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A novel uterine lipocalin supporting pregnancy in equids

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Abstract. Horses, donkeys, and therefore, probably all equids, secrete a nonglycosylated, progesterone-dependent, 19-kDa protein (P19) into the uterine lumen during early pregnancy, and significant quantities of it are taken up by the developing conceptus. Sequence analysis and structural modelling have identified P19 as a lipocalin with greatest similarity to the murine major urinary protein lipocalins. However, lack of strong identity with any particular group of lipocalins and several unusual structural features, including a unique amino acid triplet within one of the invariant domains and an unusual external tryptophan residue, classify it as a new member of the lipocalin family. P19 is therefore likely to be a transport protein involved in supporting early embryonic development. Preliminary evidence using recombinant-derived P19 and fluorescently tagged ligands suggests that it may transport a fatty acid or retinol-like molecule. Although an initial search failed to identify homologues of P19 in other mammals, they may nevertheless exist but are synthesised and secreted in much smaller quantities, making them difficult to detect. Equids appear to need particularly large quantities of the protein during early pregnancy because of the unusually late implantation in this species and the presence of a capsule surrounding the conceptus until about day 23 of gestation.

Key words. Lipocalin; equine; uterus; pregnancy; embryo.

Introduction

Prior to implantation and development of the placenta, the mammalian conceptus is bathed in uterine secretions (histotrophe) from which it must obtain all of its nutrient and oxygen requirements. The duration of the preimplantation period varies considerably between species—from just a few days in rodents and humans to several weeks in the larger domestic species. For obvious reasons, those species with long pre-implantation periods produce more copious and complex uterine secretions during early pregnancy than do those with short preimplantation periods, and the endometrial lining of their uteri contain many more glands, the major source of the proteins within the secretions. Furthermore, in some species, particularly those with non-invasive placentae, endometrial histotrophe continues to play a role in nutritional support of the fetus to term. In all mammals, the steroid hormone progesterone is required for pregnancy maintenance, and one of its important functions is to stimulate the production of endometrial histotrophe. Uteroglobin was one of the first progesterone-dependent endometrial proteins to be studied in detail. However, although it is produced in large quantities during early pregnancy in many species, particularly rabbits, its function remains unclear [1]. Gene knockout studies in mice led to severe glomerular disease due to renal fibronectin deposition [2], but since the null mice were fertile, uteroglobin does not appear to be essential for pregnancy, at least in mice. This probably also applies to humans where, like mice, implantation is very early and the placenta is invasive. On the other hand, a very recent study has demonstrated that uteroglobin is synthesised in the endometrial glands of women and that it is stimulated by progesterone [3].

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The most important progesterone-dependent proteins in endometrial histotrophe are proteins that are required for the transport of hormones, vitamins and minerals to the embryo. The best known of these is retinol binding protein (RBP), which has been studied in many species, including cows [4], sheep [5], pigs [6] and horses [7]. It is synthesised by the endometrium, and in pigs there is a novel family of progesterone-induced endometrial RBPs [6]. However, since there is significant transudation of serum proteins into the uterine lumen, some of the RBP present in uterine secretions is almost certainly serum derived. Since retinol, the principal serum transport form of vitamin A, is so important in embryonic development, uterine RBPs are assumed to play a vital role in pregnancy, particularly in those species with a long preimplantation period such as pigs, sheep, cattle and particularly horses.

Uteroferrin, the uterine iron transporter, has also been studied in several species. It was originally described in the pig, where it has a distinct purple colour and was initially referred to as 'purple protein'. Uteroferrin has been partially characterised in the horse and shown to be very similar to that in the pig, with the same distinctive purple coloration, acid phosphatase activity and a molecular mass of 35 kDa [8].

