosen assay conditions.

Galectin-3 showed a strong to very strong epidermal expression during the whole cycle, except day 0, with the highest intensity around day 5 to 18, i.e. anagen IV to V (Figs. 2b,c, 6). The ORS also contained high levels of galectin-3 from day 8 to 19 (anagen V to catagen VIII), with slight decreases being seen at days 17 and 18 (Fig. 2b,c). Matrix cells were labeled weakly from day 12 (anagen VI) to 17 (catagen IV) and dermal papilla cells between day 10 to 25 (anagen V to telogen). The late anagen staining of dermal papilla cells was not a constant feature to be found in all follicles.

In sebocytes, galectin-1 binding sites were observed at an almost constant level during the hair cycle (Fig. 1a) with the strongest staining between day 17 to 19 (catagen I-VIII). Galectin-3 binding sites were consistently found in sebocytes with a weak to moderate intensity (Fig. 1b).

Sebocytes were stained weakly for galectin-1 around day 0, but showed a strong staining intensity from day 1 to 19 (anagen I to catagen VIII). Sebocytes gave a very pronounced reactivity for galectin-3 between day 0 to 5 (postepilation telogen, anagen I-V) and a pronounced reactivity from day 5 to 25 (anagen V to telogen).

The dermal connective tissue disclosed a weak to moderate expression of galectin-1 binding sites from day 0 to 19 (postepilation telogen, anagen, catagen), but was completely negative on day 25 (telogen). Interestingly, the subdermal muscles (panniculus carnosus) also showed a cycle-dependent lectin-reactivity with

