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Galectin fingerprinting in human endometrium and decidua during the menstrual cycle and in early gestation

M.von Wolff[1,3], X.Wang[1], H.-J.Gabius[2] and T.Strowitzki[1]

[1]Department of Gynecological Endocrinology and Reproductive Medicine, Universitätsklinikum Heidelberg, Voßstrasse 9, 69115 Heidelberg and [2]Institute of Physiological Chemistry, Faculty of Veterinary Medicine, Ludwig-Maximilians-University, Veterinärstr. 13, München, Germany

[3]To whom correspondence should be addressed at: Voßstrasse 9, 69115 Heidelberg, Germany.
E-mail: michael.von.wolff@med.uni-heidelberg.de

The emerging functionality of the sugar code via cell surface glycans and endogenous lectins ascribes pertinent roles in cell physiology to the carbohydrate signals of cellular glycoconjugates. To initiate monitoring of endogenous lectins in human endometrium, we focused on a family of growth/adhesion-regulatory lectins, i.e. galectins. Comprehensive fingerprinting was performed on samples throughout the menstrual cycle and in decidua. The endometrium ($n = 30$) and decidua ($n = 7$) were collected from patients undergoing hysterectomy for benign reasons and from induced abortions. Measurements by RT–PCR and then by multiprobe RNase protection assay with total endometrial and decidual tissue and with epithelial cells, stromal cells and CD45-positive cell fractions ($n = 16$), isolated by the use of antibody-coated magnetic beads, revealed a predominant expression of galectins-1 and -3. Protein analysis was performed by immunocytochemistry with monoclonal and polyclonal antibodies ($n = 40$). Galectin-1 was localized mainly in stromal cells, whereas galectin-3 was predominantly found in epithelial cells. Expression of galectin-1 increased significantly in the late secretory phase endometrium and in the decidual tissue. Expression of galectin-3 increased significantly during the secretory phase of the menstrual cycle. Cycle-dependent expression of galectin-1 in stromal cells and galectin-3 in epithelial cells suggest these lectins to be involved in the regulation of different endometrial cellular functions.

Key words: adhesion/decidua/endometrium/galectins/implantation

Introduction

Galectins are members of a large family of lectins, which contain one or more carbohydrate recognition domains with affinity for beta-galactosides (Leffler *et al.*, 2004).

Carbohydrates surpass any other class of biomolecule in their capacity to store biological information (Laine, 1997). Fittingly, a multitude of glycan epitopes are presented on the cell surface as signals produced by a complex enzymatic machinery in glycosylation, calling for biochemical means to read these 'messages' (Reuter and Gabius, 1999). The emerging functionality of endogenous lectins has already raised convincing evidence for the physiological action of the sugar code (Gabius, 2000; Gabius *et al.*, 2004). Regarding mammalian reproduction, the glycomic profile has been mapped to undergo substantial changes (Kimber, 1990, 2000). In distinct cases such as the H(type1)-antigen, a finely tuned hormonal control of the expression of the glycosyltransferase responsible could be delineated in mice (White and Kimber, 1994; Sidhu and Kimber, 1999). Such results draw attention to the detection of endogenous lectins and monitoring of their expression.

Diverse biological functions have been demonstrated for various galectins *in vitro* and *in vivo*, which in part suggest galectins also to be expressed in human endometrium and to play a role in endometrial regulation.

Galectins-1 and -3 have been shown to activate various cell types through cross-linkage of appropriate cell surface glycoproteins, and to modulate cell adhesion, probably through interaction with cell surface glycoproteins (Rabinovich, 1999; Liu, 2000). Galectin-3 has been shown to induce differentiation and angiogenesis of endothelial cells (Nangia-Makker *et al.*, 2000). Galectin-3 is also a chemoattractant for monocytes (Sano *et al.*, 2000) and endothelial cells (Nangia-Makker *et al.*, 2000). Furthermore, galectin-3 expression increases from the hyperplastic to the cancerous state of the endometrial tissue (Brustmann *et al.*, 2003), suggesting that it plays a role in tumour cell function.

