Immunohistochemical localization of bovine placental retinol-binding protein

KUIXIONG GAO, KAUNG H. LIU and JAMES D. GODKIN

Department of Animal Science, The University of Tennessee, Knoxville, USA

ABSTRACT

An immunogold staining method was used in combination with epipolarization microscopic detection to demonstrate the presence of bovine placental retinol-binding protein in bovine extraembryonic membranes. Amnion, chorion and allantois were fixed in Bouin fixation fluid and embedded in polyethylene glycol 1500. Sections (5 mm) were cut and transferred onto Digene silanated slides and immunostained using rabbit antiserum raised against bovine placental retinol-binding protein followed by goat anti-rabbit IgG labeled with 1 nm gold. Gold particles after silver enhancement were viewed and photographed under epipolarization microscopy. Epithelial cells of all three membranes (i.e. amniotic ectoderm, chorionic trophoderm, and allantoic endoderm) were immunoreactive, while mesodermal cells, collagen, and blood cells were not. These data, together with our previous observation that these three placental membranes synthesize and secrete retinol-binding protein, indicate that epithelial cells lining the amnion, chorion and allantois are the major sources of this protein. The presence of retinol-binding protein in placental membranes and their fluids may be indicative of an important role for retinol in placental differentiation and development.

KEYWORDS: retinol-binding protein, PEG, placenta and immunohistochemistry

It has been recognized for over half a century that vitamin A (retinol) is essential for normal fetal growth and development (Evans, 1928; Wolbach and Howe, 1933; Thompson et al., 1964; O'Toole et al., 1974) as well as for the maintenance of pregnancy (Moore, 1957; Howell et al., 1963). However, until recently the mechanism of action caused by vitamin A in these processes has been largely unknown. Retinoids, a family of vitamin A metabolites and analogs (Lotan, 1980; Sporn and Roberts, 1984; Dawson et al., 1985) were known to modulate differentiation and growth of several cell types (Sherman, 1986; Elias, 1987; Harper, 1988; Keeble and Maden, 1989) and influence production of many cell surface molecules, extracellular matrix and cytoskeleton components (De Luca, 1977; Strickland et al., 1980; Fuchs and Green, 1981; Jetten, 1981; Shapiro and Mott, 1981; Yuspa et al., 1982). Retinoids also influence expression of some growth factors (Jetten, 1981; Rizzino and Bowen-Pope, 1985) and their receptors (Rees et al., 1979; Mercola et al., 1990; Mummery et al., 1990). Retinoic acid, a natural metabolite of retinol, has been identified as a morphogen in induction of pattern formation in the chick limb bud (Tickle, et al., 1975; Maden, 1982; Eichele et al., 1985). Retinol is transported in plasma complexed to retinol-binding protein (RBP). RBP is the only known plasma transport protein that delivers retinol from its source (mainly liver) to target cells (Smith and Goodman, 1971). Within the cell, the mechanism of vitamin A action has been proposed to involve the interaction with distinct retinoid-binding proteins in the cytosol (Chytil and Ong, 1984) and nuclear receptors (Giguere et al., 1987; Petrovich et al., 1987).

Recently, we have demonstrated the synthesis and secretion of RBP by bovine extraembryonic membranes (Liu et al., 1990). The protein has been identified as a major low molecular weight acidic protein (LMWAP) synthesized by amnion, chorion and allantois in organ culture and a major component of amniotic and allantoic sac fluids (Liu et al., 1990). It is not glycosylated and composed of three isotypes (Godkin et al., 1988). Sequence analysis of the first 43 NH2-terminal amino acids of the protein demonstrated that they were identical to those of bovine plasma RBP (Berni et al., 1990) and >90% homologous with human and rabbit plasma RBP (Liu et al., 1990). The objective of the present study was to identify the cellular source of this protein, which we refer to as bovine placental RBP, or, bpRBP (Liu et al., 1990), in amnion, chorion, and allantois.

