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—Original Article—

The luteotrophic function of galectin-1 by binding to the glycans on vascular endothelial growth factor receptor-2 in bovine luteal cells

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Abstract. The corpus luteum (CL) is a temporary endocrine gland producing a large amount of progesterone, which is essential for the establishment and maintenance of pregnancy. Galectin-1 is a β-galactose-binding protein that can modify functions of membrane glycoproteins and is expressed in the CL of mice and women. However, the physiological role of galectin-1 in the CL is unclear. In the present study, we investigated the expression and localization of galectin-1 in the bovine CL and the effect of galectin-1 on cultured luteal steroidogenic cells (LSCs) with special reference to its binding to the glycans on vascular endothelial growth factor receptor-2 (VEGFR-2). Galectin-1 protein was highly expressed at the mid and late luteal stages in the membrane fraction of bovine CL tissue and was localized to the surface of LSCs in a carbohydrate-dependent manner. Galectin-1 increased the viability in cultured LSCs. However, the viability of LSCs was decreased by addition of β-lactose, a competitive carbohydrate inhibitor of galectin-1 binding activity. VEGFR-2 protein, like galectin-1, is also highly expressed in the mid CL, and it was modified by multi-antennary glycans, which can be recognized by galectin-1. An overlay assay using biotinylated galectin-1 revealed that galectin-1 directly binds to asparagine-linked glycans (*N*-glycans) on VEGFR-2. Enhancement of LSC viability by galectin-1 was suppressed by a selective inhibitor of VEGFR-2. The overall findings suggest that galectin-1 plays a role as a survival factor in the bovine CL, possibly by binding to *N*-glycans on VEGFR-2.

Key words: Corpus luteum, Galectin-1, Lectin, Ovary, vascular endothelial growth factor receptor (VEGFR)

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The corpus luteum (CL) is a transient endocrine gland that is essential for the regulation of ovarian cycles in mammals. The development and secretory function of the bovine CL are controlled by many factors, such as growth factors, hormones and steroids [1], and progesterone secreted by luteal cells is essential for the establishment and maintenance of pregnancy in many mammals, including cattle [2–5]. If pregnancy does not occur, regression of the ruminant CL, called luteolysis, is initiated by endometrial prostaglandin $F_{2\alpha}$ [6, 7]. Luteal regression is characterized by a reduction in progesterone (functional luteolysis) and tissue degeneration by apoptosis (structural luteolysis) [8–10].

Glycosylation modulates a variety of physicochemical and biological properties of proteins, such as protein folding, stability, targeting, dynamics and ligand binding [11–16]. Most of the membrane proteins including cell surface receptors are glycosylated, and glycosylation status is important for their function [17, 18]. However, the role of glycosylation in CL function is not clear.

Galectins are intra- and extracellularly distributed carbohydrate-binding proteins. Their carbohydrate-binding specificity for β-galactoside is conserved by evolutionarily preserved carbohydrate-recognition domains [19]. Extracellular galectins interact with cell surface oligosaccharides and form lattices that enhance the residency time of glycoproteins at the cell surface [20]. Among the 15 galectins that have so far been identified, galectin-1 is one of the most broadly distributed galectins in mammalian tissues and modulates a variety of cell functions, e.g., immune function [21, 22], angiogenesis [23] and tumor growth [24]. It has so far been detected in the CL of mice [25] and humans [26]. We previously reported that galectin-3 decreases the viability of luteal steroidogenic cells (LSCs) and is only expressed at the regressing luteal stage in the bovine CL [27]. However, the expression and function of galectin-1 in the bovine CL are unclear.

Vascular endothelial growth factor (VEGF) is a major angiogenic factor secreted from LSCs in the CL of various animals [28–30]. VEGF binds its receptors (VEGFRs) and activates a number of intracellular signaling pathways, which are involved in cell proliferation, migration and survival [31]. VEGF, produced from LSCs in the CL, acts not only as an angiogenic factor by binding to receptors expressing on endothelial cells but also as a non-angiogenic luteotrophic factor affecting LSCs themselves via autocrine and/or paracrine manners. In fact, VEGF increased the progesterone production from LSCs in cows and horses [32–35], and the actions of VEGF mediated by VEGFR-2 in the CL is required to maintain CL functions during pregnancy [36].

VEGFR is modified by glycans that are required for its function [37]. Recently, VEGFR-2 was reported to be a ligand for galectin-1

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Table 1. Primers for real-time PCR

Gene	Forward and reverse primers (5'-3')	Accession no.	Product (bp)
VEGFR-2	F: TGGCCCAACAATCAGAGCAG R: GAACGGAGCCCATGTCAGTG	X94298	154
VEGFA	F: ATTTTCAAGCCGTCCTGTGT R: TATGTGCTGGCTTTGGTGAG	M32976	138
NRP-1	F: CCAGAAGCCAGAGGAGTACG R: CTTTTCCGATTTCACCCTCA	XM_586711	137
MRPL4	F: GGCTCAAGACCTTCAACCTG R: GCGTGTAACGTGAGTCATGC	BC108102	138

regulating angiogenesis in cancer [38, 39]. There is increasing evidence that galectins are involved in the regulation of cell surface expression and activity of VEGFRs, especially for VEGFR-2 in endothelial cells [40, 41]. On the other hand, neuropilin (NRP)-1, which is a neuronal receptor [42], functions as a coreceptor of VEGFRs in endothelial cells [43]. However, the relationships among galectin-1, VEGFR-2 and NRP-1 in the CL are unclear. Here, we attempted to better understand the physiological functions of galectin-1 and VEGFR-2 in the bovine CL. Specifically, we investigated the expression of galectin-1, VEGFR-2, VEGFA and NRP-1 in the bovine CL throughout the estrous cycle and the effects of galectin-1 on the viability, progesterone production and VEGFR-2, VEGFA and NRP-1 mRNA expression in cultured LSCs. We also examined whether the effect of galectin-1 is regulated via its binding to VEGFR-2 or NRP-1 in a carbohydrate-dependent manner.