Studies on galectins-1 and -3 deficient mice have been set out to analyse which of these *in vitro* functions might also be relevant *in vivo* (Poirier, 2002). Although galectins-1 and -3 are dispensable for survival and fertilization of mice under house conditions, clear but more subtle and transient defects have been found after careful analysis. The galectin-1 null mice are deficient in the development of a subset of olfactory neurons and transiently in muscle development. Galectin-3 null mutant mice show some effect on the survival or maintenance of neutrophils and macrophages at an inflammatory site (Poirier, 2002) and delayed phagocytosis (Sano *et al.*, 2003). As double galectins-1 and -3 mutants are alive, it has been suggested that these galectins may be 'optimizing molecules', indicating that these molecules are not required for absolute function, but for a most efficient function (Poirier, 2002).

Several parallels in the function of galectins and the regulation of human endometrium suggest that galectins might also be expressed in the endometrium. First, several reports have shown that galectins-1 and -3 are important mediators of inflammation

(Almkvist and Karlsson, 2004). As endometrial function and implantation involve many inflammatory mediators, galectins might contribute to the modulation of the endometrial immune system. Second, galectins have been shown to play an important role in cell adhesion, migration and chemotaxis (Almkvist and Karlsson, 2004). As adequate endometrial function is dependent on leukocyte migration and as implantation is characterized by the accumulation of immune cells around the implantation site, galectins can be expected to play a role in the regulation of endometrial leukocytes. Third, galectins have been characterized as major players in the defence against invading micro-organisms (Almkvist and Karlsson, 2004). As the endometrial immune system is constantly challenged by bacteria ascending through the cervix, galectins might contribute to the protection of the endometrium against bacterial infection.

To support our hypothesis that galectins might play a role in the regulation of endometrial function, we critically investigated the temporal mRNA and protein expression of all well-characterized human galectins (gal-1–4, gal-7–9, gal-12) in human endometrium throughout the menstrual cycle and in decidua of early gestation.

Materials and methods

Collection of material

Endometrial tissues ($n = 30$) from regularly cycling women (35–45 years of age) were collected at different phases of the menstrual cycle after hysterectomy. Hysterectomy was performed for benign reasons such as myoma or hypermenorrhoea. None of the patients were hormonally stimulated. Decidual tissue ($n = 7$) was obtained from induced abortions, performed in the 6th to 9th week of pregnancy for social reasons (18–30 years of age). Curettage was performed without the application of prostaglandins. Ethical approval according to the principles defined in the 'Declaration of Helsinki' and written consent from each patient were obtained, to allow collection of the tissue samples. All specimens were dated according to the last menstrual period, the histology (Noyes and Hertig, 1950) and the serum levels of estradiol, progesterone and LH. The menstrual cycle was divided into four phases: the proliferative phase (day 1–14, 11 tissue samples), the early secretory phase (day 15–19, 7 tissue samples), the mid-secretory phase (day 20–23, 5 tissue samples) and the late secretory phase (day 24–28, 7 tissue samples).

Separation of endometrial epithelial and stromal cells and leukocytes

Separation of cells ($n = 16$) was performed as described elsewhere (von Wolff *et al.*, 2001). In brief, epithelial glands were separated from single stromal and immune cells by mechanical dissociation and collagenase digestion, followed by filtration through a 40 μm sieve. Epithelial glands trapped in the sieve were thoroughly washed and purified by the separation of immune and endothelial cells using CD14-, CD56-, CD45- and CD31-bearing magnetic Dynabeads (Dynal, Oslo, Norway). The single stromal cells that passed the 40 μm sieve were purified with the same panel of magnetic beads, including Dynabeads against the human epithelial antigen EpCAM to remove contaminating epithelial cells.

CD45-positive cells were isolated from the cell suspension that had passed through a 40 μm sieve by positive selection using CD45 Dynabeads (Dynal). Separated cells were immediately snap frozen to prevent RNA degradation or stimulation by binding to the antibodies on the beads.

RT–PCR

RNA isolation and DNA digestion were performed with High Pure RNA Isolation Kits (Roche Diagnostics, Mannheim, Germany). First strand cDNA was synthesized using First Strand Synthesis Kits (Roche Diagnostics) followed by PCR (PCR Core Kit, Roche Diagnostics). The housekeeping gene cytochrome C oxidase subunit I was co-amplified in a companion tube. PCR for the human galectins-1, -2, -3, -4, -7, -8 and -9 was performed with primers, described by Lahm *et al.* (2004). Note that so far no human orthologues of rat/mouse galectins-5 and -6 are known. PCR for galectin-12 was performed with primers, described by Yang *et al.*, (2001). Twenty-five cycles were used for cytochrome C oxidase subunit I, and 40 cycles for galectins. PCR conditions were the same as previously described. The PCR products were separated electrophoretically in a 1% agarose gel.

