Production of Retinol-binding Protein by Caprine Conceptus during the Time Period of Maternal Recognition of Pregnancy*

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ABSTRACT : The purpose of the study were to characterize the proteins secreted by elongating caprine conceptus, to identify a group of low molecular weight proteins as retinol-binding protein (RBP), to identify RBP cell-specific localization in conceptus tissue, and to demonstrate that the conceptuses secreted continuously RBP during the time period maternal recognition of pregnancy. Caprine conceptuses were removed from the uterus between days 16 and 22 of pregnancy, the time period maternal recognition of pregnancy. Isolated conceptuses were cultured in a modified minimum essential medium in the presence of radiolabeled amino acids. Proteins synthesized and secreted into medium were analyzed by fluorography of two-dimensional polyacrylamide gel electrophoresis and fluorography. At least five proteins showed consistently a grouping of spots with characteristic location on two-dimensional gels. A major low molecular weight protein consisted of two major isoforms (pI 5.3-6.0) of similar molecular mass (21 kDa) was identified as RBP by using antiserum against RBP. Presence of RBP in conceptus culture medium and uterine flushings between days 16 and 22 of pregnancy were determined by immunoprecipitation and Western blotting using anti-RBP serum. In immunocytochemical study, strong immunostaining for RBP was localized in trophectoderm and endoderm of conceptus. These results clearly demonstrated that the caprine conceptus was active in protein synthesis as early as day 16 of pregnancy. Secretion of RBP by caprine conceptuses (days 16-22) coincident with the rapid transformation of the conceptus from a spherical blastocyst to a filamentous structure. Production of RBP by the elongating conceptuses may be indicative of an important role for conceptus RBP in the transport, availability and metabolism of retinol during maternal recognition of pregnancy. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 7 : 962-967)***

Key Words : Goat, Conceptus, Vitamin A

INTRODUCTION

In caprine, communication between the developing conceptus and maternal tissues is essential for a successful pregnancy. If there is no presence of conceptuses in the uterus, regression of the corpora lutea occurs at the end of the estrus. Gnatek et al. (1989) demonstrated that removal of conceptuses on days 13 and 15 after mating did not alter interestrus intervals. Removal of conceptuses on day 17 and times thereafter resulted in extension of interestrus intervals until approximately day 28 (Gnatek et al., 1989). The critical period for signaling by the conceptus to block luteolysis, to maintain luteal progesterone production, and to allow pregnancy to be established is called maternal recognition of pregnancy (Geisert and Malayer, 2000). The phenomenon of maternal recognition of pregnancy in the goats occurs between days 15-17 of pregnancy (Gnatek et al., 1989). Maternal recognition of pregnancy in caprine is mediated by conceptus secretory protein, caprine trophoblast protein-1 (cTP-1). The cTP-1 is a major secretory product of conceptuses from day 16 through day 21 of pregnancy (Gnatek et al., 1989). The cTP-1 has physical and immunological characteristics similar to bovine trophoblast protein-1 and ovine throphoblast-1. These trophoblast proteins have been classified as interferon-τ (Bartlol et al., 1985; Godkin et al., 1988a,b; Gnatek et al., 1989).

Besides the interferon-τ signal for maternal recognition of pregnancy, the signal for the rapid growth and differentiation of caprine conceptus has not been established. Vitamin A (retinol) and it metabolites, are potent regulators of cell differentiation (Wolf, 1984) that most likely play a role in early embryonic development. In plasma, retinol is transported by plasma retinon-binding protein (RBP) secreted by liver (Goodman, 1984). However, extrahepatic tissue sites of RBP have been identified in caprine. Recently, RBP was demonstrated to be a product of caprine extraembryonic membranes (chorion, allantois and amnion) (Liu et al., 1985). Prior to placentation, the caprine conceptus undergoes a transformation from spherical to filamentous morphology during the time period of maternal recognition of pregnancy. It is likely that RBP may also be a secretory product of elongating conceptuses in caprine. The RBP originating from the conceptus may serve to transport retinol locally from the uterus to embryonic tissues. Objectives of the present study were to characterize more thoroughly the proteins produced by elongating caprine conceptus. A group of low molecular weight proteins was identified as caprine conceptus RBP. The RBP cell-specific localization in conceptus was determined by immunocytochemistry. In addition, this study demonstrated

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that the conceptuses secreted continuously RBP during the time period maternal recognition of pregnancy.