Materials and Methods

Collection of bovine CLs

Ovaries were collected from Holstein cows at a local slaughterhouse within 10–20 min after exsanguinations. The stage of the estrous cycle was defined as described previously [44]. 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 according to their morphology. After determination of these stages, CL tissues were immediately separated from the ovaries, frozen rapidly in liquid nitrogen, and then stored at –80 C until processing for studies of mRNA and protein expression. For histological analysis, the CLs were immersed in formalin for 24 h, dehydrated with ethanol, and embedded in paraffin according to the conventional method. For cell culture, ovaries with CLs at the mid luteal stage were submerged in ice-cold physiological saline and transported to the laboratory.

Reverse transcription (RT) and quantitative PCR

Total RNA was extracted from the CL tissues and cultured LSCs using TRIsure (BIO-38032; BIOLINE, London, UK) according to the manufacturer's directions. Total RNA (1 μg) was reverse transcribed using a ThermoScript™ RT-PCR System (11146-016; Invitrogen, Carlsbad, CA, USA). The primers used for quantitative PCR are listed in Table 1. Quantification of mRNA expression was performed with a QuantiTect™ SYBR Green PCR system (Qiagen, Hilden, Germany) starting with 2 ng of reverse-transcribed total RNA. For quantification of the mRNA expression levels, 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. Use of the QuantiTect™ SYBR Green PCR system at elevated temperatures resulted in reliable and sensitive quantification of the RT-PCR products with high linearity (Pearson correlation coefficient: $r > 0.99$). *MRPL4* mRNA expression was used as an internal control as described previously [27], and the expression of each gene was evaluated on the basis of the *MRPL4* mRNA expression in the individual samples.

Immunohistochemistry

Five micrometer sections were cut from the paraffin-embedded bovine CLs, dewaxed and washed in phosphate-buffered saline (PBS). 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, Burlingame, CA, USA) for 15 min for each reagent. Then the sections were incubated with 10% normal donkey serum for 60 min at room temperature followed by goat anti-human galectin-1 antibody (1:1000; AF1152, R&D Systems, Minneapolis, MN, USA) at 4 C overnight. Control sections were incubated with PBS. After washing twice in PBS, the sections were incubated with biotinylated anti-goat IgG (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 60 min at room temperature. The reaction sites were visualized using a Vectastain ABC Elite kit (Vector Laboratories) for 60 min at room temperature and ImmPACT® DAB (3, 3'-diaminobenzidine) Peroxidase Substrate Kit (Vector Laboratories) for 5 min. The sections were counterstained for 2 min with hemotoxylin and observed under a light microscope (BX51, Olympus, Tokyo, Japan).

Protein fractionation and extraction

CL tissues for western blotting were homogenized on ice in homogenization buffer (300 mM sucrose, 32.5 mM Tris-HCl, 2 mM EDTA, pH 7.4) by a tissue homogenizer (Physcotron, NS-50; Microtec, 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 × *g* for 5 min. The resultant supernatant was fractionated into membrane and cytoplasmic cell fractions by centrifugation at 100,000 × *g* for 60 min. Protein concentrations in the lysates were determined by using bovine serum albumin (BSA; 23209; Life Technologies, Grand Island, NY, USA.) as a standard. 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 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 β-actin (A2228; Sigma-Aldrich, St. Louis, MO, USA) as a cytoplasm marker (data not shown).

Cell isolation and culture

CLs classified in the mid luteal stage were collected for cell culture. Luteal tissue was enzymatically dissociated, and LSCs were cultured as described previously [45]. Dissociated LSCs from CLs were pooled and suspended in a culture medium, Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 medium (D/F; 1:1 [vol/vol]; D8900; Sigma-Aldrich) containing 5% calf serum (16170–078; Life Technologies) and 20 μg/ml gentamicin (G1397; Sigma-Aldrich). Cell viability was greater than 85% as assessed by trypan blue exclusion. The cells in the cell suspension consisted of about 70% small LSCs, 20% large LSCs, 10% endothelial cells or fibrocytes, and no erythrocytes. Thus they mainly consisted of LSCs.

Dispersed LSCs (2.0×10^5 /ml) were cultured in D/F medium containing 5% calf serum in 10 cm^2 culture dishes (664160; Greiner Bio-One, Frickenhausen, Germany) for determination of galectin-1 and VEGFR-2 protein expressions and in 96-well culture dishes (3860–096; Iwaki, Chiba, Japan) for determination of cell viability. After 24 h of culture, the medium was replaced with phenol red-free D/F medium (D2906; Sigma-Aldrich) containing 0.1% BSA, 5 ng/ml sodium selenite (S5261; Sigma-Aldrich), and 5 μg/ml holo-transferrin (T3400; Sigma-Aldrich), and the following experiments were carried out.

SDS-PAGE and protein staining

The membrane fraction of the CLs (30 μg protein) was subjected to SDS-PAGE. Electrophoresis was carried out at a constant current of 25 mA for 1 h. Staining and destaining were carried out by the standard Coomassie Brilliant Blue (CBB) staining procedure in order to show a similar distribution and intensity of bands for the membrane fraction.

