REPRODUCTION
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Interferon-τ upregulates prolactin receptor mRNA in the ovine endometrium during the peri-implantation period

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Abstract

Our objective was to determine the effect of ovine interferon-τ (IFN-τ) on prolactin receptor (PRL-R) gene expression in the ovine endometrium. IFN-τ is an embryonic cytokine which, via its paracrine anti-luteolytic activity, plays a critical role in maternal recognition of pregnancy in ruminants. Using ribonuclease protection assay procedures, we compared endometrial PRL-R mRNA levels in ewes that were intrauterine injected with either 2 mg bovine serum albumin or 2 mg recombinant ovine IFN-τ on day 10 of the oestrous cycle (day 0 = day of oestrus). IFN treatment significantly increased the abundance of both the long and short forms of PRL-R mRNA in the ovine uterus, but had no effect on the long:short form ratio. *In situ* hybridization experiments revealed that the increase in abundance of PRL-R mRNA in the uterus was localized to the glandular compartment of the endometrium. In pregnant ewes, a similar increase in PRL-R mRNA abundance was found to occur in ovine endometrium on days 14–15 post conception. Collectively, these data provided strong evidence that IFN-τ modulates the level of lactogenic hormone receptor mRNA in the ovine uterus. Whether the effect of IFN-τ on PRL-R expression is mediated directly or influenced, at least in part, by progesterone remains to be elucidated.

Reproduction (2004) **128** 99–105

Introduction

Implantation of the conceptus is a critical developmental step in mammalian reproduction and involves a complex interaction between embryonic trophectodermal cells and receptive maternal endometrium. During implantation, the uterus undergoes remodelling to create an optimized environment for embryonic and foetal growth. The generation of this remodelled receptive endometrium is dictated by various factors including peptides, steroid hormones, cytokines and growth factors, which are sequentially secreted by both embryonic and maternal tissues. A major messenger in the communication that occurs between the conceptus and the endometrium of sheep is the type I interferon-τ (IFN-τ), which is anti-luteolytic and transiently secreted by the extra-embryonic trophectoderm during the peri-implantation period (days 12–21 of pregnancy) (Martal *et al.* 1979, Hansen *et al.* 1988). IFN-τ exerts a paracrine effect on the uterine epithelium to suppress oestrogen and oxytocin receptor gene expression, which prevents pulsatile production of prostaglandin $F_{2\alpha}$ and subsequent luteolysis (Bazer *et al.* 1996, Martal *et al.* 1997, 1998).

In sheep, implantation occurs by day 15 of pregnancy and is coincident with onset of binucleate-trophectodermal cell production of the prolactin (PRL)- and growth hormone (GH)-related hormone, placental lactogen (PL) (Martal *et al.* 1977, Wooding *et al.* 1992). Moreover, local production of PRL and the presence of PRL receptors (PRL-R) in the pregnant uterus of a number of species, including sheep (Cassy *et al.* 1999), provide the opportunity for PRL to have a direct effect on the uterus. The precise role of lactogenic hormones (PRL/PL) in the foeto–maternal environment is currently unknown. One hypothesis is that lactogenic hormones are not only involved in endometrial–glandular–epithelial hypertrophy and differentiation (Chilton & Daniel 1985, Gray *et al.* 2001*a*), but also in influencing uterine secretions (Young *et al.* 1989, Randall *et al.* 1991). Because implantation is a process involving a precise synchronization between blastocyst development and uterine receptivity, lactogenic hormones may constitute essential factors in the dialogue between mother and conceptus during this critical period. Indeed, Ormandy *et al.* (1997) demonstrated, using PRL-R knockout mice, that lactogenic hormones play a major role in embryonic development and in the implantation process.