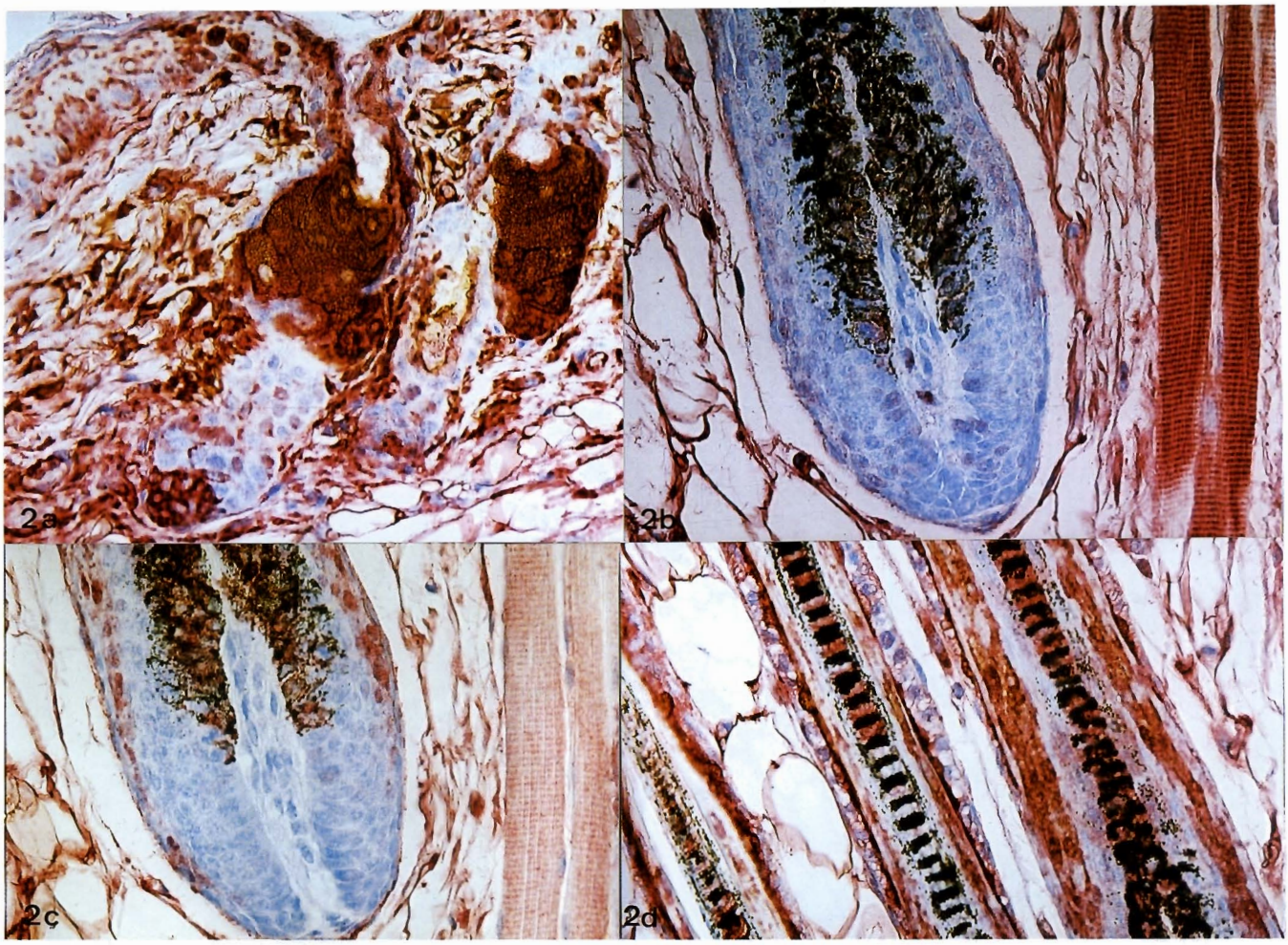


Fig. 2. Immunolocalization of galectins in murine skin. **a.** Galectin-1, day 3: faint nuclear epidermal staining, stronger decoration in the infundibular part. Marked staining of the sebaceous glands and to a lesser extent of the dermal papilla and connective tissue. **b.** Galectin-1, day 12: Decoration of the outer root sheath, faint labeling of some matrix cells, marked staining of selected dermal papilla cells and the panniculus carnosus. **c and d.** Immunolocalization of galectin-3 in murine skin, day 12 (anagen VI). c. The hair bulb shows anti-galectin-3 binding in the outer root sheath epithelium and is very faint in some matrix cells. The dermal papilla is negative. The panniculus carnosus weakly expresses galectin-3. d. Weak staining of the outer root sheath except the innermost layer, which strongly expresses galectin-3. Moderate to strong expression of galectin-3 in the inner hair root sheath. x 460

strongest binding at days 17 to 19 (catagen) and inconstant binding or weak binding between day 0 and 12 (anagen) (Fig.1a). The dermal connective tissue showed a variable extent of expression of galectin-3 binding sites, which was strongest around day 5 (anagen IV-V). The subdermal muscles presented a weak expression of galectin-3 binding sites at day 1 (anagen I), and between day 17 to 19 (catagen). The binding intensity was slightly increased at day 19.

The dermal connective tissue gave a weak to moderate staining for galectin-1 between day 1 and 18 (anagen I to catagen VII). Subdermal muscles were labeled strongly to very strongly between day 3 and 18 (anagen II to catagen VII). The dermal connective tissue showed a weak reaction for galectin-3 between days 5 to 12 (anagen IV-V). Subdermal muscles gave a weak signal between days 3 to 8 (anagen II-V).

Discussion

Carbohydrate recognition mechanisms have been implicated in specific cellular interactions that occur during cell growth and differentiation (Gabius and Gabius, 1997). Consequently, impairment of display of glycans should affect regulatory processes. Human diseases linked to aberrant or defective glycosylation strongly support this concept (Brockhausen et al., 1998). By disrupting or overexpressing genes for enzymes of glycan synthesis the relevance of their products can be delineated. As shown by transgenic mouse lines with ectopic expression of α1,3-galactosyltransferase, glycan epitopes resulting from its activity seem to be involved in morphogenesis of hair follicles. These mice tended to an impaired hair growth (Ikematsu et al., 1993). The role(s) of endogenous lectins and their accessible bindings sites in hair follicle morphogenesis and cycling have not been investigated so far.