Polyethylene glycol 1500 (PEG) was used as a water soluble embedding medium. PEG embedded tissue sections were transferred onto silanated slides for immunohistochemistry (Gao and...
Figs. 1-7. Histochemical and immunohistochemical staining of bovine extraembryonic membranes. (1) Bovine chorionic membrane. Tissue was fixed in Bouin's fixative solution and embedded in PEG1500. 5μm section was stained with hematoxylin and eosin (H&E). AC, apical cytosol; Ne, nuclei of epithelial cells; Nm, nucleus of mesenchymal cells; L, lumen (x330, Bar 0.05 mm). (2) Keratin immunostain in chorionic membrane. Rabbit anti-keratin 1:1000, goat anti-rabbit IgG labeled with 1 nm colloidal gold (IgG-Au) 1:30, silver enhancement (IGS) and counterstained with H&E, observed with transmission light (TM), x330. K, keratin filament; Ne, nucleus of epithelial cell; v, vacuol. Transmission light (TM) x330. (3) Localization of bpRBP in bovine allantois. Anti-bpRBP 1:300, IgG-Au 1:30, IGSS, H&E. Photograph was taken with epipolarization illumination (EP). M, mesenchymal cells; E, epithelium; L, lumen, x264, Bar 0.05 mm. (4) Allantois treated with pre-immune serum (1:300 as negative control for Fig. 3), IgG-Au, 1:30, IGSS. Photograph was taken with TM and EP, x264. (5) Chorionic membrane, Immunostained with pre-immune rabbit serum (1:100) followed by IgG-Au (1:30), H&E (as a negative control for Fig. 8). L, lumen; E, epithelium; M, mesenchyme; TM plus EP, x264. (6) Immunostain of bpRBP antigenic sites in bovine amniotic membrane. Anti-bpRBP 1:800, IgG-Au 1:100, IGSS and observed with EP, E, epithelium; L, lumen; M, mesenchym. x264. (7) Nonspecific fluorescence in chorionic epithelium. Photographed with FITC filter. Comparison of Figs. 7 and 8 demonstrates the specific immunostaining detected only with the BH2-DM BIGS filter (Fig. 8).

Godkin, 1991). The method provided excellent morphological preservation of cell structure as well as good sensitivity for antigen detection by immunostaining (Figs. 1-8).

Chorion consisted of an apical monolayer of cells that appeared as a pseudostratified epithelium due to nuclei arranged at different distances from the basement substratum (Fig. 1). Fig. 1 is a micrograph of normal bovine chorionic membrane stained with hematoxylin and eosin (H&E). Nuclei of chorionic epithelial cells (Ne) are larger and more spherical than nuclei of underlying mesenchymal cells (Nm). Fig. 2 illustrates the pattern of keratin immunostaining in chorionic membrane with H&E counterstaining. The brown reaction product localized cytokeratin primarily at the apical and basolateral compartments. Rod-like branches of vertical keratin filaments, deeply rooted basolaterally, were often connected to apical filaments. In contrast, bpRBP antigenic sites in chorionic epithelium were clustered in the apical region of these.
cells and diffused basolaterally (Fig. 8). Nuclei and vacuoles were not stained. No immunostaining was observed when pre-immune rabbit serum was applied in place of the anti-bpRBP (Fig. 5). Fig. 7 is a photograph of chorionic epithelium after both bpRBP immunostain and H&E counterstain but without epipolarization illumination and observed with an FITC filter to show non-specific fluorescence in tissue. Comparison of Figs. 7 and 8 demonstrates the specific immunostaining (immunogold enhanced by silver stain) detected with the BH2-DM BIGS filter (Fig. 8).

Immunolocalization of bpRBP antigenic sites in allantois is illustrated in Fig. 3. The entire epithelial lining of this membrane was intensely stained with the exception of nuclei. Some diffuse staining was observed in the underlying mesoderm. For comparison, Fig. 4 shows allantoic membrane treated with pre-immune rabbit serum (1:100) in place of anti-bpRBP serum followed with IgG-Au (1:30) and IGSS and viewed with TM and EP (x264). No immunostaining was apparent.

The presence of bpRBP in amnion is illustrated in Fig. 6. Similar to the observation in allantois, localization of the bpRBP in amnion was restricted predominantly to the epithelial lining of this membrane. Control sections (not shown) were totally negative for immunostaining.

Retinol-binding protein is the only known transport protein for retinol in plasma. It forms a 1:1 complex with retinol and this complex generally is bound non-covalently to transthyretin (Sherman, 1986). Liver serves as a major site of RBP synthesis; however, several extrahepatic tissue sites of RBP and/or RBP mRNA synthesis have recently been identified (Soprano et al., 1986). Identification of RBP and/or its mRNA in placental tissue of the rat (Soprano et al., 1986), cow (Liu et al., 1990), pig (Harney et al., 1990), sheep (Liu et al., 1991) and goat (Liu and Godkin, unpublished observations) suggest that the placenta may play an important role in regulating vitamin A storage, transport and metabolism during pregnancy.

Results from the present study identified and localized bpRBP in the epithelial cells lining the amnion, allantois and chorion. The bpRBP was sometimes more concentrated in the apical regions of these cells. Based on these findings and our previous demonstration (Liu et al., 1990; Gao and Godkin, 1991) that these placental membranes synthesize and release the protein, we suggest that bpRBP is synthesized and secreted by epithelia of the chorion, allantois, and amnion.

