### ROLES OF GALECTINS AND GLYCANS IN BOVINE CORPUS LUTEUM

**March 2017** 

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### PREFACE

The experiments described in this dissertation were carried out at the Graduate School of Environmental and Life Science (Doctor's course), Okayama University, Japan, from April 2014 to March 2017, under the supervision of Professor Kiyoshi OKUDA (April, 2014-December, 2015) and Professor Koji KIMURA (January, 2016-March, 2017).

This dissertation has not been submitted previously in whole or in part to a council, university or any other professional institution for a degree, diploma or other professional qualifications.

Kazuhisa HASHIBA March, 2017

### ACKNOWLEDGMENTS

I wish to express my deep gratitude to Kiyoshi OKUDA, DVM, Ph.D., Professor emeritus of Okayama University, Okayama, Japan and President of Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan, and Koji KIMURA, Ph.D., Professor of Graduate School of Environmental and Life Science, Okayama University for their encouragement, guidance, constructive criticisms and excellent supervision. I also wish to be grateful to Masahiro SANO, Ph.D., Research staff of Hirosaki Industrial Research Institute, Aomori Prefectural Industrial Technology Research Center, Aomori Prefectural Government and Junko NIO-KOBAYASHI, DVM, Ph.D., Assistant Professor of Hokkaido University, Yoshinobu KIMURA, Ph.D., Professor of Okayama University, Megumi MAEDA, Ph.D., Assistant Professor of Okayama University, Yuki YAMAMOTO, DVM, Ph.D., Assistant Professor of Okayama University their supports, advices, encouragement to conduct the present study. It is a pleasure to express my thanks to all the members of Laboratory of Animal Reproductive Physiology for helping me during carrying out this study.

### ABSTRACT

In mammals, the corpus luteum (CL) is an essential endocrine gland for the establishment and maintenance of pregnancy. If pregnancy is not established, the CL regresses and disappears rapidly from the ovary (luteolysis). Luteolysis is initiated by the uterine prostaglandin F2 $\alpha$  (PGF) in ruminants, and luteolysis is characterized by a reduction in progesterone (P4) production (functional luteolysis) followed by luteal cell death (structural luteolysis). The major event that causes structural luteolysis is luteal cell death (apoptosis). Apoptosis is a highly conserved mechanism that allows an organism to tightly control cell numbers and tissue size. Luteal regression is triggered by complex factors. Many cytokines, including tumor necrosis factor  $\alpha$  and interferon  $\gamma$  secreted by immune cells, and nitric oxide produced by luteal endothelial cells directly induce CL cells to undergo apoptosis. These reports indicate that many molecules are involved in structural luteolysis. However, the indirect effects of the above luteolytic factors on luteolysis are not well understood.

Glycosylation modulates a variety of physicochemical and biological properties of proteins such as protein folding, stability, targeting, and ligand binding. Most membrane proteins including cell surface receptors are glycosylated, and glycosylation status is important for their function. However, the role of glycosylation in the CL is not clear.

Galectins are carbohydrate-binding proteins distributed intra- and extracellularly. Their carbohydrate-binding specificity for  $\beta$ -galactoside is conserved by evolutionarily preserved carbohydrate-recognition domains. Extracellular galectins interact with cell surface oligosaccharides and form lattices that enhance the resident time of glycoproteins at the cell surface.

Galectin-3, a ubiquitously expressed protein involved in many cellular processes, serves as an anti-apoptotic and/or pro-apoptotic factor in various cell types. Although galectin-3 is detected in the bovine CL, its role remains unclear. The expression of galectin-3 in the bovine CL was highest at the regressed stage, and galectin-3 was localized on luteal steroidogenic cells (LSCs). When cultured LSCs were exposed to PGF for 48 h, the expression and secretion of galectin-3 increased. When the cultured LSCs were treated with galectin-3 for 24 h, cleaved caspase-3 expression was increased and the cell viability was decreased, whereas P4 production did not change.  $\beta$ 1 integrin, a target protein of galectin-3, was expressed in bovine CL and possessed glycans which galectin-3 binds. Furthermore, galectin-3 bound to glycans of luteal  $\beta$ 1 integrin. The decreased cell viability of cultured LSCs by galectin-3 was suppressed by  $\beta$ 1 integrin

antibody. The overall findings suggest that the secreted galectin-3 stimulated by PGF plays a role in structural luteolysis by binding to  $\beta$ 1 integrin.

Galectin-1 is expressed in the functional CL of cows, and increases the viability of bovine LSCs by modifying the functions of membrane glycoproteins. The binding of galectin-1 to glycoproteins is blocked by  $\alpha 2,6$ -sialylation of the terminal galactose residues of glycoconjugates, which is catalyzed by a sialyltransferase (ST6Gal-I). However, the physiological role of  $\alpha 2$ ,6-sialic acid in the bovine CL is unclear. The level of  $\alpha 2,6$ -sialylation of the bovine CL were higher during the regressed-luteal stage than other luteal stages. Lectin histochemistry revealed that  $\alpha 2,6$ -sialylated glycoconjugates were localized to luteal endothelial cells throughout the estrous cycle. In addition,  $\alpha 2,6$ -sialylated glycoconjugates concentrated to the membrane of LSCs during the regressed-luteal stage. PGF treatment for 72 h enhanced the expression of ST6Gal-I mRNA and the level of  $\alpha 2,6$ -sialylated glycoproteins in mid-LSCs. The level of a2,6-sialylated glycoproteins of late-LSCs (Days 15-17 after ovulation) was higher than that of mid-LSCs (Days 8-12 after ovulation), and galectin-1 increased the viability of mid-LSCs but not that of late-LSCs. Furthermore, galectin-1 increased the viability of late-LSCs when  $\alpha 2,6$ -sialic acid residues were removed by neuraminidase. The overall findings suggest that  $\alpha 2.6$ -sialylation stimulated by PGF contributes to luteolysis by inhibiting the luteotrophic effects of galectin-1 in the bovine CL.

My study shows that the interaction between galectins and glycan play a critical role in the regression of bovine CL. Galectin-3 increased by PGF induced the apoptosis of bovine LSCs, and  $\alpha$ 2,6-sialylated glycoproteins inductively expressed by stimulation with PGF2 $\alpha$  inhibits the interaction between galectin-1 and the *N*-glycoproteins on the cell surfaces, contribute to the regulation of luteolysis in co

### CHAPTER 1 Galectin-3 contributes to luteolysis by binding to β1 integrin in the bovine corpus luteum

### **INTRODUCTION**

The corpus luteum (CL) is a transient endocrine gland that is essential for the regulation of ovarian cycles as well as for the establishment of pregnancy. If pregnancy does not occur, regression of the CL (luteolysis) is initiated by the uterine prostaglandin  $F2\alpha$  (PGF) in ruminants [1]. Luteolysis is characterized by a reduction in progesterone (P4) production (functional luteolysis) followed by luteal cell death (structural luteolysis) [2]. The major event that causes structural luteolysis is luteal cell death (apoptosis) [3-5]. Apoptosis is a highly conserved mechanism that allows an organism to tightly control cell numbers and tissue size [6]. Luteal regression is triggered by complex factors. Since progesterone is one of the survival factors in the bovine corpus luteum [7], functional luteolysis could be a cause of structural luteolysis. Many cytokines, including tumor necrosis factor  $\alpha$  (TNF) and interferon  $\gamma$  (IFNG) secreted by immune cells, and nitric oxide produced by luteal endothelial cells directly induce CL cells to undergo apoptosis [8-10]. Nitric oxide is also known to play a crucial role in functional luteolysis [11]. These reports indicate that many molecules are involved in structural luteolysis. However, the indirect effects of the above luteolytic factors on luteolysis are not well understood.

Galectin-3 is one of the  $\beta$ -galactoside-binding proteins that bind to target molecules through conserved carbohydrate recognition domains [12]. In various cell types, galectin-3 is expressed in the nucleus or cytoplasm, on the cell membrane, and in the extracellular matrix [13]. Galectin-3 is involved in many cellular processes such as apoptosis and proliferation [14-16]. Intracellular galectin-3 generally mediates anti-apoptotic effects through carbohydrate-independent processes [17-20], whereas extracellular galectin-3 binds to cell surface oligosaccharides, and thereby induces apoptosis [14, 21, 22].

Integrins are cell surface glycoproteins that mediate adhesion to the extracellular matrix, and integrin signaling regulates cell proliferation, differentiation and apoptosis [23]. In mammals, they are composed of 18  $\alpha$ -subunits and 8  $\beta$ -subunits

[24]. Among these integrins, 12 members containing the  $\beta$ 1 subunit are involved in various biological functions [25].  $\beta$ 1 integrin is a glycan to which galectin-3 binds [26] and extracellular galectin-3 induces apoptosis by binding to  $\beta$ 1 integrin in T cells and tumor cells [14, 22]. Therefore, interaction between galectin-3 and  $\beta$ 1 integrin might also play a role in inducing apoptosis of luteal cells.

In cow, galectin-3 has been identified in the uterus, cervix, oviduct, atretic follicles and CL [27]. Galectin-3 protein localization has been immunohistochemically detected in the regressing CL [27], but its physiological role is still unclear. Based on these reports, we hypothesized that galectin-3 is involved in the apoptosis of luteal cells during luteolysis. In the present study, to test this hypothesis, we examined 1) cyclic changes in the galectin-3 mRNA and protein expressions, 2) effect of luteolytic factors on expression of galectin-3 mRNA and protein in cultured mid luteal steroidogenic cells (LSCs), 3) subcellular localization of galectin-3 in the regressed CL and effect of PGF on the secretion of galectin-3 in LSCs, 4) effect of galectin-3 on P4 production and cell viability in LSCs, 5) expression and glycan structure of  $\beta$ 1 integrin in bovine CL throughout the estrous cycle, 6) interaction between  $\beta$ 1 integrin and galectin-3 in LSCs.