In the present study, occurrence of two endogenous ß-galactoside binding lectins (galectins) and their accessible binding sites has been studied in cycling murine hair follicles. We observed a cycle-dependent expression of galectin-1 and -3 during hair cycle. Hair growth may be influenced by hypodermal structures. In a functional *in vitro* assay it was demonstrated recently,

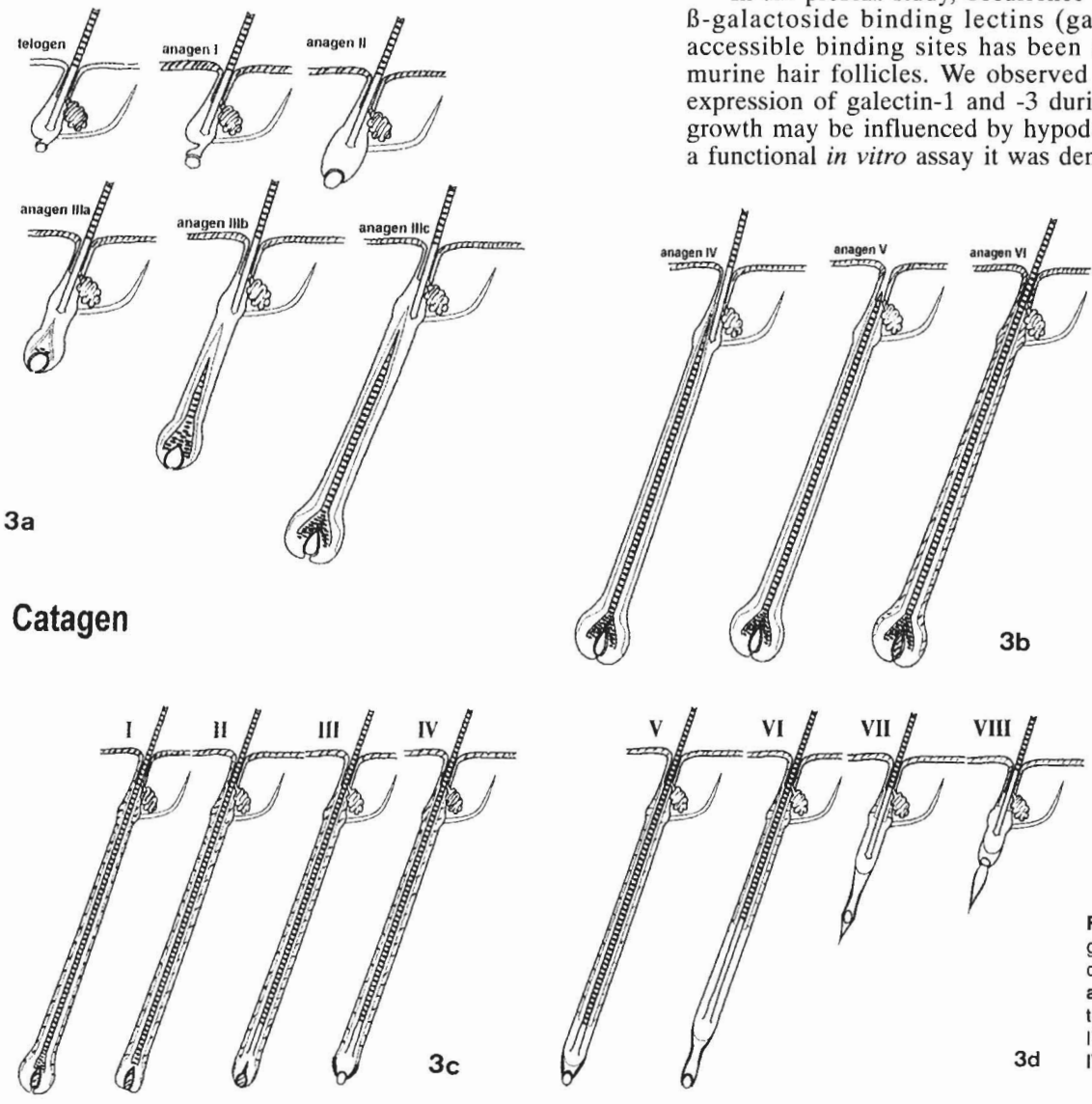


Fig. 3. Expression of galectin-1 (hatched area) during the hair cycle. **a.** Post-epilation telogen to anagen III **b.** Anagen IV to V. **c.** Catagen I to IV. **d.** Catagen V to VIII.

that rat hair follicle growth can be inhibited by co-culture with rat fat cells, but differentiation is accelerated (Misago et al., 1998). We also observed cycle-dependent changes of galectin and galectin-binding site expression on the dermal extracellular matrix and subdermal muscles (panniculus carnosus). This might be another example of possible regulatory involvement of hypodermal structures in the hair cycle.

