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## PrP-C and PrP-Sc at the Fetal-Maternal Interface\*

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**Scrapie is a naturally occurring prion (PrP) disease causing a fatal neurodegenerative disorder in sheep and goats. Previous studies suggest that scrapie is transmitted naturally through exposure to the scrapie agent in wasted placentas of infected ewes. This study determined the distribution and biochemical properties of PrP cellular (PrP-C) and the distribution of PrP scrapie (PrP-Sc) in reproductive, placental, and selected fetal tissues and fetal fluids in sheep. Glycosylated, N-terminally truncated, proteinase K-sensitive PrP-C with apparent molecular masses of 23–37 kDa was present in reproductive, placental, and fetal tissues and fetal fluids. PrP-C was low or undetectable in intercotyledonary chorioallantois, amnion, urachus, amniotic fluid, and fetal urine. In pregnant ewes, cotyledonary chorioallantois, allantoic fluid, and caruncular endometrium contained higher levels of PrP-C than did intercaruncular endometrium, myometrium, oviduct, ovary, fetal bladder, or fetal kidney. Caruncular endometrial PrP-C was up-regulated during pregnancy. Despite the wide distribution of PrP-C in reproductive, placental, and selected fetal tissues and fetal fluid, PrP-Sc was detected only in caruncular endometrium and cotyledonary chorioallantois of pregnant scrapie-infected ewes. The embryo/fetus may not be exposed to scrapie *in utero* because it is separated physically from PrP-positive allantois and chorioallantois by PrP-negative amnion.**

Scrapie in sheep and goats is a naturally occurring fatal neurodegenerative disorder characterized by accumulation of the abnormally folded, PK<sup>1</sup>-resistant prion protein (PrP-Sc) (1, 2). In contrast to the ubiquitous distribution of the cellular, PK-sensitive prion protein (PrP-C) (3, 4), PrP-Sc accumulation in sheep is limited to the central and peripheral nervous system (5, 6), the lymphoreticular system (6), and placenta (7–9). PrP-C is synthesized primarily as a glycosylphosphatidylinositol-anchored glycoprotein found in the cell membrane of a variety of cell types (10). A secretory form of PrP-C lacking the glycosylphosphatidylinositol anchor is present in cell-free translation systems, cell cultures, human cerebrospinal fluid,

and platelets (11–15). Previous studies demonstrated that PrP-C is the substrate for conversion to PrP-Sc and is required for scrapie infection (16–19).

Scrapie can be transmitted experimentally through multiple routes (20). However, natural scrapie transmission may be associated primarily with exposure to the infectious agent in the placenta. Pattison *et al.* (7, 8) reported that oral inoculation with placental membranes derived from scrapie-infected ewes caused scrapie in both sheep and goats. Race *et al.* (9) recently showed that PrP-Sc was detected by Western blotting in the placenta from scrapie-infected ewes and scrapie infectivity in tissue homogenates of placental membranes. Under natural conditions, fetal fluids and placental tissues are expelled during or shortly after parturition and contaminate the body surface of the dam and the area where lambing takes place. Lambs may be exposed to scrapie by ingesting the transmissible agent during contact with contaminated wool and mammary gland of the dam. Adult sheep in the same flock may also be exposed orally through contaminated feed sources.

In the present study, we investigated the distribution of PrP-C and biochemical properties of PrP-C and distribution of PrP-Sc in the ovine female reproductive, placental, and selected fetal tissues and fetal fluids of infected and uninfected ewes. In contrast to the ubiquitous distribution of PrP-C in the female reproductive tract and conceptuses, PrP-Sc was present only in the uterine caruncular endometrium and placental cotyledonary chorioallantois of pregnant infected ewes. N-terminally truncated, glycosylated, PK-sensitive PrP-C with apparent molecular masses of 23–37 kDa was present in cotyledonary chorioallantois, allantoic fluid, fetal bladder, and fetal kidney as well as endometrium, myometrium, oviduct, and ovary of pregnant and nonpregnant uninfected ewes. PrP-C levels were low or undetectable in intercotyledonary chorioallantois, amnion, urachus, amniotic fluid, and fetal urine. In pregnant ewes, cotyledonary chorioallantois, allantoic fluid, or caruncular endometrium contained higher ( $p < 0.05$ ) levels of PrP-C than did intercaruncular endometrium, myometrium, oviduct, ovary, fetal bladder, or fetal kidney. PrP-C expression in caruncular endometrium was up-regulated ( $p < 0.05$ ) during pregnancy when compared with caruncular endometrial PrP-C of nonpregnant ewes. Pregnancy status had no effect on PrP-C expression in the brain, myometrium, oviduct, and ovary. PrP-C detected in uninfected ewes had a protein core of ~18–22 kDa in tissues and ~16.5 kDa in allantoic fluid.

### EXPERIMENTAL PROCEDURES

**Animals and Tissue Collection**—Uninfected ( $n = 10$ ) and scrapie-infected ( $n = 4$ ) Suffolk sheep were used. All uninfected ewes and rams were from flocks with no reported history of scrapie and negative for PrP-Sc by live animal testing (immunohistochemistry of third eyelid lymphoid tissue) prior to mating, and no PrP-Sc was detected in brain or lymphoid tissues by immunohistochemistry and Western blotting

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<sup>1</sup> The abbreviations used are: PK, proteinase K; PrP, prion; PrP-C, PrP cellular; PrP-Sc, PrP scrapie; roPrP, recombinant ovine PrP; mAb, monoclonal antibody; PNGase F, peptidase-N-glycosidase F.

