Galectin-9: A New Endometrial Epithelial Marker for the Mid- and Late-Secretory and Decidual Phases in Humans

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Context: The galectin family has been reported to play a role in the regulation of cell growth, cell adhesion, apoptosis, inflammation, and immunomodulation, all of which are important for endometrial function, as well as implantation.

Objective: The objective of the study was to investigate the expression and regulation of galectin-9, a β-galactoside-binding lectin in the human endometrium.

Design: Galectin-9 mRNA and protein were analyzed in dated endometrial biopsies throughout the menstrual cycle and in human early-pregnancy decidua, as well as in the different endometrial cell compartments. Regulation of galectin-9 by estradiol, progesterone, epidermal growth factor, and interferon-γ in endometrial epithelial cells *in vitro* was studied.

Results: Galectin-9 mRNA analyzed by RNase protection assay is expressed in the human endometrium, specifically in the human endometrial epithelial cells but not in stromal or immune cells. It is expressed at very low concentrations during the proliferative phase and the early-secretory phase and shows a sharp and significant increase in the mid- and late-secretory phases, the window of implantation, as well as in the decidua. Accordingly, galectin-9 protein is also exclusively increased in human endometrial epithelial cells during the mid- and late-secretory phases and in the decidua, however, not in endometrial stromal cells or decidualized cells *in vivo* or *in vitro*. A regulation *in vitro* by estradiol, progesterone, epidermal growth factor, and interferon-γ could not be detected.

Conclusions: Based on these findings and on the functional studies of other galectins, we suggest galectin-9 as a novel endometrial marker for the mid- and late-secretory and decidual phases. (*J Clin Endocrinol Metab* **90:** 6170–6176, 2005)

S TARTING AND RETAINING pregnancy in the human requires a well-functioning endometrium. The human endometrium, a unique tissue because of its morphology and function, only allows implantation of the blastocyst at a special time, *i.e.* during the window of implantation (1). During this postovulatory phase, under the influence of progesterone, glandular secretion and stromal decidualization, as well as changes in the immune cells, become the dominant features of the endometrium (2). In fertile cycles, the blastocyst has to attach and invade the epithelial barrier, which necessitates finely tuned adhesion molecules and immune functions (3). Despite extensive research, the phenomenon explaining why the blastocyst is not rejected like a semiallograft during the window of implantation is still not understood.

Several functions that have been described for the galectin family are important features that play a role in endometrial regulation. First, galectin-9 has been reported to be an important mediator in immunomodulation (4, 5). Because endometrial function and implantation involve many inflammatory mediators, galectin-9 might contribute to the modulation of the endometrial immune system. Second, galectins of the tandem-repeat type, such as galectin-9, play an important role in the process of cell adhesion and chemotaxis

(4, 6–8). Because adequate endometrial function is dependent on leukocyte migration and because 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 defense against invading microorganisms (8). Because 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.

Galectins are a rapidly growing family of β-galactoside-binding proteins. Of the 15 members known, many have been described to have specific functions in the regulation of cell growth, cell adhesion, apoptosis, and immunomodulation (4, 6, 9, 10). Paroutaud *et al.* (11) observed homologies concerning the structure of galectins in different species, suggesting a common ancestor gene. Furthermore, galectins can be found in nearly all organisms, from sponges and nematodes to higher vertebrates (12), indicating their important role throughout evolution.

Recently, Gray *et al.* (9) discovered a new member of the galectin family, named galectin-15 (or OVGAL 11), as a secreted substance in the uterus of sheep, and could show that galectin-15 was expressed specifically in the endometrial luminal epithelium, induced by progesterone and up-regulated during pregnancy. In human endometrium, cycle-dependent expression of galectin-1 in stromal cells and galectin-3 in epithelial cells suggests that these lectins are involved in the regulation of different endometrial cellular functions (13).

In an initial study leading to the publication of the report by von Wolff *et al.* (13), we analyzed the endometrial expression of eight different galectins. Among these, galectin-9 was expressed at increasing concentrations, suggesting that galectin-9 plays a role in the regulation of the periimplantation endometrium.

Human galectin-9 was first cloned from tissue of a patient with Hodgkin's disease (14). It consists of two carbohydrate-recognition domains connected by a linker peptide and has previously been found to be highly expressed in tissues of the immune system (7, 15). Chabot *et al.* (5) distinguished three isoforms of galectin-9 in Jurkat T cells, differing only in the length of the linker peptide and having a calculated molecular weight of 34.7, 35.9, and 39.5 kDa, respectively.

Galectin-9 plays an important role in immunomodulation because it induces T cell apoptosis through the calcium-calpain-caspase-1 pathway (16). It is also produced by activated T cells as an eosinophile chemoattractant (17) and could play a role in adhesion because it is able to bind two different glycosylated ligands with its two nonidentical carbohydrate-recognition domains (18, 19).