MATERIALS AND METHODS

Materials

Eagle's minimal essential medium (MEM) and other supplies for tissue culture were purchased from GIBCO. [^{35}S]-methionine and [^{3}H]-leucine were obtained from New England Nuclear. X-Omat AR film was a product of Eastman Kodak. Immunoblot Assay Kit was from Bio-Rad Laboratories. Reagents for immunoperoxidase staining were obtained from BioGenex. Supplies for polyacrylamide gel electrophoresis and immune complex precipitation were as described by Lifsey et al. (1989). All other chemicals were of reagent grade or better and were products of Sigma Chemical Co.

Culture of conceptuses and collection of uterine flushings

Modified MEM with 20 μCi/ml [^{35}S]-methionine and 20 μCi/ml [^{3}H]-leucine were prepared. Adult crossbred female goats, primarily of Taiwan black (Capra aegagrus hircus), Alpine and Nubian breeding, were checked twice daily for estrus with vasectomized bucks. Estrous does (estrus=day 0) were mated by intact bucks. The reproductive tract was exposed via midventral laparotomy under aseptic condition and conceptuses were collected as previously described (Gnatek et al., 1989). Days 16 (n=4), 19 (n=4), and 22 (n=4) conceptuses were incubated at 37°C on a rocking platform in a gaseous atmosphere of 50% O_2, 45% N_2, and 5% CO_2, (by volume). After 36 h, culture was terminated by centrifuging at 12,000×g for 15 min at 4°C to separate tissue from medium. Conditioned medium samples were dialyzed against 10 mM Tris-HCl buffer, pH 7.6. Uterine flushings were collected according to procedures described by Bazer et al. (1978).

Protein electrophoresis

The method of Roberts et el. (1984) was used for two-dimensional (2D) SDS-PAGE. Aliquots of dialyzed medium (200,000 cpm) from individual embryonic tissues were lyophilized. Dried samples were dissolved in 75 μl of 5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol for 2D SDS-PAGE. The proteins were separated by isoelectric focusing in the first dimension and in 12% polyacrylamide gels in the second dimension. Fluorographs were exposed to dried gel for four weeks. One-dimensional SDS-PAGE was performed according to the method of Laemmli (1970) in 12.5% (w/v) polyacrylamide gels and 5% of stacking gel. Following electrophoresis, Coomassie blue R-250-stained gels were dried after impregnation with 1 M sodium salicylate (Chamberlain, 1979). Fluorographs were prepared and radiolabeled proteins were detected using Kodak film.

Immunoprecipitation

Anti-bovine placental RBP antiserum (50 μl) or non-immune serum (50 μl) (Liu et al., 1990) was added to 1 ml of ^{35}S- and ^{3}H-labeled conceptus tissue culture medium and incubated over night at 4°C. Immune complex was collected onto protein-A Sepharose CL-4B in 40 mM Tris-HCl (pH 7.5). Immune complexes were solublized by boiling at 100°C for 5 min. Proteins contained in the supernatant were analyzed by 2-D SDS-PAGE or 1D-SDS-PAGE. Following electrophoresis, Coomassie blue R-250-stained gels were dried after impregnation with 1 M sodium salicylate (1979). Fluorographs were prepared and radiolabeled proteins were detected using Kodak X-ray film.

Immunocytochemistry

Day 16 conceptus tissues were immersion-fixed in Bouin's solution for 2 h and washed with water. Tissues were dehydrated and embedded in paraffin. Tissue sections (6 μm thick) were prepared. A modified immunoperoxidase method was used to detect RBP in these issues. Briefly, it consisted of the following steps:

Sections were completely covered with 3% hydrogen peroxide and incubated for 10 min at room temperature (RT, 20-23°C) to block endogenous peroxidase activity.

After rising well with phosphate buffered saline (PBS), sections were treated with 1% bovine serum albumin for 20 min at 37°C. Sections were blotted with paper to remove excess liquid.

Anti-RBP serum raised against RBP purified from bovine allantoic membrane culture medium and immune-absorbed serum (negative control) (Liu et al., 1990) were prepared (1:500 dilution in PBS). After blotting the slides, anti-RBP antiserum or negative control serum were applied to sections for 30 min at 37°C.

After blotting the slides, the link antibody (anti-immunoglobulin serum) was applied to sections for 20 min at 37°C.

Sections were washed with PBS and incubated in peroxidase-antiperoxidase solution in PBS with carrier protein (labeling antibody) for 10 min at 37°C.

Sections were then washed with PBS and covered with 5% hydrogen peroxide for 10 min at RT.

Finally, sections were covered with substrate solutions (aminoethylcarbazole) for 10-20 min at RT. Then they were washed with PBS, mounted with glycerol and examined under a microscope.