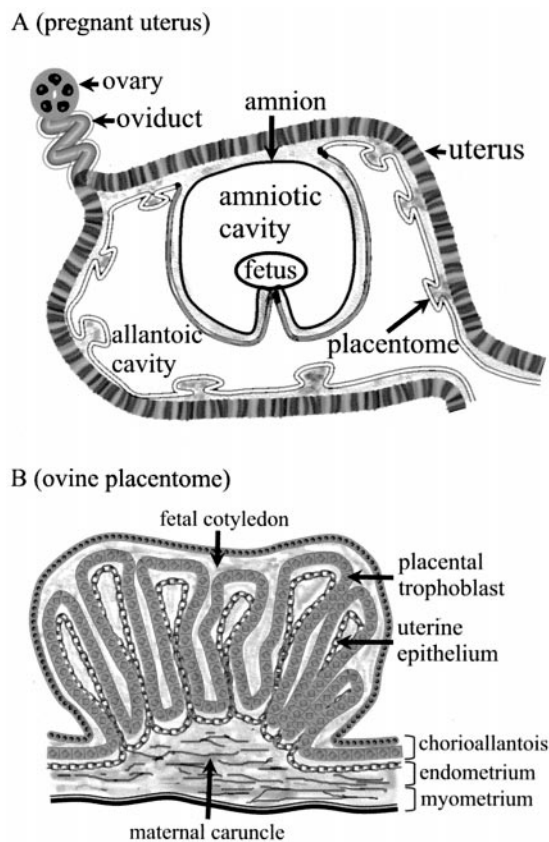


FIG. 1. A schematic representation of the ovine pregnant uterus with a fetus and associated membranes and the placenta. A, pregnant uterus with fetus and fetal-associated membranes; B, an ovine placenta. Sheep and goats possess a synepitheliochorial placenta that is formed by interdigitation of villi of the uterine caruncular endometrium and placental cotyledonary chorioallantois. The intercotyledonary portion of chorioallantois forms only diffuse attachment with the apposing uterine intercaruncular endometrial epithelium.

following necropsy (21, 22). Scrapie-infected ewes were identified by positive third eyelid testing, and scrapie was confirmed by immunohistochemistry and Western blotting of brain and lymphoid tissue following necropsy. All sheep were homozygous for glutamine at codon 171 of the PrP gene (23). Pregnant scrapie-infected ewes ( $n = 4$ ) were mated to rams at the United States Department of Agriculture research support facility in Pullman and estimated to be near term pregnancy at euthanasia. The fetuses of these infected ewes were also homozygous for glutamine at codon 171 of the PrP gene. Uninfected ewes ( $n = 6$ ) were checked daily for estrus in the presence of vasectomized rams and the first day of exhibiting estrous behavior is referred to as Day 0 of the estrous cycle. All ewes were mated to intact rams two to three times at 12-h intervals starting on Day 0 of the second estrous cycle. All pregnant uninfected ewes used in this study were 135–140 days pregnant (full term pregnancy for sheep is 144–150 days) at euthanasia. Reproductive tissues from nonpregnant uninfected ewes ( $n = 4$ ) were also examined. Sheep were fed with hay and alfalfa and had free access to water. All sheep were euthanized immediately prior to necropsy. Maternal tissues (brain, uterine caruncular endometrium, and intercaruncular endometrium, uterine myometrium, oviduct, and ovary), fetal tissues (brain, kidney, and bladder), placenta and fetal membranes (cotyledonary and intercotyledonary chorioallantois, amnion, and urachus), and fetal fluids (allantoic fluid, amniotic fluid, and fetal urine) were collected, frozen, and stored at  $-80^{\circ}\text{C}$  until use (Fig. 1). Animal care, handling, and use in this study were approved by the Institutional Animal Care and Use Committee of Washington State University.

**Antibodies**—mAb 97/99 (recognizing an epitope between amino acid residues 217 and 231 near the C terminus of the ovine PrP) and mAb 8H4 (recognizing epitopes between residues 146 and 200) were used to identify PrP in ovine reproductive, fetal, and placental tissues and fetal fluids (22, 24, 25). mAbs 5B2 and 8B4 (recognizing epitopes located at amino acid residues 34–52 and 36–43) were used to identify the presence of the N terminus of PrP-C (24, 25).

**Tissue Homogenate Preparation**—Tissue homogenates were prepared with (for PrP-Sc detection) or without (for PrP-C detection) the use of 10% Sarkosyl and ultracentrifugation. Tissue homogenates obtained in the absence of Sarkosyl (direct tissue homogenates) were prepared from either fresh or frozen tissues. Briefly, tissues (100 mg) were minced with scissors in microcentrifuge tubes or homogenized with disposable tissue grinders. Homogenates were incubated at room temperature for 1 h in 900  $\mu\text{l}$  of lysis buffer containing 10 mM Tris (pH 7.5), 0.5% Nonidet P-40, and 0.5% sodium deoxycholate. Tissue homogenates or fetal fluids were digested with or without PK (Roche Diagnostic Inc., Indianapolis, IN) at 20  $\mu\text{g}/\text{ml}$  for 30 min at  $37^{\circ}\text{C}$ . PK digestion was stopped by the addition of a protease inhibitor, Pefabloc (Roche Diagnostic Inc., Indianapolis, IN), to a final concentration of 4 mM, and the reaction mixtures were stored at  $-20^{\circ}\text{C}$  until analyzed by electrophoresis and Western blotting. Tissue homogenates without PK digestion were prepared in the presence of protease inhibitor mixture following the manufacturer's instructions (complete, Roche Diagnostic Inc., Indianapolis, IN).