The known functions of galectin-9 in regulating the immune system, its possible role in cell adhesion, and our preliminary results on galectins in the endometrium have prompted us to systematically analyze galectin-9 expression in human endometrium at various stages across the menstrual cycle and in early-pregnancy decidua. Galectin-9 has been shown to be regulated by interferon (IFN)-γ in human umbilical-vein endothelial cells (20), as well as in lung fibroblasts (21). This regulation could be relevant in the endometrium, as well as in important cycle-dependent hormones, such as estradiol and progesterone, or growth factors, such as epidermal growth factor (EGF). We analyzed the possible regulation of galectin-9 by these factors. We have also examined galectin-9 expression in the different endometrial cell types in an effort to elucidate the role of galectin-9 in endometrial function.

Patients and Methods

Patients and samples

All samples were obtained after informed consent of all patients, and the protocol was approved by the Ethical Committee of Heidelberg University. Endometrium was collected either from endometrial biopsies (Women's University Hospital, Heidelberg; n = 10) or from hysterectomy (St. Elisabeth Klinik, Heidelberg; n = 36) at different phases of the menstrual cycle of patients having a regular cycle, without hormonal stimulation and without suspect of malignancy (n = 46). Endometrial biopsies were obtained from women undergoing diagnostic hysteroscopy during the proliferative phase for reasons of uterine myoma (80%), septal uterus (10%), or polycystic ovary syndrome (10%). Average age was 34.7 ± 4.5 yr. Because only the functionalis layer could be obtained, we used this tissue solely for epithelial- and stromal cell cultures. Patients undergoing hysterectomy were 37.8 ± 7.3 yr. Indications for surgery were uterine myoma (75%) and uterine descensus (25%). Here we could obtain full-thickness endometrium; therefore, we used this tissue for cell isolation, immunohistochemistry, Western blot, and RNase protection assay.

Decidua were obtained at the time of elective first-trimester pregnancy termination of uncomplicated, unwanted pregnancies (n = 8). Median age here was 24.4 ± 5.6 yr. Samples were dated according to the last menstrual period, histology, and serum levels of estradiol, progesterone, and LH.

Separation of endometrial and decidual epithelial and stromal cells and leukocytes

For cell culture, endometrial stromal and epithelial cells (n = 7) were separated by enzymatic digestion as described previously (22). Briefly, the endometrial tissue was digested for 1 h with collagenase IV (Life Technologies, Inc., Carlsbad, CA), hyaluronidase IV (Sigma-Aldrich, St. Louis, MO), and DNase I (Roche Diagnostics, Indianapolis, IN).

Stromal and epithelial cells were separated by sieving through a 40-μm filter and consecutive centrifugation; stromal cells were plated in DMEM/MCDB-105 medium containing 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT), 5 μg/ml insulin (Sigma-Aldrich), 10 μg/ml gentamycin (ccpro, Neustadt/W, Germany), 100 U/ml penicillin (ccpro), 100 μg/ml streptomycin (ccpro), and 100 U/ml nystatin (Life Technologies, Inc.) and cultured at 37 C and 5% CO_2 until confluent. Purity of cultures was determined to be over 99%, as published previously (22).

Epithelial cells were cultured on Matrigel without phenol red or growth factors in 10% fetal calf serum DMEM/F12 medium. For RNA isolation, separation of endometrial and decidual epithelial and stromal cells and CD45-positive leukocytes was performed as described elsewhere (23). Stromal and epithelial cells were separated as described above.

CD45-positive cells were isolated from the stromal and immune cells by positive selection with CD45 Dynabeads (Dynal Biotech, Inc., Lake Success, NY), and stromal cells were purified by negative selection with a mixture of magnetic beads against CD14, CD56, CD45, and CD31 and Dynabeads against epithelial cells (all from Dynal Biotech Inc.).

CD45-positive cells were washed and snap frozen in liquid nitrogen in TRIzol. Stromal cells were cultured for 1 h at 37 C and 5% CO_2 to allow adherence to culture flask. After rinsing off nonadherent cells, such as erythrocytes, stromal cells were trypsinized, washed with 1× PBS, and snap frozen in liquid nitrogen in TRIzol.

Decidualization

Endometrial stromal cells (n = 4) were obtained as previously described (22). When confluent, they were treated with serum-free DMEM/MCDB-105 medium, 17β-estradiol (10 nM; Sigma-Aldrich) and/or progesterone (1 μM; Sigma-Aldrich), and EGF (20 ng/ml; Sigma-Aldrich) for up to 16 d. Medium was changed every 2 d, and decidualization was confirmed by measuring prolactin in the conditioned medium.

