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Chorioamnionitis and increased galectin-1 expression in PPROM –an anti-inflammatory response in the fetal membranes?

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Abstract

Problem—Galectin-1 can regulate immune responses upon infection and inflammation. We determined galectin-1 expression in the chorioamniotic membranes and its changes during histological chorioamnionitis.

Methods of Study—Chorioamniotic membranes were obtained from women with normal pregnancy (n=5) and from patients with pre-term pre-labor rupture of the membranes (PPROM) with (n=8) and without histological chorioamnionitis (n=8). Galectin-1 mRNA and protein were localized by *in situ* hybridization and immunohistochemistry. Galectin-1 mRNA expression was also determined by quantitative RT-PCR.

Results—Galectin-1 mRNA and protein were detected in the amnion epithelium, chorioamniotic fibroblasts/myofibroblasts and macrophages, chorionic trophoblasts, and decidual stromal cells. In patients with PPROM, galectin-1 mRNA expression in the fetal membranes was higher (2.07-fold, p=0.002) in those with chorioamnionitis than in those without. Moreover, chorioamnionitis was associated with a strong galectin-1 immunostaining in amniotic epithelium, chorioamniotic mesodermal cells, and apoptotic bodies.

Conclusions—Chorioamnionitis is associated with an increased galectin-1 mRNA expression and strong immunoreactivity of the chorioamniotic membranes; thus, galectin-1 may be involved in the regulation of the inflammatory responses to chorioamniotic infection.

Keywords

chorioamniotic fibroblast/myofibroblast and macrophage; chorioamniotic membranes; inflammation; lectin; prelabour rupture of membranes; preterm delivery

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INTRODUCTION

Preterm prelabor rupture of the membranes (PPROM) complicates 2–3.5% of pregnancies, accounts for 30–40% of preterm births, and is considered an obstetrical syndrome characterized by multiple etiologies.^{1–5} Intra-amniotic infection and inflammation (IAI) is a major underlying mechanism of the disease in 25–39% of the cases.^{3–5} The histological signs of IAI are detected in the chorioamniotic membranes, including the infiltration of inflammatory cells, edema, fibrin deposition, necrosis and reparative responses.⁶ This may include the maternal inflammatory response manifested in chorionitis and chorioamnionitis as well as the fetal inflammatory response characterized by chorionic vasculitis and funisitis.^{6–8}

Chorioamniotic membranes do not only represent a mechanical barrier against microorganisms but can possibly sense⁹ and inhibit bacterial growth.¹⁰ These local processes are characterized by the activation of the innate immune system,^{4,11} including pattern recognition molecules [e.g. Toll-like receptors (TLRs)],⁹ C-type lectins,⁴ pro-inflammatory cytokines [e.g. interleukin (IL)-6],¹² and chemokines (e.g. IL-8).^{11,13} IL-10 was implicated in countering inflammation associated with preterm labor in placental and chorioamniotic membranes explants *in vitro*¹⁴ and appeared to be effective in preventing endotoxin-induced preterm birth in pregnant rats.¹⁵ Hence, there is only limited information available on how other anti-inflammatory molecules participate in the regulation of inflammation in the chorioamniotic membranes.

Galectin-1 is an anti-inflammatory protein expressed by chorioamniotic membranes and the decidua.^{16–20} Like other members of the galectin family,²¹ galectin-1 has pleiotropic intra- and extracellular functions;²² it regulates cell-cell and cell-extracellular matrix (ECM) adhesions, cell fate, immune cell homeostasis and host-pathogen interactions.²³ Galectin-1 is overexpressed in activated immune and endothelial cells^{24–26} as well as in sites of inflammation, such as fibroblasts in chronic pancreatitis.²⁷ Due to its potent immunomodulatory properties (summarized in recent reviews),^{22,28} galectin-1 was suggested to regulate the extent of the adaptive and innate immune response.²³

The changes in galectin-1 expression in chorioamniotic membranes upon inflammation have not yet been investigated. This study aimed to determine the cellular localization of galectin-1 in normal term chorioamniotic membranes and its expression in the membranes in patients with PPRM with and without histological chorioamnionitis.

PATIENTS AND METHODS

Study design and population

A cross-sectional study was conducted by searching our bank of biological samples and clinical databases of the Perinatology Research Branch, including patients in the following groups: 1) normal pregnant women at term, without labor and histological chorioamnionitis (n=5), and 2) women with PPRM with (n=8) and without chorioamnionitis (n=8). Women in the PPRM groups were matched for gestational age at delivery within two weeks of gestation. Patients presenting with labor, medical complications, multiple pregnancies or fetal congenital or chromosomal abnormalities were excluded.