Tissue homogenates enriched for PrP-Sc (PrP-Sc-enriched tissue homogenates) by differential solubilization and ultracentrifugation obtained in the presence of 10% Sarkosyl were prepared as described by Race *et al.* (9) with minor modifications. Briefly, fresh or frozen tissue (up to 1 g) was minced, homogenized, and incubated at room temperature for 1 h in 10 mM Tris-HCl buffer (pH 7.5) containing 5 mM  $\text{MgCl}_2$ . An equal volume of Sarkosyl solution (20% Sarkosyl in 10 mM Tris-HCl buffer (pH 7.5)) was added to tissue homogenates, which were centrifuged at  $6,000 \times g$  for 10 min; the resultant supernatants were collected and centrifuged at  $348,000 \times g$  for 50 min at room temperature. The pellets were dissolved in 10 mM Tris-HCl (pH 7.5) and treated with or without PK (Roche Diagnostics Inc., Indianapolis, IN) as described above. The preparations were centrifuged at  $279,000 \times g$  for 30 min, and the pellets were resuspended in 10 mM Tris-HCl (pH 7.5) and boiled for 5 min in sample loading buffer prior to analysis by Western blotting.

**Western Blot Analysis**—Tissue homogenates and fetal fluids were analyzed by 14% SDS-polyacrylamide gel electrophoresis minigels (Invitrogen) followed by transfer using a semidry transblotter to polyvinylidene difluoride membranes (Millipore) prior to detection by mAbs 97/99, 8H4, 8B4, or 5B2. The membranes were incubated with goat anti-mouse IgG-horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL), developed with a chemiluminescence substrate (Amersham Pharmacia Biotech), and exposed to x-ray film (Eastman Kodak Co.) for 1–10 min.

The relative levels of PrP-C in tissue homogenates and fetal fluids were determined by densitometric analysis of films of Western blotting using mAb 99/97. Briefly, fixed amounts of tissue homogenates (1.5 mg/lane of wet tissue equivalent) and fetal fluids (7.5  $\mu\text{l}/\text{lane}$  of allantoic, amniotic, or fetal urine) were analyzed by Western blotting as described above. Brain tissue homogenate was analyzed at 0.15 mg/lane, due to high levels of PrP-C present in the brain. All films were exposed to Western blots for 1 min, and densitometry was performed using a digital imaging system (ChemImager 4000, Alpha Innotech Corp., San Leandro, CA). The sensitivity of the assay was determined by detecting serial dilutions of roPrP (21) (ranging from 1 to 600 ng/lane) suspended in tissue homogenates. Twenty-five nanograms of roPrP were loaded on one lane of each gel to normalize the relative levels of PrP-C in tissues and fluids. Results are expressed as relative units, representing the ratio between densitometric values of sample PrP-C and roPrP (25 ng).

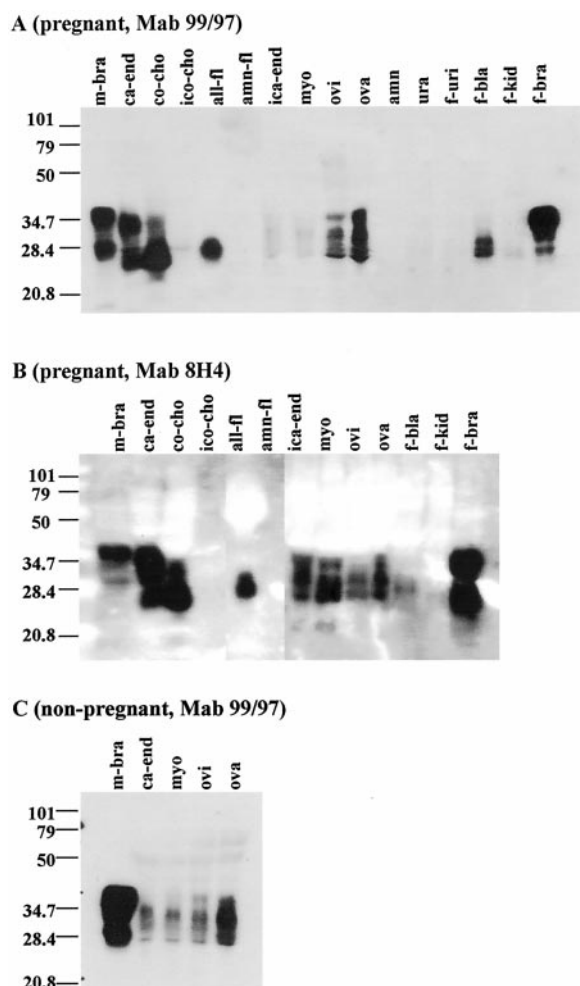
**Deglycosylation**—Deglycosylation was conducted using a commercial kit following the manufacturer's instructions (Prozyme, Inc., San Leandro, CA). Briefly, 2  $\mu\text{l}$  of  $5\times$  PNGase F buffer and 2  $\mu\text{l}$  of denaturation solution were added to 1  $\mu\text{l}$  of brain homogenate plus 5  $\mu\text{l}$  of water or 6  $\mu\text{l}$  of extraneural tissue homogenate as prepared above (200 mg wet tissue in 1 ml of lysis buffer), followed by incubation at  $100^{\circ}\text{C}$  for 5 min. After the reaction was cooled to room temperature, 1  $\mu\text{l}$  of Triton X-100 and 1  $\mu\text{l}$  of PNGase F were added and incubated at  $37^{\circ}\text{C}$  overnight. The reaction mixture was boiled for 5 min in sample loading buffer prior to analyses by electrophoresis and Western blotting.