Stimulation of endometrial epithelial cells in culture

Endometrial epithelial cells (n = 8) from the late-proliferative phase (cycle d 10–13) were cultured on Matrigel without phenol red or growth factors for 48 h in 10% fetal calf serum DMEM/F12 medium for cell adhesion. Cells from each patient were divided into four inlets.

After 48 h, medium was changed to serum-free DMEM/F12 medium for 24 h to prepare for the stimulation experiment. Then medium was changed again in all inlets. The stimulation was performed for 24 h with estradiol (10 nM), progesterone (1 μM), and estradiol and progesterone (in the known conditions) (n = 4), as well as with EGF (20 ng/ml and 40 ng/ml) and IFN-γ (1 ng/ml) (n = 4). Serum-free medium served only as negative control. After 24 h, supernatant was snap frozen in liquid nitrogen, and cells were snap frozen in 0.5 ml of TRIzol.

Immunohistochemistry

Tissue (n = 22) was embedded in OCT compound (Tissue Tek, Elkhart, IN) and frozen in liquid nitrogen. Five samples were from the proliferative phase (d 3–14), four samples from the early-secretory phase (d 15–18), four samples from the mid-secretory phase (d 19–23), five samples from the late-secretory phase (d 24–28), and four samples from early-pregnancy decidua. Cryostatic sections (7-μm thick) were mounted on slides, fixed with ice-cold 100% acetone (Sigma-Aldrich) for 10 min, and dried. After blocking the background with 10% swine serum (DAKO Corp., Carpentaria, CA) diluted in 1× PBS, samples were incubated with goat antihuman galectin-9 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibody and negative controls with goat-IgG (1:125; DAKO Corp.), respectively, overnight for 14 h at 4 C.

After four washings with $1\times$ PBS and incubation for 10 min at 21 C with a secondary antibody and 1% multilink swine (DAKO Corp.), immunohistochemistry was continued according to the instructions of the manufacturer with the commercially available Histostain-Plus Kit (Zymed Laboratories, San Francisco, CA).

Western blot analysis

Tissue was obtained from a total of 12 patients. Four samples were from the proliferative phase, four samples from the mid- to late-secretory phase, and four samples from young patients with first-trimester pregnancy termination.

We obtained cell lysates by adding radioimmunoprecipitation assay lysis buffer including freshly added phenylmethylsulfonyl fluoride, protease inhibitor cocktail, and sodium orthovanadate (all from Santa Cruz Biotechnology) to cell pellets or tissue and homogenized them. Samples were placed on ice for 30 min and then centrifuged ($10,000 \times g$, 4 C, 10 min). Proteins were found in the supernatant, and pellets could be discarded.

Samples were mixed with an equal volume of $2\times$ electrophoresis sample buffer (Santa Cruz Biotechnology) and boiled for 3 min; then samples were run on a 12% acrylamide-sodium dodecyl sulfate gel and electrotransferred onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ).

After blocking of nonspecific binding by 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST), membranes were incubated for 14 h with polyclonal rabbit antigalectin-9 (Dr. Preuss, Universitätsklinikum Homburg/Saar, Germany) diluted 1:100 in 5% skim milk in TBST. Membranes were then washed, followed by incubation with horseradish peroxidase-conjugated antirabbit antibody (Amersham Biosciences) diluted 1:7000 in TBST for 45 min.

Finally, membranes were washed in TBST and immersed into enhanced chemiluminescence (Amersham Biosciences) for 1 min, and immunoblotting could be visualized by exposing the membrane to a Kodak BioMax MR-1 film for 6 min. Equal loading was carried out with polyclonal goat-anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) 1:100 in 5% skim milk in TBST after stripping.

Multiprobe RNase protection assay

RNase protection assay was performed as described elsewhere (24). Using the BD Multiprobe RiboQuant Kit (PharMingen, San Diego, CA) allowed us to simultaneously analyze the expressions of galectin-9, the implantation marker PP14, and the housekeeping genes in one experiment. All samples were analyzed in one experiment for reduction of assay variability.

This experiment was carried out with RNA isolated from complete endometrial tissue. We used 12 samples from the proliferative phase, eight samples from the early-secretory phase, five samples from the mid-secretory phase, eight samples from the late-secretory phase, and seven samples from early-pregnancy decidua.

Furthermore, RNA had been isolated from endometrial stromal and epithelial cells, decidua, decidual epithelial, and stromal cells and CD45-positive immune cells and trophoblast using TRIzol according to the instructions of the manufacturer. These cells were isolated each from proliferative tissue ($n = 6$), from secretory tissue ($n = 6$), and from early-pregnancy decidua ($n = 5$).

The probe synthesis was performed, and the probe was diluted with hybridization buffer to a concentration of 4000 Cherencov counts/μl, respectively, followed by addition of 2 μl of probe to each RNA sample.