RNase protection assay

RNA isolation and RNase protection assay (RPA) were performed as described elsewhere (von Wolff *et al.*, 2001). RPA was performed using the RiboQuant Kit (Pharmingen, San Diego, CA). Probes for the galectins and placenta protein 14 were tested; negative controls were also purchased from Pharmingen. To reduce assay variability, the complete sample panel was analysed in one experiment. RNA were resolved on a denaturing 6% acrylamide sequencing gel. Unprotected probes were loaded as size markers. Identity of the bands protected from RNase degradation was established by comparing their size with the size of the bands of the positive control samples. For negative controls, endometrial RNA was substituted with 5 μg yeast RNA. Semi-quantification was achieved by normalizing the optical densities of the specific bands to the optical densities of two housekeeping genes, i.e. glyceraldehyde-3-phosphatedehydrogenase (GAPDH) and ribosomal protein L-32 (L32). The optical density of the specifically protected bands was expressed as relative values. A 2-fold increase of the relative optical density values corresponded, on an average, to a 1.8-fold increase of the specific RNA, as determined in several dilution series of RPA with different concentrations of the total RNA.

Immunohistochemical staining

Immunohistochemical staining was performed in duplicate as described elsewhere (von Wolff *et al.*, 2001). Frozen sections (10 μm thick) were stained using commercially available kits (Histostain-SP Kits, Zymed Laboratories, San Francisco, CA). The sections were incubated with polyclonal goat anti-galectin-1 antibody (R&D Systems, Minneapolis, MN) at a concentration of 1:200, with polyclonal rabbit anti-galectin-3 antibody (Kaltner *et al.*, 2002) at a concentration of 1:20 and with monoclonal anti-CD45 antibody (Dako, Hamburg, Germany) at a concentration of 1:100. Double immunostaining was performed using commercially available kits (Histostain DS Double-staining Kit, Zymed Laboratories), Fluorescein isothiocyanate-labelled goat anti-mouse antibody (Dako) at a dilution of 1:50 and tetramethylrhodamine isothiocyanate-labelled swine anti-rabbit antibody (Dako) at a dilution of 1:50 were applied as second-step reagents. Control experiments included staining without the primary antibody to assess the level of antigen-independent binding and substitution of galectin-1 antibody by goat immunoglobulin G (IgG) (Jackson ImmunoResearch, West Grove, PA), galectin-3 antibody by rabbit IgG (Dako) and CD45 antibody by mouse IgG at the same concentration as the primary antibody. The intensity of the immunohistochemical staining was semi-quantitatively assessed (M.von Wolff, X.Wang).

Statistical analysis

The Mann–Whitney test was used for statistical evaluation. Significance was established at the $P < 0.05$ level. Error bars in Figures 2–4 represent SD variation of samples.

Results

Endometrial expression of galectins in human endometrium

Endometrial tissue was first screened for galectin mRNA by RT–PCR. Primer pairs were applied for a comprehensive panel of human lectins including the proto-type galectins-1, -2 and -7, the tandem-repeat-type galectins-4, -8, -9 and -12 and the chimera-type galectin-3. RT–PCR revealed expression of galectins-1–4, -9 and -12, whereas galectins-7 and -8 were not expressed. Multiprobe RPA was used to assess mRNA expression of the RT–PCR-positive cases semi-quantitatively (Figure 1). Evaluation by RPA showed weak levels of expression for galectins-2, -4, -9 and -12 and strong expression of galectins-1 and -3 in the proliferative phase. During the secretory phase and in decidua, the extent of mRNA expression