Definitions

Normal pregnant women delivered a healthy infant at term (≥ 37 weeks of gestation) whose birthweight was between the 10–90th percentiles for gestational age.²⁹ PPRM was diagnosed before 37 weeks of gestation in the presence of vaginal pooling and positive nitrazine or ferning tests documented by a sterile speculum examination at admission. Labor

was defined as the presence of regular uterine contractions that occurred at a frequency of at least 2 every 10 minutes associated with cervical changes. Histological chorioamnionitis was diagnosed in the presence of acute inflammatory changes in the chorioamniotic membranes, chorionic plate of the placenta or umbilical cord using criteria previously described.^{6,8}

All patients were enrolled at Hutzel Women's Hospital, Detroit, MI, USA, and provided written informed consent prior to the collection of samples. The collection and utilization of samples for research purposes was approved by the Institutional Review Boards of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD/NIH/DHHS) and Wayne State University. Many of these samples have been employed to study the biology of inflammation.

Tissue samples and histopathological examinations

Tissue samples collected after spontaneous vaginal delivery or cesarean section were either snap-frozen and stored at -80°C , or fixed in 10% neutral buffered formalin overnight and embedded in paraffin. Five μm -thick paraffin sections were stained with hematoxylin and eosin, and examined using bright-field light microscopy. Histopathological examinations were performed by three pathologists who were blinded to the clinical information based on the diagnostic criteria previously described.^{6,8}

mRNA *in situ* hybridization

The 123bp fragment of human galectin-1 cDNA generated by PCR (forward primer: CATCTCTCTCGGGTGGAGTC, reverse primer: GAAGGCACTCTCCAGGTTTG) was subcloned into pGEM-T Easy vector (Promega Corp., Madison, WI, USA) containing SP6 and T7 polymerase promoters. Digoxigenin-labeled anti-sense and sense riboprobes were generated with SP6 and T7 polymerases after linearization of the plasmid with Bam HI and Hind III, respectively. Five μm -thick paraffin sections were deparaffinized, rehydrated in xylene and graded series of ethanol and then treated with proteinase K (15 $\mu\text{g}/\text{ml}$) in 0.1 M Tris buffer (pH 8.0) and 50 mM EDTA for 10min at 37°C . Slides were fixed with 4% paraformaldehyde for 20 minutes and acetic anhydride for 10 minutes. Sections were incubated in a hybridization buffer containing digoxigenin-tagged galectin-1 riboprobe (2 $\mu\text{g}/\text{ml}$). Hybridization was carried out in a humidity chamber overnight at 55°C . After repeated post-hybridization washes, sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics, Indianapolis, IN) for 1 hour at room temperature. Nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt were used for the detection of the hybridization signal.

Immunohistochemistry

Immunostaining was performed on 5 μm -thick paraffin sections with goat anti-human galectin-1 IgG (R&D Systems, Minneapolis, MN) using the Discovery autostainer (Ventana Medical Systems Inc., Tucson, AZ). Sections were incubated for 60 minutes at a 1:50 dilution following antigen retrieval in a citrate-based buffer (pH 6.0). A biotinylated horse anti-goat IgG (Vector Laboratories Inc., Burlingame, CA) at a 1:400 dilution, DAB MAP HRP Kit and 3-3'diaminobenzidine (Ventana Medical Systems Inc., Tucson, AZ) were used for the detection. The antibodies and the detection systems used for subsequent double stainings are shown in Table I.

Total RNA extraction

Total RNA was isolated from snap-frozen chorioamniotic membranes using TRIzol reagent (Gibco BRL, Grand Island, NY) and then Qiagen RNeasy kit (Qiagen, Valencia, CA, USA)

according to the manufacturers' recommendations. The 28S/18S ratio and the RNA integrity number (RIN) were assessed using a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE, USA). An $A_{260\text{nm}}/A_{280\text{nm}}$ ratio of 1.8, a 28S/18S ratio of 1.3, and a RIN of 6 were minimum requirements for inclusion in expression analysis.

Quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR)

Total RNA was reverse transcribed with a TaqMan Reverse Transcription Reagent kit using random hexamers (Applied Biosystems, Foster City, CA, USA). The standard curve was run with the *LGALS1* TaqMan Gene Expression Assay (Hs00169327_m1; Applied Biosystems, Foster City, CA, USA) to determine the quantity of cDNA needed for an approximate cycle threshold (Ct) of 25. The human *RPLPO* (large ribosomal protein) TaqMan Endogenous Control (part number: 4326314E) was used as the housekeeping gene for relative quantification. The *LGALS1* gene and the *RPLPO* housekeeping gene were then run in triplicate for each case to allow for the assessment of technical variability.

