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Effect of early pregnancy on the expression of progesterone receptor and progesterone-induced blocking factor in ovine lymph node

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ABSTRACT

Lymph nodes are the sites where the immune reaction or suppression takes place. Progesterone (P4) exerts an essential effect of the immunomodulation on the maternal uterus during early pregnancy in ruminants. At present study, the inguinal lymph nodes were obtained at day 16 of non-pregnancy, days 13, 16 and 25 of pregnancy (n = 3 for each group) in ewes, and RT-PCR assay, western blot and immunohistochemistry analysis were used to analyze to the effect of early pregnancy on the expression of P4 receptor (PGR) and progesterone-induced blocking factor (PIBF) in the lymph nodes. Our results showed that the PGR and PIBF mRNA were up-regulated in the lymph nodes in pregnant ewes, and the PGR isoform (60 kDa) and the PIBF variant (75 kDa) were expressed constantly in the lymph nodes. However, there was no expression of the PGR isoform (40 kDa) and the PIBF variant (48 kDa) at day 16 of the estrous cycle. The immunohistochemistry results confirmed that the PGR and PIBF proteins were limited to the subcapsular sinus and trabeculae in the cortex, medullary sinuses, and were localized in the cytoplasm of the specific cells. This paper reports for the first time that early pregnancy exerts its effect on the specific cells in the lymph nodes through P4, which results in the up-regulated expression of the PGR mRNA and 40 kDa isoform, the PIBF mRNA and 48 kDa variant, and is involved in the immunoregulation of the lymph nodes through a cytosolic pathway in ewes.

1. Introduction

During early pregnancy in ruminants, the elongating peri-implantation conceptus secretes the primary signal for maternal recognition of pregnancy, interferon tau (IFNT), which prolongs the lifespan of the corpus luteum (CL) [1–3]. Progesterone (P4) is an endogenous steroid, is primarily produced by the CL, and involved in the oestrous cycle, pregnancy, and mammal embryogenesis, and critical to the immunomodulation in mammals [4–6]. It is also reported that P4 regulates the blastocyst growth and conceptus elongation indirectly via the endometrium, which is essential for ovine conceptus survival and growth [7]. P4 regulates reproductive functions through binding to P4 receptors (PGR) which include nuclear PGR (PGR-A and PGR-B) and membrane PGR (mPGR) [8]. P4 binds to PGR, which results in the formation of new mRNA, and the mRNA is translated to the specific proteins by ribosomes. PGR is involved in the control of transcriptional network in the uterine endometrium [9]. Progesterone-induced blocking factor (PIBF) is a protein that plays a role in the immunologic and proliferative actions, is induced by P4 through intracellular PGR in U373 cells [10]. A characteristic feature of normal pregnancy is high concentration of PIBF in the urine, and PIBF is mostly secreted by activated maternal lymphocytes, which exerts an immunomodulatory function and contributes to the maintenance of pregnancy in women [11].

Lymph nodes are located all over the body in mammals, and the sites where the immune reaction or suppression takes place. Immune reactions include immune response to pathogenic antigens, harmless antigens and tolerance [12]. It has been demonstrated that pregnancy exerts remarkable effect on the female immune system through an endocrine style [13], and the weights of lumbar and renal lymph nodes, inguinal lymph nodes increase during pregnancy in mice [14]. The large pyronionphilic cell is related to the cell-mediated immunological reactions in regional lymphoid tissue. The large pyronionphilic cell that counts from iliac lymph nodes rises significantly in early pregnant rat, falls in late pregnancy, returns to virgin levels after delivery [15], which suggests that lymph nodes are implicated in the immunoregulation in early pregnant animals. However, it is unclear that early pregnancy

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exerts its effect on the expression of PGR and PIBF in inguinal lymph nodes during early pregnancy in sheep. At present study, the inguinal lymph nodes from non-pregnant and early pregnant ewes were sampled to study the expression of PGR and PIBF, which may be beneficial to understand the effects of early pregnancy on the immunomodulation of the lymph node in sheep.

2. Materials and methods

2.1. Animals and experimental design

Small Tail Han ewes approximately 18 months of age were housed at the farm of Handan Boyuan Animal Husbandry Co. Ltd. in China, and all procedures were approved by Hebei University of Engineering Animal Care and Use Committee. The ewes with normal oestrous cycles were observed daily for estrus using vasectomized rams, mated twice with intact rams at 12-h intervals after the detection of sexual receptivity. Twenty-four ewes were randomly divided into four groups (n = 6 for each group), and each group was ensured at least three normal pregnant ewes or normal non-pregnant ewes (n = 3 for each group) [16]. The day of coition was counted as day 0 of pregnancy or non-pregnancy. The ewes assigned to the non-pregnant group were not mated with intact ram. The inguinal lymph nodes were obtained from ewes on days 13, 16 and 25 of pregnancy, day 16 of non-pregnancy at the time of slaughter. Pregnancy was confirmed through observing the present of conceptus in the uterus, and the day 13 of pregnant ewes were identified through detecting the expression of interferon-stimulated gene 15 in the endometrium by western blot [17]. The transverse pieces of lymph node (0.3 cm^3) were fixed in fresh 4% (w/v) paraformaldehyde in PBS buffer (pH 7.4), and the remaining portions of lymph nodes were frozen in liquid nitrogen for subsequent quantitative Real Time PCR (qRT-PCR) and protein analysis.