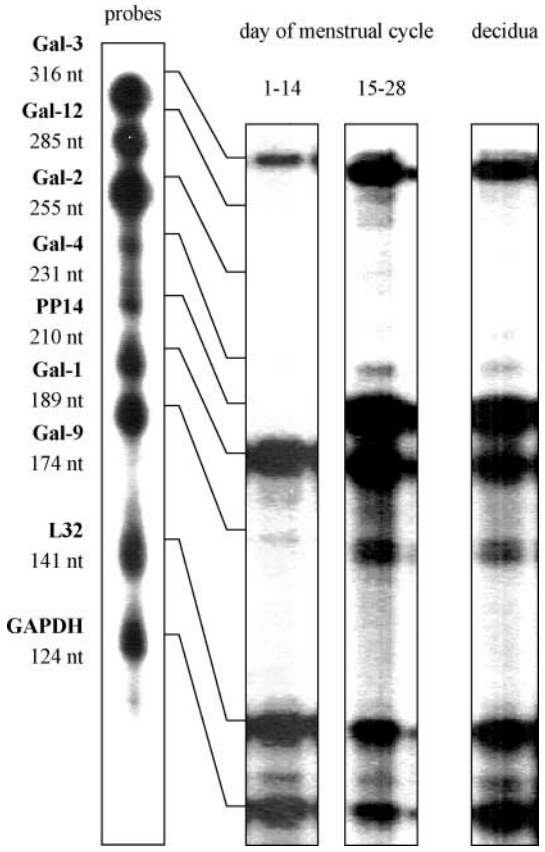

Figure 1. Comprehensive multiprobe RPA of human galectins, detected by RT–PCR in human endometrium in representative tissue samples from the proliferative and secretory phases of the menstrual cycle and in decidua. Placenta protein 14 was added as a marker of secretory-phase endometrium. The relative optical density values of the mRNA bands were normalized to the relative optical density of the housekeeping genes L32 and GAPDH. Representative samples revealed strong staining of galectins-1 and -3, whereas other galectins were expressed at relatively low levels or not present.

of all galectins increased slightly with the exception of galectin-3. Its mRNA was abundantly present. These experiments show presence of galectins-1 and -3 at the mRNA level in the analysed samples. When comparing the other signals, these two galectins are the prevalent members of this family of endogenous lectins in human endometrium. Thus, these results give us reason to focus on galectins-1 and -3 in the following experiments.

Galectins-1 and -3-specific mRNA and protein expression throughout the menstrual cycle and in decidua

Analysis of the total endometrium and decidua by RPA revealed moderate expression of galectin-1 mRNA in the proliferative phase and early/mid-secretory phases (Figure 2). The expression of galectin-1 increased significantly in the late secretory phase ($P < 0.05$) and in decidua of the 6th to 9th week of pregnancy ($P < 0.05$). Increase of GLUT1 mRNA expression was approximately 2-fold in decidua in comparison to the mid-secretory phase. Semi-quantitative comparison between expression levels of galectins-1 and -3 revealed increased mRNA expression of galectin-1 in the proliferative phase and in decidua, whereas expression was similar during the secretory phase (Figure 2). In comparison, mRNA expression of galectin-3 was weak in the proliferative phase (Figure 2). Expression of

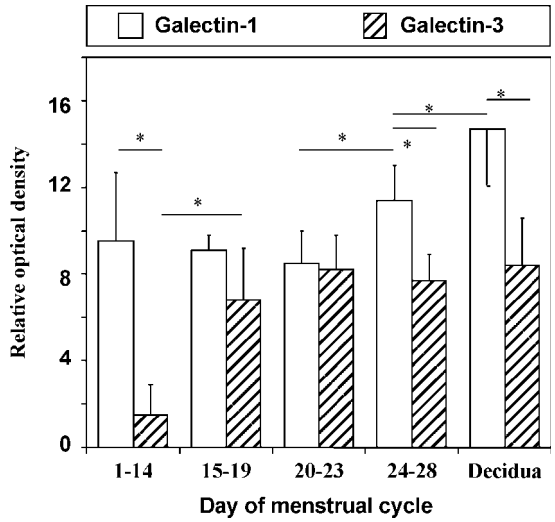

Figure 2. Galectins-1 and -3 mRNA expression in the total endometrium throughout the menstrual cycle ($n = 30$) and in decidua from the 6th to 9th week of pregnancy ($n = 7$). The expression levels of GLUT1 mRNA and the housekeeping genes L32 and GAPDH were examined by RPA in specimens from normal subjects. The relative optical density values of the mRNA bands were normalized to the relative optical density for L32 and GAPDH. A 2-fold increase of the relative optical density values corresponded, on an average, to a 1.8-fold increase of the specific RNA. Galectin-1 expression increased in the late secretory-phase and in decidua, whereas expression of galectin-3 was low in the proliferative phase and increased thereafter.

galectin-3 increased around 3-fold in the secretory phase and in decidua ($P < 0.05$).