### **MATERIALS AND METHODS**

### **Collection of bovine CLs**

Ovaries were collected from Holstein cows at a local slaughter-house within 10–20 min after exsanguinations. The stage of the estrous cycle was defined as described previously [28]. Ovaries with CLs were classified into the early (Days 2-3 after ovulation), developing (Days 5-6), mid (Days 8-12), late (Days 15-17), and regressed (Days 19-21) luteal stages. After determination of the stages, CL tissues were immediately separated from the ovaries, frozen rapidly in liquid nitrogen, and then stored at  $-80^{\circ}$ C until processed for mRNA and protein analyses. For cell culture, ovaries with CLs were submerged in ice-cold physiological saline and transported to the laboratory.

#### Luteal cell isolation

Luteal tissue from mid- and late-luteal stage CLs was enzymatically dissociated, and luteal cells were cultured as described previously [29]. Dissociated luteal cells from CLs were pooled. The luteal cells were suspended in a culture medium, Dulbecco modified Eagle medium and Ham F-12 medium (D/F; 1:1 [vol/vol]; D8900; Sigma-Aldrich Corp., St. Louis, MO, USA) containing 5% bovine serum (16170–078; Thermo Fisher Scientific, MA, U.S.A.) and 20  $\mu$ g/ml gentamicin (15750–060; Thermo Fisher Scientific, MA, U.S.A.) under 5% CO<sub>2</sub> in air. Cell viability was greater than 85% as assessed by trypan blue exclusion. The cells consisted of approximately 70% small LSCs, 20% large LSCs, 10% endothelial cells or fibrocytes, and no erythrocytes. In this study, these cells were defined as LSCs.

### **Cell culture**

Dispersed luteal cells  $(2.0 \times 10^5 \text{ cells/ml})$  were cultured in D/F medium containing 5% bovine serum in 24-well plates  $(2.0 \times 10^5 \text{ cells/well}, 662160;$  Greiner Bio-One, Frickenhausen, Germany) for Quantitative RT-PCR, or 75-cm<sup>2</sup> culture flasks  $(12 \times 10^5 \text{ cells/flask}, 658175;$  Greiner Bio-One, Frickenhausen, Germany) for western blotting, or 96-well culture dishes  $(0.2 \times 10^5 \text{ cells/well}, 3860-096;$  Iwaki, Chiba, Japan) for cell viability test and EIA. The medium was replaced with D/F medium without phenol red (D2906; Sigma-Aldrich Corp., St. Louis, MO, USA) containing 0.1% bovine serum albumin (BSA; 23209; Thermo Fisher Scientific, MA, U.S.A.), 5 ng/ml sodium selenite (S5261; Sigma-Aldrich Corp., St. Louis, MO, USA), and 5  $\mu$ g/ml holo-transferrin (T3400; Sigma-Aldrich Corp., St. Louis, MO, USA), and the following experiments were carried out.

### **Quantitative RT-PCR**

Total RNA was extracted from CL tissues and cells using TRIsure (BIO-38032; BIOLINE) according to the manufacturer's directions. One µg of each total RNA was reverse transcribed using a ThermoScriptTM RT-PCR System (no. 11146-016; Invitrogen, Carlsbad, CA, USA), and 10% of the reaction mixture was used in each PCR using specific primers for galectin-3 from the bovine sequence (Table 1). Quantification of mRNA expression was determined by iQ SYBR Green Supermix (no. 170-8880; Bio-Rad Laboratories, Inc, Berkeley, CA, USA) starting with 2 ng of reverse-transcribed total RNA. For quantification of the mRNA expression levels, and PCR was performed under the following conditions: 95°C for 15 min, followed by 45 cycles of 94°C for 15 sec, 60°C for 20 sec and 72°C for 15 sec. MRPL4 mRNA expression was used as an internal control. To analyze the relative level of expression of each mRNA, the 2-  $\Delta\Delta$ CT method was used [34]. In the present study, we compared four housekeeping genes in the CL by real-time PCR. The four genes evaluated were mitochondrial ribosomal protein L4 (MRPL4), cytochrome c oxidase subunit IV (COX4), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tubulin beta 2 (TUBB) (Table 1). To determine the most stable transcribed housekeeping gene, Normfinder software (download at http://moma.dk/normfinder-software) was used. This software calculates the gene expression stability measure (M) and determines the most stable housekeeping gene via a stepwise exclusion or ranking process resulting in the selection of the most stable housekeeping genes for the specific tissue. Since MRPL4 was the most suitable, with an average expression stability of M = 0.171, followed by COX4 (M =0.261) > GAPDH (M = 0.762) > TUBB (M = 0.280), MRPL4 was used as a housekeeping gene for further experiments.

#### Western blotting

Protein concentrations in the lysates were determined by using BSA as a standard. The proteins were then solubilized in SDS gel-loading buffer (50 mM Tris-HCl, 2% SDS [31607-94, Nacalai Tesque, Tokyo, Japan], 10% glycerol, 1%  $\beta$ -mercaptoethanol [137-06862; Wako Pure Chemical Industries, Ltd.], pH 6.8), and

heated at 95°C for 10 min. Samples (30 µg protein/lane) were separated on SDS-PAGE, and then transferred to a PVDF membrane (RPN303F; GE Healthcare., Little Chalfont, Buckinghamshire, UK). The membrane was washed in TBS-T (0.1% Tween 20 in TBS [25 mM Tris-HCl, 137 mM NaCl, pH 7.5]), incubated in blocking buffer (5% nonfat dry milk in TBS-T) for 1 h at room temperature. After washing, the membranes were incubated with galectin-3 antibody diluted at 1:1000 (sc-20157; Santa Cruz Biotechnology Inc., CA, USA), cleaved caspase-3 antibody (#9661; Cell Signaling Technology Inc., Beverly, MA, USA) diluted at 1:1000, tumor necrosis factor receptor 1 (TNFR1) antibody (ab19139; Abcam, Cambridge, UK) diluted at 1:1000, β1 integrin antibody (MAB2000; Millipore, Billerica, MA, USA) diluted at 1:1000 or ACTB antibody (A2228; Sigma-Aldrich Corp., St. Louis, MO, USA) diluted at 1:10000 for overnight at 4°C. After washing with TBS-T, the membrane was incubated with the appropriate secondary antibodies (anti-rabbit Ig, HRP-linked whole antibody produced by donkey [NA934; Amersham Biosciences Corp., San Francisco, CA, USA; 1:10000] for galectin-3, cleaved caspase-3 and TNFR1 protein; anti-mouse Ig, HRP-linked whole antibody produced by sheep [no. NA931; Amersham Biosciences Corp.; 1:10000 for  $\beta$ 1 integrin or 1:40000 for ACTB]) for 1 h at room temperature. After washing again with TBS-T, the signal was detected by chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate (P36599; Millipore, Billerica, MA, USA). ACTB protein expression was used as an internal control. The intensity of the immunological reaction in the tissues was estimated by measuring the optical density in the defined area by computerized densitometry using Image LabTM Software version 4.0 (Bio-Rad Laboratories, Inc, Berkeley, CA, USA).

#### Immunohistochemistry

The sections, at 5 µm thickness, were de-waxed and washed in phosphate-buffered saline. Subsequently the sections were incubated at room temperature with 3% hydrogen peroxide in distilled water for 20 min and Avidin/Biotin blocking solution (Vector Laboratories Inc., Burlingame, CA, USA) for 15 min for each reagent. Then the sections were incubated with normal goat serum for 60 min at room temperature followed by galectin-3 antibody (1:200) at 4°C overnight. After washing twice in PBS, the sections were incubated with biotinylated anti-rabbit IgG (1:500; Vector laboratories Inc., Burlingame, CA, USA) for 60 min at room temperature. The reaction sites were visualized using Vectastain ABC Elite kit (Vector Laboratories Inc.,

Burlingame, CA, USA) for 60 min at room temperature and ImmPACT® 3, 3'-Diaminobenzidine (DAB) Peroxidase Substrate Kit (Vector Laboratories Inc., Burlingame, CA, USA) for 5 min. The sections were counterstained for 2 min with haematoxylin and observed under a light microscope (BX51; Olympus Corporation, Tokyo, Japan).

### WST-1 assay

WST-1, a kind of MTT [3-(4,5-dimethyl-2 thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide], is a yellow tetrazolium salt that is reduced to formazan by live cells containing active mitochondria. The culture medium was replaced with 100  $\mu$ l D/F without phenol red medium-BSA, and a 10  $\mu$ l aliquot (0.3% WST-1, 0.2 mM 1-methoxy PMS in PBS, pH 7.4) was added to each well. The cells were then incubated for 4 h at 38°C. The absorbance was read at 450 nm using a microplate reader (Model 450; Bio-Rad, Hercules, CA). In this assay, data were expressed as percentages of the appropriate control values.

#### P4 determination

Concentrations of P4 were determined directly from the cell culture media with an enzyme immunoassay, as described previously [30]. The standard curve ranged from 0.391 to 100 ng/ml, and the median effective dose (ED50) of the assay was 3.5 ng/ml. The intra- and interassay coefficients of variation were 5.8% and 9.3%, respectively.

### Lectin blotting

Protein samples were subsequently separated by SDS-PAGE. Separated proteins were transferred onto PVDF membrane. The membrane was blocked with 5% BSA in TBS-T, and then reacted with 1.0  $\mu$ g/ml of HRP-conjugated L4-PHA lectin (J212; J-OIL MILLS, Tokyo, Japan) in TBS-T for 1 h. After washing again with TBS-T, the reactive protein bands were detected by chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate.

### Galectin-3 overlay assay

Cell lysates (2.0 mg of protein) were immunoprecipitated with  $\beta$ 1 integrin antibody and protein-G-beads, as described above. Precipitated proteins were separated by SDS-PAGE and subsequently transferred to a PVDF membrane. The membrane was blocked with 5% BSA in TBS-T, and then exposed to biotin-conjugated galectin-3. Biotinylated label of glectin-3 was performed using EasyLink Biotin Conjugation Kit (ab102865; Abcam), according to the manufacture's instruction. After washing with TBS-T, the membrane was incubated with streptavidin conjugated to HRP. After washing again with TBS-T, the reactive protein bands were detected by chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate.