This review will describe a newly discovered progesterone-dependent transport protein, P19, which is produced in large quantities by the mare and other equids during early pregnancy. It is a lipocalin, and therefore closely related to RBP. However, it has been classified as a new member of the lipocalin family, and there is no clear indication yet as to what it might be transporting to the embryo. It is thought that equids secrete particularly large quantities of P19 during early pregnancy because, in addition to the zona pellucida, their embryos are enveloped by a second coat, the embryonic capsule, through which all maternal nutrients must pass. The embryonic capsule forms beneath the zona pellucida at about 6 days after ovulation. After the zona has disappeared at day 7, the capsule expands and develops along with the conceptus until about day 22, but then it fragments and disappears [9]. Furthermore, other than species that display true delayed implantation during which embryonic development is arrested, equids have the longest preimplantation period known for any mammal. This is because the true chorioallantoic placenta, which provides haemotrophic nutrition for the embryo, does not start to form until the 6th week of gestation (term $\approx$ 11–12 months). Nevertheless, early equine embryonic development proceeds at a very similar rate to that in other large mammals, including humans.

Discovery of P19

P19 was initially isolated and partially characterised from the culture medium in which horse embryos had been incubated, suggesting that the protein was taken up in significant quantities by the embryo in vivo and then released into the medium [10]. The purpose of these culture experiments was to identify new embryonic proteins by incubating early (day 11–14) equine embryos in serum-free medium overnight with or without their capsules and analysing the medium by SDS-polyacrylamide gel electrophoresis (PAGE). The medium from the embryos intact within their capsules contained a dominant protein band, estimated to be around 18 kDa in size, whereas the medium from embryos without their capsules contained no obvious 18-kDa band. N-terminal sequencing of the first 18 residues of a sample excised from a gel failed to identify the protein and suggested that it was a new protein. At this stage the protein was called P18 (based on the original estimate of its size), and it was assumed to be embryonic in origin. However, when a dominant protein of the same size was noticed on PAGE gels of mare uterine secretions, it was also sequenced and shown to have an identical N-terminal sequence. This suggested that the protein was maternal in origin and that it was produced preparative to the arrival of a fertilised ovum. Additional investigations showed that nonpregnant mares secreted the protein during the luteal phase of the estrous cycle, i.e. when progesterone levels were high, indicating that the protein was progesterone-dependent. Levels of the protein in the uterine lumen were then measured semiquantitatively during the estrous cycle and early pregnancy and shown to correlate well with levels of progesterone in maternal blood. Exogenous progesterone treatment also stimulated P19 secretion in anestrous mares. However, the protein became undetectable on PAGE gels after about day 23 of gestation, even though progesterone levels remained elevated, suggesting that an additional mechanism operates during pregnancy to downregulate secretion [10]. Subsequent studies have shown that although the protein is undetectable in uterine secretions after day 23, a small amount of immunoreactive protein is detectable in the endometrial glands as late as 250 days and in the fetal trophoblast cells overlying these glands, the so-called intercotyledonary regions of the placenta [11]. This suggests that P19 may continue to play a role in supporting the equine fetus to term. A similar pattern of secretion was noted in female donkeys, indicating that all equids secrete the protein during the luteal phase of the estrous cycle and pregnancy. In order to obtain more information about the origin and function of this protein, the complementary DNA (cDNA) was cloned and sequenced.

Cloning the cDNA for P19

The cDNA for the newly discovered protein was cloned from an equine endometrial cDNA library [12]. Initially, a mixture of oligos based on the back-translation of the N-terminal sequence was shown to hybridise to a single band on Northern blots of equine endometrial RNA samples. A cDNA library was then made from one of the samples and screened with the mixture of oligos. Although this did not yield any positive clones, the oligos were used in a polymerase chain reaction (PCR), along with a vector primer, to obtain a small 5′ fragment of the cDNA covering the signal peptide and most of the amino acid residues identified in the initial N-terminal sequencing experiments. The PCR product was used to screen the library, and several full-length cDNA clones were obtained. Theoretical translation and analysis of the sequence revealed a pI of 9.72 and a molecular mass of 18,793 Da. Since previous studies had shown that the protein was not glycosylated [10], this theoretical calculation of almost 19 kDa was considered to be a fairly accurate estimate of its mass, and the protein was renamed P19. The cDNA clone was also used in Northern blots of RNA from a number of organs and tissues (including the conceptus) to show that the endometrium was the only site of expression in the mare. Oligonucleotides were then used in in situ hybridisation experiments to show that messenger RNA (mRNA) for P19 was specifically localised to the epithelium of the glands within the endometrium. This site of synthesis is very much in keeping with secreted endometrial proteins. Southern blots using the cDNA clone showed that a single gene encodes P19, the exons of which span approximately 4.5 kb [12]. This is consistent with the other members of the lipocalin family, which have a conserved structure consisting of seven exons spread over 6–25 kb [13].