Statistical analysis

Clinical and demographic characteristics were compared among the groups with the Kruskal-Wallis test followed by the Mann-Whitney U-test for continuous variables, as well as the Chi square test and the Fisher exact test for proportions. For the analysis of qRT-PCR data, pair-wise group comparisons were performed using “Generalized estimating equations”, a model-based method that allows for the analysis of correlated qRT-PCR data. In parallel, t-test was also applied to average over the three technical replicates (Ct values) of each subject. To determine the influence of the gestational age on *LGALS1* gene expression within the groups, a stepwise linear model was fitted in which the gestational age was used as a predictor of the Ct values. The R statistical software (www.r-project.org) including required libraries, and SPSS version 12.0 (SPSS Inc., Chicago, IL) were used for the analyses. A p value of <0.05 was considered statistically significant.

RESULTS

Table II displays the demographic and clinical characteristics of the study groups. Gestational age at delivery and birthweight were significantly higher in the term, not in labor group than in the PPRM groups. The rate of Cesarean deliveries was not different among the groups. The histopathologic examination revealed marked maternal neutrophilic infiltration of the chorioamniotic membranes in cases of PPRM with chorioamnionitis. Chorionic vasculitis, umbilical phlebitis or arteritis characterized by fetal neutrophil infiltrates were also diagnosed in four patients of PPRM with chorioamnionitis. Amniocentesis was performed at admission to the hospital in five patients with PPRM (three with and two without chorioamnionitis), and amniotic fluid culture was positive for *C. albicans* in one case. Two patients had group B Streptococcus and one had *C. trachomatis* infection in the PPRM with chorioamnionitis group.

Localization of galectin-1 mRNA and protein in normal term chorioamniotic membranes

Galectin-1 mRNA hybridization signals were readily detectable, and the pattern was concordant with galectin-1 immunostaining. Galectin-1 mRNA was detected in amnion epithelial cells, chorioamniotic mesodermal cells, chorionic trophoblasts, and decidual stromal cells (Figure 1A). The cytoplasm of amnion epithelial cells was consistently positive for galectin-1, while the apical membrane and the nuclei of these cells stained variably. Chorionic trophoblasts exhibited similar staining pattern. The cytoplasm of chorioamniotic fibroblasts/myofibroblasts and macrophages, as well as decidual stromal cells were moderately galectin-1 positive (Figure 1B).

Galectin-1 expression is increased in the presence of inflammation

Galectin-1 expression was markedly increased in chorioamniotic membranes with histological signs of inflammation. In PPRM without chorioamnionitis, the expression pattern of galectin-1 was similar to those seen in normal pregnant women (Figure 2A). In PPRM with histological chorioamnionitis, amnion epithelial cells, and chorioamniotic fibroblasts/myofibroblasts and macrophages expressed increased amounts of galectin-1 (Figure 2B). Patients with PPRM and chorioamnionitis had a significantly higher galectin-1 mRNA expression (2.07-fold, $p=0.002$) in the chorioamniotic membranes than those without chorioamnionitis (Figure 2C). There was no correlation between galectin-1 mRNA expression and gestational age in these samples:

Galectin-1 expression changes with the sequence of inflammatory responses in chorioamnionitis

The spatial and temporal changes in galectin-1 expression were analyzed according to the sequence and timing of inflammatory responses in chorioamnionitis:^{30,31}

In *acute chorionitis*, maternal neutrophils invading the chorion and aggregating at the border of the chorioamniotic mesodermal layer stained weakly for galectin-1. Fibroblasts/myofibroblasts and macrophages in the chorioamniotic mesodermal layer stained strongly for galectin-1; however, no remarkable change in galectin-1 staining of the chorionic trophoblasts was detected (Figure 3A).

In *acute chorioamnionitis*, maternal neutrophils in the chorion and the chorioamniotic mesodermal layer were moderately immunopositive. Chorioamniotic fibroblasts/myofibroblasts and macrophages exhibited strong galectin-1 staining. The nuclei and cytoplasm of amnion epithelial cells were also strongly galectin-1 positive. There was no similar change in the staining of chorionic trophoblasts (Figure 3B). In an advanced stage of inflammation, maternal neutrophils that were trapped in the mesodermal layer, chorioamniotic mesodermal cells, amnion epithelial cells and apoptotic bodies stained strongly for galectin-1 (Figure 3C).

In *necrotizing chorioamnionitis*, the amnion epithelium underwent necrosis. Galectin-1 positive neutrophils, chorioamniotic mesodermal cells, apoptotic bodies and cell debris were seen in the chorioamniotic mesodermal layer (Figure 3D).