#### Experiment 1: Cyclic changes in the galectin-3 mRNA and protein expressions

Galectin-3 mRNA and protein expressions of in each CL tissues (Early n=4, Developing n=4, Mid n=4, Late n=4, Regressed n=4,) were examined by quantitative RT-PCR and western blotting. Galectin-3 protein localization of in each CL tissues (Early; n=3, Mid; n=3, Regressed; n=3) was analyzed by immunohistochemistry.

### **Experiment 2: Effect of luteolytic factors on expression of galectin-3 mRNA and protein in cultured mid LSCs**

To clarify regulatory factors of galectin-3 mRNA and protein in cultured mid LSCs, the cells were exposed to 2.3 nM recombinant human TNF (Dainippon Pharmaceutical, Osaka, Japan) and/or 2.5 nM recombinant bovine IFNG (kindly donated by Dr. S. Inumaru, NIAH, Ibaraki, Japan) or 1.0  $\mu$ M PGF (P7652; Sigma-Aldrich Corp., St. Louis, MO, USA) for 12, 24, 48 and 72 h. The doses for treatments were selected based on previous reports [30, 31]. After the culture, the expression of galectin-3 mRNA was determined by quantitative RT-PCR. The galectin-3 protein expression was assessed by western blotting in cells treated with PGF for 48 h.

### **Experiment 3: Subcellular localization of galectin-3 in the regressed CL and effect of PGF on the secretion of galectin-3 in cultured mid LSCs**

To elucidate the subcellular localization of galectin-3 in the regressed CL, the regressed CL tissue was fractionated into membrane and cytoplasm fractions. The CL tissues were homogenized on ice in the homogenization buffer by a tissue homogenizer (Physcotron, NS-50; NITI-ON Inc., Chiba, Japan), followed by a filtration with a metal wire mesh (150  $\mu$ m). For protein analysis, nuclei were removed from the tissue homogenates by centrifugation at 700 x g for 5 min. The resultant supernatant was fractionated into membrane and cytoplasmic cell fractions by centrifugation at 20,000 x

g for 1 h. The membrane and cytoplasm fractions were detected expression of galectin-3 protein by western blotting. Validation of the cell fractionation was performed by reprobing with TNFR1 antibody as a plasma membrane marker and ACTB antibody as a cytoplasm marker. Since TNFR1 is expressed in the bovine CL [32] and generally known as a membrane receptor, TNFR1 antibody was used for plasma membrane marker.

To examine whether PGF induces secretion of the galectin-3 in LSCs, after 48 h of treatment with PGF (1.0  $\mu$ M), cultured LSCs were treated with a non-competing saccharide, 0.1 M sucrose and a competing saccharide, 0.1 M  $\beta$ -lactose for 4 h. LSCs were collected and subsequently analyzed galectin-3 protein by western blotting.

# **Experiment 4: Effect of galectin-3 on P4 production and cell viability in cultured mid LSCs**

To reveal the effect of galectin-3 on P4 production and cell viability in LSCs, the cells were exposed to recombinant galectin-3 (0.01, 0.1, 1.0, 5.0  $\mu$ g/ml; R&D Systems, Inc., Minneapolis, MN, USA) for 24 h. The cell viability was determined by Dojindo Cell Counting Kit including WST-1 (No. 345-06463; Dojindo, Kumamoto, Japan) as described previously [33]. Progesterone concentrations in the media of LSCs were measured by enzyme immunoassay. Expression of cleaved caspase-3 was analyzed by western blotting in cells treated with recombinant galectin-3 (1.0  $\mu$ g/ml) for 24 h.

# Experiment 5: Expression and glycan structure of $\beta$ 1 integrin in bovine CL throughout the estrous cycle

The membrane fractions of the CLs and the LSCs were lysed in a lysis buffer consisting of 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1.0% Triton X 100, 10% Glycerol and protease inhibitor cocktail (11 697 498 001; Roche; Basel, Switzerland). The lysates (2.0 mg of protein) were incubated with  $\beta$ 1 integrin antibody and protein-G-beads (MAB2000; Millipore, Billerica, MA, USA) at 4°C for 24 h. The beads were washed with lysis buffer three times and immunoprecipitated proteins were analyzed by lectin blotting.

# Experiment 6: Interaction between $\beta 1$ integrin and galectin-3 in cultured mid LSCs

To investigate whether galectin-3 decreases cell viability via  $\beta$ 1 integrin in LSCs, after 1 h of treatment with  $\beta$ 1 integrin neutralizing antibody (MAB1965; Millipore, Billerica, MA, USA) or anti-mouse IgG diluted at 1:1000, cultured LSCs were treated with recombinant galectin-3 (1.0 µg/ml) for 24 h and the cell viability was determined by WST-1.

### Statistical analysis

All experimental data are shown as the mean  $\pm$  SEM. The statistical significance of differences in the expression of galectin-3 mRNA and protein, cleaved caspase-3 protein, P4 production and the viability of LSCs were assessed by analysis of a one-way ANOVA followed by Bonferroni's multiple comparison test. Probabilities less than 5% (P<0.05) were considered significant.

### RESULTS

### Experiment 1: Cyclic changes in the galectin-3 mRNA and protein expressions

The expressions of galectin-3 mRNA and protein in the bovine CL were higher at the regressed luteal stage than at the other luteal stages (Fig. 1A, B; P<0.05). Galectin-3 was mainly localized in LSCs and macrophage (Fig. 2).

### Experiment 2: Effect of luteolytic factors on expression of galectin-3 mRNA and protein in cultured mid LSCs

The expression of *galectin-3* mRNA was significantly increased by a treatment with PGF for 48 h (Fig. 3A; P<0.05), but not by TNF and/or IFNG, compared with control for 12, 24, 48 and 72 h. The expression of galectin-3 protein was also significantly increased by a treatment with PGF for 48 h (Fig. 3B; P<0.05).

# **Experiment 3: Subcellular localization of galectin-3 in the regressed CL and effect of PGF on the secretion of galectin-3 in cultured mid LSCs**

The expression of galectin-3 protein was mainly detected in membrane fraction. ACTB was mostly detected in the cytoplasmic fraction and TNFR1 was detected in the membrane fraction (Fig. 4A).

The expression of galectin-3 protein was significantly increased by PGF. PGF-induced galectin-3 expression was significantly decreased by  $\beta$ -lactose (Fig. 4B, P<0.05).

## **Experiment 4: Effect of galectin-3 on P4 production and cell viability in cultured mid LSCs**

Galectin-3 did not affect P4 production (Fig. 5A), whereas galectin-3 (1.0, 5.0  $\mu$ g/ml) significantly decreased the cell viability (Fig. 5B; P<0.05). Furthermore, the protein expression of cleaved caspase-3 was significantly increased by galectin-3 (1.0  $\mu$ g/ml) (Fig. 5C; P<0.05).

# Experiment 5: Expression and glycan structure of $\beta 1$ integrin in bovine CL throughout the estrous cycle

 $\beta$ 1 integrin mRNA and protein in bovine CL were expressed throughout the estrous cycle (Fig. 6A, B).  $\beta$ 1 integrin in both bovine CL and cultured mid LSCs

possessed glycans which galectin-3 binds as revealed by lectin blotting using L4-PHA (Fig. 6C).

# Experiment 6: Interaction between $\beta 1$ integrin and galectin-3 in cultured mid LSCs

Galectin-3 bound directly to  $\beta$ 1 integrin in the isolated mid luteal cells (Fig.7 A). Although cultured LSC viability was decreased by galectin-3, the viability of the LSC treated with  $\beta$ 1 neutralizing integrin antibody was not changed by galectin-3 compared with control (Fig. 7 B P<0.05).



**Figure 1.** Changes in the relative amounts of galectin-3 expressions. (A) Comparison of relative amounts of galectin-3 mRNA determined by quantitative RT-PCR in bovine CL tissue throughout the estrous cycle (Early, Days 2-3; Developing, Days 5-6; Mid, Days 8-12; Late, Days 15-17; Regressed luteal stages [Regress], Days 19-21). Data are the mean  $\pm$  SEM for four samples/stage and are expressed as the relative ratio of galectin-3 mRNA to MRPL4 mRNA. (B) Representative western blot bands for galectin-3 and ACTB. Densitometrically analyzed western blot results in the bovine CL tissue during different luteal phases. Data are the mean  $\pm$  SEM for four samples/stage and are the mean  $\pm$  SEM for four samples/stage and are the mean  $\pm$  SEM for four samples/stage and are the mean  $\pm$  SEM for four samples/stage and are four samples/stage and are the bovine CL tissue during different luteal phases. Data are the mean  $\pm$  SEM for four samples/stage and are expressed as the relative ratio of galectin-3 protein to ACTB protein. Different superscript letters indicate significant difference (P<0.05), as determined by ANOVA followed by Bonferroni's multiple comparison test.



Figure 2. Representative images of the localization of galectin-3 protein in bovine CL throughout the estrous cycle. Black arrows indicate macrophages. Scale bars represent  $50 \mu m$ .



**Figure 3.** Effects of luteolytic factors on the expression of galectin-3 mRNA and protein in LSCs. (A) LSCs were treated with PGF (1.0  $\mu$ M) or TNF (2.3 nM) and/or IFNG (2.5 nM) for 12, 24, 48 and 72 h. Data are expressed as the relative ratio of galectin-3 mRNA to MRPL4 mRNA levels. (B) Representative western blot bands for galectin-3 and ACTB in the cells treated with PGF (1.0  $\mu$ M) for 48 h. The resultant signal was detected by chemiluminescence and quantitated by computer-assisted densitometry. Data are expressed as the relative ratio of galectin-3 protein to ACTB protein. All values are the means ± SEM. All of the experiments were repeated more than three times. The data were statistically analyzed using ANOVA followed by Bonferroni's multiple comparison test. Asterisks indicate significant differences compared with control for 48 h (P<0.05).



A

В





**Figure 4.** (A) Subcellular localization of galectin-3 in the regressed CL. Regressed CL was fractionated into the membrane and cytoplasm fractions. Galectin-3 is shown in the upper lane. Staining for ACTB and TNFR1 was used to assess the purity of the cytoplasm and membrane fractions, respectively. (B) Effect of PGF on the secretion of galectin-3 in cultured mid LSCs. Cultured mid LSCs were treated with 0.1 M sucrose and 0.1 M  $\beta$ -lactose (Lactose) for 4 h after treated PGF (1.0  $\mu$ M) for 48 h. The galectin-3 protein expression was assessed by western blotting analysis. Representative western blot bands for galectin-3 and ACTB are shown. All values are the means  $\pm$  SEM. All of the experiments were repeated more than three times and different superscript letters indicate significant difference (P<0.05), as determined by ANOVA followed by Bonferroni's multiple comparison test.