After incubating the samples overnight for a minimum of 16 h at 45 C, RNase treatment took place. Then a 4.75% prerun acrylamide-gel was loaded with the protected probes and run at 55 W constant power for 2 h. Finally, the gel was dried under vacuum for 2 h at 80 C and placed on a Kodak BioMax MR-1 film in a cassette with intensifying screen for 1–3 d at -70 C. The identity of bands protected from RNase degradation was established by comparing their size with the size of the positive-control samples. The negative control was 5 μg of yeast RNA.

Densitometry

Autoradiographs of bands on RNase protection assays for galectin-9, PP14, and L32 were scanned on a Hewlett Packard desk scanner and analyzed on the Bio-Rad Quantity One program (Bio-Rad, Hercules, CA). The integrated areas under the absorbance curves were measured for each band and used to determine the relative amounts of mRNA. A 2.1-fold increase of the relative OD values corresponded on average to a 2-fold increase of the specific RNA, as determined in several dilution series of RNase protection assays with different known concentrations of total RNA.

Chemiluminescence bands on Western blots for galectin-9 and GAPDH were also scanned, and relative amounts of specific protein production were determined by measuring the density of the bands. A 1.8-fold increase of the relative OD values corresponded to a 2-fold increase of the specific protein.

Statistical analysis

All experimental variables were tested in five or more different patients. Cultures were tested in duplicate in each experiment. Specific galectin-9 and PP14 densitometry values, normalized to L32, were used to calculate mean values $\pm$ SEM in the RNase protection assay. In Western blots, levels of galectin-9 protein normalized to GAPDH equal loading were used to calculate mean values $\pm$ SEM. Data were analyzed by ANOVA using the StatView software (Abacus Concepts, Berkley, CA). Significance between treatment groups was determined using Fisher's protected least-significant difference *post hoc* test, with $P < 0.001$ taken as significant. Spearman correlation coefficient was calculated for the correlation between progesterone and staining intensity of the immunohistochemistry samples shown (1, weak; 2, medium; 3, strong staining).

Results

Identification of galectin-9 mRNA in human endometrium

The identification of galectin-9 in human endometrium was conducted by RNase protection assay and Western blot and immunohistochemical analysis. The expression of galectin-9 in dated total endometrial samples from individual patients that were taken during the menstrual cycle, as well as from early-pregnancy decidua, was investigated. Shown in Fig. 1A is a representative autoradiograph of total endometrial samples from proliferative (d 1–14), early-secretory (d 15–18), mid-secretory (d 19–23), and late-secretory (d 24–28) phases, as well as decidua. Galectin-9 and galectin-1 expression, as well as the expression of glycodelin (PP14), L32, and GAPDH, is depicted. The expression of galectin-9 normalized to L32, a common ribosomal housekeeping gene, was analyzed by densitometry, and the results are shown in Fig. 1B. Galectin-9 mRNA is barely detectable in the proliferative phase. Densitometric analysis of galectin-9-specific bands on RNase protection blots from at least five different patients shows a distinctive increase of galectin-9 in the early- to mid-secretory phase and a further significant ($P < 0.001$) increase during the late-secretory phase. Early-pregnancy decidua also shows a highly increased expression of galectin-9, which does not correlate with the week of pregnancy of the herein-examined samples (wk 7–12) but is expressed consistently throughout early pregnancy. The RNase protection probe also included a probe for PP14 mRNA as an internal control and marker for endometrial epithelial mRNA expression. PP14 mRNA was expressed in epithelial cells in the same pattern as galectin-9 mRNA in all of our experiments. Koopman *et al.* (25) could show by microarray assay that PP14 was also expressed in uterine decidual natural killer (NK) cells, which we could prove in our RNase protection assay as well. In contrast, galectin-9 is expressed only in secretory endometrial and decidual epithelial cells
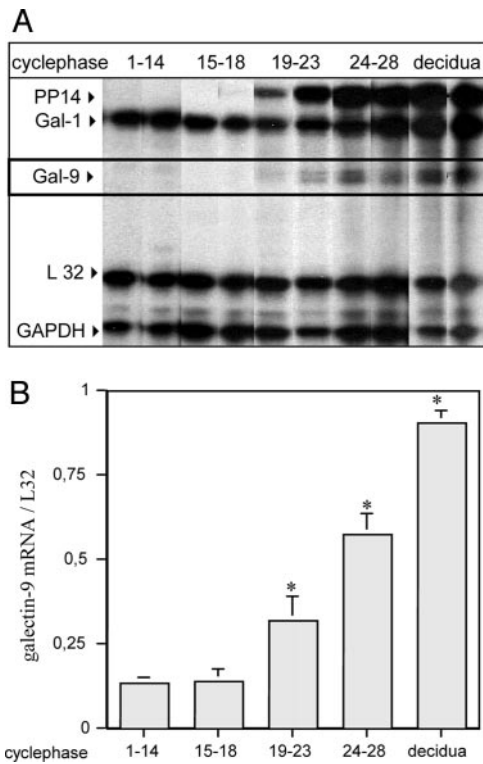
FIG. 1. Expression of galectin-9 mRNA in human endometrium from different cycle phases (proliferative phase, n = 12; early-secretory phase, n = 8; mid-secretory phase, n = 5; and late-secretory phase, n = 8) and from early-pregnancy decidua (n = 7). A, Representative RNase protection assay showing PP14, galectin-1, galectin-9, L32, and GAPDH mRNA expression in endometrium from proliferative, early-, middle-, and late-secretory phase and human early-pregnancy decidua. The two lanes in each group are independent samples from two different patients. B, Densitometric analysis of galectin-9 mRNA, normalized to the OD of L32, a common ribosomal housekeeping gene. Data represent the mean ± SEM of at least five independent patient samples in each group. *, Significant difference ($P < 0.001$) of galectin-9 mRNA expression between late-secretory endometrium *vs.* proliferative endometrium and decidua *vs.* proliferative endometrium.