To analyse which cell types were responsible for the increase of mRNA expression for a galectin in the total endometrium and in decidua, we separated endometrial tissue and decidua by collagenase digestion, filtration and by bead separation into epithelial cells, stromal cells and CD45-positive leukocytes. Analysis by RPA yielded low level expression of galectin-1 in glandular epithelial cells and CD45-positive leukocytes (Figure 3) throughout the menstrual cycle

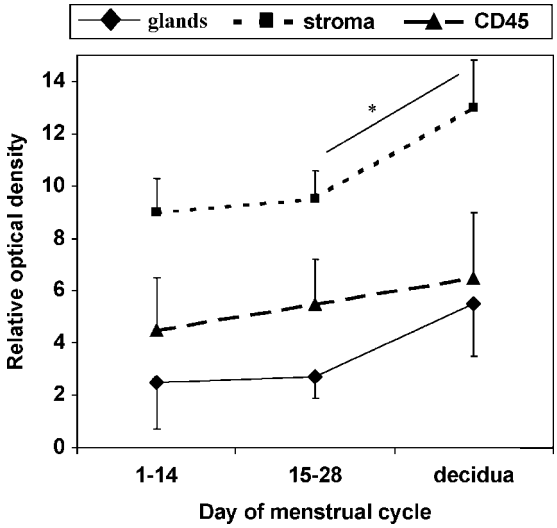

Figure 3. Galectin-1 mRNA expression in endometrial and decidual cells, epithelial cells, stromal cells, and CD45-positive leukocytes. Cells were separated from the total endometrium and decidua by enzymatic digestion and use of antibody-coated magnetic beads. They were analysed by RPA. Galectin-1 expression was high in stromal cells during the proliferative phase and increased further during the secretory phase and in decidua.

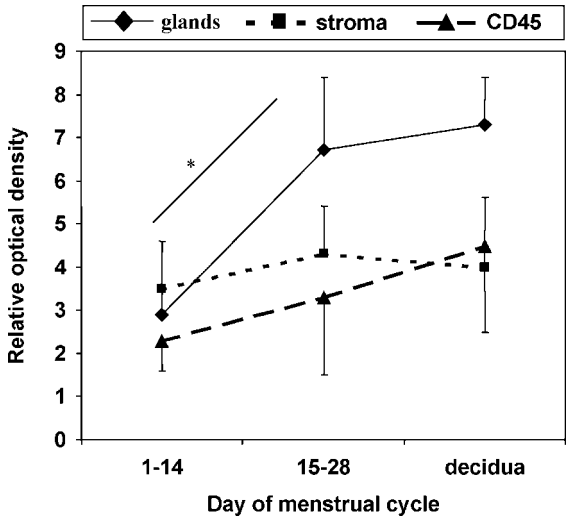

Figure 4. Galectin-3 mRNA expression in endometrial and decidual, epithelial and stromal cells, and CD45-positive leukocytes. Cells were separated from the total endometrium and decidua by enzymatic digestion and use of antibody-coated magnetic beads. They were analysed by RPA. Galectin-3 expression was low during the proliferative phase and increased in endometrial glandular cells during the secretory phase and in decidua.

and in decidua. In contrast, mRNA levels for galectin-1 in stromal cells were constantly high and increased further in decidual stromal cells ($P < 0.05$), suggesting galectin-1 expression in stromal cells to be responsible for the increase of galectin-1 in the total endometrium in the late secretory phase and in decidua (Figure 2). Revealing a distinct profile of regulation, mRNA expression for galectin-3 was consistently low in the tested cell fractions in the proliferative phase (Figure 4). Expression in stromal cells and leukocytes remained low during the secretory phase and in the decidua. Expression in glandular epithelial cells increased significantly in the secretory phase and in decidua ($P < 0.05$), pointing to a relation between galectin-3 expression in the glandular epithelial cells and galectin-3 expression in the total endometrium during the secretory phase (Figure 2).

To determine the expression patterns of galectins-1 and -3 in human endometrium and in decidua at the protein level we performed immunohistochemistry (Figure 5). In tissue samples taken during the proliferative phase, only weak reactivity of galectin-1 was observed in the stromal compartment of the endometrium (Figure 5a). As already shown by RPA, stromal reactivity increased in the late secretory phase (Figure 5b) with moderate staining in glandular epithelial cells. Immunostaining with anti-CD45 antibodies (Figure 5c) and double immunostaining with anti-galectin-1 and anti-CD45 antibodies (Figure 5d) documented that staining of the stromal compartment was mainly due to decidualized stromal cells (green), whereas CD45-positive leukocytes (red) were not stained by anti-galectin-1 antibodies. Immunohistochemistry of galectin-3 confirmed strong staining of endometrial glands in the secretory phase and weak staining in stromal cells (Figure 5e), hereby confirming the results at the mRNA level.