In the present study, we have hypothesized that IFN-τ may be involved in the regulation of lactogenic hormone receptor mRNA levels in the ovine endometrium during early pregnancy. We thus provide the first *in vivo* evidence that IFN-τ is able to upregulate PRL-R gene

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/rep.1.00158
Online version via www.reproduction-online.org

expression in the ovine endometrium. A quantitative analysis of the two forms of PRL-R mRNA levels was first conducted by ribonuclease protection assay, and then spatially confirmed by *in situ* hybridization. Moreover, using ribonuclease protection assay procedures we report that transcription levels of both long and short PRL-R forms increase during the time of maternal recognition of pregnancy.

Materials and Methods

Animals, surgery and experimental procedure

All procedures relating to the care and use of animals were approved by the French Ministry of Agriculture in accordance with the French regulations for animal experimentation (guideline 19/04/1988). This study was performed using 18 primiparous Préalpes du Sud ewes. Oestrous cycles were synchronized as previously described (Cassy *et al.* 1998). Ewes were assigned to the following experimental groups. (1) Ewes were slaughtered on days 7–8 ($n = 4$) or 14–15 ($n = 5$) following a fertile mating. At the time of slaughter, the presence of a conceptus was identified to confirm that the ewes were pregnant. (2) Control ewes ($n = 3$) were exposed to non-fertile rams and received a single intrauterine injection of 2 mg bovine serum albumin (BSA) diluted in 0.9% NaCl (1 ml) on day 10 of the oestrous cycle. (3) Ewes in the treated group (IFN-τ; $n = 6$) received a single intrauterine injection of 0.9% NaCl (1 ml) with 2 mg recombinant IFN-τ (Institut National de la Recherche Agronomique, Dijon, France; 10^8 IU/mg) + 2 mg BSA in the uterine horn adjacent to the corpus luteum on day 10 of the oestrous cycle and were slaughtered 6 days later when uterine horns were collected and prepared for subsequent analysis. To verify the maintenance of luteal function, daily blood samples were collected by jugular venipuncture and progesterone concentrations were measured by radioimmunoassay as described by Heyman *et al.* (1984).

Preparation of the hybridization probes

Two recombinant Bluescript SK(−) transcription vectors (Stratagene, Amsterdam, The Netherlands) containing either the 324 bp AflII-XbaI fragment of the ovine PRL-R cDNA as described by Cassy *et al.* (1998) or the full-length ovine PRL-R cDNA were used to perform, respectively, ribonuclease protection assay and *in situ* hybridization. After linearization, antisense and sense cRNA probes were further transcribed using either T3 or T7 RNA polymerases (Invitrogen, Cergy-Pontoise, France) and labelled with [α-^{35}S]UTP (>1000 Ci/mmol; Amersham, Saclay, France) for *in situ* hybridization or [α-^{32}P]UTP (800 Ci/mmol; Amersham) for ribonuclease protection assay. Ovine full-length PRL-R sense cRNA probe was used as a negative control for *in situ* hybridization. For ribonuclease protection assays, all results were normalized using a pGEM-T (Promega, Charbonnières-les-Bains, France) vector containing a 110 bp fragment of the ovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene (Cassy *et al.* 2000).

Tissue preparation and RNA extraction

Total RNA extraction from ovine endometrium was performed by the guanidium–isothiocyanate method as previously described by Chomczynski & Sacchi (1987) and as modified by Puissant & Houdebine (1990). Poly(A) + RNA was then isolated using the poly(A) + RNA isolation kit (R&D System, Abingdon, Kent, UK) according to the manufacturer's instructions. For *in situ* hybridization, intercaruncular endometrial tissue was cut and fixed in 1% paraformaldehyde for 24 h at 4 °C, cryoprotected in a 15% sucrose solution overnight, and frozen with Tissue-Tek (Leica, Rueil Malmaison, France) at −45 °C in isopentane cooled on dry ice. Tissues were then stored at 80 °C until use.