Western blotting

Cultured LSCs or the membrane fraction of the CLs (30 μg protein) were separated on SDS-PAGE as described above and then transferred to a PVDF membrane (RPN303F; GE Healthcare, Little Chalfont, UK). The membrane was washed in TBS-T (25 mM Tris-HCl, 137 mM NaCl, pH 7.5, containing 0.1% Tween 20), incubated in blocking buffer (5% nonfat dry milk in TBS-T) for 1 h at room temperature and reacted with galectin-1 antibody diluted at 1:4000, VEGFR-2 antibody diluted at 1:500 (ab39256; Abcam), TNFR1 antibody diluted at 1:1000 or β-actin antibody diluted at 1:10000 overnight at 4 C. After washing with TBS-T, the membrane was incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). 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. The intensity of the immunological reaction on the membranes was estimated by measuring the optical density in the defined area by computerized densitometry using Image Lab Software version 4.0 (Bio-Rad, Hercules, CA, USA).

Effects of galectin-1 on cell viability and progesterone secretion in cultured LSCs

Cultured LSCs were exposed to recombinant galectin-1 (10–1000 ng/ml; 1152-GA-050/CF; R&D Systems) in phenol red-free D/F medium containing 0.1% BSA, 5 ng/ml sodium selenite, and 5 μg/ml holo-transferrin for 24 h of culture. The cell viability was determined with a Dojindo Cell Counting Kit including WST-1 (345-06463; Dojindo, Kumamoto, Japan). Briefly, 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 collected for progesterone determination and replaced with 100 μl phenol red-free D/F medium-BSA, and a 10 μl 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 680; Bio-Rad). In this assay, data were expressed as percentages of the appropriate control values. The concentrations of progesterone in the culture medium diluted at 1:100 were assayed using a direct enzyme immunoassay (EIA) according to the method described previously [46]. The progesterone standard curve ranged from 0.39 to 25 ng/ml.

Lactose inhibition assay

To investigate whether galectin-1 interacts with proteins on the plasma membrane in a carbohydrate-dependent manner, after 24 h of culture in D/F medium containing 5% calf serum, cultured LSCs were treated for 24 h with a noncompeting saccharide, 0.1 M sucrose (Suc) and a competing saccharide, 0.1 M β-lactose (Lac). After treatment, LSCs were collected, and subsequently analyzed by western blotting.

Immunoprecipitation of VEGFR-2 and lectin blot

The membrane fractions of CLs at different stages were lysed in cold 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). The lysates (0.5 mg of protein) were incubated with anti-VEGFR-2 antibody and protein G beads (sc-2002; Santa Cruz Biotechnology, Dallas TX, USA) at 4 C overnight while rocking. The beads were washed with lysis buffer three times, and the same amounts of immunoprecipitated VEGFR-2 proteins 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 overnight at 4 C with the VEGFR-2 antibody diluted at 1:500. After washing with TBS-T, the membrane was incubated with the appropriate secondary antibodies conjugated to HRP. After washing again with TBS-T, the reactive protein bands were detected as described above. Subsequently, the membrane was stripped with Restore Plus Western Blot Stripping Buffer (46430; Thermo Scientific, Waltham, MA, USA) and reprobed with 1.0 μg/ml of HRP-conjugated plant lectin, *Datura stramonium* (DSA) (J405; J-OIL MILLS, Tokyo, Japan), in TBS-T for 1 h. After washing with

TBS-T, the reactive protein bands were detected as described above.

Galectin-1 overlay assay

Recombinant galectin-1 was labeled with biotin using an EasyLink Biotin Conjugation Kit (ab102865; Abcam), according to the manufacturer's instructions. Proteins immunoprecipitated with anti-VEGFR-2 or NRP-1 antibody as described above were used for the galectin-1 overlay assay. Some immunoprecipitated VEGFR-2 proteins from the lysates for the mid CL were digested with 25 U/ml of PNGase F (1365169; Roche) according to the manufacturer's instructions to release asparagine-linked glycans (*N*-glycans) from glycoproteins.

Immunoprecipitated VEGFR-2 proteins with or without PNGase F treatment were separated by SDS-PAGE and subsequently transferred to a PVDF membrane. The early CL was used as a negative control. The membrane was blocked with 5% BSA in TBS-T and then exposed to biotin-conjugated recombinant galectin-1 diluted at 1:300 for 2 h at room temperature. After washing with TBS-T, the membrane was incubated with HRP-conjugated streptavidin, and the reactive protein bands were detected as described above.

Effect of galectin-1 through the VEGFR-2 pathway in cultured LSCs

To test whether VEGFR-2 participates in the cell survival effect of galectin-1 on cultured LSCs, LSCs were incubated with VEGFR-2 kinase inhibitor I (676480; Calbiochem, San Diego, CA, USA, 70–280 nM) with or without galectin-1 (1000 ng/ml) for 24 h. The inhibitor is highly selective for the receptor tyrosine kinase (RTK) of VEGFR-2 (IC50 = 70 nM) and does not inhibit platelet-derived growth factor receptor, epidermal growth factor receptor and insulin-like growth factor-1 RTK activities [47]. At the end of the incubation, a cell viability assay was performed with WST-1 as described above.

Statistical analysis

All experimental data are shown as the mean ± SEM. The statistical significances of differences were assessed by analysis of one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test or the Student's *t*-test using the GraphPad Prism ver 6.0 software. The statistical analysis of results obtained from each experiment is described in the figure legends. In all analyses, a value of $P < 0.05$ was considered significant.

Results

Expression and localization of galectin-1 in the bovine CL

The expression of galectin-1 protein in the membrane fraction of the CL was higher at the mid and late luteal stages than at the early and regressed luteal stages (Fig. 1A and B), whereas the distributions and band intensities of proteins in the membrane fraction of the CL during the estrous cycle were similar (data not shown). Immunohistochemically, galectin-1 was mainly localized in large LSCs, and galectin-1 positive large LSCs were increased in number at the mid and late luteal stages (Fig. 1C).