and not in CD56 NK cells (data not shown) or CD45 immune cells (Fig. 2; see next paragraph).

To characterize the cells that express galectin-9 in human endometrium, we isolated endometrial epithelial cells, endometrial stromal cells, and endometrial CD45-positive cells by enzymatic digestion and magnetic bead-separation techniques, isolated the total RNA, and analyzed it by RNase protection assay as described in *Patients and Methods*. Densitometric measurements of bands for galectin-9 were normalized to L32, and the results are shown in Fig. 2. Galectin-9 mRNA is mainly expressed in endometrial epithelial cells, whereas it is not expressed in endometrial stromal cells, during the cycle. Also, endometrial immune cells express no detectable galectin-9. Galectin-9 mRNA expression in endometrial epithelial cells is significantly up-regulated during the secretory phase and in early-pregnancy decidua. As shown, decidualization in the secretory phase does not regulate galectin-9 mRNA expression in endometrial stromal cells *in vivo*. This was also confirmed in endometrial stromal cells in culture, which were decidualized with estradiol and progesterone *in vitro* (data not shown).
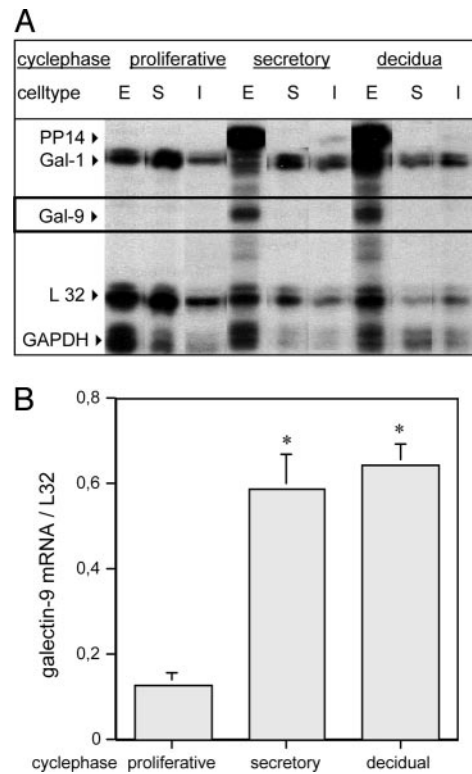

FIG. 2. Expression of galectin-9 mRNA of cell fractions isolated from endometrium in the proliferative (n = 6) and secretory phase (n = 6), as well as from early-pregnancy decidua (n = 5). A, Representative RNase protection assay showing PP14, galectin-1, galectin-9, L32, and GAPDH mRNA expression in human endometrial epithelial (E), stromal (S), and CD45-positive immune (I) cells from proliferative and secretory phase and early-pregnancy decidua. B, Densitometric analysis of galectin-9 mRNA normalized to the OD of L32, a common ribosomal housekeeping gene. Data represent the mean ± SEM of at least five independent samples in each group. *, Significant difference ($P < 0.001$) of galectin-9 mRNA expression between secretory and decidual epithelial cells *vs.* proliferative epithelial cells

Primary cultures of human endometrial epithelial cells were established on Matrigel, and the effect of estradiol and progesterone, as well as of EFG and IFN-γ, on the galectin-9 mRNA expression was analyzed by RNase protection assay. No regulation by estradiol alone, progesterone alone, or the combination of estradiol with progesterone was seen after 24 h of treatment (data not shown), neither was any effect of EGF at either 20 ng/ml or 40 ng/ml after 24 h seen on the RNA expression of galectin-9 in this epithelial monoculture system. IFN-γ also did not increase galectin-9 expression (data not shown).