The RBP producing chorionic epithelium is the trophoblast which is intimately associated with uterine epithelium and the site
of fetal-maternal attachment (placentaion) and nutritive exchange (for review of placental development see Stevens, 1975). RBP is apparently released from the trophoderm toward the uterine epithelium where it may act in a paracrine manner. Amnion develops from folding of the chorion around the embryonic disc with chorionic ectoderm (trophoderm) on the inside and mesoderm on the outside. Our results suggest that RBP is secreted from ectoderm into the amniotic fluid that surrounds the developing fetus. The amnion is an outgrowth of the hind gut composed of endoderm on the inside covered with vascular mesoderm which comes in opposition to and eventually fuses with chorionic mesoderm. Production of bpRBP is from the endoderm. Fluids of both the amniotic and allantoic sacs contain high concentrations of RBP.

Although it is clear that each function of RBP is the transport of retinol, defining a role for retinol in bovine reproductive processes is problematic due, in part, to its pleiotropic and synergistic effects. Given the importance of vitamin A as a potential morphogen and differentiation factor, regulation of its transport and storage appears to be an important placental function mediated through RBP production.

Experimental Procedures

Production of anti-bpRBP serum

Purification of bpRBP, production of rabbit-anti-bpRBP and characterization of the antisera were as previously described by Liu et al. (1990).

Tissue sample preparation

Bovine extraembryonic membranes (chorion, allantois, and amnion) from a 35 cm fetus (135 days of pregnancy) were fixed in Bouin fixation fluid (EN Diagnostic System, Inc., Gibbstown, NJ) for 30 min at room temperature and washed thoroughly with 70% ethanol. After rehydration in phosphate buffered saline (PBS), pH 7.3, membranes were treated with a permeabilization solution containing 1% Surfact-Amps X-100 and 1% bovine serum albumin (BSA), 5% normal goat serum (NGS) and 0.1% NH4CI in PBS for 10 min at room temperature. After a brief wash with distilled water, samples were infiltrated with increasing concentrations (25%, 50%, 75%) of polyethylene glycol 1500 (PEG) (Aldrich Chemical Co., Milwaukee, WI) in water at 55°C for 20 min at each concentration. Samples were then infiltrated with molten PEG 1500 at 55°C (3 changes, 20 min each) and embedded in molten PEG 1500. Samples were allowed to cool to room temperature to form blocks which were further hardened at 4°C for 15 min. Sections (5 mm) were cut with a microtome (American Optical Co., Buffalo, NY) equipped with an Accu-Edge microtome blade (Miles Laboratories Inc., Elkhart, IN). Sections were transferred to Digenesilanated slides (Digene Diagnostic Inc., Silver Spring, MD) according to the minute agarose block method (Gao and Godkin, 1991).

Immunohistochemistry

Immunohistochemistry was performed according to the method reported previously (Gao and Godkin, 1991). Briefly, it consisted of the following steps:

(a) Sections were first permeabilized with 1% Surface Amps X-100 and 1% NH4CI in PBS for 15 min at room temperature.

(b) Sections were treated with a PBS based blocking medium containing 1% bovine serum albumin (BSA), 5% normal goat serum (NGS) and 0.1% cold water fish gelatin (Sigma Chemical Co., St. Louis, MO) for 45 min at room temperature.

(c) Sections were incubated in a medium similar to blocking medium as in (a) but with 1% NGS. Concentrations of first antibodies added to the incubation medium were as follows: anti-bpRBP 1:100, 1:300, and 1:800; preimmune serum (negative control) 1:100; rabbit anti keratin wide spectum overnight screening. (Accurate Chemical and Scientific Corp. Westbury, NY) 1:1000.

(d) Sections were then washed with PBS, incubated in diluted (1:30) 1 nm gold labeled goat anti-rabbit IgG (Janssenn Life Sciences Products, Olen, Belgium), washed with PBS and fixed with 2% glutaraldehyde (EM grade, Polysciences Inc., Warrington, PA) in PBS. Silver enhancement procedures were according to manufacturer's directions using a freshly prepared mixture of IntenSEm (Janssenn Life Sciences Products, Olen, Belgium).

(e) Finally, sections were stained with Ehrlich hematoxylin and eosin Y (M and E stain) and mounted with Visio-Bond light sensitive and instantly curable resin (ESPE-Primer Sales Corp., Norristown, PA) according to the method of Gao and Peng (1987).

Epipolarization microscopy

Slides were viewed under a Olympus BH-2 epi-illumination fluorescence microscope equipped with BH-DM BIGS filter set which was specially designed for epipolarization detection of silver enhanced colloidal gold particles at the light microscopic level (Olympus Optical Co., Ltd., Tokyo, Japan, New York Branch).

Either TM or a combination of EP and TM or epifluorescence was used. Photographs were taken using Kodak Ektachrome 160 Tungsten 35 mm color slide films. A green filter (IF550) was inserted in the TM light path and combined with EP illumination as compensation for color temperature for photography. In some cases, both EP and TM light were used to visualize tissue profile and fluorescence-like antigenic sites simultaneously.

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References


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