Effects of galectin-1 on cell viability, progesterone secretion and VEGFR-2, VEGFA and NRP-1 mRNA expression in cultured LSCs

Although the viability of non-treated LSCs used as control was significantly decreased during culture (data not shown), galectin-1 (10, 100 and 1000 ng/ml) significantly increased the viability of LSCs compared with the control (Fig. 2A). Galectin-1 did not change progesterone production by cultured LSCs (Fig. 2B).

Galectin-1 (1000 ng/ml) significantly increased *VEGFR-2* and *NRP-1* but not *VEGFA* mRNA expression in the LSCs (Fig 2C, D and E).

Effect of saccharides on the expression of galectin-1 and viability in cultured LSCs

To determine whether the galectin-1-glycan interaction is essential for the localization of galectin-1 on the surface of cultured bovine LSCs, we treated cells with a noncompeting saccharide (sucrose) or a competing saccharide (β-lactose) and examined the galectin-1 expression in the membrane fraction of cultured LSCs. Galectin-1 was not detectable in cultured LSCs treated with β-lactose as revealed by western blot, whereas it was detected in the LSCs treated with sucrose (Fig. 3A), indicating that galectin-1 is expressed on the surface of cultured LSCs in a carbohydrate-dependent manner. Furthermore, β-lactose significantly decreased the viability of cultured LSCs compared with both the non-treated control LSCs (Fig. 3B; $P < 0.01$) and sucrose-treated LSCs (Fig. 3B; $P < 0.05$), suggesting that galectin-1 binding to the glycans on the cell surface has a positive effect on the viability of LSCs.

Interaction between galectin-1 and VEGFR-2 or NRP-1

VEGFR-2 mRNA was expressed in the CL throughout the estrous cycle without any significant differences (Fig. 4A), whereas VEGFR-2 protein expression in the membrane fraction of the CL was higher at the mid luteal stage than at the early and regressed luteal stages (Fig. 4B and C). To examine whether VEGFR-2 is glycosylated or not, we carried out lectin blot analysis using DSA plant lectin, which specifically binds to multi-antennary oligosaccharides [48]. DSA lectin blot analysis using immunoprecipitated VEGFR-2 protein from the bovine CL demonstrated that VEGFR-2 possessed multi-antennary oligosaccharides, which could be recognized by galectin-1, and that the glycosylation status of VEGFR-2 did not change during the developing, mid, and late luteal stages (Fig. 4D).

To determine whether galectin-1 directly binds to VEGFR-2 in the CL, we performed a galectin-1 overlay assay. Binding of galectin-1 to immunoprecipitated VEGFR-2 was found only in the CL at the mid luteal stage (Fig. 5A). Furthermore, we analyzed whether the binding of galectin-1 and VEGFR-2 in mid CL is dependent on carbohydrate recognition by galectin-1. Deglycosylation of immunoprecipitated VEGFR-2 by pretreatment with PNGase F, which removes *N*-glycans from glycoproteins, reduced its molecular weight and the binding of galectin-1 to VEGFR-2 compared with the control (Fig. 5B). These results indicate that galectin-1 can bind to *N*-glycans on VEGFR-2.

While *NRP-1* mRNA was expressed in the CL throughout the estrous cycle (Fig. 1E), binding of galectin-1 and NRP-1 was not detected in an overlay assay (Fig. 5C).