Structure of P19

Further analysis of the P19 sequence showed that it is a lipocalin with highest identity to the mouse (34%) and rat (32%) major urinary proteins (MUPs) and 20% identity to horse RBP [12]. It was therefore clearly a new member of the family. The most unusual substitutions in P19, compared with the other lipocalins, were Val-Asp-Pro instead of Thr-Asp-Tyr in the second of the three major lipocalin structural motifs [14] and a tryptophan in the third motif. Secondary structural predictions indicated eight β strands and two α helixes, which aligned very well with the consensus lipocalin structure. Also, modelling of the P19 sequence to the known crystal structure of a MUP (Protein Data Bank accession 1MUP) gave an excellent fit [15] and provided the three-dimensional (3D) model shown in figure 1. As with all lipocalins, the eight β strands form a cup-shaped β barrel to create a binding pocket for small hydrophobic molecules. The unique tryptophan on the large a helix is exposed and may be involved in a receptor binding site.

Most of the liopcalins are transport proteins, although it is now clear that they perform many other functions, including controlling enzyme synthesis, regulating immune responses and maintaining cell homoeostasis [14, 16]. Although the overall sequence similarity between P19 and the MUPs gave the highest score, and P19 is obviously structurally very similar to the MUPs, it is very unlikely that P19 is a member of this group of lipocalins or that it binds a pheromone; P19 is not expressed in horse kidneys, and pheromones have never been described in endometrial secretions. The closest lipocalin to P19, in terms of site of production and possible function, is placental protein 14 (PP14). It was discovered as a member of a large group of soluble human placental proteins [17]. However, it was later discovered that like P19, PP14 is synthesised and secreted by the epithelial cells of endometrial glands in women [18] and is progesterone-dependent [19]. PP14 may therefore have a similar transport function to P19, but since the sequence identity between them is only 19%, they may transport different molecules. Interest-

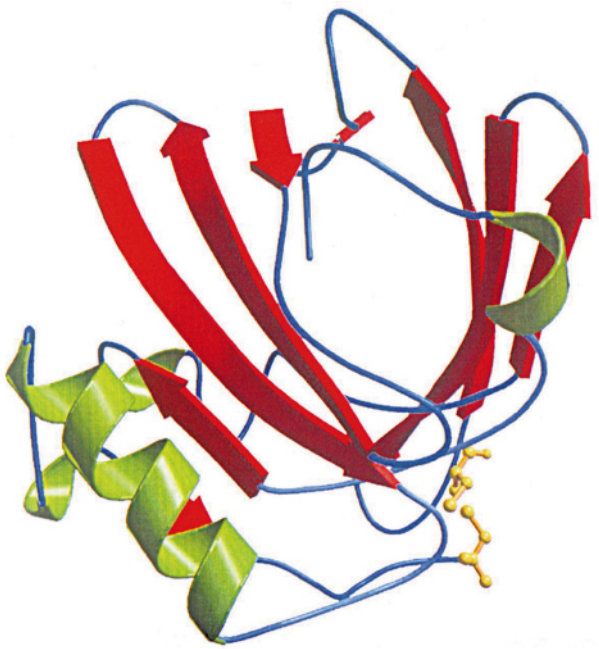

Figure 1. Predicted tertiary structure of P19, based on the crystal structure of mouse major urinary protein 1 (Protein Data Bank accession 1MUP), showing an end-on view of the eight-stranded β-barrel fold (red) that forms a cup-shaped binding pocket in all lipocalins. The α helices are shown in green, and the side chains of the two conserved cysteine residues (forming a disulphide bond) are shown in yellow. The unusually positioned tryptophan residue appears to project directly into solvent from the helix (not shown).

ingly, although PP14 has quite high sequence identity (around 44%) with β-lactoglobulin, the retinol transport protein in milk, PP14 does not appear to bind retinol or retinoic acid [20].