**Statistics**—Data were analyzed by one-way analysis of variance with Student-Newman-Keuls multiple comparisons test. Probability values less than 0.05 were considered statistically significant.

## RESULTS

**PrP-C Is Detected in the Uterine Endometrium, Myometrium, Oviduct, and Ovary of Pregnant and Nonpregnant Normal Ewes and Cotyledonary Chorioallantois, Fetal Bladder, and Allantoic Fluid of Pregnant Normal Ewes**—Representative





**FIG. 2. Representative Western blot analysis of PrP-C in maternal brain and reproductive tissues of pregnant ( $n = 6$ ) and nonpregnant ( $n = 4$ ) ewes and in fetal and placental tissues and fetal fluids (six fetal units were analyzed).** A, tissues and fetal fluids from a pregnant uninfected ewe analyzed with mAb 99/97; B, tissues and fetal fluids from a pregnant uninfected ewe analyzed with mAb 8H4; C, tissues from a nonpregnant uninfected ewe analyzed with mAb 99/97. *m-bra*, maternal brain; *ca-end*, caruncular endometrium; *co-cho*, cotyledonary chorioallantois; *ico-cho*, intercotyledonary chorioallantois; *all-fl*, allantoic fluid; *amn-fl*, amniotic fluid; *ica-end*, intercaruncular endometrium; *myo*, myometrium; *ovi*, oviduct; *ova*, ovary; *amn*, amnion; *ura*, urachus; *f-uri*, fetal urine; *f-bla*, fetal bladder; *f-kid*, fetal kidney; and *f-bra*, fetal brain. One to one and a half mg of tissue homogenates (wet tissue equivalent) or 7–9  $\mu$ l of fetal fluids per lane were used.

Western blot results of PrP-C in brain, reproductive, placental, and selected fetal tissues and fetal fluids of six pregnant uninfected and four nonpregnant uninfected ewes are shown in Fig. 2, A–C. PrP-C was not detectable when the primary mAbs were replaced by isotype control mAbs, nor when mAb 99/97 was preabsorbed with the immunogen peptide to which mAb 99/97 was generated (data not shown).

Fetal brain (Fig. 2, A and B, lane *f-bra*) contained PrP-C at an apparent molecular mass of ~25–38 kDa, similar to that in maternal brain (Fig. 2, A–C, lane *m-bra*) when probed with mAb 99/97 or 8H4. The molecular masses of these immunoreactive bands are consistent with the predicted molecular masses of PrP-C in sheep (4).

PrP-C isoforms with apparent molecular masses of ~23–37 kDa were also detected by both mAbs 97/99 and 8H4 in tissue homogenates of maternal reproductive tissues of both pregnant and nonpregnant ewes (uterine intercaruncular and caruncular endometria, myometrium, oviduct, and ovary; Fig. 2, A–C,

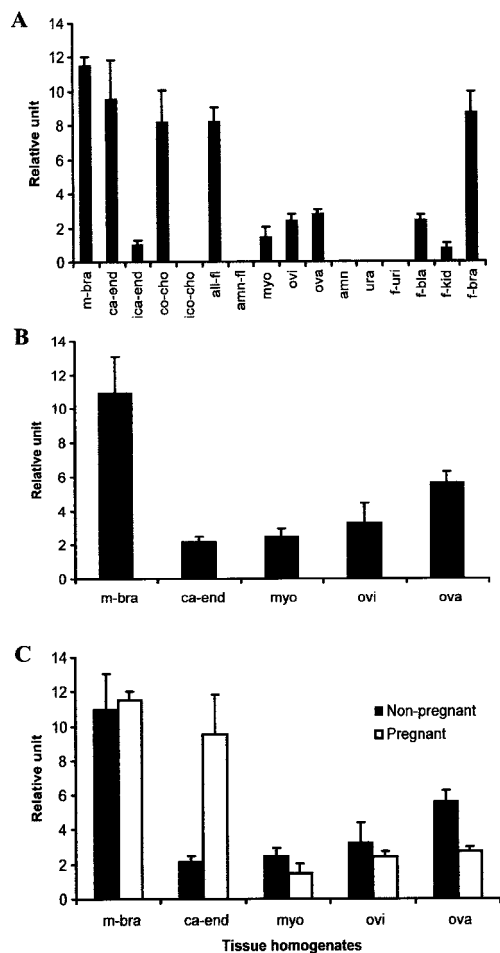
lanes *ica-end*, *ca-end*, *myo*, *ovi*, and *ova*), fetal placenta (cotyledonary chorioallantois; Fig. 2, A and B, lane *co-cho*), fetal fluid (allantoic fluid; Fig. 2, A and B, lane *all-fl*), and selected fetal tissues (fetal bladder; Fig. 2, A and B, lane *f-bla*). These PrP-C isoforms were either very low or undetectable in the intercotyledonary chorioallantois, urachus, amnion, amniotic fluid, fetal kidney, and fetal urine (Fig. 2, A and B, lanes *ico-cho*, *ura*, *amn*, *amn-fl*, *f-kid*, and *f-uri*). One predominant species of PrP-C was detected in cotyledonary chorioallantois (~26 kDa), allantoic fluid (27 kDa), and fetal bladder (27 kDa) (Fig. 2, A and B, lanes *co-cho*, *all-fl*, and *f-bla*).