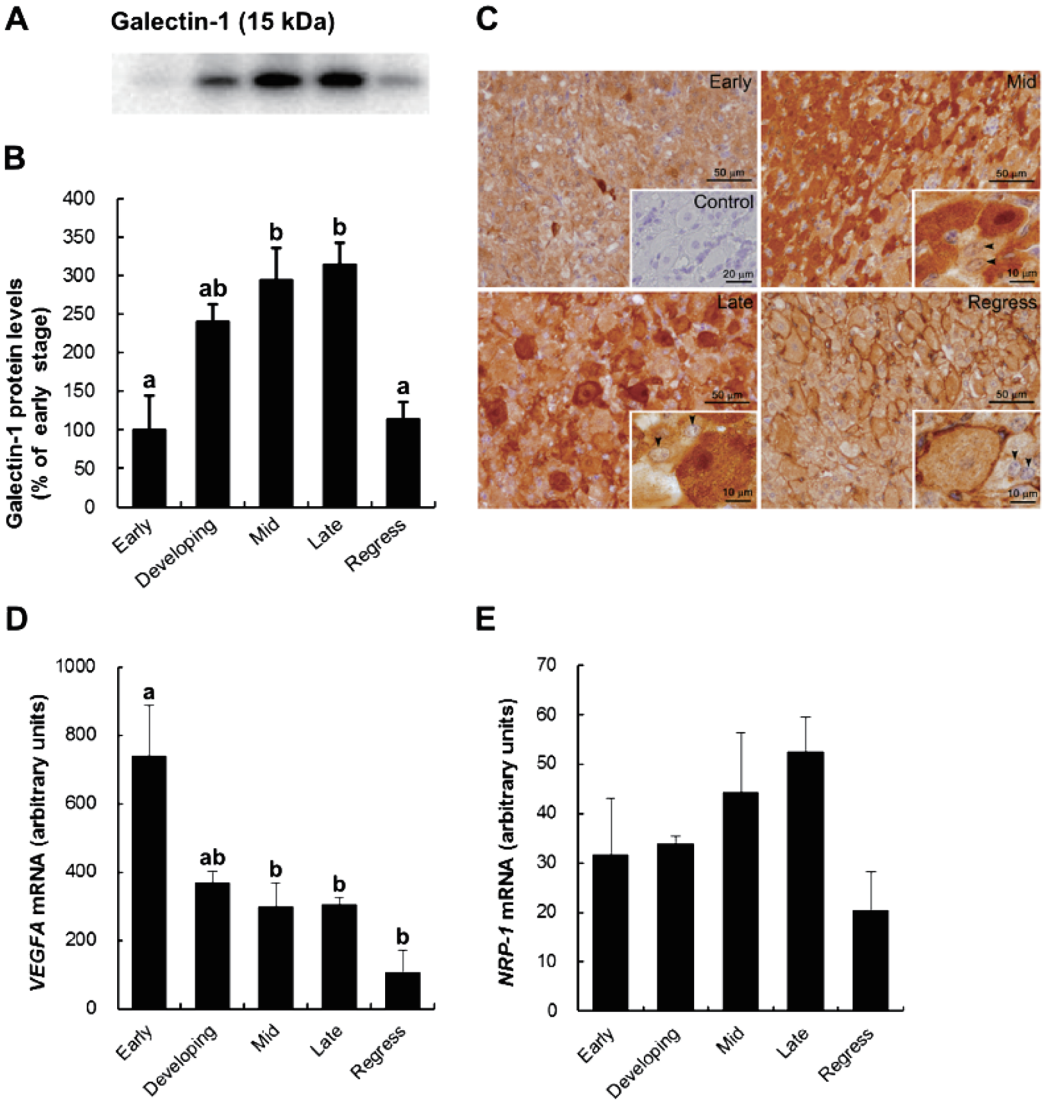

Fig. 1. Galectin-1 protein and *VEGFA* and *NRP-1* mRNA expression in the bovine CL throughout the estrous cycle. (A) Representative western blot bands and (B) densitometrically analyzed western blot results showing galectin-1 protein expression in the membrane fraction of the bovine CL throughout the estrous cycle (early, developing, mid, late and regressed [regress] luteal stages, days 2–3, days 5–6, days 8–12, days 15–17 and days 19–21, respectively). Data are the mean ± SEM for four samples of each stage and are expressed as the ratio of galectin-1 protein relative to that in the early stage. (C) Representative immunohistochemical images of the localization of galectin-1 protein in the bovine CL throughout the estrous cycle. Galectin-1 is localized in the cytoplasm or cell membrane of large luteal steroidogenic cells. Black arrowheads in the inserts indicate small luteal cells negative for galectin-1 immunoreaction. The insert in the image for the early luteal stage is an image of a control section in which the primary antibody was omitted. (D and E) Change in the relative amounts of *VEGFA* and *NRP-1* mRNA expressions, respectively. Comparison of the relative amounts of *VEGFA* and *NRP-1* mRNA determined by quantitative RT-PCR in the bovine CL tissue throughout the estrous cycle are shown. Data are the mean ± SEM for four samples of each stage and are expressed as the relative ratio of *VEGFA* or *NRP-1* mRNA to *MRPL4* mRNA. Different superscript letters indicate significant differences ($P < 0.05$), as determined by a one-way ANOVA followed by *Tukey's multiple comparison* test.

Inhibition of VEGFR-2 signaling reduced galectin-1-promoted cell viability in cultured LSCs

We analyzed whether the effect of galectin-1 on the viability of LSCs is through the binding to VEGFR-2. Although the viability of cultured LSCs was not affected by a VEGFR-2 kinase inhibitor alone, the increased cell viability caused by galectin-1 was suppressed by addition of a selective VEGFR-2 kinase inhibitor (Fig. 6; $P < 0.05$), suggesting that galectin-1 increased the cell viability through the VEGF signal pathway.

Discussion

Galectins are intra- and extracellularly distributed carbohydrate-binding proteins, and galectin-1 modulates a variety of cell functions [21–24]. In the mouse, both galectin-1 and galectin-3 are expressed in the regressing CL, which suggests that they are involved in luteolysis