DISCUSSION

Principal findings of this study

1) Galectin-1 mRNA and protein were ubiquitously expressed in the chorioamniotic membranes of women with normal pregnancy at term, and a similar pattern was found in women with PPRM without chorioamnionitis. 2) In patients with PPRM, galectin-1 mRNA expression in the fetal membranes was significantly higher in those with chorioamnionitis than in those without. 3) Galectin-1 immunoreactivity of the amnion epithelium and chorioamniotic mesodermal cells was stronger in the membranes of patients with PPRM with histological chorioamnionitis than in those without. 4) The pattern of galectin-1 immunostaining changed with the severity of histological chorioamnionitis.

Galectin-1 is abundantly expressed in the chorioamniotic membranes in the third trimester

This is the first study that in parallel localized galectin-1 mRNA and protein expression in human chorioamniotic membranes. Previous studies reported galectin-1 mRNA and protein expression in the endometrium and decidua^{16–19} as well as galectin-1 immunostaining of the umbilical cord, amniotic epithelium¹⁶ and extravillous trophoblasts (EVTs)^{16–18,32} in

humans. Galectin-1 mRNA expression increased in endometrial stromal and epithelial cells in the late secretory phase and in the decidua.¹⁹ This menstrual cycle-dependent endometrial expression suggested that galectin-1 might be regulated by sex steroids.¹⁹ Similarly, galectin-1 was found to be under the regulation of estrogen and progesterone during the estrous cycle and during implantation in the murine endometrium and decidua.³³ Moreover, evidence for a synergy between galectin-1 and progesterone in the maintenance of pregnancy in mice has been presented recently.³⁴ The results of these murine experiments suggested that galectin-1 may have an important role in regulating endometrial functions, blastocyst-uterine interactions, and immune-endocrine crosstalk.^{19:33:34}

This study localized galectin-1 mRNA and protein in all cell types of the chorioamniotic membranes in normal pregnant women at term. Galectin-1 immunostaining was moderately strong in chorioamniotic mesodermal cells and decidual stromal cells; however, chorionic trophoblasts and amnion epithelial cells stained less intensely. In general, galectin-1 is more abundant in cells of mesenchymal origin²² that can be the explanation for this observation. As galectin-1 mRNA hybridization signals were similarly intense in all these cell types, it is possible that the differences in galectin-1 production may originate from a differential posttranscriptional regulation of galectin-1. In fact, the *LGALS1* gene promoter was shown to contain a complex functional architecture with two evolutionary conserved alternative transcription initiation sites. One of these directs the transcription of a longer mRNA with extremely GC-rich 5' end terminus that is capable of folding into a relative stable hairpin, and thus, to differentially regulate the translation of galectin-1 in various tissues.³⁵ An alternative explanation for the relative difference in galectin-1 mRNA signal and protein staining in the amnion epithelium is that galectin-1 may be continuously secreted into the amniotic fluid.

In fact, galectin-1 was localized to the apical membrane of the amnion epithelium (Figure 1B), which is consistent with recent reports that revealed the translocation of cytoplasmic galectin-1 to the extracellular side of the cell membrane²² as well as the staining of the syncytiotrophoblast apical membrane in the third trimester.³⁶ Of note, similar to the syncytiotrophoblast, the apical surface of the amnion epithelium is also covered by microvilli,³⁷ which may suggest a common mechanism for the sub-localization of galectin-1 in these distinct fetal cells. The nuclear localization of galectin-1 in chorionic trophoblasts and amnion epithelial cells may indicate an active splicing process in these cells as nuclear galectin-1 is involved in the spliceosome assembly pathway and direct pre-mRNA splicing.²²

What is the role of galectin-1 in the chorioamniotic membranes?

Previous reports have determined the role of galectin-1 in tissue development, cell-cell and cell-ECM interactions, immune tolerance and counter-regulation of the inflammatory response. We propose that galectin-1 may have a role in these processes in the chorioamniotic membranes, as well.

Tissue development and cell-ECM interactions—In normal pregnant women at term, chorioamniotic mesodermal cells and decidual stromal cells were positive for galectin-1, which is in accord with the general abundance of galectin-1 in cells of mesenchymal origin and their ECM.²² In fact, many cell types, especially normal and malignant mesenchymal cells, secrete galectin-1 into the ECM, where it binds to adhesion and ECM molecules, including laminin, fibronectin and integrins, and thus modulates cell adhesion, migration, invasion, assembly and remodeling of the ECM.²² The de-novo synthesis and the reorganization of the ECM is an important feature of decidualization, thus, galectin-1 may play role in these processes.

Previous studies have shown galectin-1 staining of the EVT's and their ECM in the mid and distal but not in the proximal columns.^{16,18} It was suggested that the expression of galectin-1 is dependent on the differentiation stage of the EVT's, and galectin-1 is involved in the organization of their ECM upon differentiation.¹⁸ Based on our findings, galectin-1 may also have a similar function in the development and maintenance of the chorionic ECM, as well.