The two galectins, galectin-1 and -3, were expressed on a high and nearly constant level in sebocytes, whereas the expression showed remarkable variations in the ORS. The only decrease of immunoreactivity in sebocytes was found at the end of anagen (anagen IV). It has been speculated recently that sebocytes may be involved in hair cycle control by the secretion of latent transforming growth factor-ß (TGF-ß) binding protein (LTBP) (Wollina et al., 1996, 1997), which plays a role in the secretion of the TGF-ß complex and the compartmentalization of TGF-ß in the extracellular matrix (Paus et al., 1997). The expression of LTBP by sebocytes was abrogated at the anagen-catagen transition (Wollina et al., 1996). Notably, the decrease of galectin expression in sebaceous glands occurs at the turning point of anagen-catagen transition of the hair follicle. It is of potential interest to point out that galectin-1 has been shown to harbor TGF-activity after intramolecular disulfide bridge formation (Yamaoka et al., 1996).

Regulation of the apoptosis-driven regression phase of the hair follicle is of substantial clinical interest (Lindner et al., 1997) because the majority of human hair growth disorders show abnormalities in the anagen-catagen transition (Paus, 1996). During this short period, both galectin-1 and galectin-3 become visible in the ORS. At the onset of catagen, an increase of the galectin binding sites is notable, raising the possibility of some kind of autoregulation (Adams et al., 1996). There is a body of evidence that one role of galectins is to function as accessory adhesion molecules (Gabius, 1997b; Hirabayashi, 1997; Hughes, 1997; Kaltner and Stierstorfer, 1998). Important ligands of galectins are laminin, fibronectin, several ß1-containing integrins and other poly-N-acetyllactosamine-containing glycoproteins (Gabius et al., 1986a; Gabius, 1997b; Ohannesian and Lotan, 1997). Laminin is expressed by the dermal sheath