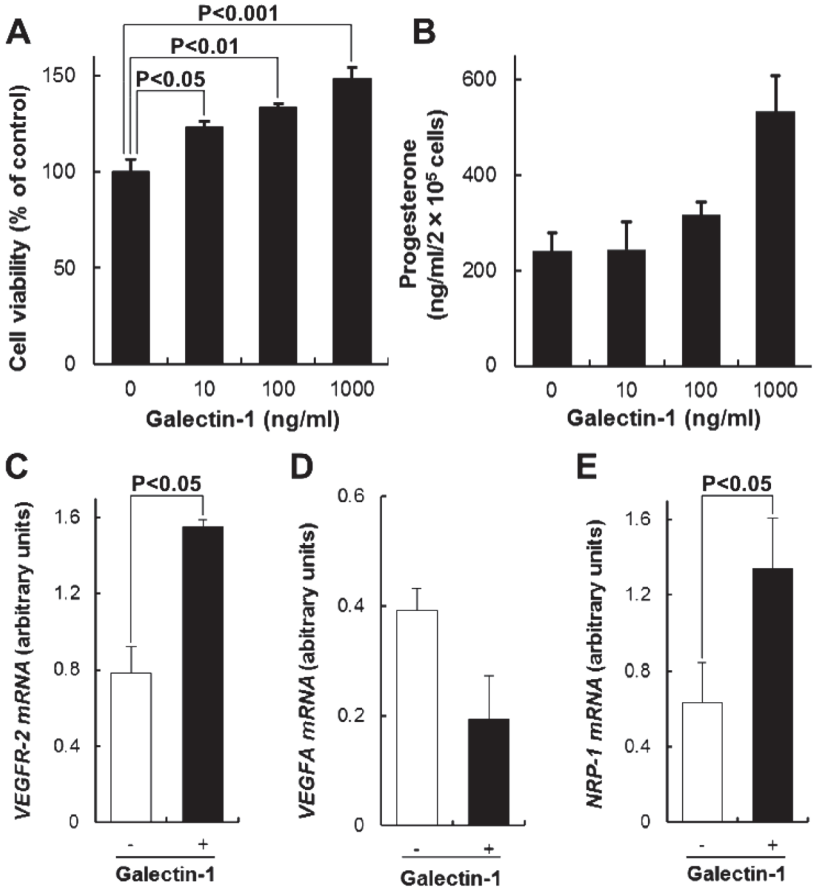

Fig. 2. Effect of galectin-1 on cell viability, progesterone production and *VEGFR-2*, *VEGFA* and *NRP-1* mRNA expression in cultured LSCs. (A and B) The cells were treated with recombinant galectin-1 (0, 10, 100, 1000 ng/ml) for 24 h. (A) Cell viability determined by WST-1 assay. Data are expressed as a percentage of control. (B) Progesterone concentration measured by EIA assay. The data for Fig. 2 A and B were statistically analyzed using a one-way ANOVA followed by Dunnett's multiple comparison test. Results represent means ± SEM of more than four separate experiments, each performed in triplicate. (C, D and E) The cells were treated with recombinant galectin-1 (1000 ng/ml) for 24 h. Comparisons of the relative amounts of *VEGFR-2* (C), *VEGFA* (D) and *NRP-1* (E) mRNA determined by quantitative RT-PCR in cultured cells are shown. Results represent means ± SEM of three separate experiments, each performed in duplicate, and are expressed as the relative ratio of *VEGFR-2*, *VEGFA* or *NRP-1* mRNA to *MRPL4* mRNA. The data were statistically analyzed using the Student's *t*-test.

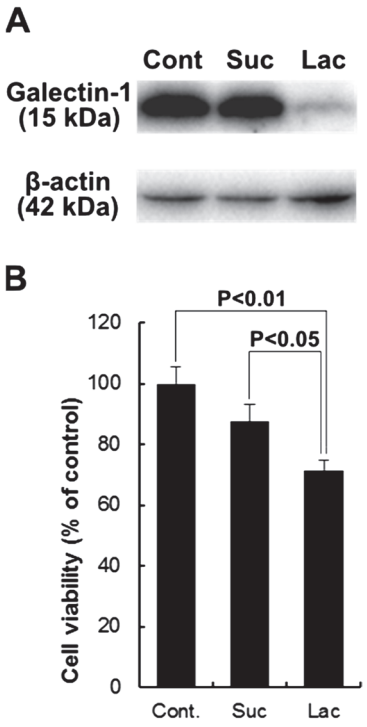

Fig. 3. Effects of saccharides on the expression of galectin-1 and viability in cultured LSCs. The cells were treated for 24 h with a noncompeting saccharide, 0.1 M sucrose (Suc), or a competing saccharide, 0.1 M β-lactose (Lac). (A) Localization of galectin-1 protein in cultured mid LSCs. Galectin-1 protein expression was assessed by western blotting analysis. Representative western blot bands for galectin-1 and β-actin are shown. This experiment was repeated three times. (B) Cell viability determined by WST-1 assay. Data are expressed as a percentage of the control (Cont). Results represent means ± SEM of more than six separate experiments, each performed in triplicate. The data were statistically analyzed using the Student's *t*-test.

[49]. A possible luteolytic role of galectin-3 in the bovine CL was recently reported by us [27]. On the other hand, another group reported the differential expression of galectin-1 and galectin-3 in the CL in women: galectin-1 was expressed in the healthy functional CL, while galectin-3 increased during luteolysis [26]. The expression and localization of galectin-1 in the functional CL of cows revealed in this study (Fig. 1) largely corresponded to its expression and localization in women, which raises the possibility that galectin-1 has a luteotrophic function in the CL of cows as well as women. However, the exact role of galectin-1and identification of its ligand glycoproteins require further investigation.

Galectin-1 has been shown to affect cell proliferation and survival in different cell types [24, 50, 51]. The mean galectin-1 level in serum of healthy females was approximately 100 ng/ml [52]. However, since interactions of galectins with glycoproteins and glycolipids form a complex lattice at the cell surface [53], the concentrations of galectin-1 is thought to be higher on the cell surface than in serum due to the enrichment effect of galectin-1 caused by lattice formation. In the present study, galectin-1 (10, 100 and 1000 ng/ml) significantly increased the viability of cultured LSCs, but it did not affect P4 production by cultured LSCs (Fig. 2A and B), and its expression was higher in the functional CL than the regressing CL (Fig. 1A). Based on the above findings, galectin-1 may contribute to luteoprotective roles in the bovine CL.