In cases of PPRM without histological chorioamnionitis, the pattern of galectin-1 staining was similar to those in normal term pregnancy; however, in cases with histological chorioamnionitis, the up-regulation of galectin-1 was observed in the membranes. Of note, the overexpression of galectin-1 in smooth muscle cells was shown to decrease the incorporation of its ligands (vitronectin and chondroitin sulphate) into the ECM.³⁸ Moreover, in intracranial aneurysms, which bear a considerable inflammatory component, the overexpression of galectin-1 seemed to critically influence tissue remodelling.³⁹ Furthermore, microarray analysis revealed that treatment with galectin-1 induced the up-regulation of genes encoding for matrix metalloproteinase (MMP)-1, MMP-10, MMP-12, and tissue plasminogen activator in dendritic cells, and this was related to an increased migratory activity of these antigen presenting cells.⁴⁰ We propose that the overexpression of galectin-1 in the chorioamniotic membranes may be a possible link between inflammation, tissue remodeling, and a subsequent membrane weakening that may contribute to the rupture of the membranes.

Anti-microbial effects—The presence of activated macrophages in the chorioamniotic mesodermal layer has previously been reported^{41–43} in association with fetal inflammatory response upon infection. Our finding on the increased expression of galectin-1 by chorioamniotic fibroblasts/myofibroblasts and macrophages upon inflammation is novel and may suggest that galectin-1 has a role in the active barrier functions of the chorioamniotic membranes, protecting the fetus from bacterial infection and maternal inflammatory cells.^{9,10} There is a solid body of evidence that chorioamniotic membranes and amniotic fluid have antimicrobial properties mediated by antimicrobial peptides, including neutrophil defensins, bactericidal/permeability-increasing protein and calprotectin;⁴⁴ however, currently there is no data regarding the association between galectin-1 and these peptides. Of importance, there is an up-regulation of galectin-1 expression in activated macrophages,²⁶ where its expression has been suggested to regulate macrophage effector functions in an autocrine way during the development of the immune responses.⁴⁵ *In vitro* experiments demonstrated that recombinant galectin-1 decreases iNOS expression and strongly inhibits lipopolysaccharide (LPS)-induced NO metabolism in macrophages in a dose- and time-dependent manner.⁴⁶ Furthermore, low concentrations of galectin-1 up-regulate the constitutive cell surface expression of FcγRI on macrophages, while it suppresses INFγ-induced FcγRI and MHC-II expression in a dose-dependent manner, parallel to the inhibition of FcγRI-dependent phagocytosis and antigen-presentation.⁴⁵ We propose that the changes in galectin-1 expression in chorioamniotic macrophages found in this study may be part of the host response to pathogenic insults, which in turn can modify the extent of the inflammation in the chorioamniotic membranes.

Immune tolerance and counter-regulation of the inflammatory response—This study demonstrated a substantial galectin-1 staining of the amnion epithelium, its apical membrane, and chorioamniotic mesodermal cells in the connective tissue beneath. Of interest, this innermost layer of the chorioamniotic membranes has important immunosuppressive properties that are implemented in surgery and ophthalmology.⁴⁷ Indeed, the amniotic membrane was first used for skin transplantation in 1910 and then applied as a biological bandage for burned or ulcerated skin, surgical reconstruction of the vagina, repair of abdominal hernia, closure of the pericardium, or the prevention of surgical

adhesions.⁴⁷ The use of the amniotic membrane in the treatment of ocular surface disorders has only recently become common.⁴⁷ Since then, several investigations have shown that inflammation is markedly reduced in the area covered by the membrane graft, and no rejection occurs, while wound healing is promoted in amniotic membrane transplants.⁴⁸ Moreover, cultured amniotic epithelial cells were shown to secrete soluble factors capable of suppressing innate and adaptive immune responses, such as the migration of neutrophils and macrophages, T and B cell proliferation, and to induce T cell apoptosis.⁴⁸ In fact, mono- and polymorphonuclear cell infiltration was reduced by the amniotic membrane *in vivo*, and selective trapping, sequestering and apoptosis of monocytes and neutrophils were demonstrated in the stroma.^{49:50} These results demonstrated that the amniotic membrane mitigated inflammatory cell influx by enhancing their adherence and subsequent apoptosis and, thus, excluded monocytes and neutrophils from the basal membrane and the amnion epithelium.^{49:50} These observations can explain why neutrophils in the amniotic fluid are reported to be of fetal but not maternal origin.⁵¹