**Levels of PrP-C in Reproductive, Placental, and Fetal Tissues and Fetal Fluids of Pregnant and Nonpregnant Uninfected Ewes**—Relative levels of PrP-C in fixed amounts of tissue homogenates of maternal brain, caruncular endometrium, cotyledonary chorioallantois, intercotyledonary chorioallantois, allantoic fluid, amniotic fluid, intercaruncular endometrium, myometrium, oviduct, ovary, amnion, urachus, fetal urine, fetal bladder, fetal kidney, and fetal brain of pregnant ewes and brain, caruncular endometrium, myometrium, oviduct, and ovary of nonpregnant ewes were determined by Western blotting and densitometry (Fig. 3). The Western blot assay was linear over a range of 3–200 ng of roPrP per lane in the presence or absence of 10% tissue homogenates (data not shown).

Levels of PrP-C in brains of pregnant, nonpregnant, and fetal sheep were similar ( $p > 0.05$ ), but were higher ( $p < 0.001$ ) than those of PrP-C in extraneural tissues and fetal fluids (Fig. 3, A–C). In pregnant ewes, similar ( $p > 0.05$ ) levels of PrP-C were detected in caruncular endometrium, cotyledonary chorioallantois, and allantoic fluid (Fig. 3A). Likewise, intercaruncular endometrium, myometrium, oviduct, ovary, fetal bladder, and fetal kidney also had similar ( $p > 0.05$ ) levels of PrP-C (Fig. 3A). However, PrP-C levels in caruncular endometrium, cotyledonary chorioallantois, and allantoic fluid were higher ( $p < 0.05$ ) than those in intercaruncular endometrium, myometrium, oviduct, ovary, fetal bladder, and fetal kidney. PrP-C in intercotyledonary chorioallantois, amniotic fluid, amnion, urachus, and fetal urine was either very low or undetectable (Fig. 3A). In nonpregnant ewes, levels of PrP-C in endometrium, myometrium, oviduct, and ovary were similar ( $p > 0.05$ ) (Fig. 3B). PrP-C in pregnant caruncular endometrium was higher ( $p < 0.05$ ) by almost 4-fold than PrP-C in nonpregnant caruncular endometrium (Fig. 3C). Pregnancy had no effect on PrP-C levels in myometrium, oviduct, and ovary ( $p > 0.05$ ) (Fig. 3C).

**PrP-C of the Uterus, Oviduct, Ovary, Cotyledonary Chorioallantois, Fetal Kidney, Fetal Bladder, and Allantoic Fluid Is Differentially Glycosylated and N-terminal Truncated**—PrP-C in the ovine brain was consistently detected as multiple bands with apparent molecular masses between 25 and 38 kDa (Fig. 2). However, the apparent molecular masses of PrP-C in reproductive, fetal, and placental tissues and fetal fluid were variable between 23 and 37 kDa (Fig. 2). To determine whether apparent variation of molecular masses of PrP-C isoforms in brain, uterus, oviduct, ovary, cotyledonary chorioallantois, fetal kidney, fetal bladder, and allantoic fluid was a result of differential glycosylation, tissue homogenates and allantoic fluid were treated with endoglycosidase (PNGase F) to remove the carbohydrate structures to assess the molecular mass of PrP-C protein core (Fig. 4).

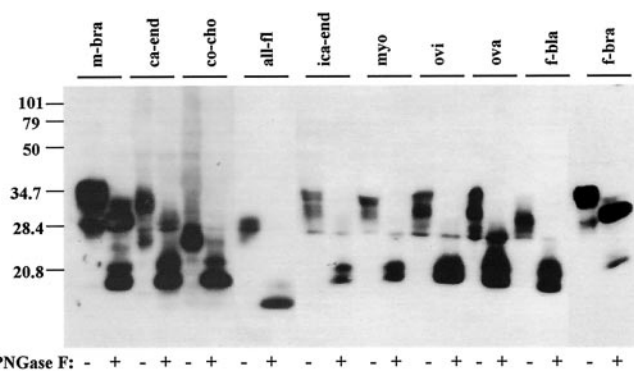
Treatment with endoglycosidase reduced the apparent molecular mass of fetal and maternal brain PrP-C to ~18–22 and ~29 kDa (Fig. 4, lanes *m-bra* and *f-bra*). Endoglycosidase treatment of tissue homogenates of the uterine caruncular endometrium, cotyledonary chorioallantois, intercaruncular endometrium, myometrium, oviduct, ovary, and fetal bladder