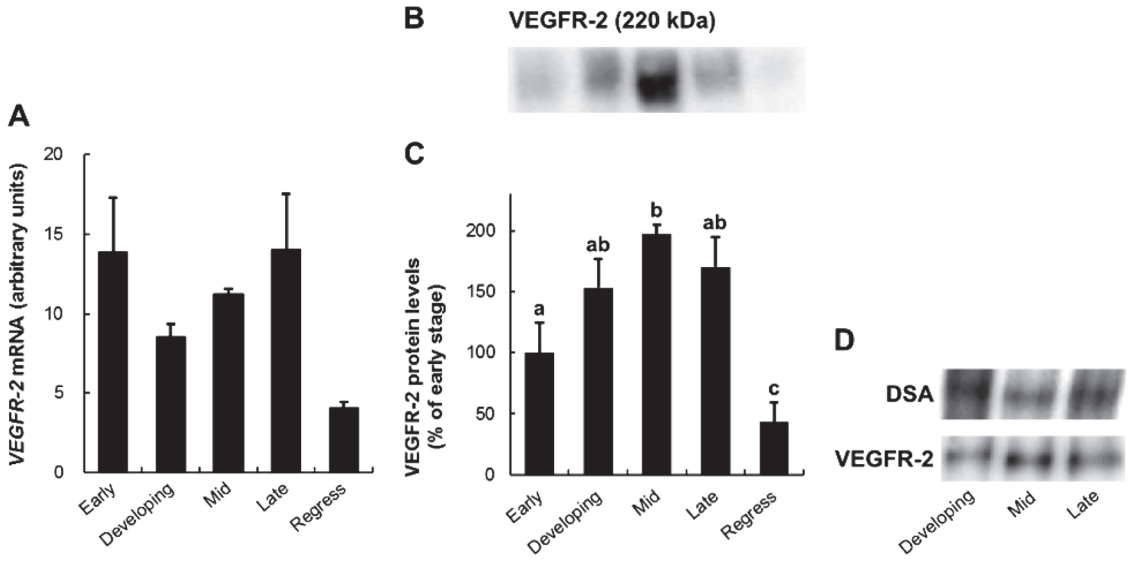

Fig. 4. Expression and glycosylation status of VEGFR-2 in the bovine CL. (A) Comparison of relative amounts of *VEGFR-2* mRNA determined by quantitative RT-PCR in bovine CL tissue throughout the estrous cycle. Data are the mean ± SEM for three or four samples of each stage and are expressed as the relative ratio of *VEGFR-2* mRNA to *MRPL4* mRNA. (B) Representative western blot bands and (C) densitometrically analyzed western blot results for VEGFR-2 proteins in the membrane fraction of the bovine CL tissue during different luteal phases. Data are the mean ± SEM for six samples of each stage and are expressed as the ratio of VEGFR-2 protein relative to that in the early stage. Different superscript letters indicate significant differences ($P < 0.05$), as determined by a one-way ANOVA followed by Tukey's multiple comparison test. (D) DSA lectin blot analysis of glycans on VEGFR-2. Purified VEGFR-2 proteins were subjected to blotting analysis and probed with either VEGFR-2 antibody or plant lectin (DSA for detecting oligomers of GlcNAc or Galβ1-4GlcNAc). The images are representative of at least three experiments.

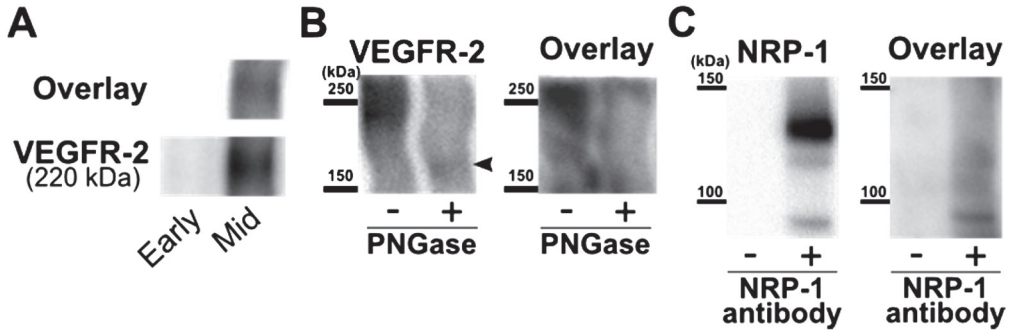

Fig. 5. Galectin-1 overlay assay to reveal the binding of galectin-1 to the glycans on VEGFR-2 and NRP-1. Immunoprecipitated VEGFR-2 or NRP-1 proteins from the membrane fraction of the bovine CL were subjected to blotting analysis and probed with either VEGFR-2 or NRP-1 antibody or biotinylated galectin-1. (A) VEGFR-2 expression and galectin-1 binding in the bovine CL tissue at the early and mid luteal stages. Positive bands representing galectin-1 binding to VEGFR-2 are observed only in the CL at the mid luteal stage at a molecular weight of approximately 220 kDa. In the early CL, VEGFR-2 protein is not expressed, and no binding of biotinylated galectin-1 is observed. (B) Effect of PNGase F treatment. Purified VEGFR-2 proteins were treated with PNGase F to remove *N*-glycans. Deglycosylated VEGFR-2 shows a decreased molecular weight of approximately 160 kDa (arrowhead in B) compared with VEGFR-2 without PNGase F treatment. Galectin-1 binding to VEGFR-2 protein disappears as a result of PNGase F treatment. (C) Immunoprecipitations were carried out with (+) or without (–) the NRP-1 antibody. With the NRP-1 antibody, the NRP-1 protein band is observed, but no binding of biotinylated galectin-1 is observed. All of the experiments were repeated more than three times.

In the present study, we revealed that galectin-1 is localized on the surface of LSCs in a carbohydrate-dependent manner and that removal of galectin-1 from the cell surface reduced the viability of cultured LSCs (Fig. 3). These findings, together with the finding that galectin interactions with glycans on cell surface proteins play some important roles in signaling and cell-cell junction formations [54–56], suggest that the binding of galectin-1 to cell surface glycosylated receptors such as VEGFR-2 is involved in the luteotrophic function of galectin-1. Interestingly, the enhancement of cell viability by galectin-1 was suppressed by addition of a selective inhibitor of VEGFR-2 in cultured LSCs (Fig. 6). This and the finding that galectin-1 upregulates VEGFR signaling in trophoblast tumor cells

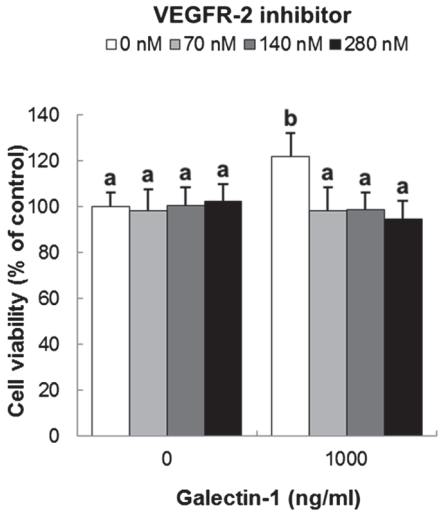

Fig. 6. Effect of a selective VEGFR-2 inhibitor on the galectin-1-promoted viability of LSCs. The cells were treated with recombinant galectin-1 (1000 ng/ml) and/or a selective VEGFR-2 kinase inhibitor (0, 70, 140, 280 nM) for 24 h. Cell viability was determined by WST-1 assay. Data are expressed as a percentage of non-treated control cells. Results represent means ± SEM of more than eight separate experiments, each performed in triplicate. Different superscript letters indicate significant differences ($P < 0.05$), as determined by a one-way ANOVA followed by Dunnett's multiple comparison test.