**Figure 5.** Effects of galectin-3 on P4 production (A) and cell viability (B) in LSCs. The cells were treated with recombinant galectin-3 (0, 0.01, 0.1, 1.0, 5.0 µg/ml) for 24 h. (C) Representative western blot bands for cleaved caspase-3 and ACTB in the cells treated with recombinant galectin-3 (1.0 µg/ml) for 24 h. The resultant signal was detected by chemiluminescence and quantitated by computer-assisted densitometry. Protein data are expressed as the relative ratio of cleaved caspase-3 protein to ACTB protein. All values are the means  $\pm$  SEM. All of the experiments were repeated more than three times. The data were statistically analyzed using ANOVA followed by Bonferroni's multiple comparison test (\* P<0.05).



**Figure 6.** Changes in the relative amounts and glycan structure of  $\beta 1$  integrin. (A) Comparison of relative amounts of  $\beta 1$  integrin mRNA determined by quantitative RT-PCR in bovine CL tissue throughout the estrous cycle. Data are the mean  $\pm$  SEM for four samples/stage and are expressed as the relative ratio of  $\beta 1$  integrin mRNA to MRPL4 mRNA. (B) Representative western blot bands for  $\beta 1$  integrin and ACTB. Densitometrically analyzed western blot results in the bovine CL tissue during different luteal phases. (C) L4-PHA lectin blot analysis of glycans attached to  $\beta 1$  integrin. Purified  $\beta 1$  integrin proteins were subjected to blotting analysis probed either  $\beta 1$  integrin antibody or plant lectin (L4-PHA for detecting oligomers of D-GalNac). Data are the mean  $\pm$  SEM for four samples/stage and are expressed as the relative ratio of galectin-3 protein to ACTB protein.



**Figure 7.** Interaction between  $\beta$ 1 integrin and galectin-3 in cultured mid LSCs. (A) Galectin-3 overlay assay of glycans attached to  $\beta$ 1 integrin. Immunoprecipitated  $\beta$ 1 integrin proteins were subjected to blotting analysis probed either  $\beta$ 1 integrin antibody or Biotinylated galectin-3. As shown, biotin-conjugated galection-3 bound to  $\beta$ 1 integrin from the mid LSCs. (B) Cultured mid LSCs were treated with recombinant galectin-3 (1.0 µg/ml) for 24 h after treated with  $\beta$ 1 integrin function blocking antibody or mouse IgG (diluted at 1:1000) for 1 h. All values are the means ± SEM. All of the experiments were repeated more than three times, and different superscript letters indicate significant difference (P<0.05), as determined by ANOVA followed by Bonferroni's multiple comparison test.

Gene	Forward and reverse primers (5'-3')	Accession no.	Product (bp)
LCAIS 2	F:TCGCATGCTGATAACAATCC	DC149126	106
LGALS-3	R:GAAGCGTGGGTTAAAGTGGA	BC148130	
ITCDI	F:GCAGTTTGTGGATCCCTGAT	DC114107	109
IIGBI	R:AAACGATGCCACCAAGTTTC	BC114107	
	F:GGCTCAAGACCTTCAACCTG	BC108102	138
MKPL4	R:GCGTGTAACGTGAGTCATGC	BC108102	
COVA	F: AAGGAGAAGGCTTCCTGGAG	P.C102722	118
<i>COA4</i>	F: CCACCGTCTTCCACTCATTT	BC102755	
CADDU	F: CACCCTCAAGATTGTCAGCA	DC102590	103
GAPDH	R: GGTCA TAAGTCCCTCCA CGA	BC102589	
TUDD	F: GAGGCCACCGGTAACAAGTA	PC105401	129
IUDD	F: ACTCTGGCCAAACACGAAGT	BC105401	

Table 1. Primers for Quantitative RT-PCR

#### DISCUSSION

Galectin-3 is a ubiquitously expressed protein that is involved in cell survival and death in many cell types [35]. In the mice, since regressing CL consisted of galectin-3-positive luteal cells, galectin-3 is suggested to be involved in luteal regression [36]. Although galectin-3 has been detected immunohistochemically in the bovine CL throughout the estrous cycle [27], its regulatory factor, target protein and roles in LSCs are unclear. Galectin-3 is present in the cell nucleus or cytoplasm, on the cell surface, and also in the extracellular environment [15]. Galectin-3 has been demonstrated to play the conflicting roles in cell viability, i.e. cell survival and apoptosis [15]. The two different actions of galectin-3 on cell viability depend on its subcellular localization [13]. Intracellular galectin-3 generally protects cells against apoptosis through carbohydrate-independent mechanisms, whereas extracellular galectin-3 binds to cell surface oligosaccharides and induces apoptosis [14, 21, 22, 37]. The major event that causes the structural luteolysis is death of LSCs by apoptosis [5]. In the present study, the expression of galectin-3 in the bovine CL was higher at the regressed luteal stage than at the other luteal stages, and that its protein was localized in LSCs and macrophages (Fig. 1, 2). As galectin-3 is commonly used as a macrophage maker [38], galectin-3 positive cells besides LSCs were judged as macrophages. Since the bone marrow-derived macrophages of galectin-3-deficient mouse exhibited defective phagocytosis of apoptotic cells, galectin-3 in macrophages is thought to play important roles of phagocytosis [39]. Moreover, luteolysis involves phagocytic elimination of apoptotic luteal cells by luteal macrophages in rat [40]. Based on the above findings, galectin-3 may also be required for phagocytosis by macrophages in the regressing CL in cattle.

The localization of galectin-3 on the membrane might not completely be supported, because a faint band of  $\beta$ -actin was detected in plasma membrane fraction (Fig. 4A). Therefore, I further investigated whether galectin-3 was localized on LSCs stimulated by PGF (a mimic luteal regression) (Fig 4B). Galectin-3 binds to N-acetyllactosamine (LacNAc) structure in glycans, and lactose is a saccharide which has structure similar to LacNAc and inhibits the binding to galectin-3 [13]. On the other hand, since sucrose is a saccharide which does not bind to galectin-3, sucrose is commonly used as a negative control of lactose [22]. In the present study, the expression of galectin-3 protein stimulated by PGF was decreased by lactose but not sucrose (Fig 4B), and the western blot band of membrane fraction was obviously stronger than that of cytosol fraction (Fig. 4A). The above findings strongly suggest that galectin-3 is mainly localized on the plasma membrane in the regressed CL.

In various cells types, galectin expression is induced by cytokines as TNF, IFNG and interleukin 1  $\beta$  [41-44]. In luteolysis, TNF and IFNG produced by immune cells play an important role [5]. Not only cytokines (TNF and IFNG) but also PGF are known as important luteolytic factors [5, 33]. Since galectin-3 expression was higher at the regressed luteal stage (Fig. 1, 2), I hypothesized that TNF, IFNG or PGF affect the expression and secretion of galectin-3 in LSCs. In the present study, the expression and secretion of galectin-3 in LSCs was stimulated by PGF at 48 h. PGF is elevated as a series of pulses in uterine venous blood at days 18-19 after ovulation [1]. In addition, galectin-3 expression in bovine CL was increased at the regressed stage (Fig. 1). Based on the above findings, PGF which triggers the induction of luteolysis during days 18-19 [1] may stimulate the expression and secretion of galectin-3 localized on plasma membrane in the regressed CL may be the result of secretion of galectin-3 from LSCs in response to PGF.

Luteolysis is characterized by a phase of inhibited P4 production (functional luteolysis), followed by a phase of decreasing in luteal size (structural luteolysis), when cells of the CL undergo apoptosis [1]. P4 production by LSCs treated with galectin-3 did not change in spite of decreasing LSC viability. There are no reports that galectin-3 affects a hormone production by any types of cells. One might only suppose that the cell death of LSCs started occurring at around 24 h after galectin-3 treatment. Galectin-3 may contribute to only structural luteolysis, not to functional luteolysis. Galectin-3 binds to glycoproteins such as integrins, neural cell adhesion molecule and CD98 [35]. In addition, integrins are expressed in luteal cells of rodents, and granulosa and theca cells of ruminants [23].  $\beta$ 1 integrin is reported to be involved in apoptosis induced by galectin-3 in colonic epithelial cells [22]. Based on the above findings, I hypothesized that the apoptosis in LSCs induced by galectin-3 requires  $\beta$ 1 integrin. As expected,  $\beta$ 1 integrin was expressed and possesses glycans which galectin-3 binds in bovine CL throughout the estrous cycle. However,  $\beta 1$  integrin expression and glycan structure did not change throughout the estrous cycle (Fig. 6). This may be the reason why luteolysis is controlled by the change of galectin-3 expression during the estrous cycle, but not by changes in the expression or glycan structure of  $\beta$ 1 integrin. I confirmed that galectin-3 induces LSC death by binding to  $\beta$ 1 integrin (Fig. 7), as shown in colon epithelial cells

and T cells [14, 22]. Integrins including  $\beta$ 1 integrin bind to extracellular matrix (fibronectin, collagen), and galectin-3 inhibits the binding of the integrins to the extracellular matrix [45]. A variety of cell types undergo apoptosis by lack of extracellular matrix attachment, which is termed anoikis [24]. These findings suggest that galectin-3 produced by LSCs inhibits binding of integrins to extracellular matrix, and induces anoikis in LSCs.

The overall findings suggest that galectin-3 increased by PGF induces apoptosis in bovine LSCs via  $\beta$ 1 integrin, contributing to structural luteolysis.