Identification of galectin-9 protein in human endometrium

To verify the results of the RNase protection assay, we conducted immunohistological analysis using a peroxidase technique, as described in *Patients and Methods*, on dated endometrial biopsies from the proliferative and secretory phase of the cycle, as well as from early-pregnancy decidua, and confirmed the findings of the RNase protection assay.

The immunohistochemistry results are depicted in Fig. 3A. Row A shows the proliferative phase; row B, the secretory phase; and row C, decidua. The samples in lanes 1 and 2 are
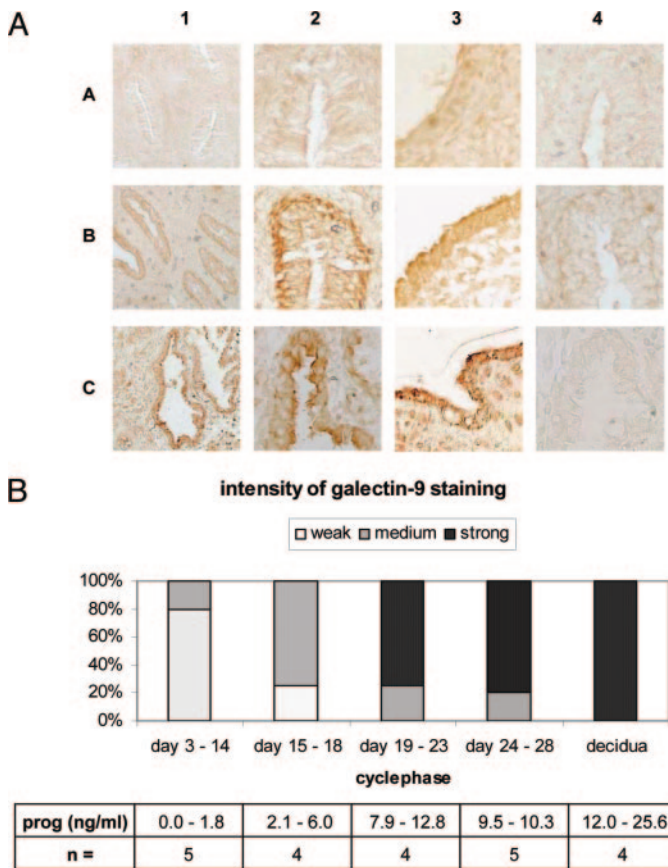
Fig. 3. Immunohistochemical staining for galectin-9 during the menstrual cycle. A, Immunohistochemical staining for galectin-9 in human proliferative endometrium (row A), secretory endometrium (row B), and early-pregnancy decidua (row C). Lane 1 shows specific staining for galectin-9 in low magnification ($\times$200); lane 2, galectin-9 staining of glandular epithelial cells (magnification, $\times$400); and lane 3, galectin-9 staining of luminal epithelial cells (magnification, $\times$400). Lane 4 shows the negative control with goat-IgG (magnification $\times$400). Similar staining was observed with tissue from at least 21 other endometrial and decidual samples. B, Intensity of galectin-9 staining according to the phase of the cycle, as well as the corresponding progesterone levels in the serum of the patient taken on the same day as the biopsies; proliferative phase (d 3–14; n = 5), early-secretory phase (d 15–18; n = 4), mid-secretory phase (d 19–23; n = 4), late-secretory phase (d 24–28; n = 5), and early-pregnancy decidua (n = 4). Spearman correlation coefficient between progesterone and staining intensity equals 0.861 with $P < 0.001$.

at $\times$200 and $\times$400 magnification, respectively, and were stained with polyclonal galectin-9 antibody. Lane 3 shows luminal epithelium stained with polyclonal galectin-9 antibody ($\times$400), and lane 4 was incubated with goat antiserum as a negative control ($\times$400). Galectin-9 was not produced in the glandular or luminal epithelium during the proliferative phase nor in the endometrial stromal cells.

During the secretory phase, galectin-9 was detected and significantly up-regulated in glandular epithelium of both basal and functional layers, as well as in luminal epithelium. It was produced both in the cytoplasm and on the cell membrane of the endometrial glands. The endometrial stromal cells of the secretory phase, however, did not produce galectin-9 either. In early-pregnancy decidua, strong immunohistochemical staining could be detected in the luminal and

glandular epithelium, whereas stromal or immune cells did not stain positively for galectin-9 protein.

The intensity of galectin-9 staining in biopsies from different phases of the cycle is shown in Fig. 3B. Increased staining was seen starting from d 19, with a maximum in the early-pregnancy decidua. A tight correlation of the intensity of staining with the progesterone level in the respective patient serum taken on the same day as the biopsy could be detected (Spearman correlation coefficient 0.861 with $P < 0.001$).