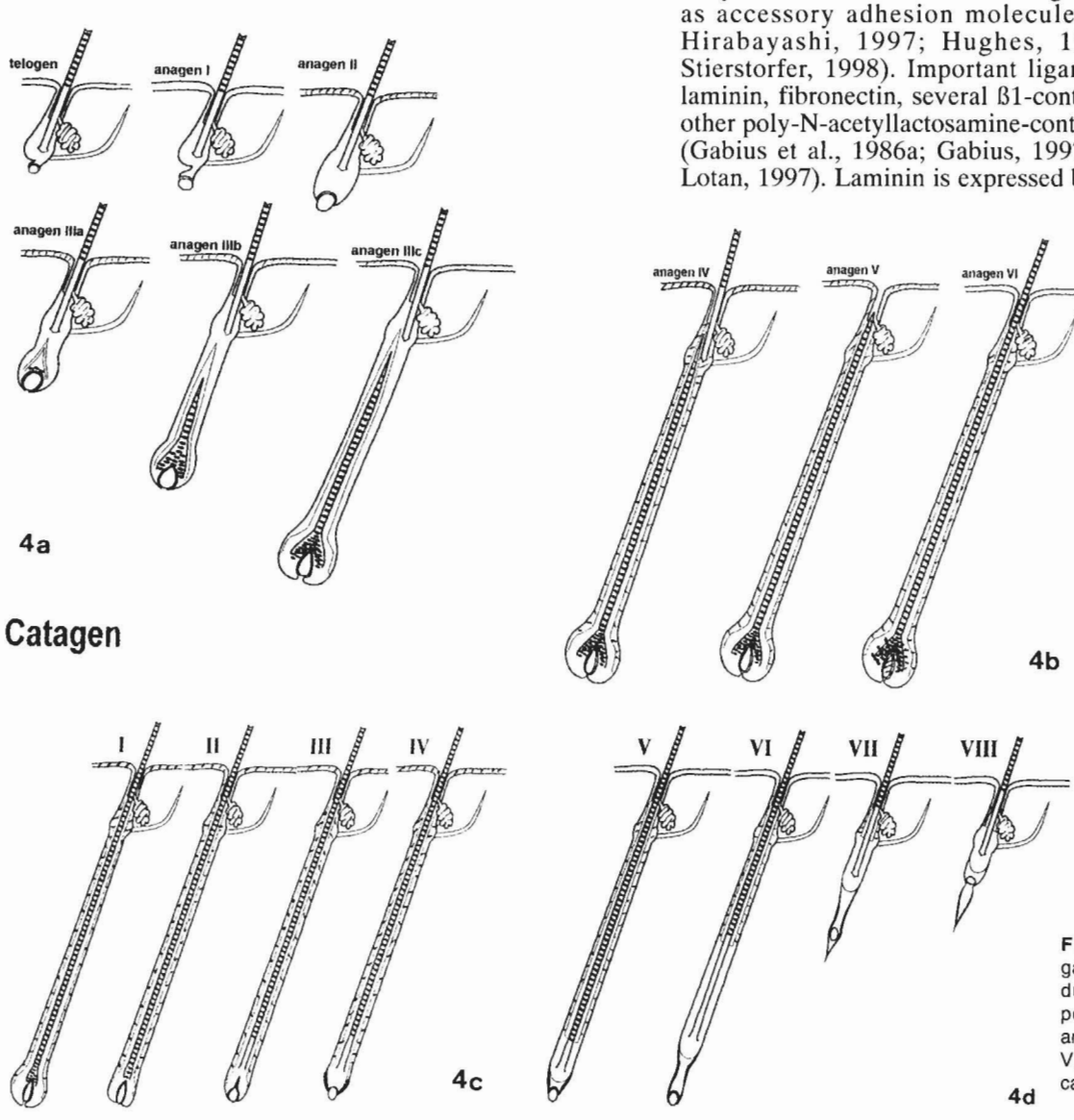
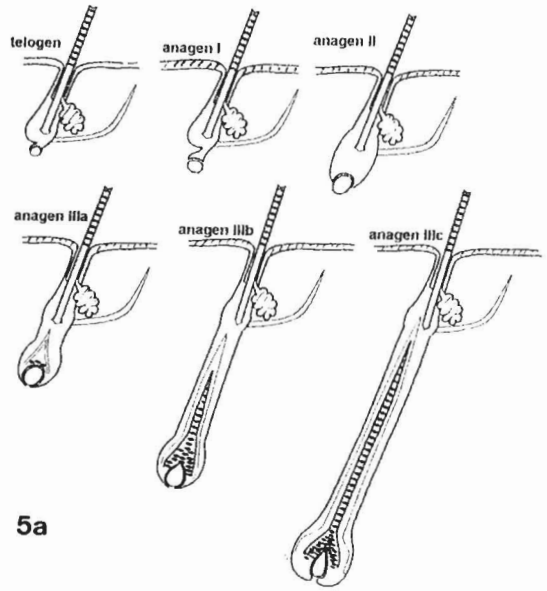


Fig. 4. Expression of galectin-3 (hatched area) during the hair cycle: **a.** post-epilation telogen to anagen III, **b.** anagen IV to V, **c.** catagen I to IV, **d.** catagen V to VIII.

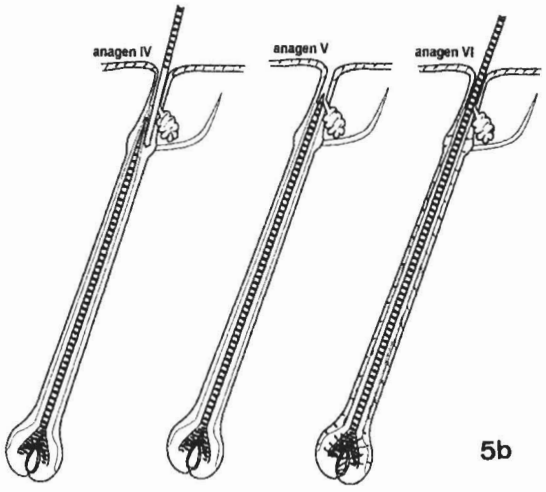
throughout anagen and catagen (Messenger et al., 1991). During early anagen, increased laminin deposition in the dermal papilla has been noted (Couchman and Gibson, 1985). Galectin-1 is known to bind to integrins like $\alpha_7\beta_1$ of myogenic cells thereby interfering selectively with its binding to laminin (Gu et al., 1994). Galectin-3 also associates to integrin glycans, i.e. from $\alpha 1\beta 1$ integrins and the α-subunit of the integrin CD11b/CD18 (Dong and Hughes, 1997; Ochieng et al., 1998), possibly also affecting adhesion of certain cell types to extracellular matrix proteins. Presence of galectin at this site is therefore of potential relevance, as the interplay between laminin and integrins can be modulated by galectins.