#### SUMMARY

Luteolysis is characterized by a reduction in progesterone (P4) production (functional luteolysis) and tissue degeneration (structural luteolysis) in the corpus luteum (CL). One of major events during structural luteolysis is luteal cell death. Galectin-3, a ubiquitously expressed protein involved in many cellular processes, serves as an anti-apoptotic and/or pro-apoptotic factor in various cell types. Although galectin-3 is detected in the bovine CL, its role remains unclear. The expression of galectin-3 in the bovine CL was higher at the regressed stage than at the other luteal stages. Galectin-3 was localized on luteal steroidogenic cells (LSCs). When cultured LSCs were exposed to prostaglandin F2a (PGF) for 48 h, the expression and secretion of galectin-3 increased. When the cultured LSCs were treated with galectin-3 for 24 h, cleaved caspase-3 expression was increased and the cell viability was decreased, whereas P4 production did not change.  $\beta$ 1 integrin, a target protein of galectin-3, was expressed in bovine CL and possessed glycans which galectin-3 binds. Furthermore, galectin-3 bound to glycans of luteal  $\beta$ 1 integrin. The decreased cell viability of cultured LSCs by galectin-3 was suppressed by  $\beta 1$  integrin antibody. The overall findings suggest that the secreted galectin-3 stimulated by PGF plays a role in structural luteolysis by binding to  $\beta$ 1 integrin.

### CHAPTER 2 Possible contribution of α2,6-sialylation to luteolysis in cows by inhibiting the luteotrophic effects of galectin-1

### **INTRODUCTION**

Luteolysis is characterized by a reduction in progesterone production (functional luteolysis) followed by a rapid decrease of the size of the corps luteum (CL) (structural luteolysis) [2]. The major cause of structural luteolysis is luteal cell death (apoptosis) [1-5]. During structural luteolysis, many cytokines from macrophages and neutrophils [9], and nitric oxide from luteal endothelial cells in response to prostaglandin F2 $\alpha$  (PGF) directly induce apoptosis of cells in the CL [2, 8-11]. However, other factors besides these key molecules may also be involved in luteolysis.

Glycosylation modulates a variety of physicochemical and biological properties of proteins such as protein folding, stability, targeting, and ligand binding [47-52]. Most membrane proteins including cell surface receptors are glycosylated, and glycosylation status is important for their function [53, 54]. However, the role of glycosylation in the CL is not clear. Galectins are carbohydrate-binding proteins distributed intra- and extra-cellularly [55]. Their carbohydrate-binding specificity for  $\beta$ -galactoside is conserved by evolutionarily preserved carbohydrate-recognition domains [55]. Extracellular galectins interact with cell surface oligosaccharides and form lattices that enhance the resident time of glycoproteins at the cell surface [56]. Among 15 members of galectins, galectin-1 is one of the most broadly distributed galectins in mammalian tissues, in which it modulates cell proliferation and survival [57-59]. Galectin-1 binds to N-acetyllactosamine (LacNAc) residues, which consist of galactose  $\beta$ -linked to N-acetylglucosamine, whereas its binding is completely inhibited by  $\alpha 2,6$ -sialylation of galactose [60-62]. ST6Gal-I is a sialyltransferase that catalyzes the binding of an  $\alpha 2,6$ -sialic acid to galactose on glycan branch ends through an  $\alpha$ 2,6-linkage [63, 64]. The addition of  $\alpha$ 2,6-sialic acid to the terminal galactose of glycans masks the galectin recognition sites, and inhibits the role of galectin-1 in adhesion, migration, and apoptosis [65]. We recently demonstrated that galectin-1 binds to the glycans on the surface of bovine luteal steroidogenic cells (LSCs) in the bovine mid CL and increases the viability of LSCs, suggesting that galectin-1 has a luteotrophic function in the bovine CL [66]. The protein expression of galectin-1 in the cell membrane fraction is high in the CL during mid-luteal stages while it decreases in the regressed CL [66]. The loss of luteotrophic galectin-1 from the cell membrane may be due to a decreased ligand-binding ability and accompanied by modifications of ligand glycoconjugates such as  $\alpha 2$ ,6-sialylation during luteolysis.

I hypothesized that the level of  $\alpha 2,6$ -sialylation is increased by luteolytic factors and that this increase inhibits the binding of galectin-1 to glycan, thereby weakening the survival signal of galectin-1 during luteolysis. To test this hypothesis, I examined 1) cyclic changes in the expression of *ST6Gal-I* mRNA and the level of  $\alpha 2,6$ -sialylation of glycoproteins in the bovine CL, 2) the effects of luteolytic factors on the expression of *ST6Gal-I* mRNA and the level of  $\alpha 2,6$ -sialylation in cultured mid-LSCs, and 3) Effect of  $\alpha 2,6$ -sialylation on galactin-1-enhanced viability of cultured mid-(Days 8-12 after ovulation) and late- (Days 15-17 after ovulation) LSCs.

### MATERIALS AND METHODS

### Collection of bovine CLs, luteal cell isolation and cell culture

Collection of bovine CLs, luteal cell isolation and cell culture were conducted as described in MATERIALS AND METHODS of chapter 1 (Page 8).

### **Protein fractionation and extraction**

The CL tissues for western blotting were homogenized on ice in the homogenization buffer (300 mM sucrose, 32.5 mM Tris-HCl, 2 mM EDTA, pH 7.4) by a tissue homogenizer (Physcotron, NS-50; NITI-ON Inc., Chiba, Japan), followed by filtration with a metal wire mesh (grid size 150 µm). For protein analysis, nuclei were removed from the tissue homogenates by centrifugation at 700 xg for 5 min. The resultant supernatant was fractionated into membrane and cytoplasmic fractions by centrifugation at 20,000 xg for 60 min. For protein analysis using LSCs, total cell lysates were used. After culture, LSCs were collected and lysed in lysis buffer consisting of 20-mM Tris-HCl, pH 7.4, 150-mM NaCl, 1-mM EDTA, 1.0% Triton X-100, 10% glycerol, and protease inhibitor cocktail (1697498; Roche, Mannheim, Germany). Their protein concentrations were determined by BCA method [67]. The proteins were then solubilized in SDS gel-loading buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, pH 6.8), and heated at 95°C for 10 min. Validation of the subcellular fractionation was performed by western blotting using the antibodies against tumor necrosis factor receptor 1 (TNFR1; ab19139; Abcam, Cambridge, UK) as a plasma membrane marker and  $\beta$ -actin (A2228; Sigma-Aldrich, MO, USA) as a cytoplasm marker (data not shown).

### **Quantitative RT-PCR**

Quantitative RT-PCR was conducted as described in MATERIALS AND METHODS of chapter 1 (Page 9) using specific primers for ST6Gal-I from the bovine sequence (Table 1).

### Western blotting

Proteins were solubilized in SDS gel-loading buffer, and heated at 95°C for 10 min. Samples (20 µg protein/lane) were separated on SDS-PAGE, and then transferred to a PVDF membrane (RPN303F; GE Healthcare., Little Chalfont, Buckinghamshire,

UK). The membrane was washed in TBS-T (0.1% Tween 20 in TBS [25 mM Tris-HCl, 137 mM NaCl, pH 7.5]) and incubated in blocking buffer (5% nonfat dry milk in TBS-T) for 1 h at room temperature. After washing, the membranes were incubated with galectin-1 antibody at 1:4,000 (AF1152, R&D Systems, MN, USA) or β-actin (ACTB) antibody diluted at 1:10,000 (A2228; Sigma-Aldrich, MO, USA) for overnight at 4°C. After washing with TBS-T, the membrane was incubated with the appropriate secondary antibodies (HRP-linked donkey anti-goat IgG [no.sc-2020; Santa Cruz Biotechnology; 1:5,000 for galectin-1] or HRP-linked sheep anti-mouse IgG [no. NA931; Amersham Biosciences Corp.; 1:40,000 for ACTB]) for 1 h at room temperature. After washing again with TBS-T, the signal was detected by chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate (P36599; Millipore, Billerica, MA, USA). β-Actin protein expression was used as an internal control for the experiments using total cell lysates. The intensity of the immunological reaction in the tissues or cells was estimated by measuring the optical density in the defined area by computerized densitometry using Image LabTM Software version 4.0 (Bio-Rad Laboratories, Inc, Berkeley, CA, USA).

### Lectin blotting

Protein samples (5  $\mu$ g/lane) were subsequently separated by SDS-PAGE. Separated proteins were transferred onto a PVDF membrane. The membrane was blocked with 5% BSA in TBS-T, and then reacted with biotin-conjugated SSA (0.17  $\mu$ g/ml) lectin in TBS-T for 1 h. After washing again with TBS-T, the membrane was incubated with 0.17  $\mu$ g/ml of streptavidin conjugated to HRP (#21130; Pierce Biotechnology, IL, USA). After washing again with TBS-T, the reactive protein bands were detected by chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate as described above.

### Lectin histochemistry

The CL tissues (2.0-3.0 g) are classified as early-luteal (n=3), mid-luteal (n=4), late-luteal (n=3), and regressing stages (n=5), and fixed with 4% paraformaldehyde for histological analysis. The fixed tissues were embedded into paraffin according to the conventional method. Five- $\mu$ m-thick sections were de-waxed and washed in phosphate-buffered saline. Subsequently the sections were incubated at room temperature with 3% hydrogen peroxide in distilled water for 20 min, and Avidin/Biotin

blocking solution (Vector Laboratories Inc., Burlingame, CA, USA) for 15 min for each reagent, followed by Carbo-free blocking solution (Vector Laboratories In.) for 60 min. Then the sections were incubated with biotin-conjugated SSA lectin ( $1.0 \mu g/ml$ ) at 4°C overnight. After washing twice in PBS, the reaction sites were visualized using Vectastain ABC Elite kit (Vector Laboratories Inc., Burlingame, CA, USA) for 60 min at room temperature and ImmPACT® 3, 3'-Diaminobenzidine (DAB) Peroxidase Substrate Kit (Vector Laboratories Inc., Burlingame, CA, USA) for 5 min. The sections were counterstained for 2 min with haematoxylin and observed under a light microscope (BX51; Olympus Corporation, Tokyo, Japan) using 20x object lens. Control sections were incubated with PBS instead of SSA, and a disappearance of the signals in both endothelial cells and luteal cells was confirmed. LSCs and luteal endothelial cells were identified by localization and morphology features of the cells [71, 72]. Since SSA lectin reacts against the endothelial cells.

