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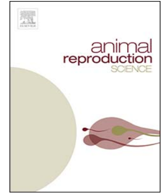

Pregnancy-associated changes in expression of progesterone receptor and progesterone-induced blocking factor genes in bone marrow of ewes

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ABSTRACT

Progesterone (P4) regulates reproductive and immune functions through binding to the progesterone receptor (PGR), and the effects of P4 are partly mediated by a progesterone-induced blocking factor (PIBF). Bone marrow (BM) is a key component of the lymphatic system and has an important role in immune response. In this study, BM was harvested from femurs on days 13, 16 and 25 of pregnancy and day 16 of the estrous cycles without mated by intact rams, and a qRT-PCR assay, Western blot and an immunohistochemistry analysis were used to analyze the expression of PGR and PIBF genes in BM. The results showed that there was an increase in relative abundance of *PGR* and *PIBF* mRNA in BM during early pregnancy, and PGR-B and the full-length PIBF genes were up-regulated in pregnant ewes. Immunohistochemistry results confirmed that the PGR and PIBF proteins were localized in both the cytoplasm and nuclei of adipocytes and the cells in the stroma and capillaries. This is the first study reporting an up-regulated expression of PGR-B and full-length PIBF genes in BM during early pregnancy in sheep. It is suggested that the conceptus exerted its effects on the adipocytes and the cells in the stroma and capillaries in BM, which were involved in the immunoregulation of BM through both cytosolic and nuclear pathways in ewes.

1. Introduction

As an endogenous steroid, progesterone (P4) is produced primarily by the corpus luteum (CL) and is important for preparing the endometrium for implantation in a normal estrous cycle. To maintain the pregnancy, P4 is also essential for immunomodulation in mammals (Brooks et al., 2014; Micks et al., 2015). A treatment with P4 via daily injections increases blastocyst diameter and the elongation of blastocysts to a filamentous conceptus during early pregnancy in sheep (Satterfield et al., 2006). The progesterone receptor (PGR) is induced by P4, and P4 binds to PGR. The complex of P4 and PGR then binds to DNA in the nucleus, resulting in the formation of new mRNA to produce specific proteins. The PGR includes nuclear PGR (PGR-A and PGR-B) and membrane PGRs (mPGRs) in the endometrium of cattle, and P4 binds to PGR to regulate the reproductive functions during the estrous cycle and the first trimester of pregnancy (Kowalik et al., 2013). There is a change in relative abundance of *PGR* mRNA and amount of protein in the endometrial luminal epithelium and the glandular epithelium in estrous cyclic and pregnant ewes (Spencer and Bazer, 1995). The effects of P4 are partly mediated by the progesterone-induced blocking factor (PIBF). The full-length PIBF at 90 kDa is associated with the nucleus, and the shorter splice variant of PIBF is secreted by activated cells (Lachmann et al., 2004) that have a role in cytokine synthesis, NK activity and arachidonic acid metabolism (Szekeres-Bartho and Polgar, 2010). The PIBF is induced by P4 through

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intracellular PGR in U373 cells and exerts a variety of effects on the immunologic and proliferative actions (González-Arenas et al., 2014).

The immunological interaction between the maternal and fetal systems is suppressed to inhibit the maternal immune rejection of the fetal semi-allograft during ruminant pregnancies (Hansen, 2011; Yang et al., 2014; Zhang et al., 2015). Bone marrow (BM) generates lymphocytes from immature hematopoietic progenitor cells, which is an important component of the lymphatic system. The numbers of pre/pro and immature B cells were gradually reduced in the BM, which resulted in a decreased influx of B cells in the blood and spleens of pregnant mice (Muzzio et al., 2014). The expression of PGR and PIBF genes in BM during early pregnancy in sheep is, however, unclear. In the present study, the BM from non-pregnant and early pregnant ewes were sampled to explore the expression of PGR and PIBF genes in ewes, which may be beneficial to increasing the understanding of how immune suppression is regulated by P4 during early pregnancy 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 the Handan Boyuan Animal Husbandry Co. Ltd. in China. The ewes were observed daily for estrus using vasectomized rams and mated twice with intact rams at 12 h intervals after the detection of sexual receptivity. The experimental protocol was approved by the Hebei University of Engineering Animal Ethical Committee, and humane animal care and handling procedures were followed throughout the experiment. The ewes were randomly divided into four groups ($n = 6$ for each group), and the ewes that were assigned to the non-pregnant group were not mated with intact rams. The day of detection of sexual receptivity was assigned as day 0 of pregnancy or non-pregnancy. The BM was harvested from femurs on days 13, 16 and 25 of pregnancy in ewes in which pregnancy was confirmed and day 16 of estrous cycle in non-pregnant ewes. Pregnancy was confirmed through observing the presence of conceptus in the uterus. Several sections of BM (0.3 cm^3) were fixed in fresh 4% (w/v) paraformaldehyde in PBS buffer (pH 7.4), and the remaining portions of BM were frozen in liquid nitrogen for subsequent quantitative Real Time PCR (qRT-PCR) and protein analysis.

2.2. RNA extraction and qRT-PCR assay

The TRIzol method was used to extract the total RNA from the BM samples, and FastQuant RT Kit (Tiangen Biotech Co., Ltd., Beijing) was used to synthesize the cDNA, and SuperReal PreMix Plus Kit (Tiangen Biotech Co., Ltd., Beijing) was used for qRT-PCR. The primer sequences of *PGR*, *PIBF* and *GAPDH* were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Table 1). The relative expression value was set as 1 in the BM (on day 16 of the estrous cycle) of the non-pregnant ewes.

2.3. Western blot

The total proteins of the BM samples were extracted by RIPA Lysis Buffer (Biosharp, BL504A), and the protein concentration was measured using a BCA Protein Assay Kit (Tiangen Biotech Co., Ltd., Beijing, PA115) with bovine serum albumin as the standard. An equal amount of total protein (10 μg/lane) was separated using 12% SDS-PAGE, and the proteins were transferred to 0.22 μm polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). 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) were used to detect PGR and PIBF proteins. Secondary goat anti-rabbit IgG-HRP (Biosharp, BL003A) was diluted to 1:5000. Pro-light HRP chemiluminescence detection reagent (Tiangen Biotech Co., Ltd., Beijing) was used to detect immunoreactive bands. Sample loading was monitored with the GAPDH antibody (Santa Cruz Biotechnology, Inc., sc-20357) at a dilution of 1:1000. The intensity of blots was quantified using Quantity One V452 (Bio-Rad Laboratories), and the relative abundances were calculated using the internal control protein (GAPDH).

2.4. Immunohistochemistry analysis

The fixed tissue samples were embedded in paraffin, and paraffin-embedded sections were deparaffinized in xylene and

Table 1
Primers used for qRT-PCR.

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

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