Although several molecules have been investigated (e.g. Fas ligand, tumor necrosis factor-related apoptosis-inducing ligand, macrophage migration-inhibitory factor, transforming growth factor- β),⁴⁸ factors that can be responsible for the wide spectrum of anti-inflammatory effects of the amniotic membrane, and can also maintain immune privilege, have not yet been identified. However, we note that the anti-inflammatory effects^{22:23:25:28:45:52-57} of galectin-1 (Table III) and the reported immunosuppressive features of the amniotic membrane have extensive similarities. Of importance, galectin-1 was proposed to be a major molecule of tumor immune surveillance²² and transplantation tolerance,⁵⁴ and recent clinical and experimental data implicated its role in maternal-fetal tolerance.^{34:36} In fact, galectin-1 is over-expressed in the human placenta in severe preeclampsia, a condition similar to allograft rejection, and its increased expression was proposed to be a local fetal response to the exaggerated systemic maternal inflammation.³⁶ Moreover, galectin-1-null mutant mice have higher rates of fetal loss than wild type mice, and treatment with recombinant galectin-1 prevents fetal loss and reestablishes maternal-fetal tolerance through several actions, such as promoting the generation of tolerogenic dendritic cells and the expansion of IL-10-secreting regulatory T cells.³⁴ Based on the extensive functional data on galectin-1 and the results of this study, we propose that galectin-1 is one of the immunosuppressive agents expressed by the human chorioamniotic membrane that is involved in the regulation of inflammation and the establishment of maternal-fetal immune tolerance in chorioamniotic membranes.

A model of the role of galectin-1 in chorioamnionitis

By the previously proposed sequence of inflammatory responses in histological chorioamnionitis,^{30:31} maternal neutrophils first assault the chorion and then the mesodermal layer of the chorioamnion (Figures 3A and 4B).^{6:31} In an early phase of inflammation, neutrophils in the chorion stained weakly for galectin-1, which is in accord with the data on transmigrated neutrophils in a rat model of experimental peritonitis that contained decreased amounts of galectin-1 compared to non-migrated or intravascular cells in the early stages of the inflammatory response.^{26:58} In parallel, chorioamniotic fibroblasts/myofibroblasts and macrophages stained moderately for galectin-1. In a later phase of inflammation, maternal neutrophils increased their galectin-1 content and spread into the chorioamniotic mesodermal layer. Chorioamniotic mesodermal cells that were in close proximity to these neutrophils also exhibited increased galectin-1 staining (Figures 3B and 4C).

Similarly, there was an augmented galectin-1 expression in transmigrating neutrophils and activated macrophages within 24–48 hours of the induction of inflammation in a rat model of peritonitis,²⁶ where galectin-1 regulated the inflammatory response and neutrophil

turnover by inducing phosphatidylserine surface flip on activated neutrophils,²⁶ promoting their recognition and phagocytotic removal by macrophages *in vitro*⁵⁹ and *in vivo*.²⁶ In fact, a subset of macrophages has anti-inflammatory properties characterized by high IL-10 production,⁶⁰ preferentially binds and internalizes apoptotic cells,⁶⁰ and controls acute inflammation.⁶¹

The findings presented herein concur with these previous reports, as in acute chorioamnionitis, maternal neutrophils that underwent apoptosis and fetal chorioamniotic macrophages that possibly engulfed these neutrophils were strongly positive for galectin-1. Of note, there is an increased proportion of macrophages amongst chorioamniotic mesodermal cells upon inflammation due to a phenotypic switch that was suggested to be an effective mechanisms of uterine mucosal immune responses.⁶² Amnion epithelial cells also increased their galectin-1 content upon maternal neutrophil infiltration, suggesting an active role for this epithelial layer in the regulation of inflammation in the chorioamniotic membranes. Importantly, maternal neutrophils were trapped in the connective tissue, similar to previous *in vitro* results with the amniotic membrane^{49:50} (Figures 3C and 4D). Based on these findings, we propose that the role of galectin-1 in the inflamed or infected chorioamniotic membrane is similar to that demonstrated in the rat model of peritonitis.²⁶

The immune responses to infections and transplantation grafts were proposed to be analogous⁶³ and also involve mechanisms that may help to avoid the damage of the grafts or infected tissues. The “Danger model” of immunity suggested that the immune system is more concerned with tissue damage than with foreignness, and it is activated by alarm signals from injured tissues rather than by the recognition of non-self antigens.⁶⁴ This model also implied that injured tissues may control the activation and resolution of the immune responses,⁶⁴ and the local interactions between feto-placental tissues and maternal immune cells also came into the focus of interest in the past few years.⁶⁵ Based on our results, we propose that galectin-1, a “cell stress sensor”, may also participate in retaining tissue integrity and participate in the local inflammatory processes in human chorioamniotic membranes.