RESULTS AND DISCUSSION

Secretory protein products of the caprine conceptus are

Table 1. Proteins identified in cultures of day 16 conceptuses

Protein	kDa[a]	pI[b]
1	55-65	6.8-6.4
2	70-75	7.4-6.8
3	23-27	7.7-5.3
4	21-22	6.0-5.3
5	17-20	5.3-5.0

[a] Apparent molecular mass (k Da) was estimated for each protein based on its relative mobility compared with protein standards in the second dimension electrophoresis.

[b] Apparent pI was estimated for each protein based on its relative mobility compared with isoelectric focusing in the first dimension electrophoresis.

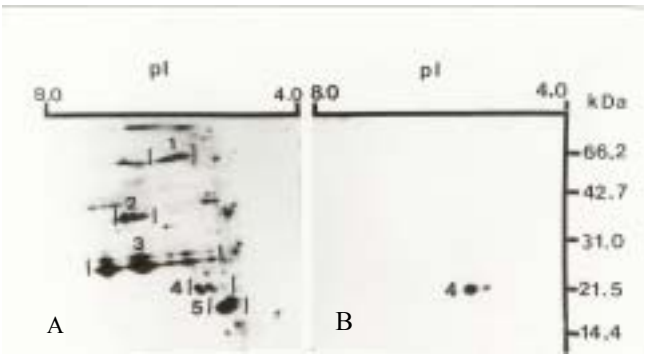

Figure 1. Fluorogram of 2D-PAGE gel for analysis of [^{35}S] methionine- and [^{3}H] leucine-labeled synthesized and secreted by caprine day 16 conceptuses (A). Fluorogram resulting from 2D-PAGE analysis of radiolabeled immunoprecipitated with anti-RBP serum indentified that protein 4 was a conceptus RBP (B). Exposure time was six weeks.

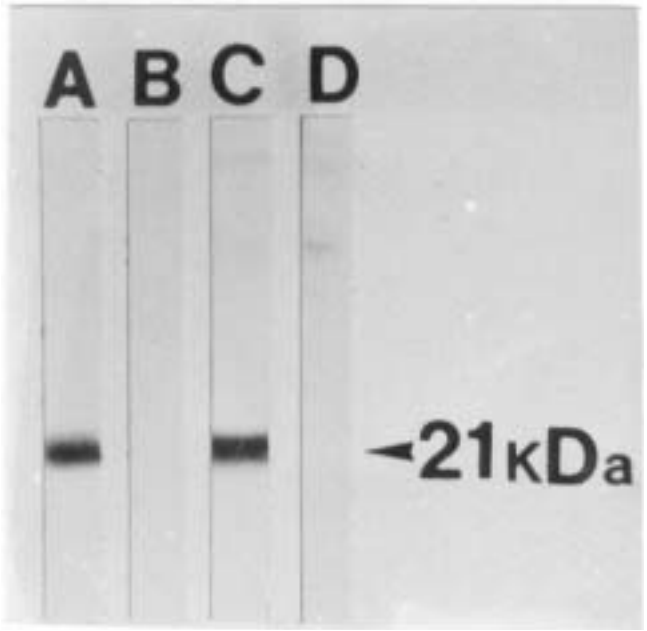

Figure 2. Identification of RBP secreted by days 19-22 conceptuses. Proteins in culture medium conditioned by days 19 (A) and 22 (C) conceptuses were immunoprecipitated with anti-RBP serum, and analyzed by 1D-SDS-PAGE and fluorography. Lanes B and D are controls with non-immune serum for lanes A and C, respectively. Exposure time was four weeks.

believed to play important roles in maternal-fetal interactions and embryonic development in caprine. A representative fluorograph of 2D-PAGE analysis of radiolabeled proteins synthesized and secreted by day 16 conceptus is shown in Figure 1. These fluorographic images indicated that day 16 conceptus incorporated sufficient radiolabeled amino acids into secretory proteins. The caprine conceptus was active in protein synthesis as early as day 16 of pregnancy. Table 1 lists the major proteins (numbered 1-5) which showed consistently a grouping of spots with characteristic location on 2D gels. The 17 kDa protein (numbered 5) has been identified as cTP-1 by Gnatek et al. (1989). This protein was observed to be a major secretory product from Day 16 through Day 21 of pregnancy (Gnatek et al. 1989). Embryo removal and transfer studies have shown that maternal recognition of pregnancy occurs between days 12 and 13 in ewes (Moor and Rowson, 1966), days 16 and 17 in cows (Betteriodge et al., 1980; Northey and French, 1980) and days 15 and 17 in goats (Gnatek et al., 1989). Ovine, bovine and caprine maternal recognition of pregnancy are thought to be initiated by conceptus secretory proteins, ovine trophoblast protein-l (oTP-1) (Godkin et al., 1982), bovine TP-1 (bTP-1) (Barton et al., 1985) and cTP-1 (Gnatek et al., 1989), respectively.