Immunolocalisation of P19

Initial studies showed that the capsule surrounding the early horse conceptus bound significant quantities of P19 in vivo [10], but in order to investigate whether it was transferred through the capsule and into the embryo itself, antisera were raised against the protein. The first P19 antiserum was raised against a C-terminal peptide [21], which worked very well on Western blots and was used to detect P19 in the capsule, the fetal membranes and the yolk sac fluid of day-16 horse embryos. It failed, however, to detect P19 in histological sections, presumably because it was unable to recognise the native protein in tissue sections. A high-titre rabbit antiserum was then raised to recombinant-derived P19 (P19-GST fusion protein), which worked well on both Western blots and on tissue sections. This antiserum demonstrated the presence of P19 in the endometrial glands of the mare and in the capsule, the membranes and the yolk sac fluid of early horse conceptuses. Immunostaining of the membranes showed localisation of P19 to the apical surface of the trophoblast cells, but no staining on the embryonic disc, suggesting specific binding to trophoblast cells [21]. Figure 2 shows the localisation of P19 by immunostaining in an endometrial biopsy from a mare at day 14 of gestation and part of the conceptus recovered from the same mare. Positive staining can be seen in the glands of the endometrium and in the trophoblast cells of the conceptus.

These studies provided good evidence that P19 is taken up by the highly absorptive trophoblast cells of the early horse conceptus and that some of it passes through into the yolk sac fluid. A third polyclonal antiserum, raised against a preparation of aggregated, insoluble recombinant-derived P19 from *Escherechia coli* inclusion bodies, gave a much higher titre antiserum which was used in an additional study to show that P19 synthesis and secretion is disrupted in mares with age-related degenerative changes in their endometrium [11]. This study showed that small amounts of P19 are also detectable in the endometrial glands and trophoblast cells of the pregnant mare as late as day 250 of gestation. Thus, although P19 cannot be detected in the uterine secretions after the first month of gestation, small amounts are probably secreted and taken up by the placenta throughout gestation.

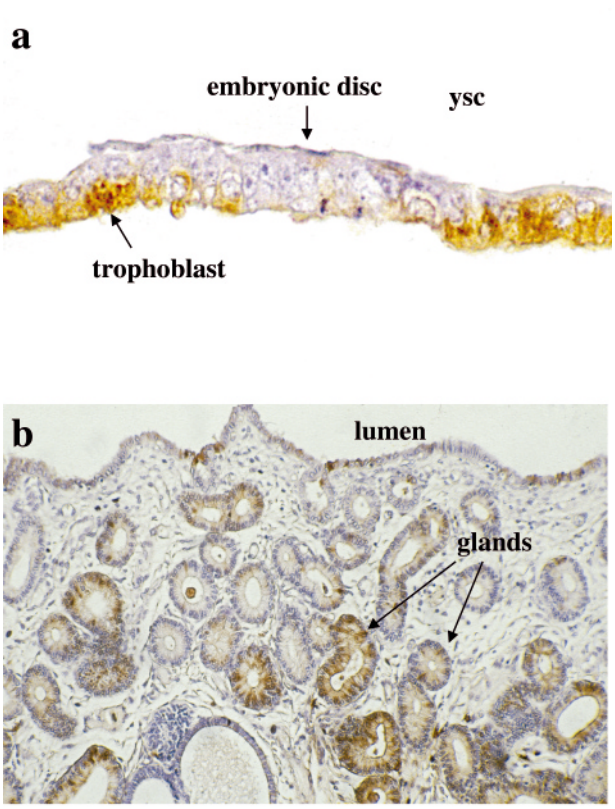

Figure 2. Immunostaining (brown colour) for P19 in (*a*) the conceptus and (*b*) the endometrium of a mare at day 14 of gestation. P19 is produced by the epithelial cells of the endometrial glands, secreted into the lumen of the uterus and then taken up by the trophoblast cells of the developing conceptus. The polar trophoblast cells overlying the embryonic disc have almost disappeared by this stage of gestation, thus explaining the lack of staining in this region. ysc, yolk sac cavity.