Conclusions

We report for the first time the localization of galectin-1 mRNA and protein in the chorioamniotic membranes and demonstrate their abundant expression in the third trimester. This study has revealed that histologic chorioamnionitis is associated with an increased galectin-1 mRNA expression and strong immunoreactivity of the chorioamniotic membranes in patients with PPROM. We propose that galectin-1 may be involved in the fetal anti-inflammatory responses in human chorioamniotic membranes.

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Figure 1. Localization of galectin-1 mRNA and protein in normal, term chorioamniotic membranes

(A) Galectin-1 mRNA was detectable in amnion epithelial cells, chorioamniotic mesodermal cells, chorionic trophoblasts, and decidual stromal cells (*In situ* hybridization, 200-magnification). **Inset:** amniotic epithelium, chorioamniotic mesodermal cells and chorionic trophoblasts (400-magnification). (B) The cytoplasm of amnion epithelial cells was consistently, while the nuclei were variably immunopositive for galectin-1. Chorionic trophoblasts were cytokeratin-7+ (blue), and exhibited steady cytoplasmic and inconsistent nuclear galectin-1 immunostaining. The cytoplasm of chorioamniotic mesodermal cells and decidual stromal cells were moderately galectin-1 positive (cytokeratin-7/galectin-1 double

stain, hematoxylin counterstain; 200· magnification). **Inset:** apical membrane staining of amnion epithelial cells (400· magnification). Ae: amnion epithelium; me: chorioamniotic mesoderm; ct: chorionic trophoblast; de: decidua.

Figure 2. Galectin-1 expression and immunostaining in the chorioamniotic membranes of PPROM patients

(A) *PPROM without chorioamnionitis*: the cytoplasm of amnion epithelial cells was consistently immunopositive, while the nuclei were variably immunopositive for galectin-1. Chorionic trophoblasts exhibited moderate cytoplasmic and variable nuclear immunostaining. Chorioamniotic mesodermal cells and decidua stromal cells had moderate galectin-1 immunostaining. (B) *PPROM with acute chorioamnionitis*: amnion epithelial cells and chorioamniotic mesodermal cells expressed increased amounts of galectin-1. Hematoxylin counterstain; 200× magnifications. Ae: amnion epithelium; me: chorioamniotic mesoderm; ct: chorionic trophoblast; de: decidua. (C) Galectin-1 mRNA expression was

significantly higher in chorioamniotic membranes of PPRM patients with chorioamnionitis than in those without (fold-change: 2.07).

Figure 3. Galectin-1 immunostaining changes with the sequence of inflammatory responses in chorioamnionitis

(A) *Acute chorionitis*: invading maternal neutrophils in the chorion were weakly galectin-1 positive. Chorioamniotic fibroblasts/myofibroblasts and macrophages stained strongly for galectin-1. **Inset**: neutrophils aggregated at the border of chorion and chorioamniotic mesodermal layer. (B) *Acute chorioamnionitis*: maternal neutrophils in the chorion and chorioamniotic mesodermal layer were moderately immunopositive. Chorioamniotic mesodermal cells and amnion epithelial cells had increased galectin-1 content. **Inset**: neutrophils in the chorion and mesodermal layer. (C) *Acute chorioamnionitis, advanced phase*: maternal neutrophils trapped in the mesodermal layer, amnion epithelial cells, chorioamniotic mesodermal cells, and apoptotic bodies stained strongly for galectin-1. **Inset**: neutrophils and apoptotic bodies. (D) *Necrotizing chorioamnionitis*: Amnion epithelium underwent necrosis. Galectin-1 positive neutrophils, chorioamniotic mesodermal cells, apoptotic bodies and cell debris were seen in the mesodermal layer. **Inset**: HLA-DR+ (red)/galectin-1+ fetal macrophages. **Figures A–D**: Galectin-1 stain, hematoxylin counterstain; 200· magnifications. **Insets A–C**: hematoxylin-eosin stain. **Inset D**: galectin-1/ HLA-DR double stain, hematoxylin counterstain. 400· magnification. Ae: amnion epithelium; me: chorioamniotic mesoderm; ct: chorionic trophoblast; de: decidua. Neutrophils are shown with arrows, chorioamniotic mesodermal cells with arrowheads and apoptotic bodies with open triangles.

Figure 4. A model of the role of galectin-1 in chorioamnionitis

(A) Chorioamnionic membranes without inflammation. (B) Maternal neutrophils spread into the chorion, where they show marginating pattern. Amnion epithelial cells, chorioamnionic fibroblasts/myofibroblasts and macrophages increase their galectin-1 production. (C) Maternal neutrophils spread into the chorioamnionic mesoderm and express increased amounts of galectin-1. Chorioamnionic mesodermal cells further increase their galectin-1 production. Galectin-1 possibly contributes to neutrophil turnover by inducing phosphatidylserine surface flipping on activated neutrophils and by promoting their recognition and phagocytotic removal by macrophages. (D) Maternal neutrophils are trapped in the chorioamnionic mesoderm. Those neutrophils that underwent apoptosis or karyorrhexis, and fetal macrophages that possibly had engulfed neutrophils are strongly galectin-1 positive.