Discussion

The present study provides, for the first time, comprehensive information on the endometrial and decidual expression of human galectins. Our study showed that among the known human members of this lectin family galectins-1 and -3 are expressed rather abundantly. Obviously, galectin expression is tightly controlled by regulatory mechanisms, assigning distinct functions to certain galectins with restricted expression profile. Of note, the expression of galectins-1 and -3 is dependent on the menstrual cycle, and both levels of expression of galectins peak in different endometrial cell types.

The expression pattern and the broad spectrum of their documented functions suggest distinct galectins to play a role in the regulation of endometrial function. These two galectins have been

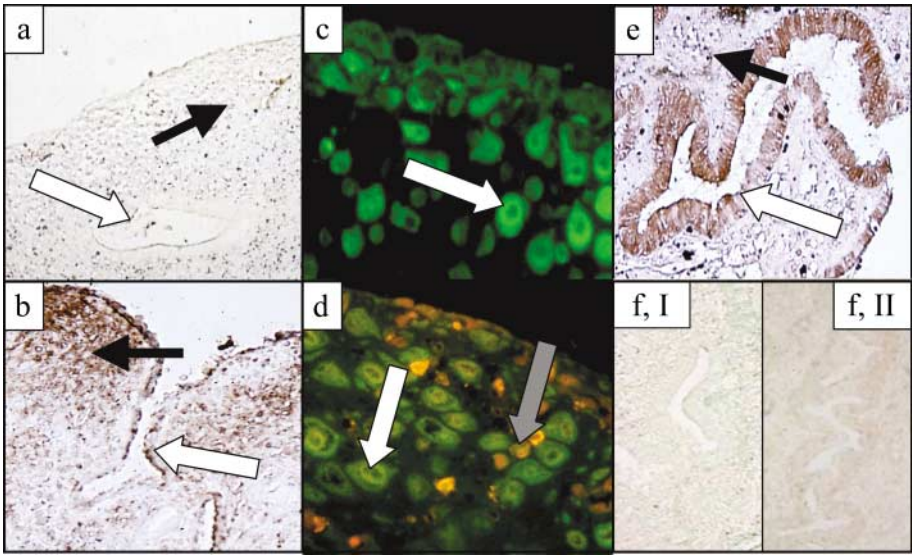

Figure 5. Immunostaining of galectins-1 and -3 in human endometrium and decidua. Staining for galectin-1 was weak in stromal cells (**a**, black arrow) and in epithelial cells (**a**, white arrow) during the proliferative phase, but strong during the secretory phase and in the decidua in the stromal compartment (**b**, black arrow) and moderate in epithelial cells (**b**, white arrow). Staining for galectin-1 in the stromal compartment was mainly due to staining of decidualized stromal cells (**c**, **d**; green cells, white arrows), whereas CD45-positive leukocytes (**d**, red cells, grey arrow) were not positive for galectin-1 as shown by double immunostaining. Staining for galectin-3 was weak during the proliferative phase in stromal and epithelial cells and increased in epithelial cells during the secretory phase (**e**, white arrow), whereas stromal staining remained weak (**e**, black arrow). Negative controls for galectin-1 (**f**, **I**) and negative control for galectin-3 (**f**, **II**), in which the primary antibody was substituted by goat IgG or rabbit IgG, revealed very weak background staining. **a**, **b**, **e**, **f**: × 200; **c**, **d**: × 640).

shown to play a pivotal role in mechanisms involved in the control of cell adhesion, chemotaxis, antigen presentation and apoptosis, as outlined in the introduction, all of which are intimately involved in endometrial regulation. In concert with endometrial cytokines such as interleukin-6 (Tseng *et al.*, 1996; von Wolff *et al.*, 2000), these two galectins could thus participate in immune regulation as pro- and anti-inflammatory 'cytokine-like' mediators, depending on the presence of cell targets such as CD7, a crucial ligand for galectin-1's activity as a pro-apoptotic signal on T leukaemic cells in Sézary syndrome (Rappl *et al.*, 2002). With the potential to compete for the same ligand and act functionally divergent to target distinct glycans, the clearly different expression profiles intimate distinct functions.