2.2. RNA extraction and qRT-PCR assay

Samples were crushed into fine powders in liquid nitrogen, and the powders were digested in TRIzol, and the total RNA was extracted according to the manufacturer's instructions (Invitrogen, California, USA). The cDNA was synthesized with the FastQuant RT Kit (Tiangen Biotech Co., Ltd., Beijing), and the SuperReal PreMix Plus Kit (Tiangen Biotech Co., Ltd., Beijing) was employed for qRT-PCR. The primer sequences of PGR, PIBF and GAPDH were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Table 1). The $2^{-\Delta\Delta Ct}$ analysis method was used to calculate the relative expression values for the qRT-PCR assay, with GAPDH as the endogenous control [18]. The relative expression value was set as 1 for the group of day 16 of non-pregnancy.

2.3. Western blot

The total proteins of the lymph nodes were extracted by RIPA Lysis Buffer (Biosharp, BL504A). A BCA Protein Assay Kit (Tiangen Biotech Co., Ltd., Beijing) was used to measure the protein concentration with bovine serum albumin as the standard. Equal amounts of total proteins (10 μg/lane) were separated using 12% SDS-PAGE, and the proteins were transferred to 0.22 μm polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). PGR and PIBF were detected by western blot using the rabbit anti-PGR polyclonal antibody (Santa Cruz Biotechnology, Inc., sc-538, 1:1000) and rabbit anti-PIBF polyclonal antibody (Santa Cruz Biotechnology, Inc., sc-99129, 1:1000). Secondary goat anti-rabbit IgG-HRP (Biosharp, BL003A) was diluted to 1:2000. The Pro-light HRP chemiluminescence detection reagent (Tiangen Biotech Co., Ltd., Beijing) was used to detect the immunoreactive bands. Sample loading was monitored with the GAPDH antibody (Santa Cruz Biotechnology, Inc., sc-20357) at a dilution of 1:1000, and secondary goat anti-mouse IgG-HRP was diluted to 1:2000. The intensity of blots were quantified using Quantity One V452 (Bio-Rad Laboratories), and the relative levels were calculated using the internal control protein (GAPDH).

Table 1
The primer sequence for quantitative Real Time PCR.

Gene	Primer	Sequence	Product (bp)
PGR	Forward	CAACAGCAAACCTGATACCT	183
	Reverse	CCATCCTAGTCCAAATACCATT	
PIBF	Forward	CCAGGCAGCTAATTGAACGG	189
	Reverse	GGGCTAGTACCTGCTTCTGG	
GAPDH	Forward	GGGTCATCATCTCTGCACCT	176
	Reverse	GGTCATAAGTCCCTCCACGA	

2.4. Immunohistochemistry analysis

The fixed lymph samples were embedded in paraffin, and paraffin-embedded sections were deparaffinized in xylene and rehydrated in ethanol. The rehydrated sections were treated with 3% H_2O_2 to quench endogenous peroxidase activity, and reduced non-specific binding with 5% normal goat serum in PBS. Immunohistochemical localizations of PGR and PIBF in the lymph tissue were performed using the rabbit anti-PGR polyclonal antibody (1:100) and rabbit anti-PIBF polyclonal antibody (1:100), respectively. For a negative control, non-immune goat serum was used in place of primary antibody. DAB kit (Tiangen Biotech Co., Ltd., Beijing) was used to visualize the antibody binding sites in the tissue sections. Finally, the images were captured using a light microscope (Nikon Eclipse E800, Japan) and a digital camera AxioCam ERc 5s, and the intensity of staining and density of stained cells were analyzed through the images.

2.5. Statistical analyses

The data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System Package version 9.1 for Windows (SAS Institute, Cary, NC, USA). Experimental sample groups consisted of at least three biological replicates. Groups were considered significantly different at $P < 0.05$.

3. Results

3.1. Relative expression levels of PGR and PIBF mRNA in lymph nodes

The qRT-PCR assay revealed (Fig. 1) that the relative expression level of PGR mRNA was lower in the lymph nodes at day 16 of non-pregnancy than that at early pregnancy ($P < 0.05$), but there was no significant difference in the expression level of PGR mRNA among the lymph nodes from pregnant ewes ($P > 0.05$). Furthermore, the relative expression level of PIBF mRNA was almost similar to that of PGR mRNA. The relative expression level of PIBF mRNA was higher in the lymph nodes from early pregnant ewes compared to that from day 16 of non-pregnant ewes ($P < 0.05$).

3.2. Expression of PGR and PIBF proteins in lymph nodes

Western blot analysis revealed that the PGR proteins were expressed in the lymph nodes (Fig. 2), and the isoform of PGR with a molecular weight of approximately 60 kDa was expressed