**FIG. 3. Levels of PrP-C in reproductive, placental, fetal tissues, and fetal fluids were determined in a fixed amount of tissue homogenates or fetal fluids of pregnant and nonpregnant uninfected ewes.** Affinity-purified roPrP (25 ng/lane), tissue homogenates prepared from brain (0.15 mg/lane wet tissue equivalent), all other tissues (1.5 mg/lane wet tissue equivalent), and fetal fluids (7–9  $\mu$ l/lane) were analyzed by Western blotting using mAb 99/97. Films exposed to Western blots for 1 min were analyzed by a digital imaging system. The results are expressed as relative units, representing the ratio between densitometric values of sample PrP-C and roPrP (25 ng). Data were analyzed by one-way analysis of variance with Student-Newman-Keuls multiple comparisons test. *A*, levels of PrP-C in tissues/fluids of pregnant uninfected ewes (tissues and fluids from six ewes and fetal units were analyzed). *B*, levels of PrP-C in tissues of nonpregnant ewes (tissues from four ewes were analyzed). *C*, comparison of PrP-C levels in reproductive tissues of pregnant ( $n = 6$ ) and nonpregnant ( $n = 4$ ) ewes. See Fig. 2 legend for explanation of abbreviations.

reduced the apparent molecular mass of PrP-C in these tissues to ~18–22 kDa with a minor species of 29 kDa in some tissues (Fig. 4, lanes *ca-end*, *co-cho*, *ica-end*, *myo*, *ovi*, *ova*, and *f-bla*). Allantoic fluid PrP-C was reduced by endoglycosidase treatment to ~16.5 kDa (Fig. 4, lane *all-fl*). Similar results were also shown for PrP-C in tissues collected from nonpregnant ewes (data not shown).

N-terminal truncation is common for PrP-C (26, 27) and, therefore, was also investigated in this study. mAb 5B2 or 8B4, which recognizes the N terminus of PrP, reacted to the full-length PrP-C in maternal or fetal brain, but lacked reactivity to isoforms of PrP-C present in uterine caruncular endometrium, cotyledonary chorioallantois, allantoic fluid, uterine intercaruncular endometrium, myometrium, oviduct, ovary, fetal bladder, and fetal kidney (data not shown). Neither mAb 99/97, 5B2, nor 8B4 detected PrP-C in intercotyledonary chorioallantois, amniotic fluid, amnion, urachus, or fetal urine (data not



**FIG. 4. Representative results of deglycosylation of PrP-C in brain, reproductive, fetal, and placental tissues and allantoic fluid of pregnant uninfected ewes ( $n = 6$ ).** Tissue homogenates (1–1.5 mg/lane wet tissue equivalent) or allantoic fluid (7–9  $\mu$ l/lane) was treated with or without PNGase F at 37 °C overnight followed by Western blot analysis using mAb 99/97. Similar results were also shown for PrP-C in tissues from nonpregnant ewes (data not shown). See Fig. 2 legend for explanation of abbreviations.

shown), further confirming the absence of PrP-C in these tissues/fluids.

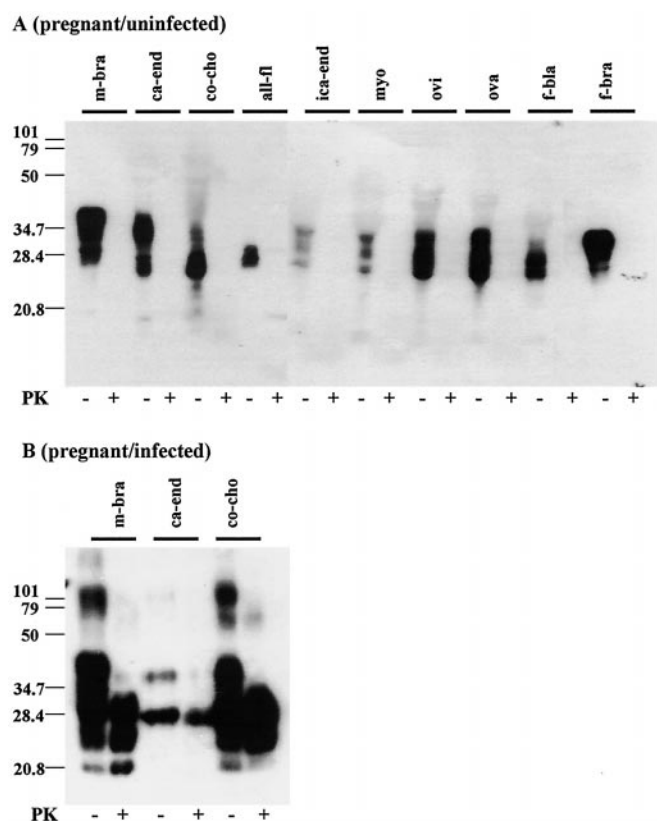
**PK Susceptibility of PrP-C and PrP-Sc in the Reproductive, Fetal, and Placental Tissues and Fetal Fluids of Normal and Scrapie-infected Ewes**—To determine whether the PrP detected in these tissues was susceptible to PK digestion, all direct tissue homogenates, PrP-Sc-enriched tissue homogenates, and fetal fluids of uninfected and scrapie-infected ewes were digested with PK and analyzed by Western blotting (Fig. 5). Results showed that PrP-C from fetal fluid and direct tissue homogenates of pregnant uninfected ewes was susceptible to PK digestion (Fig. 5A). Similar results were also shown for PrP-C in tissues collected from nonpregnant ewes (data not shown). No PrP-Sc was detected when tissues and fluids of pregnant uninfected ewes were extracted with the procedure used to prepare PrP-Sc-enriched tissue homogenates (data not shown).