#### Viability assay

WST-8, a kind of MTT, is a yellow tetrazolium salt that is reduced to formazan by live cells containing active mitochondria. The culture medium was replaced with 100  $\mu$ l D/F without phenol red medium-BSA, and a 10  $\mu$ l WST-8 was added to each well. Cells were then incubated for 4 h at 38.5°C. The absorbance was read at 450 nm using a microplate reader (Model 450; Bio-Rad, Hercules, CA).

# Experiment 1: Cyclic changes in the expression of ST6Gal-I mRNA and the level of α2,6-sialylation of glycoproteins in the bovine CL

ST6Gal-I mRNA and  $\alpha 2,6$ -sialic acid expressions of in each CL tissues and membrane fraction of CL tissues (early n=4, developing n=4, mid n=4, late n=4, regressed n=4,) were examined by quantitative RT-PCR and lectin blotting using Sambucus Sieboldiana Agglutinin (SSA; J218; J-OIL MILLS, Tokyo, Japan). Localization of  $\alpha 2,6$ -sialic acid in each CL tissues (Early; n=3, mid; n=3, Regressed; n=3) was analyzed by lectin histochemistry using SSA.

Experiment 2: Effect of luteolytic factors on the expression of ST6Gal-I mRNA and the level of α2,6-sialylation of cultured mid-LSCs To clarify whether luteolytic factors play a role in the expression of ST6Gal-I mRNA and the level of  $\alpha$ 2,6-sialylation in the bovine CL, the cultured mid-LSCs were exposed to 2.3 nM recombinant human tumor necrosis factor  $\alpha$  (TNF $\alpha$ ; Kindly donated by Dainippon Pharmaceutical, Osaka, Japan), 2.5 nM recombinant bovine interferon  $\gamma$  (IFNG; Kindly donated by Dr. S. Inumaru, NIAH, Ibaraki, Japan), 1.0  $\mu$ M PGF (P7652; Sigma-Aldrich Corp., St. Louis, MO, USA) alone, or TNF $\alpha$  in combination with IFNG for 24, 48 and 72 h. Additionally, the cultured mid-LSCs were exposed to 10 ng/ml luteinizing hormone (LH: USDA-bLH-B6) and 1.0  $\mu$ M prostaglandin E2 (PGE: no. P0409; Sigma) for 24 h. The doses for treatments were selected based on previous reports [30, 31, 68, 69]. After the culture, the expression of ST6Gal-I mRNA was determined by quantitative RT-PCR. TNF (2.3 nM) and IFNG (2.5 nM) induces the expression of FAS mRNA in cultured LSCs [34]. The induction of the expression of Fas mRNA by TNF (2.3 nM) and IFNG (2.5 nM) was confirmed in this experiment. The expression of  $\alpha$ 2,6-sialic acid in total cell lysates was assessed by SSA lectin blotting only in cells treated with PGF for 72 h.

# Experiment 3: Effect of α2,6-sialylation on galactin-1-enhanced viability of cultured mid- and late-LSCs

The level of  $\alpha 2,6$ -sialylation of mid- and late-LSCs in total cell lysates was measured by SSA lectin blotting. To investigate the effect of galectin-1 on cell viability of mid- and late-LSCs, these cells were incubated with or without recombinant galectin-1 (1,000 ng/ml; 1152-GA-050/CF; R&D Systems, Inc, Minneapolis, MN) for 24 h. The cell viability was determined by Methyl tetrazolium (MTT) assay. In my previous study, galectin-1 (10, 100, and 1,000 ng/ml) significantly increased the viability of cultured LSCs and the greatest increase in the viability of LSCs was observed by the treatment with galectin-1 (1,000 ng/ml) [66]. Thus, I used 1,000 ng/ml of galectin-1 in the present study.

To remove sialic acid from cell surface glycoproteins, late-LSCs were treated with or without neuraminidase (0.1, 1.0, 10 units/ml; P0720S; New England BioLabs Inc., Ipswich, MA) for 1 h and washed. Then, cells were further incubated for 24 h and  $\alpha$ 2,6-sialic acid expression was analyzed by SSA lectin blotting to confirm a reduction in the level of  $\alpha$ 2,6-sialylation by neuraminidase treatment. To examine whether  $\alpha$ 2,6-sialic acid inhibits the effect of galectin-1 on the viability of late-LSCs, cells were incubated with or without neuraminidase (10 units/ml) for 1 h and washed. Then cells

were further incubated with or without recombinant galectin-1 (1000 ng/ml) for 24 h. The cell viability was determined using a WST-8 cell proliferation assay.

### **Statistical analysis**

All experimental data are shown as the mean  $\pm$  SEM. The statistical significances of differences were assessed by analysis of Student's *t*-test, one-way ANOVA followed by Dunnett's multiple comparison test or two-way ANOVA followed by Tukey's multiple comparison test using the GraphPad Prism ver 6.0 (GraphPad software, San Diego, CA). Probabilities less than 5% (P<0.05) were considered statistically significant.

#### RESULTS

# Experiment 1: Cyclic changes in the expression of ST6Gal-I mRNA and the level of $\alpha$ 2,6-sialylation of glycoproteins in the bovine CL

The expression of ST6Gal-I mRNA in the bovine CL was significantly higher during the regressed-luteal stage than during the developing and mid-luteal stages (Fig. 8A). The level of  $\alpha 2,6$ -sialylation in the membrane fraction was higher during the regressed-luteal stage than during the other luteal stages (Fig. 8B). Lectin histochemistry revealed that  $\alpha 2,6$ -sialylated glycoconjugates were localized to luteal endothelial cells throughout the estrous cycle (arrows in Fig. 9). In the regressed-luteal stage,  $\alpha 2,6$ -sialylated glycoconjugates concentrated to the plasma membrane of LSCs (Fig. 9).

# Experiment 2: Effect of luteolytic factors on the expression of ST6Gal-I mRNA and the level of α2,6-sialylation of cultured mid-LSCs

The expression of ST6Gal-I mRNA was significantly increased by treatment with PGF for 72 h, but it was not affected by treatment with TNF $\alpha$ , IFNG, or a combination of TNF $\alpha$  and IFNG for 24, 48 or 72 h (Fig. 10A). Moreover, the expression of ST6Gal-I mRNA was not affected by treatment with LH or PGE for 24 h (Fig. 14). The level of  $\alpha$ 2,6-sialylated glycoproteins in total cell lysates was also significantly increased by treatment with PGF for 72 h (Fig. 10B).

## Experiment 3: Effect of $\alpha 2,6$ -sialylation on galactin-1-enhanced viability of cultured mid- and late-LSCs

The level of  $\alpha 2,6$ -sialylated glycoproteins of late-LSCs was higher than that of mid-LSCs (Fig. 11A). Galectin-1 (1.0 µg/ml) increased the viability of mid-LSCs, while it did not affect the viability of late-LSCs (Fig. 11B, C). Neuraminidase treatment (1.0 and 10 units/ml) decreased the level of  $\alpha 2,6$ -sialylation in total cell lysates of late-LSCs (Fig. 12A). Pretreatment of late-LSCs with neuraminidase (10 units/ml) restored the ability of galectin-1 to increase their viability (Fig. 12B).



**Figure 8.** Changes in the relative amounts of the expression of ST6Gal-I mRNA and  $\alpha 2,6$ -sialylated glycoproteins. (A) Comparison of relative amounts of ST6Gal-I mRNA determined by quantitative RT-PCR in bovine CL tissue throughout the estrous cycle (early [E], Days 2-3; developing [D], Days 5-6; mid [M], Days 8-12; late [L], Days 15-17; regressed luteal stages [R], Days 19-21). Data are the mean  $\pm$  SEM for four samples/stage and are expressed as the relative ratio of ST6Gal-I mRNA to MRPL4 mRNA. (B) Representative  $\alpha 2,6$ -sialic acid specific SSA lectin blotting of membrane glycoproteins from CL. Different superscript letters indicate significant difference (P<0.05), as determined by ANOVA followed by Tukey's multiple comparison test.



Figure 9. Representative images of the localization  $\alpha 2$ ,6-sialylated glycoconjugates on the bovine CL throughout the estrous cycle revealed by SSA lectin histochemistry. Black arrows indicate luteal endothelial cells. The mid-CL tissues were used in the control sections.



**Figure 10.** Effects of luteolytic factors on the expression of ST6Gal-I mRNA and the level of  $\alpha 2,6$ -sialylated glycoproteins in total cell lysates of mid-LSCs. (A) LSCs were treated with TNF $\alpha$  (2.3 nM), IFNG (2.5 nM), TNF $\alpha$  and IFNG or PGF (1.0  $\mu$ M) for 24, 48, and 72 h. Data are expressed as the relative ratio of ST6Gal-I mRNA to MRPL4 mRNA levels. (B) Representative SSA lectin blot analysis for  $\alpha 2,6$ -sialic acid and ACTB in cells treated with PGF (1.0  $\mu$ M) for 72 h. The resultant signal was detected by chemiluminescence and quantitated by computer-assisted densitometry. Data are expressed as the relative ratio of  $\alpha 2,6$ -sialylated proteins to ACTB protein. All values are the means  $\pm$  SEM. All of the experiments were repeated more than three times, and the data were statistically analyzed using student *t*-test and ANOVA followed by Dunnett's multiple comparison test. Asterisks indicate significant differences compared with control for 72 h (P<0.05). T+I: TNF $\alpha$ +IFNG treatment.



**Figure 11.** Difference in  $\alpha$ 2,6-sialylation and the effect of galectin-1 in cultured midand late-LSCs. (A) Representative SSA lectin blotting of glycoproteins and ACTB in total cell lysates of mid- and late-LSCs. (B, C) Mid- and late-LSCs were incubated with or without galectin-1 (1.0 µg/ml) for 24 h. All values are the means ± SEM. The data were statistically analyzed using student t-tests. Asterisks indicate significant differences compared with control (P<0.05).