The innermost layer of the ORS has been characterized ultrastructurally and immunohistochemically as a specialized epithelial compartment with particular kinetics and directions of cell movement that are thought to be responsible for a regulated interplay between the ORS and the IRS (Ito, 1986; Miyauchi et al., 1990; Wollina, 1997). The prominent decoration of the innermost layer of the ORS by galectin-3 furnishes further evidence for the concept of its role in cell-matrix interactions. Furthermore and intriguingly, this distinct layer appears to be a particular target for endogenous galectin -3. The expression of galectin-1 and-3 seemed to be less restricted during the murine hair cycle than the expression of accessible ligand sites. Hence the target structures possibly control the galectin-effects by varying the density of binding sites. This is particulary obvious for the innermost layer of the ORS, which strongly expressed galectin-3 ligand sites only around day 12 when the hair shaft formation was initiated. The IRS completely negative for galectin-3, expressed galectin-3 ligand sites in late anagen and early catagen. The innermost layer of the ORS represents a reasonable source for galectin ligands.

The massive, controlled programmed cell death (apoptosis) which drives hair follicle involution during catagen (Paus, 1996) must be stringently regulated. Since galectin-3 has anti-apoptotic activity in the T-cell system and has been described to harbor a potentially functional domain of the Bcl-2 family (Yang et al., 1996; Akahani et al., 1997), it is reasonable to speculate that it may exert similar functions during catagen by providing a survival signal for infrainfundibular ORS during

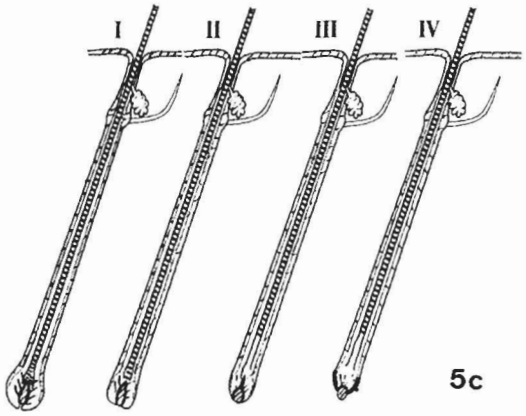


5a



5b

Catagen



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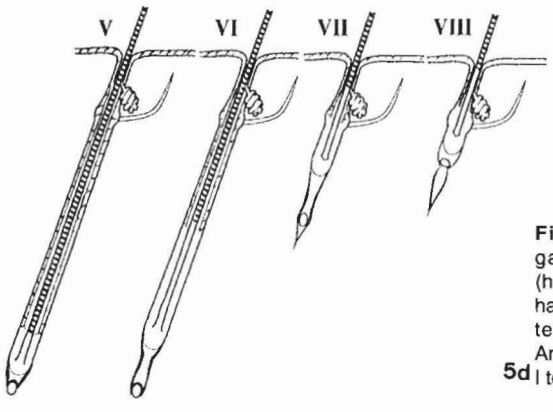

5d

Fig. 5. Expression of galectin-1 binding sites (hatched area) during the hair cycle. **a.** Post-epilation telogen to anagen III. **b.** Anagen IV to V. **c.** Catagen I to IV. **d.** catagen V to VIII.