PK-resistant PrP-Sc was detected in enriched tissue homogenates of maternal brain, uterine caruncular endometrium, and placental cotyledonary chorioallantois (Fig. 5B, lanes *m-bra*, *ca-end*, and *co-cho*), but not in intercaruncular endometrium, myometrium, oviduct, ovary, fetal spleen, fetal bladder, fetal kidney, and fetal brain (data not shown) from pregnant scrapie-infected ewes and their conceptuses. The detection of allantoic PrP-Sc was inconsistent due to the presence of endogenous protease inhibitors in allantoic fluid (data not shown) (28, 29).

## DISCUSSION

The present study demonstrated that PrP-C was differentially expressed by tissues and fluids of the female reproductive tract and conceptuses. Despite the wide distribution of PrP-C in the reproductive, placental, and fetal tissues and fetal fluids, PrP-Sc accumulation was only detected in the caruncular endometrium and cotyledonary chorioallantois of the placentome from pregnant infected ewes. These results may implicate a role of PrP-C in reproductive and fetal physiology and facilitate a further understanding of prenatal, perinatal, and postnatal transmission of scrapie under field conditions.

The biochemical properties of PrP-C in ovine reproductive, fetal, and placental tissues and fetal fluids have not been reported. This study used mAbs recognizing nonoverlapping epitopes of PrP to characterize PrP-C in these tissues and fluids. mAb binding showed that PrP-C in these tissues and fluids had an intact C terminus; however, the N terminus of PrP-C in reproductive, placental, and selected fetal tissues and



**FIG. 5. Representative results of PK susceptibility of PrP in brain, reproductive, fetal, and placental tissues and fetal fluids of uninfected ( $n = 6$ ) and scrapie-infected ( $n = 4$ ) ewes.** Direct tissue homogenates (1–1.5 mg/lane wet tissue equivalent), PrP-Sc-enriched tissue homogenates (15–30 mg/lane wet tissue equivalent), or allantoic fluid (7–9  $\mu$ l/lane) were incubated with or without PK (20  $\mu$ g/ml) at 37 °C for 30 min, followed by Western blot analysis using mAb 99/97. **A**, PK treatment and Western blot analysis of direct tissue homogenates of reproductive, fetal, and placental tissues and allantoic fluid from a pregnant uninfected ewe; similar results were also shown for PrP-C in tissues from nonpregnant uninfected ewes (data not shown). **B**, PK treatment and Western blot analysis of PrP-Sc-enriched tissue homogenates of caruncular endometrium and cotyledonary chorioallantois from a pregnant scrapie-infected ewe; PrP-Sc in other tissue homogenates was not detected (data not shown). See Fig. 2 legend for explanation of abbreviations.

allantoic fluid was differentially truncated. After the *N*-linked carbohydrate structures of PrP-C were removed, the protein core of ~29 kDa may represent the full-length PrP-C and that of ~18–22 kDa may represent the *N*-terminally truncated, potentially PK-resistant, C-terminal fragment of PrP-C (24, 26). These results suggest that PrP-C in these tissues and allantoic fluid is differentially glycosylated and truncated, giving rise to various apparent molecular masses. The results are consistent with the intrinsic partial protease resistance of the C-terminal portion of PrP-C in several species (27), indicating that an intermediate C-terminal fragment is present in many of the peripheral tissues as a result of limited protease digestion of PrP-C prior to a complete proteolysis. The additional truncation of PrP-C in allantoic fluid may indicate a further processing by proteases in the allantoic fluid, which is suggested by the synthesis and secretion of a number of proteases by the ovine placenta during pregnancy (30, 31).

The present study investigated the distribution of both PrP-C and PrP-Sc in the ovine reproductive tract, placental and selected fetal tissues, and fetal fluids. The findings suggest potential sites of maternal transmission of scrapie. In sheep, the ovum is released from the ovary at estrus, and it takes 3–4 days for the ovum to reach the uterus through the oviduct,

where the ovum is fertilized to form an embryo. The embryo will not be hatched from the zona pellucida, a noncellular mucoprotein structure immediately surrounding the embryo, until it is in the uterus. The present study showed that PrP-C, but not PrP-Sc, was present in the ovine ovary and oviduct. Our findings with four pregnant infected ewes are consistent with the report that scrapie infectivity was absent from ovaries of seven infected ewes (32). However, Hourrigan *et al.* (33) reported that scrapie infectivity was detected in ovaries of 4 of 14 ewes with unknown reproductive status. The presence of PrP-C or PrP-Sc in the ova, preimplantation embryos, and the zona pellucida with attached granulosa cells has not been reported. Although studies using embryo transfer to determine scrapie (34–36) or bovine spongiform encephalopathy (37) transmission by preimplantation embryos were conducted by several laboratories, the presence of PrP-Sc in embryos and disease transmission through embryos remain inconclusive (20, 38, 39). More studies with larger sample size are needed to confirm ovarian PrP-Sc using both bioassay and Western blotting.