Endometrial tissue is characterized by a sensitive network of inflammatory mediators, allowing the accumulation of a spectrum of immune cells in the secretory phase (King *et al.*, 1989) and migration of immune cells to the implantation site (Slukvin *et al.*, 2004). Endometrial tissue homeostasis is in part regulated by the cytokines with pro- and anti-inflammatory functions, which belong to the Th1- and Th2-spectrum of effectors for immune responses. In this context, the detection of galectins-1 and -3 in human endometrium suggests that they participate in cell homeostasis to regulate endometrial functions. Our results in this respect are underscored by studies in mice, delineating differential and spatio-temporal expression patterns for galectins-1 and -3 in the reproductive tract (Phillips *et al.*, 1996; Lee *et al.*, 1998). Galectin-1 was present in the tissue compartments of the uterus except for the luminal and glandular epithelium. After implantation of the embryo, the endometrium including the decidualized stromal cells and the glandular epithelium showed immunohistochemical staining for galectin-1. During murine blastocyst implantation, gene transcription for galectin-1 is under the control of ovarian steroids (Choe *et al.*, 1997). Galectin-3 expression was low in non-pregnant mice uteri and increased in the primary decidual zone and in the uterine epithelial cells adjacent to the implanting blastocyst immediately after implantation. Cubilin was defined as galectin-3-specific ligand in the murine utero-placental complex (Crider-Pirkle *et al.*, 2002). Our study extended the localization to human tissue, revealing a similar pattern of endometrial galectin expression with a strong presence of galectin-1 in decidualized stromal cells and of galectin-3 in secretory-phase epithelial glandular cells.

The glandular expression of a lectin involved in adhesion, i.e. galectin-3, and the increasing expression of galectin-1 raises the question as to whether these galectins might be involved in steps leading to implantation, as previously suggested for β3-integrin (Lessey *et al.*, 1992) and its ligand osteopontin (von Wolff *et al.*, 2001). Galectin-3 modulates cell adhesion by binding to several ligands including laminin, fibronectin and integrins after its secretion from epithelial cells (André *et al.*, 1999; Hughes, 1999). Furthermore, galectins-1 and -3 are expressed at the maternofetal interface (Bevan *et al.*, 1994) and in placental trophoblast cells (Maquoi *et al.*, 1997; Vicovac *et al.*, 1998). The invading, galectin expressing trophoblast cells interact with galectin expressing epithelial and decidualizing stromal cells. As galectins have been suggested to be involved in tumour metastasis (van den Brule *et al.*, 2004), galectins might also play a role during the invasion of the trophoblast. Finally, Blois *et al.* (2004), have reported that stress-induced abortions in mice are associated with limited decidual expression of galectin-1 and increased expression of galectin-3. Treatment with recombinant galectin-1 and anti-galectin-3 before and after stress exposure abrogated the effects of stress on the abortion rate. As galectin-1 has immunoregulatory effects by inducing a Th2 polarized immune response and as galectin-3 is involved in the Th1 immune response, it can be speculated that galectins may play an important role in the endometrial immunomodulation during the implantation process.

Targeting such extracellular matrix glycoproteins and membrane glycoproteins, which are produced by the human blastocyst and the trophoblast (Turpeenniemi-Hujanen *et al.*, 1995; Huppertz *et al.*, 1996), could be a function of galectin-3. Fittingly, the detection of galectin-3 as a stage-specific gene product in bovine pre-implantation development (Ponsuksili *et al.*, 2002) raises respective evidence. With the availability of galectin-3 as a tool to monitor the profile of accessible binding sites, a study can be planned to map the presence of cellular targets for the tissue lectin. In addition to glycans, intracellular proteins with specificity to a galectin will also be detected (Liu *et al.*, 2002; Pukrábková *et al.*, 2003).

In conclusion, we have focused on a family of multifunctional endogenous lectins. Using mRNA expression we performed comprehensive fingerprinting and detected a prevalent presence of galectins-1 and -3 in human endometrium. Their expression profiles were found to be distinct. Maximal expression of galectin-1 in decidualized stromal cells and of galectin-3 in secretory-phase epithelial cells favours a role of both galectins in the regulation of endometrial function and implantation.

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