**Figure 12.** Effect of neuraminidase on the action of galectin-1 in late-LSCs. (A) Representative images of SSA lectin blotting and ACTB in total cell lysates of late-LSCs cultured for 24 h after the pretreated with or without neuraminidase (0.1, 1.0, and 10 units/ml) for 1h. (B) Late-LSCs were pretreated with or without neuraminidase (10 units/ml) for 1h, and further incubated with or without recombinant galectin-1 (1.0  $\mu$ g/ml) for 24 h. All values are the means  $\pm$  SEM. Different superscript letters indicate significant difference (P<0.05), as determined by ANOVA followed by Tukey's multiple comparison tests.



**Figure 13.** Changes in the relative amounts of galectin-1 protein expression in total (cytoplasmic and cell membrane) fraction of the CL tissue. Representative images for western blot analysis for galectin-1 and ACTB are shown in the top. Densitometrically analyzed western blot results in both cytoplasmic and cell membrane fraction of bovine CL tissue throughout the estrous cycle (Early [E], Days 2-3; Developing [D], Days 5-6; Mid [M], Days 8-12; Late [L], Days 15-17; Regressed luteal stages [R], Days 19-21) are shown in the bottom. Data are the mean  $\pm$  SEM of three samples/stage and are expressed as the relative ratio of galectin-1 protein to ACTB protein. Different superscript letters indicate significant difference (P<0.05), as determined by ANOVA followed by Tukey's multiple comparison test.



**Figure 14.** Effects of LH or PGE on the expression of ST6Gal-I mRNA in mid-LSCs. LSCs were treated with LH (10 ng/ml) or PGE (1.0  $\mu$ M) for 24 h. Data are expressed as the relative ratio of ST6Gal-I mRNA to MRPL4 mRNA levels. All values are the means  $\pm$  SEM. All of the experiments were repeated more than three times.



**Figure 15.** Effects of TNF $\alpha$  and IFNG on the expression of Fas mRNA in mid-LSCs. LSCs were treated with TNF $\alpha$  (2.3 nM) and IFNG (2.5 nM) for 24 h. Data are expressed as the relative ratio of Fas mRNA to MRPL4 mRNA levels. All values are the means  $\pm$  SEM. The data were statistically analyzed using student t-tests. Asterisks indicate significant differences compared with control (P<0.05).

_	14010 2:11	liners for rear time r er			
	Gene	Forward and reverse primers (5'-3')	Accession no.	Product (bp)	
		F: ATTGTGTGGGACCCATCTGT	BC112480	110	
	S16Gal-I	R: TGATCAGGATGCAGCTTACG			
	E	F: TCCAGATCTCACGCAAACAG	NM174662	150	
	Fas	R: CAGTTGCCTCCCTTCATCAT			
N		F:GGCTCAAGACCTTCAACCTG	BC108102	138	
	MKPL4	R:GCGTGTAACGTGAGTCATGC			

### Table 2. Primers for real-time PCR

### DISCUSSION

The present study revealed that  $\alpha 2,6$ -sialylation increased during luteolysis in the bovine CL and that the increase was regulated by luteolytic PGF through enhancing the expression of a sialyltransferase, ST6GAL-I in LSCs. An increased  $\alpha 2,6$ -sialylation on membrane glycoprotein of LSCs during the late-luteal stage attenuated the effect of galectin-1 on luteal cell viability. Removal of  $\alpha 2,6$ -sialic acids by a treatment with neuraminidase restored the effect of galectin-1, suggesting that  $\alpha 2,6$ -sialylation is involved in the regulation of luteolysis by inhibiting the binding of galectin-1 to the glycans.

As mentioned above, extracellular galectin-1 promotes cell viability in bovine LSCs, suggesting that galectin-1 has a luteotrophic function in the bovine CL [66]. In that study, I also showed that the amount of galectin-1 on the cell membrane of the CL decreased during luteolysis. However, in the present study, the abundance of galectin-1 in both cytoplasmic and cell membrane fraction of the CL did not change during the estrous cycle. This reminds us that the change in the glycan structure to decrease the binding of galectin-1 to glycan on cell membrane may contribute to a loss of galectin-1 on the cell membrane of LSCs during luteolysis. However, the mechanism to inhibit the binding of galectin-1 to glycans during luteolysis remained unclear. Galectins have an affinity for LacNAc, whereas modifications of LacNAc structure influence the affinity for each galectin subtype. It is well known that  $\alpha 2,6$ -sialylation of the terminal galactose, which is catalyzed by ST6GAL-I, interferes with the binding of galectin-1 [60-62]. I revealed here that the level of  $\alpha 2$ ,6-sialylation increased during the late-luteal stage and was highest during the regressed-luteal stage where galectin-1 expression on cell membrane decreased [66]. These findings suggest that  $\alpha 2,6$ -sialylation on the glycan ligand for galectin-1 inhibits the binding of galectin-1 to the glycan, resulting in a decreased localization of galectin-1 on cell membrane of LSCs. Consequently, the survival signal induced by galectin-1 may become weak in LSCs during luteolysis. In ST6Gal-I-deficient mice, the vascularization is decreased, and the platelet endothelial cell adhesion molecule (PECAM) which is important for cell adhesion of endothelial cells is unable to remain on the cell surface and incompletely transduced inhibitory signals such as antiapoptotic role [74]. In the present study,  $\alpha 2,6$ -sialic acid localized at the luteal endothelial cells. These findings suggest that  $\alpha 2,6$ -sialylation plays important roles of vascularization.

Galectin-1 has a role in a variety of biological processes, such as cell adhesion,

invasion, cell migration, cell survival, and cell death [75]. In mouse, galectin-1 strongly expresses during luteolysis, and the expression of ST6Gal-I mRNA is enhanced in the regressing CL, suggesting that the interaction between galectin-1 and  $\alpha$ 2,6-sialylation plays an important role in luteolysis [76]. Galectin-1 is also likely to have a luteotrophic role in the human CL, and an inhibition of the binding of galectin-1 to glycan caused by  $\alpha$ 2,6-sialylation possibly contributes to the induction of luteolysis in women [77]. In the bovine CL, galectin-1 increases the viability of mid-LSCs but does not affect the viability of late-LSCs which contained abundant  $\alpha$ 2,6-sialylated glycoproteins, suggesting that  $\alpha$ 2,6-sialylation in late-LSCs attenuates the effect of galectin-1. When the late-LSCs were pretreated with neuraminidase to remove sialic acid residues, galectin-1 could increase the cell viability in late-LSCs, further supporting a luteolytic role of  $\alpha$ 2,6-sialic acids.

Expression of ST6GAL-I mRNA was enhanced by PGF but not by other luteolytic factors (Fig. 10). In human luteinizing granulosa cells, luteotrophic factors —PGE2 and human chorionic gonadotrophin— strongly suppress the expression of ST6Gal-I mRNA [77]. However, in the present study, PGE2 and luteinizing hormone did not suppress the expression of ST6Gal-I mRNA in bovine LSCs. In contrast, PGF significantly enhanced the expression of ST6GAL-I in bovine LSCs, whereas other luteolytic factors including TNF $\alpha$ , IFNG had no effect. PGF regulates luteal function by activating protein kinase (PK) C pathways in luteolysis [30, 78]. In addition, activation of PKC pathway was found to stimulate  $\alpha$ 2,6-sialylation in Madin-Darby canine kidney cells [79]. These findings suggest that activation of the PKC pathway by PGF contributes to the increase of  $\alpha$ 2,6-sialylation in bovine LSCs during luteolysis.

Although  $\alpha 2,6$ -sialylation on glycoproteins likely has a role in luteolysis in cows, the proteins carrying  $\alpha 2,6$ -sialic acid are unclear. We previously revealed that galectin-1 enhances the cell viability of mid-LSCs by binding to the glycans of vascular endothelial growth factor receptor-2 (VEGFR-2), and the protein expression and stability of VEGFR-2 on the cell surface require the binding of galectin-1 to glycan of VEGFR-2 in bovine LSCs [66]. Since glycosylation of receptors is important in cell surface retention of various receptors through interaction with galectins [80], inhibition of the binding of galectin-1 to the glycan of VEGFR-2 by  $\alpha 2,6$ -sialylation may decrease in the VEGFR-2-mediated survival signal in LSCs during luteolysis. However, it is unlikely that the sugar chain on VEGFR-2 contains abundant  $\alpha 2,6$ -sialic acids because I could not find a band with an approximate molecular weight for VEGFR-2 (220 kDa) in

the SSA lectin blotting analysis. Further studies are required to reveal the proteins carrying  $\alpha 2$ ,6-sialic acid residues.

The overall findings suggest that the  $\alpha 2,6$ -sialylation stimulated by PGF inhibits the interaction between galectin-1 and glycan, contributing to luteolysis by reducing the galectin-1 effect on luteal cell viability in cattle.

#### SUMMARY

The corpus luteum (CL) is essential for establishing pregnancy. If pregnancy does not occur during the estrus cycle, luteolysis is induced by prostaglandin (PG) F2 $\alpha$ secreted from the uterus. Galectin-1, a β-galactose-binding protein, is expressed in the functional CL of cows, and increases the viability of bovine luteal steroidogenic cells (LSCs) by modifying the functions of membrane glycoproteins. The binding of galectin-1 to glycoproteins is blocked by  $\alpha 2,6$ -sialylation of the terminal galactose residues of glycoconjugates, which is catalyzed by a sialyltransferase (ST6Gal-I). However, the physiological role of  $\alpha 2,6$ -sialic acid in the bovine CL is unclear. The level of  $\alpha 2,6$ -sialylation of the bovine CL were higher during the regressed-luteal stage than other luteal stages. Lectin histochemistry revealed that  $\alpha 2,6$ -sialylated glycoconjugates were localized to luteal endothelial cells throughout the estrous cycle. In addition,  $\alpha 2.6$ -sialylated glycoconjugates concentrated to the membrane of LSCs during the regressed-luteal stage. PGF2a treatment for 72 h enhanced the expression of ST6Gal-I mRNA and the level of  $\alpha 2,6$ -sialylated glycoproteins in mid-LSCs. The level of a2,6-sialylated glycoproteins of late-LSCs (Days 15-17 after ovulation) was higher than that of mid-LSCs (Days 8-12 after ovulation), and galectin-1 increased the viability of mid-LSCs but not that of late-LSCs. Furthermore, galectin-1 increased the viability of late-LSCs when  $\alpha 2,6$ -sialic acid residues were removed by neuraminidase. The overall findings suggest that  $\alpha 2,6$ -sialylation stimulated by PGF2 $\alpha$  contributes to luteolysis by inhibiting the luteotrophic effects of galectin-1 in the bovine CL.