PrP-C was abundantly present in the ovine uterus, which confirmed the previous finding (4); our study extended that observation by demonstrating that PrP-C was detectable in direct tissue homogenates of separated caruncular and intercaruncular endometria and myometrium. In addition, higher levels of PrP-C were detected in pregnant caruncular endometrium than in nonpregnant caruncular endometrium, suggesting that PrP-C is up-regulated during pregnancy. Since PrP-C up-regulation was limited to the caruncular region (caruncles) of the endometrium of pregnant ewes, it suggests that the intimate cellular contact or factor(s) synthesized and released by the apposing cotyledonary chorioallantois (cotyledon) as well as pregnancy-associated hormones and cytokines at the interface play a role in regulating PrP-C synthesis by the maternal endometrium. Since the caruncular endometrium is glandless and PrP-C was low in intercaruncular endometrium (rich in glands), PrP-C in caruncular endometrium may be primarily contributed by the surface endometrial epithelium. The present study for the first time demonstrated that PrP-Sc was present in the caruncular endometrium, but not in intercaruncular endometrium of the uterus of pregnant scrapie-infected ewes. These findings are consistent with the observation that low levels of scrapie infectivity were present in the ovine uterus, although the report provided no details on reproductive status of the animals and specific parts of the ovine uterus tested (33).

As the allantois grows during early gestation, it fuses with the chorion to form chorioallantois, which establishes extensive contact with the uterine endometrium at both caruncular and intercaruncular regions. The most intimate contact between the uterus and placenta is within the placentome (Fig. 1). In contrast to the placentation of primates and rodents, the conceptuses (fetus and associated membranes) of ruminants establish interactions with the uterus through noninvasive placentation within the uterine lumen. The cellular interactions and molecular exchange are active at the fetal-maternal interface of placentomes where leakage of maternal blood by endometrial villi and phagocytosis of maternal blood and degenerating endometrial cells by fetal trophoblast cells take place (40–42). The placental trophoblast cells may acquire nonspecifically pathogens at the interface during the physiological process of uptake nutrients during gestation. The present study and studies by others showed that scrapie infectivity (7–9, 43), PrP-Sc (9), and PrP-C (44) are present in the ovine uterus and placenta; therefore, PrP-Sc in the cotyledonary chorioallantois may be derived from direct phagocytosis of or conversion by the maternal PrP-Sc in the caruncular endometrium of infected ewes.



As the embryo develops, the amnion is formed and filled with amniotic fluid in which the embryo is suspended. The fetal urine and lung liquid secretion are the primary sources for amniotic fluid. The allantois is formed as a result of outgrowth of the hindgut and filled with allantoic fluid surrounding the amnion (Fig. 1). The allantoic fluid is primarily derived from fetal urine drained from the bladder through the urachus. Allantoic fluid may also be derived from chorioallantois secretion (45). The present study showed that high levels of soluble PrP-C were present in allantoic fluid, but not in other fetal fluids such as amniotic fluid and fetal urine. The source for allantoic fluid PrP-C was not directly determined. The absence of detectable PrP-C in fetal urine and amnion fluid shown in this study may indicate that fetal kidney, bladder, and urachus do not secrete detectable PrP-C into the urine, although PrP-C is synthesized by the kidney and bladder. Thus, we conclude that cotyledonary chorioallantois may be the primary source for allantoic PrP-C. The presence of PK-resistant allantoic PrP could not be demonstrated in the present study due to the presence of protease inhibitors in allantoic fluid.

Taken together, it appears that PrP-Sc is only present in tissues (such as caruncular endometrium and cotyledonary chorioallantois) expressing high levels of PrP-C, and the embryo/fetus is physically separated from the PrP-rich allantoic fluid and chorioallantois by the amnion, which has no detectable PrP-C. Based on the results of this study and previous reports, we propose that the ovine embryo/fetus may not be exposed to scrapie prior to parturition. The oocyte or early conceptus may not be exposed to PrP-Sc prior to hatching in the uterus, because the presence of an intact zona pellucida at this stage may protect the embryos from infection, as suggested by studies of a number of other pathogens (46). The hatched, preimplantation embryo may not be exposed to PrP-Sc due to the formation of extraembryonic membranes. After placentation, the fetus may not be exposed to PrP-Sc due to the presence of a PrP-negative zone, the amnion, around the developing fetus during the gestational period. A neonate is most likely to be exposed to PrP-Sc during or after parturition when fetal membranes are broken and chorioallantoic tissues and allantoic fluid contaminate the birth canal, body surface, and wool of both the mother and neonate, mammary glands, and the lambing area. PrP-Sc from both fetal membranes and fluids may serve as a source for horizontal transmission of scrapie under natural conditions (7). Overall, we propose that 1) the embryos/fetuses are less likely to be exposed to scrapie *in utero*, 2) lambs are likely to be exposed to scrapie at birth and during nursing, and 3) wasted placenta and possibly allantoic fluid of infected ewes may serve as a primary source for horizontal transmission.

The results of this study provide useful information for a better understanding of maternal transmission of scrapie during the prenatal, perinatal, and postnatal periods. Despite some differences in placentation among different species, ovine scrapie represents a valuable model for investigating PrP-Sc replication at the fetal-maternal interface in prion diseases such as chronic wasting disease and variant Creutzfeldt-Jakob disease, in which PrP-Sc is distributed outside of the nervous system (47, 48). These results also contribute to our understanding of the regulation of PrP-C at different stages of pregnancy and fetal development and the role of PrP-C in fetal-maternal interactions at the interface.

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