Table I

Antibodies and detection systems used for immunohistochemical double stainings				
Double staining	Primary antibody (dilution) Distributor	Secondary antibody (dilution) Distributor	Detection systems Distributor	
galectin-1 cytokeratin-7	Goat polyclonal anti-human galectin-1 (1:100) R&D Systems, Minneapolis, MN	Horse polyclonal anti-goat (1:400) Vector Laboratories, Burlingame, CA	DAB MAP HRP Kit Ventana Medical System, Tucson, AZ	
	Mouse monoclonal anti-human cytokeratin-7 (1:200) Dako, Carpinteria, CA	Horse anti-mouse (1:500) Ventana Medical System, Tucson, AZ	Blue MAP AP Kit Ventana Medical System, Tucson, AZ	
galectin-1 HLA-DR	Goat polyclonal anti-human galectin-1 (1:100) R&D Systems, Minneapolis, MN	Biotinylated rabbit anti-goat (1:400) Jackson ImmunoResearch Lab., West Grove, PA	Omni MAP anti-rabbit HRP Ventana Medical System, Tucson, AZ	
	Mouse monoclonal anti-human HLA-DR (1:100) Dako, Carpinteria, CA	-	Ultra MAP anti-mouse AP Ventana Medical System, Tucson, AZ	

Table II

Demographic and clinical characteristics of the study population

	Term not in labor (n=5)	PPROM with histological chorioamnionitis (n=8)	PPROM without histological chorioamnionitis (n=8)	p value
Maternal age (years)	27.8 (3.5)	27.4 (6.0)	26.3 (6.1)	NS
Gestational age at delivery (weeks)	38.2 (0.8)	32.1 (4.2)	33.1 (3.0)	0.004
Gravidity	3.4 (2.2)	4.5 (2.5) *	2.25 (1.2) *	NS
Parity	2.0 (2.0)	2.0 (1.8)	0.9 (1.2)	NS
Maternal body weight (kg)	95.2 (22.2)	90.4 (30.5)	93 (32.7)	NS
Body mass index (kg/m ²)	36.0 (9.3)	35.9 (12.8)	32.9 (9.3)	NS
Birth-weight (g)	3471.0 (418.7)	1859.0 (579.6)	2050.1 (616.5)	0.003
Cesarean section	5	4	6	NS

Values are presented as mean ($\pm$ SD) or number.

* Significant difference between two groups.

NS: statistically not significant.

Table III

Regulatory effects of galectin-1 on immune cells 22, 23, 28

Adaptive immune response	
B cells	Initiates pre-BCR signaling
T cells	<p>Inhibits cell growth and cell cycle arrest</p> <p>Inhibits migration across the endothelium</p> <p>Inhibits adhesion to ECM</p> <p>Triggers apoptosis of immature thymocytes and activated T cells</p> <p>Skews the cytokine balance toward a Th2 profile (decreases TNF-α, IL-2, IL-12, IFN-γ and increases IL-10 secretion)</p> <p>Suppresses T cell-mediated autoimmune diseases (encephalomyelitis, Concavalin-A induced hepatitis, collagen induced arthritis, experimental colitis, nephritis and retinal disease)</p> <p>Reduces host alloreactivity and ameliorates graft versus host disease</p> <p>Galectin-1 is a key effector molecule of activated CD4⁺ CD25⁺ regulatory T cells</p>
Innate immune response	
Basophils	Inhibits degranulation
Dendritic cells	Promotes the generation of tolerogenic dendritic cells
Eosinophils	Inhibits migration
Mast cells	Inhibits degranulation
Monocytes	Inhibits LPS induced arachidonic acid release
Macrophages	<p>Decreases iNOS expression and inhibits LPS induced NO metabolism</p> <p>Increases arginase activity</p> <p>Inhibits MHC-II expression and MHC-II dependent antigen presentation</p> <p>Regulates constitutive and inducible expression of high affinity FcγRI (CD64)</p> <p>Regulates FcγRI dependent phagocytosis</p> <p>Decreases IL-12 secretion</p>
Neutrophils	<p>Inhibits chemotaxis, transendothelial and transperitoneal migration</p> <p>Induces phosphatidylserine exposure in activated but not in resting state</p> <p>Activates respiratory burst (NADPH-oxidase activity and degranulation) in primed, extravasated cells</p>
NK cells	Galectin-1 is up-regulated in uterine NK cell subsets