### CONCLUSION

The present study investigated the possible contribution of galectins and glycan to luteolysis in cows. The first series of experiments indicate that PGF enhanced the expression of galectin-3 in cultured bovine LSCs, and galectin-3 binding to the glycan on  $\beta$ 1 integrin induces apoptosis of bovine LSCs during structural luteolysis. The second series of experiments showed that PGF increased the level of  $\alpha$ 2,6-sialylation, and increase in the level of  $\alpha$ 2,6-sialylation prevented the interaction between galectin-1 and glycan, contributing to luteolysis by reducing galectin-1 effect of luteal cell viability in cattle.

Overall results suggest that that the interaction between galectins and glycan play a critical role in the regression of bovine CL. Moreover, PGF controlled the change in the expression of galectin-3 and the level of  $\alpha 2,6$ -sialylation, contributing to structural luteolysis.

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### **ABSTRACT IN JAPANESE**

### ウシ黄体における糖鎖および糖鎖結合性タンパク質 galectin の役割に関する 研究

#### 羽柴 一久

ウシを含む多くの哺乳動物において、排卵後に形成される黄体は妊娠の維持 と成立に必須の progesterone (P4) を分泌する一過性の内分泌器官である。妊娠 が成立しなかった際には、主に子宮からの prostagrandin F2α (PGF) の作用によ り黄体は退行する。黄体の退行は P4 分泌の衰退する機能的退行と黄体組織の 死滅する構造的退行の 2 つの側面から成り、構造的退行は黄体を構成する細胞 のアポトーシスおよび卵巣外への流出により起こる。さまざまな組織において アポトーシスに免疫応答が関与することが明らかにされており、退行期のウシ 黄体において急激な免疫細胞の増加が認められることから、黄体におけるアポ トーシスに免疫応答の関与する可能性が示唆されている。構造的退行において 免疫細胞が分泌する tumor necrosis factor α (TNF) や interferon γ (IFNG) などの サイトカインおよび血管内皮細胞が分泌する NO がアポトーシスを誘導する ことが知られており、様々な因子により黄体の退行は制御されている。

糖鎖修飾は、タンパク質のフォールディング、安定性およびリガンド結合な ど生理的および生物学的な現象に関与している。細胞膜表面受容体を含む多く の細胞膜タンパク質は、糖鎖修飾を受けておりそのタンパク質の機能に重要で ある。しかしながら、黄体における糖鎖修飾の役割について詳細な報告はない。

Galectin は細胞内外に局在する糖鎖結合性のタンパク質であり、糖鎖中の β 型結合した galactose に結合するレクチンである。細胞外 galectin は細胞表面の 糖タンパク質の糖鎖に結合することで、そのタンパク質を細胞膜上に保ち、機 能を維持することが報告されている。Galectin には 15 種類のサブタイプが同定 されており、ウシおよびマウス黄体では galectin-1 および -3 が周期的な変化を 伴いながら発現することが報告されているが、その役割は明らかではない。

Galectin-3 は細胞質、細胞膜表面および細胞外マトリックスに発現し、アポト ーシスを制御している。Galectin-3 には細胞質型および細胞外型が存在し、細胞 質型 galectin-3 は糖鎖を介さずリン酸化され抗アポトーシスに働く。一方、細 胞から分泌された細胞外型 galectin-3 は β1 integrin を含む膜タンパク質受容体 の糖鎖と結合し細胞のアポトーシスを誘導することが報告されている。本研究 では、ウシ黄体における galectin-3 の役割を解明するために以下の研究を行っ た。

1) 卵巣を肉眼的所見により、排卵日を 0 日として、排卵後の日数で五つの周 期 (黄体初期: Days 2-3、黄体形成期: Days 5-6、黄体中期: Days 8-12、黄体後期: Days 15-17、黄体退行期: Days 19-21) に分類した。その後、各周期における黄体 組織の galectin-3 の mRNA およびタンパク質発現を定量的 RT-PCR 法、 Western blot 法によりそれぞれ検討した。また、発情周期を通じた黄体組織を用 いて、galectin-3 タンパク質の局在を免疫組織化学により検討した。2) ウシ中期 黄体から単離した黄体細胞を 24 h 培養後、新しい培養液と交換し以下の実験に 供した。黄体退行因子である TNF 2.3 nM、IFNG 2.5 nM、PGF 1.0 μM を単独ま たは組み合わせて添加し、さらに 12、24、48、72h 培養後の galectin-3 mRNA お よびタンパク質発現を調べた。3) Galectin-3 の分泌に及ぼす PGF の影響を検討 するために、PGF を添加し 48h 培養後、galectin-3 が結合する糖鎖と競合する lactose (100 μM) を添加し、さらに 4 h 後の galectin-3 タンパク質発現を調べた。 4) 発情周期を通じた黄体組織における β1 integrin mRNA およびタンパク質発 現を調べた。また、各発情周期における黄体組織および黄体細胞から免疫沈降 法を用いて β1 integrin タンパク質を回収後、その糖鎖構造を L4-PHA レクチン を用いて調べ、galectin-3 の β1 integrin への結合能を overlay assay により検討 した。さらに、黄体細胞に β1 integrin 抗体 (1.0 ng/ml) 添加 1 h 後、galectin-3 (1.0 µg/ml) を添加し 24 h 後の細胞生存率を調べた。

1) 発情周期を通じた黄体組織において galectin-3 mRNA およびタンパク質 発現量は、他の周期と比較して退行期において有意に高かった。また、galectin-3 タンパク質は黄体細胞に局在していた。2) Galectin-3 mRNA およびタンパク質 の発現量は、control と比較して PGF 添加 48 h 後において有意に増加した。ま た、全ての時間において galectin-3 mRNA 発現量は TNF および IFNG 添加に よって変化しなかった。3) PGF により増加した galectin-3 タンパク質発現量は lactose 添加により低下した。4) 発情周期を通じて  $\beta$ 1 integrin mRNA およびタ ンパク質発現量に変化はなく、 $\beta$ 1 integrin は galectin-3 結合性の糖鎖を有してい た。さらに、galectin-3 は  $\beta$ 1 integrin に対する結合能を有していた。細胞生存率 は galectin-3 添加区と比較して、 $\beta$ 1 integrin 抗体添加後 galectin-3 を添加した区 において有意に増加した。これらのことから、ウシでは子宮からの PGF により 黄体細胞の galectin-3 発現および分泌が促進され、galectin-3 が  $\beta$ 1 integrin の糖 鎖に結合することで黄体細胞の死を誘導する可能性が示された。

ウシにおいて、galectin-1 は機能的黄体に発現しており細胞膜糖タンパク質の 機能を調節することによって黄体細胞の生存率を増加させる。Galectin-1 の糖タ ンパク質糖鎖への結合は、糖鎖の末端に存在する galactose に sialyltransferase (ST6Gal-I) により  $\alpha$ 2,6-sialic acid が付加 ( $\alpha$ 2,6-sialylation) されることで阻害さ れる。しかしながら、ウシ黄体において  $\alpha$ 2,6-sialylation の役割について詳細は 明らかではない。本研究では、黄体細胞における  $\alpha$ 2,6-sialylation が退行因子に より増加し、増加した  $\alpha$ 2,6-sialic acid が galectin-1 および糖鎖との結合を阻害 することで、galectin-1 の生存シグナルを減少させるという仮説を立てた。この 仮説を証明するために、以下の研究を行った。

1) 発情周期を通じた黄体組織における *ST6Gal-I* mRNA 発現を定量的 RT-PCR で調べ、糖タンパク質の  $\alpha$ 2,6-sialylation レベルを SSA レクチンを用 いたレクチンブロットにより解析すると共に局在についても調べた。2) 中期黄 体細胞に黄体退行因子である TNF、IFNG、PGF を単独または組み合わせて添 加し、さらに 24、48 および 72 h 培養後の *ST6Gal-I* mRNA ならび  $\alpha$ 2,6-sialylation レベルを調べた。3) 培養中期および後期黄体細胞の  $\alpha$ 2,6-sialylation レベルを調べ、それぞれの細胞に galectin-1 (1000 ng/ml) を添加 し 24 h 後の細胞生存率を測定した。また、後期黄体細胞に sialic acid 分解酵素 である neuraminidase (10 units/ml) を添加し 1 h 後さらに galectin-1 を添加し 24 h 後の  $\alpha$ 2,6-sialylation レベルおよび細胞生存率を調べた。

1) 発情周期を通じて ST6Gal-I mRNA 発現量は、形成期および中期と比較し て退行期において増加した。糖タンパク質の  $\alpha 2,6$ -sialylation レベルもまた、退 行期に増加しており、黄体細胞の細胞膜にその局在が認められた。2) 黄体細胞 における ST6Gal-I mRNA 発現量および  $\alpha 2,6$ -sialylation レベルは PGF 添加後 72 h において有意な増加が認められたが、 TNF および IFNG の単独または組 み合わせ添加区における 24、48 および 72 h の培養では変化は認められなかっ た。3) 後期黄体細胞における  $\alpha 2,6$ -sialylation レベルは、中期黄体細胞と比較し て高かった。また、galectin-1 は中期黄体細胞の生存率を増加させたが、後期黄 体細胞の生存率は増加させなかった。Neuraminidase は、後期黄体細胞の  $\alpha 2,6$ -sialylation レベルを減少させ、galectin-1 は neuraminidase 処理した後期黄 体細胞の生存率を増加させた。これらのことから、黄体細胞において PGF によ り増加した  $\alpha 2,6$ -sialylation は、galectin-1 および糖鎖の結合を阻害することで、 galectin-1 の誘導する生存シグナルを減少させ、黄体退行に関与することが示唆 された。

本研究より、ウシにおいて galectin および糖鎖の相互作用が黄体退行を調節 していることが示された。さらに PGF は黄体における galectin-3 発現および α2,6-sialylation を増加させることで構造的黄体退行を促進することが明らかと なった。