[57] suggest that VEGFR-2 is involved in signaling in the luteotrophic action of galectin-1.

VEGF and *VEGFR-2* mRNAs are expressed in the bovine CL [30], and LSCs need the VEGF system to maintain luteal functions such as progesterone production [33–35]. In the present study, VEGFR-2 protein, like galectin-1, was intensely expressed in the functional bovine CL at the mid luteal stage, and VEGFR-2 was modified by glycans that can be recognized by galectin-1 (Fig. 4D). We also confirmed that galectin-1 directly binds to *N*-glycans on VEGFR-2 in the bovine CL by galectin-1 overlay assay (Fig. 5). Thus the direct binding of galectin-1 to *N*-glycans on VEGFR-2 may be involved in the galectin-1-promoted viability of LSCs. However, galectin-1 did not immediately affect phosphorylation of ERK1/2, a component of VEGF signaling (data not shown). The increased viability of LSCs treated with galectin-1 may be attributed to a gentle induction of VEGF signaling by a prolonged effect by galectin-1, since glycan-galectin interactions are generally weaker than protein-protein interactions such as receptor-ligand and adhesion molecule interactions [58]. Although galectin-1 interacts with various glycoproteins on the cell surface [59], VEGFR-2 may be one of the target glycoproteins of galectin-1 in the bovine CL.

In the present study, *VEGFR-2* mRNA was expressed in the CL throughout the estrous cycle without any significant changes (Fig. 4A), whereas VEGFR-2 protein expression was highest at the mid luteal stage (Fig. 4B and C). Furthermore, there was no significant difference in the expression of *VEGFA* mRNA in the CL throughout the estrous cycle, excluding the early stage (Fig. 1D). These findings, together with the finding that the concentrations of VEGF protein in the CL tissue changes little during the luteal stages [30], suggest that the regulation of luteal function depends on VEGFR-2 protein expression. The expression of a protein may differ from the expression of its mRNA as a result of posttranslational modifications such as glycosylation. Since VEGFR is glycosylated [37], the protein expression and stability of VEGFR-2 might be affected by the posttranslational glycosylation. Since glycosylation of receptors is involved in cell surface retention of various receptors through interaction with galectins [20], VEGFR-2 localization on the cell surface may be regulated through interaction between its glycans and galectins. The protein expression of VEGFR-2 paralleled that of galectin-1 in the bovine CL throughout the estrous cycle. The expressions of both proteins were quite low in the early luteal stage, while they were intense in the mid luteal stage. Since galectin-1 increases the retention of various proteins at the cell surface [60, 61], the binding of galectin-1 to VEGFR-2 may be required for the cell surface expression of VEGFR-2 in bovine LSCs. Additionally, galectin-1 increased *VEGFR-2* mRNA expression in the LSCs, but *VEGFA* mRNA was not altered by galectin-1 (Fig. 2C and D), suggesting that galectin-1 increases VEGFR-2 protein expression as well as its abundance on the cell surface.

It is possible that galectin-1 binds to other membrane receptors such as epidermal growth factor receptor (EGFR), luteinizing hormone receptor (LHR), prostaglandin $F_{2\alpha}$ receptor (PGFR) and NRP-1 which are modified by glycans [62–65], and expresses in the bovine CL [66–68] or granulosa cells [69]. We observed that EGFR protein expression was highest in the early CL and did not correlate with the expression pattern of galectin-1 protein (data not shown). Furthermore, because $PGF_{2\alpha}$ and LH were not added to the culture medium in the present study, it is apparent that the increases in viability caused by galectin-1 in LSCs were not mediated by PGFR or LHR. NRP-1 is a neuronal receptor that mediates axonal guidance in the nervous system [42] and functions in endothelial cells as a coreceptor of VEGFRs [43]. To access whether galectin-1 acts by binding to NRP-1, we analyzed the NRP-1 expression in the bovine CL throughout the estrous cycle and LSCs treated with galectin-1 and the interaction between galectin-1 and NRP-1. NRP-1 was expressed in the CL throughout the estrous cycle (Fig. 1E). However, binding of galectin-1 and NRP-1 was not detected by overlay assay (Fig. 5C). Thus, we concluded that the major target of galectin-1 could be VEGFR-2 but not NRP-1 in the CL. Because the *NRP-1* mRNA expression was increased by treatment with galectin-1 in LSCs (Fig. 2E), the viability promoted by galectin-1 might be partially due to an increase of NRP-1 expression in LSCs. Although further studies are required to determine whether galectin-1 can bind to other cell surface proteins in the CL, we consider that VEGFR-2 is one of the target glycoproteins in the bovine CL.

In conclusion, this study revealed that galectin-1 is expressed and localized in the functional bovine CL. Extracellular galectin-1 helps to promote cell viability in cultured bovine LSCs, suggesting that galectin-1 has a luteotrophic function in the bovine CL. Our results show that galectin-1 directly binds to *N*-glycans on VEGFR-2 in the CL. They also suggest that galectin-1 enhances luteal cell viability through a VEGF signaling pathway, possibly by regulating the expression of VEGFR-2 in LSCs.

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