Bovine TP-1 and cTP-1 have physical and immunological characteristics similar to oTP-1 (Gnatek et al., 1989). Ovine TP-1 appears to act locally as a paracrine hormone at the level of the uterine endometrium (Godkin et al., 1984a). Ovine TP-1 triggers a series of maternal responses to the presence of the conceptus including prevention of corpus luteum regression (Godkin et al., 1984a), changes in endometrial protein synthesis (Godkin et al., 1984b) and prostaglandin metabolism (Salamonsen et al., 1988; Vallet et al., 1987). As with oTP-1 in the ewe, bTP-1 can influence uterine prostaglandin metabolism (Knickerbocker et al., 1986), possibly by inducing an inhibitor of $PGF_{2\alpha}$ synthesis (Gross et al., 1988) and prolong the lifespan of the corpus luteum (Thatcher et al., 1989). Recently, oTP-1, bTP-1 and cTP-1 have been classed as interferon-τ. Infusion of bovine recombinant IFN-τ into the uteri of nonpregnant ewes (Stewart et al., 1989) or cows (Plante et al., 1988) can extend luteal lifespan. Furthermore, INF-τ can compete with oTP-1 for binding to endometrial receptors (Stewart et al., 1987; Hansen et al., 1989), and mimic the effects of oTP-1 endometrial protein and prostaglandin biosynthesis (Thatcher et al., 1989; Salamonsen et a1., 1988; Silcox et al., 1988). In addition, oTP-1 appears to possess antiviral (Roberts et al., 1989; Pontzer et al., 1988) and antiproliferative activities (Roberts et al., 1989) characteristic of interferon.

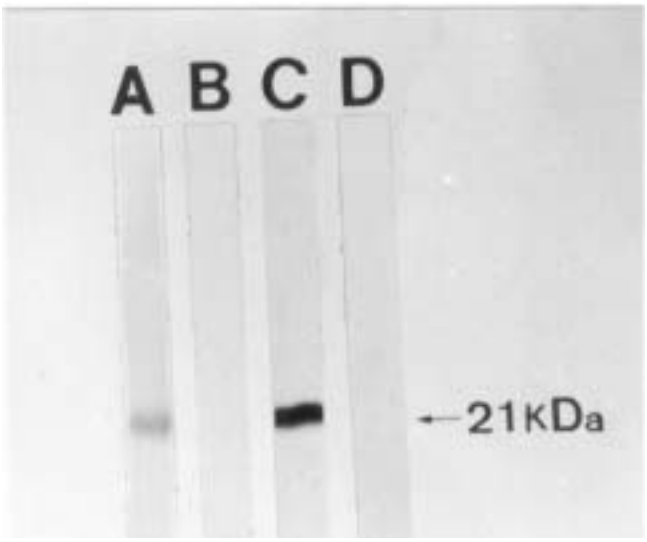

Figure 3. Identification of RBP in caprine uterine flushings collected from days 16-22 of pregnancy. Proteins in caprine uterine flushings from days 16 (A) and 22 (C) of pregnancy were analyzed by 1D Western blotting with anti-RBP serum. Lanes B and D are controls with non-immune serum for lanes A and C, respectively.

In vitro study, immune-complex precipitation with anti-RBP serum (Liu et al., 1990), followed by 2D and fluorography was used to identify the 21 kDa protein (numbered 4) with two prominent isoforms as a conceptus RBP in day 16 conceptus culture medium (Figure 1B). Molecular microheterogeneity on polyacrylamide gel systems is a general characteristic of plasma RBP (Peterson et al., 1973). Recently, Minic et al. (1997) reported that RBP prepared from human urine was composed four isoforms: two retinol-containing (holo-) and two retinol-free (apo-) species. RBP under pH 7-10 condition favoring deamidation resulted in formation of the more acidic apo-isoform. The microheterogeneity of caprine conceptus RBP shown on 2D gels (Figure 1A, 1B) is likely due to different amounts of retinol bound to the protein. In addition, the anti-RBP serum was applied to days 19 and 22 conceptus cultures (Figure 2). Immunoprecipitation analysis of proteins produced by conceptuses of days19 (Figure 2, lane A) and 22 (Figure 2, lane C) of pregnancy clearly showed that antiserum to RBP cross-reacted immunologically with a 21 kDa protein which corresponded to protein 4 on 2D-PAGE (Figure 1). In vivo study, presence of immunoreactive RBP in uterine flushings from does at days 16 (Figure 3, lane A) and 22 (Figure 3, lane C) of pregnancy was determined by Western blotting using anti-RBP serum. In immunocytochemical study, strong immunostaining for RBP was localized in trophectoderm (Figure 4, T) and endoderm (Figure 4, E) of day 16 conceptus using anti-RBP serum. Above results clearly demonstrated that RBP was synthesized and secreted by