Transporter function

Three approaches are being used to try and identify the ligand(s) for P19, (i) determination of its 3D structure by X-ray crystallography, and thus the shape and charge environment of its binding pocket, (ii) analysis of the binding affinities between recombinant-derived P19 and several potential ligands and (iii) identification of the ligand(s) by gas chromatography-mass spectrometry (GC-MS) analysis of native protein. Sufficient recombinant-derived protein has been produced for growing crystals, but solubility problems have so far hampered attempts to concentrate the protein to a sufficiently high level for crystallisation trials. However, it should be possible to overcome these problems in the near future.

Preliminary studies using recombinant P19 have shown that it binds a range of fluorescent lipids, such as the

fluorophore-tagged fatty acid 11-[(5-dimethylaminonaphthalene-1-sulphonyl)amino]undecanoic acid (DAUDA), which has been successfully used in the analysis of a range of lipid-binding proteins [22]. Displacement of DAUDA by natural, nonfluorescent lipids can therefore be used to screen for the kinds of lipid ligands that P19 may bind in vivo. To date, we have found that P19 binds oleic, arachidonic, linoleic, linolenic, docosahexaenoic and eicosapentaenoic acids. Using the dramatic change in the fluorescence emission of retinol which occurs upon binding to its carrier proteins (such as β-lactoglobulin), P19 was found to bind this lipid also. P19 therefore binds many of the small lipids which are essential to the cellular differentiation and pattern formation events involved in the development of mammalian embryos (see article by Dutta-Roy, this issue; [23]). By the time that secretion of P19 ceases, organogenesis in the horse conceptus is already well advanced, so these processes may be entirely dependent on P19. All of the lipids are poorly soluble in water, and would also be highly susceptible to degradation in an aqueous phase if not protected within a carrier protein. Moreover, a carrier protein may also have features which will direct it and its ligand more efficiently to a receptor protein or membrane than would occur by simple diffusion.

It is important to note, however, that the ligands which recombinant P19 can be demonstrated to bind in vitro may not be representative of the ligands it transports in vivo, because the secreting endometrial cells may be highly specific in the lipids with which P19 is loaded before export. We are therefore attempting to acquire sufficient purified P19 from mare uterine secretions with the aim of extracting and identifying the natural ligand(s) directly. This is, however, not an easy task, and the availability of recombinant, biochemically active P19 will be important to understanding the biochemistry and structure of the protein for the foreseeable future.

Conclusion

P19 is a novel lipocalin structurally related to, but distinct from, serum retinol-binding protein, and is probably involved in the transport of small lipids to the developing equine embryo. The reason for its abundance in the pregnant mare is probably in order to provision the conceptus with essential lipids during the particularly long preimplantation period in horses and/or to transfer lipids across the acellular embryonic capsule that surrounds the equine conceptus until day 23 of gestation. Homologues of P19 may exist in other species, particularly those with a long preimplantation such as the pig. Determining the 3D structure of P19 and identification of the ligand(s) it binds in vivo will provide new information on the structure-function relationships of lipocalins in general and might identify nutrients which are essential to early embryogenesis in mammals.