The regulation of galectin-9 protein in endometrial epithelial cells during the menstrual cycle was shown by Western blot analysis. Isolation of epithelial cells from proliferative and secretory endometrium, as well as from early-pregnancy decidua, was performed. An immunoblot of cell lysates from epithelial cells is shown in Fig. 4A. In good agreement with the results from the RNase protection assay, cells taken from the secretory phase show a major band of 39.5 kDa, which represents the large isoform of the galectin-9 protein, whereas there is only minimal galectin-9 protein in the proliferative phase. Equal loading experiments with GAPDH antibody show bands of the same OD in all lanes. We also examined the protein presence in epithelial cells of the decidua, as well as first-trimester trophoblast. Trophoblast does not produce galectin-9, whereas epithelial cells from decidua show a distinct antibody binding of galectin-9. To ascertain that the epithelial cells isolated from decidua were not contaminated by endometrial stromal cells, we cultured endometrial stromal cells and decidualized them *in vitro*. These cells did not produce galectin-9, neither on the protein nor on the RNA level (data not shown).

Discussion

In this study, we analyzed systematically, for the first time, the expression of galectin-9, a member of the lectin family, which is produced in human endometrium and in early-pregnancy decidua. We have demonstrated that galectin-9 is regulated during the menstrual cycle and that the cells responsible for this regulation are the epithelial cells of the endometrial secretory glands. In early-pregnancy decidua, galectin-9 is also expressed abundantly in epithelial cells of the surface epithelium. Contamination by endometrial stromal cells, immune cells, or trophoblast in the decidual samples could be excluded by showing that none of these cells produce galectin-9 when isolated either from histologic samples or in monocultures *in vitro*.

Galectin-9 is one of the very few epithelial markers, such as glycodelin (PP14) (26), that are strictly regulated during the menstrual cycle, with a significantly increased expression during the secretory phase. Interestingly, once pregnancy is established, there is a further increase of galectin-9, even though the relative amount of epithelial cells in the stromal-cell-rich decidua is low compared with the amount of epithelial cells in the secretory endometrium.

Galectin-9 is a glycan-binding protein and seems to play a significant role in apoptosis and immunomodulation, as well as in cell adhesion. Galectins recognize specific oligosaccharide structures on glycoproteins and glycolipids (27), bind to cell adhesion molecules, such as laminin and fi-