Figure 4. Immunocytochemical localization of RBP in the conceptus of day 16 of pregnancy. Strong immunostaining was seen in cells in the trophectoderm (T) and endoderm (E) when anti-bovine placental RBP antiserum was applied. Specific immunostaining was significantly diminished in control section when immune-absorbed serum was applied. Panel A, ×180%.

caprine (days 16-22) conceptuses during the time period of maternal recognition of pregnancy.

In caprine, the time period of secretion of conceptus RBP is coincident with the rapid transformation of the conceptus when the conceptus elongates from a spherical blastocyst to a filamentous structure. Moreover, retinol is important for the maintenance of normal cell growth and differentiation, and embryonic morphogenesis (Wolf, 1984), it is likely that conceptus RBP and its ligand (retinol) have physiological roles in the rapid transformation of the conceptus in the goat. Retinol is physiologically metabolized into retinoic acid (RA) within cells (Vieira et al., 1995). Retinoic acid is also a potent cell modifier. Specific effects of RA include promotion of differentiation, alteration in numbers and permeability of intercellular junctions, and secretion of glycoproteins and other glycoconjugates that may be involved in intercellular recognition (Shapiro, 1986). These properties of RA may have major effects on rapid elongation of the conceptus occurring through trophoblastic and endodermal cell growth, remodeling and formation of cytoskeleton and extracelluar matrix.

Although the information for RA in embryonic development has not been well understood, there are evidence linking the RA to activation of gene encoded to RA receptor-β (RAR-β) and transforming growth factor-β (TGF-β), a regulator of extracelluar matrix (Schmid et al., 1991). The RAR-β gene is a primary target for RA. The visceral endoderm contains high level of RAR-β mRNA. RA is able to induce formation of visceral endoderm from embryonal carcinoma cells (Cathleen et al., 1990). Retinoic acid can modulate gene expression of TGF-β. In the chicken, TGF-β regulates yolk formation (Kang et al., 2002). In the mouse, TGF-β genes are expressed during embryonic development and that the total levels of their specific mRNA increase with the age of the embryo (Heine et al., 1987; Miller et al., 1989a, b). TGF-β has also been

reported to stimulate proliferation of bovine trophoblastic epithelial cells (Munson, 1992). The trophectoderm and endoderm will constitute the chorion and allantois of the placental membranes in the later stage of gestation (Gnatek et al., 1989). Importantly, TGF-β activation of proteolytic enzymes such as plasminogen activator and metalloprotease may induce breakdown of the extracelluar matrix remodeling of the cells during the rapid morphological change in shape of conceptus (Geisert and Malayer, 2000). The findings of RBP in caprine trophectoderm and endoderm (this study) suggested that conceptus RBP may involved in the transport and metabolism of retinol which may provided a basis for integrated control of gene expression by RAR-β and TGF-β.

In summary, the caprine conceptus was active in protein synthesis as early as day 16 of pregnancy. Five major conceptus proteins were characterized. A 17 kDa and a 21 kDa acidic proteins were identified as interferon-τ (Gnatek et al., 1989) and RBP (this study), respectively. The precise function for the other three proteins (numbered 1-3) remains to be elucidated. Secretion of conceptus RBP was coincident with the rapid transformation of the conceptus from a spherical blastocyst to a filamentous structure. The conceptus must physically cover a large portion of maternal endometrium to regulate release of $PGF_{2\alpha}$ to prevent luteolysis (Geisert and Malayer, 2000). Additionally, conceptuses that reach the filamentous stage earliest may have competitive edge for survival over less developed conceptuses by obtaining sufficient uterine surface area necessary to support continued development (Geisert and Malayer, 2000). Thus, retinol may have an impact on these events during the time period of maternal recognition of pregnancy.

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