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1 Beier H. M. (1982). Uteroglobin and other endometrial proteins: biochemistry and biological significance in beginning pregnancy. In: Proteins and Steroids in Early Pregnancy, Beier H. M. and Karlson P. (eds), pp. 39–71, Spriger, Berlin.
2 Zhang Z., Kundu G. C., Yuan C.-J., Ward J. M., Lee E. J., De Mayo F. et al. (1997) Severe fibronectin deposit renal glomerular disease in mice lacking uteroglobin. Science **276:** 1408–1412
3 Muller-Schottle F., Classen-Linke I., Alfer J., Krusche C., Beier-Hellwig K., Sterzik K. et al. (1999) Expression of uteroglobin in the human endometrium. Mol. Human Reprod. **5:** 1155–1161
4 Thomas P. G. A., Leslie M. V. and Hansen P. J. (1992) Retinol binding protein is produced by the bovine endometrium and accumulates in uterine secretions in a progesterone-dependent manner. Anim. Reprod. Sci. **27:** 55–66
5 Harney J. P., Ott T. L., Geisert R. D. and Bazer F. W. (1993) Retinol binding protein gene expression in cyclic and pregnant endometrium of pigs, sheep and cattle. Biol. Reprod. **49:** 1006–1073
6 Clawitter J., Trout W. E., Burke M. G., Araghi S. and Roberts R. M. (1990) A novel family of progesterone-induced retinol binding proteins from uterine secretions of the pig. J. Biol. Chem. **265:** 3248–3255
7 McDowell K. J., Adams M. H., Franklin K. M. and Baker C. B. (1995) Changes in equine endometrial retinol-binding protein RNA during the estrous cycle and early pregnancy and with exogenous steroids. Biol. Reprod. **52:** 438–443
8 McDowell K. J., Sharp D. C., Fazleabas A., Roberts R. M. and Bazer F. W. (1982) Partial characterisation of the equine uteroferrin-like protein. J. Reprod. Fert. **32:** 329–334
9 Betteridge K. J., Eaglesome M. D., Mitchell D., Flood P. F. and Beriault R. (1982) Development of horse embryos up to twenty two days after ovulation: observations on fresh specimens. J. Anat. **135:** 191–209
10 Stewart F., Charleston B., Crossett B., Barker P. J. and Allen W. R. (1995) A novel endometrial protein that associates with the embryonic capsule in equids. J. Reprod. Fert. **105:** 65–70
11 Stewart F., Gerstenberg C., Suire S. & Allen W. R. (2000). Immunolocalisation of a novel endometrial protein (P19) in the endometrium of fertile and subfertile mares. J. Reprod. Fert. Suppl. 56, in press
12 Crossett B., Allen W. R. and Stewart F. (1996) A 19 kDa protein secreted by the endometrium of the mare is a novel member of the lipocalin family. Biochem. J. **320:** 137–143
13 Igarashi M., Nagata A., Toh H., Urade Y. and Hayaishi O. (1992) Structural organisation of the gene for prostaglandin-D synthase in the rat brain. Proc. Natl. Acad. Sci. USA **89:** 5376–5380
14 Flower D. R. (1995) Multiple molecular recognition properties of the lipocalin protein family. J. Mol. Rec **8:** 185–195
15 Bocskei Z., Groom C. R., Flower D. R., Wright C. E., Phillips S. E. V., Cavaggioni A. et al. (1992) Pheromone binding to two rodent urinary proteins revealed by X-ray crystallography. Nature **360:** 186–188

16 Flower D. R. (1994) The lipocalin family – a role in cell regulation. FEBS Lett. **354:** 7–11
17 Bohn H., Kraus W. and Winckler W. (1982) New soluble placental tissue proteins: their isolation, characterisation, localisation and quantification. Placenta Suppl. **4:** 67–81
18 Julkunen M., Koistinen R., Suikkari A. M., Seppala M. and Janne O. A. (1990) Identification by hybridisation histochemistry of human endometrial cells expressing m-RNAs encoding a uterine β-lactoglobulin homologue and insulin-like growth factor-binding protein 1. Mol. Endocrinol. **4:** 700–707
19 Julkunen M., Koistinen R., Sjoberg J., Rutanen E. M., Wahlstrom T. and Seppala M. (1986) Secretory endometrium synthesizes placental protein 14. Endocrinology **118:** 1782–1786
20 Seppala M., Riittinen L., Kamarainen M. and Julkunen M. (1992) A review of endometrial protein PP14 secretion during the postovulatory period and pregnancy. Reprod. Fert. Dev. **4:** 611–618
21 Crossett B., Suire S., Herrler A., Allen W. R. and Stewart F. (1998) Transfer of a uterine lipocalin from the endometrium of the mare to the developing equine conceptus. Biol. Reprod. **59:** 483–490
22 Kennedy M. W., Garside L. H., Goodrick L. E., McDermott L., Brass A., Price N. C. et al. (1997) The Ov20 protein of the parasitic nematode *Onchocerca volvulus*. A structurally novel class of small helix-rich retinol-binding protein. J. Biol. Chem. **272:** 29442–29448
23 Dutta-Roy, A. K. (2000) Cellular uptake of long chain fatty acids: role of membrane associated fatty acid binding/transport proteins. Cell. Mol. Life Sci.