FIG. 4. Characterization of galectin-9 protein staining in human epithelial cells during proliferative phase (n = 4), secretory phase (n = 4), and early-pregnancy decidua (n = 4). A, Representative Western blot (ligand analysis) of isolated endometrial epithelial cell lysates marked with a polyclonal antigalectin-9 antibody. Spleen serves as a positive control. Secretory epithelial cells incubated with nonimmune rabbit serum as primary antibody, in the same dilution as the galectin-9 antibody, serve as negative control. Galectin-9 protein staining in epithelial endometrial cells from proliferative phase (P), epithelial endometrial cells from secretory phase (S), and epithelial cells from early-pregnancy decidua (D) are shown. All lanes were loaded with 100 μg of total protein. Control of equal loading was carried out with an anti-GAPDH antibody. B, Densitometry demonstrating significant difference of galectin-9 production in epithelial cells of the proliferative phase *vs.* secretory phase and decidua ($P < 0.001$). Data are the mean values ± SEM of at least four different patient samples.
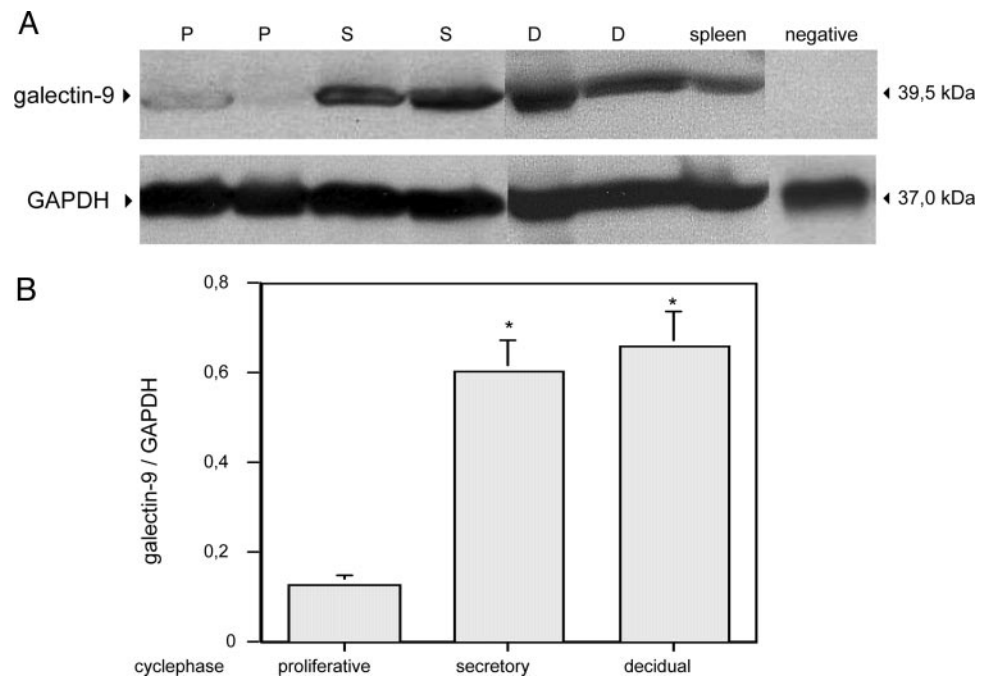
bronectin (28), and are possible candidates for supporting the binding between endometrial epithelial cells and the blastocyst. It is also known that galectins are ligands for integrins. Galectin-3 regulates binding to $\alpha_1\beta_1$-integrin on various tumor cell lines (29) and galectin-8 to $\alpha_3\beta_1$- and $\alpha_6\beta_1$-integrins on carcinoma cells (30). Hughes (19) shows that galectins can affect cell adhesion both as agonists, as well as antagonists. Integrin binding to its extracellular ligands is relatively weak; a stronger binding can be achieved by conformational changes of the integrin. Galectins can lead to these conformational changes by binding to extracellular parts of the integrins. This interaction is mostly mediated by galectins of the tandem-repeat type, such as galectin-9 (and also galectin-4, -6, and -12) (18, 31). It would be interesting to find an integrin ligand for galectin-9. Of the known galectins in the human system, it is barely detectable in the proliferative phase and then clearly and significantly increased in the late-secretory phase, with a maximum in early-pregnancy decidua, even though several other galectins are expressed in the human endometrium and have been shown to be produced differentially by different endometrial cell compartments (13). We chose EGF as the most abundant growth factor in the endometrium and IFN-γ because this cytokine has been previously reported to stimulate galectin-9 in human umbilical-vein endothelial cells (20), as well as in lung fibroblasts (21). Although we could not detect any changes in the galectin-9 expression by stimulation with estradiol, progesterone, EGF, or IFN-γ *in vitro*, the regulatory steps might be too complex to be seen in an epithelial monoculture system. Estradiol and progesterone receptors are known to be expressed in this culture system (32), and we could detect both EGF and IFN-γ receptors in our cultured cells by PCR (data not shown). *In vivo*, however, we had well-dated endometrial biopsies with respective serum samples taken on the day of the biopsy, and there was a distinct correlation of galectin-9 protein staining with rising progesterone levels in the serum of the patient. So far, no galectin-9 knockout mouse model has been described. Galectins have important functions in extracellular interactions (4). Matsumoto *et al.* (15) first described ecalectin, which is presumed to be homologous to galectin-9, to be a secreted protein. We assume that galectin-9 is secreted by an unknown mechanism from the epithelial cells because we can detect the short isoform of galectin-9 in serum from women in the secretory-cycle phase, as well as in early pregnancy (unpublished data). Gray *et al.* (9) suggest galectins as cellular and molecular markers for the endometrial receptivity. They show a distinctive regulation of galectin-15 (OVGAL 11) in sheep endometrium, which was low on d 1–10 but increased between d 10–14. The greatest homology between sheep galectin-15 is shown to human galectin-10 (44%) and galectin-8 (40%).

It is known that galectin-9 induces apoptosis of thymocytes (7) and melanoma cells (33), as in many other immune cells (12), such as T cells, B cells, and monocytes. The mechanism used is the calcium-calpain-caspase-1 pathway and differs from other proapoptotic stimuli. The β-galactoside binding, and possibly the *de novo* protein synthesis, is essential for galectin-9-induced apoptosis. It is also very interesting that galectin-9 only acts as a proapoptotic agent on activated CD4^{+} and CD8^{+} T cells but not on inactivated ones; this leads one to suspect that cell-surface-binding partners need to be present and activated before galectin-9 can actually develop its apoptotic potential. Also, it is important to note that galectin-9 does not induce apoptosis in all cells investigated; for example, it does not induce apoptosis in hepatocytes (7). It is, therefore, essential to examine galectin-9 and its effect on trophoblast, as well as on endometrial and decidual immune cells, such as NK cells. We could show that galectin-9 is not expressed by first-trimester trophoblast; however, it would be interesting to know whether the same is true for the blastocyst. Considering that galectin-9 is one of the very few proteins that has been described to be reg-

ulated in epithelial endometrial cells, it will be very intriguing to follow up with functional studies in the human endometrium. The temporal and spatial changes in galectin-9 mRNA and protein in human endometrium, combined with the functional aspects of galectin-9 and other galectins, make it an intriguing factor for cell-cell interaction in the human endometrium, as well as during human implantation. Understanding the functions of galectin-9 and its family members in the human endometrium will broaden our knowledge of the roles of galectins in endometrial function.

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