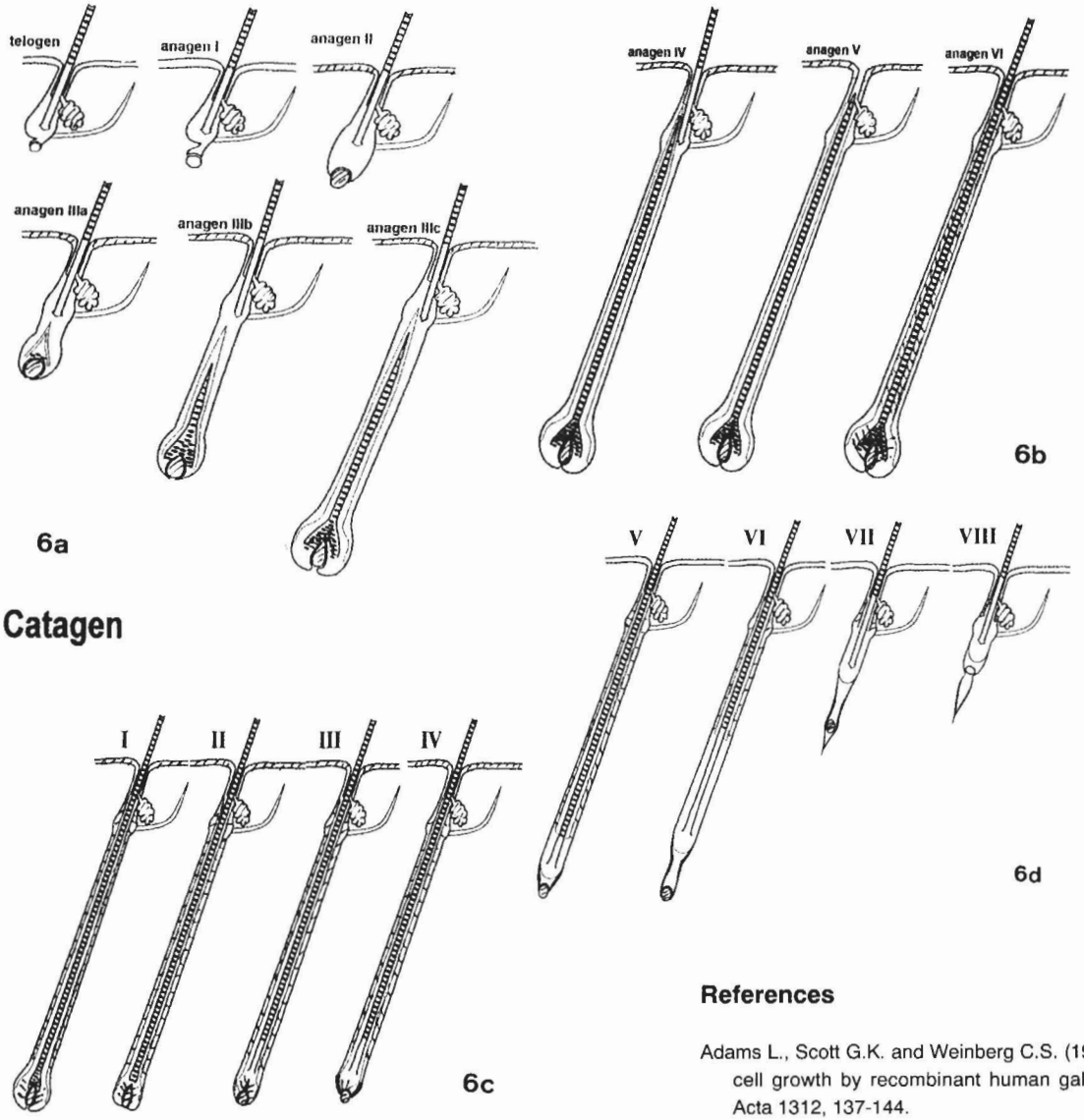

Fig. 6. Expression of galectin-3 binding sites (hatched area) during the hair cycle. **a.** Post-epilation telogen to anagen III. **b.** Anagen IV to V. **c.** Catagen I to IV, **d.** catagen V to VIII.

catagen.

In summary, herein we provide the first report of a hair cycle-dependent expression of galectin-1 and -3 binding sites and the corresponding endogenous lectins galectin-1 and -3. The data presented in the current study support the concept that endogenous lectins may be involved in hair growth regulation, for example during the clinically crucial anagen-catagen transformation of the hair follicle. Our findings have to be verified and explored by appropriate functional studies.

Acknowledgements. We thank Ruth Plieth (Berlin) and Sabine Feldrappe (Jena) for excellent technical assistance. The study was supported by a grant from Wella AG, Darmstadt (RP), the Verbund Klinische Forschung, University of Jena (UW), and the Deutsche Forschungsgemeinschaft (grant Ga 349/7-1; HJG).

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Accepted July 30, 1999