In situ *hybridization*

Seven micrometer cryostat sections of endometrium were mounted onto Superfrost/Plus slides (Prolabo, Fontenay-sous-Bois, France), postfixed in 4% paraformaldehyde, washed in 4 × SSC (1 × SSC: 150 mM sodium chloride and 15 mM sodium citrate, pH 7), acetylated in a 4 × SSC solution containing 0.25% anhydride acetic and 0.1 M triethanolamine, dehydrated in ethanol and air dried. Each slide received 50 μl of the hybridization mixture described by Cassy *et al.* (1998, 2000) and contained 2×10^7 c.p.m./ml sense or antisense cRNA probe. Slides were then incubated overnight at 55 °C in a moist chamber before a 30-min RNase A (20 μg/ml in 4 × SSC) treatment. After successive washings in decreasing concentrations of SSC at room temperature, slides were placed sequentially in two baths of 0.1 × SSC at 65 °C for 30 min each and dehydrated in ethanol. Dried slides were processed for autoradiography using NTB2 photographic emulsion (Eastman KODAK Européenne d'Imagerie Scientifique, Massy, France) and exposed for 2–4 weeks at 4 °C. Sections were then stained with toluidine blue and examined with a Reichert microscope equipped with an epi-illumination system and a polarizing filter (Leica, Rueil Malmaison, France).

Ribonuclease protection assay

The ribonuclease protection assay was carried out as described by Sambrook *et al.* (1989). Briefly, 20 μg tRNA or 5 μg poly(A) + RNA were hybridized in 30 μl hybridization buffer (80% formamide, 40 mM Pipes, pH 6.4, 0.4 M NaCl and 1 mM EDTA) containing 10^6 c.p.m. of the [α^{32}P]UTP-labelled probe. The mixture was heated at 85 °C for 10 min and incubated overnight at 37 °C. Samples were then digested with 16 μg/ml RNase A and 0.8 μg/ml RNase T1 for 1 h at 15 °C, and with 300 μg/ml proteinase K for 30 min at 37 °C. Protected RNA hybrids were

extracted, precipitated and resuspended in water. After denaturation, hybrids were loaded on a 6% acrylamide sequencing gel containing 8 M urea and visualized by autoradiography. Various controls were performed by incubating the probe with 50 μg yeast tRNA, and with or without RNase treatment. Signals were finally quantified using a STORM-860 scanner (Molecular Dynamics, Bondoufie, France) and ImageQuaNT software.

Results

Maintenance of luteal function after ovine IFN-τ injection

In order to verify the anti-luteolytic effect of IFN-τ, serum progesterone was measured in control and injected ewes on day 10 of the oestrous cycle. As shown in Fig. 1, serum progesterone concentrations dropped in the control group (ewes 1, 2 and 3) from days 14 to 15 of the oestrous cycle to less than 1 ng/ml between days 15 and 16. Intrauterine administration of 2 mg recombinant oIFN-τ had a significant effect on the maintenance of luteal function since ewes 4, 5, 6, 7, 8 and 9 exhibited a progesterone concentration higher than 3 ng/ml on day 16.

Effect of IFN-τ on PRL-R gene expression in ovine endometrium

The 324 bp AflII-XbaI cDNA fragment, including at its 3′ end the 39 bp insertion characteristic of the short form of the ovine PRL-R cDNA, enabled us to detect simultaneously both long and short forms of the ovine PRL-R. As expected, two cRNA fragments of sizes 324 and 285 bp, corresponding respectively to the short and the long forms of ovine PRL-R, were protected from RNase digestion in each ewe of the control and IFN-τ-treated groups (Fig. 2A). When the ovine PRL-R cRNA probe was hybridized with non-specific RNA (yeast tRNA) and treated with RNases, no signal was detected on the gel (Fig. 2A, lane C2). Using STORM scanning, the relative proportions of both transcripts encoding the two forms of PRL-R were quantified and normalized. As is evident in Fig. 2B, IFN-τ treatment induced a dramatic increase (~sixfold) in abundance of the long and short forms of the PRL-R mRNA. Moreover, the ratio of the long to the short

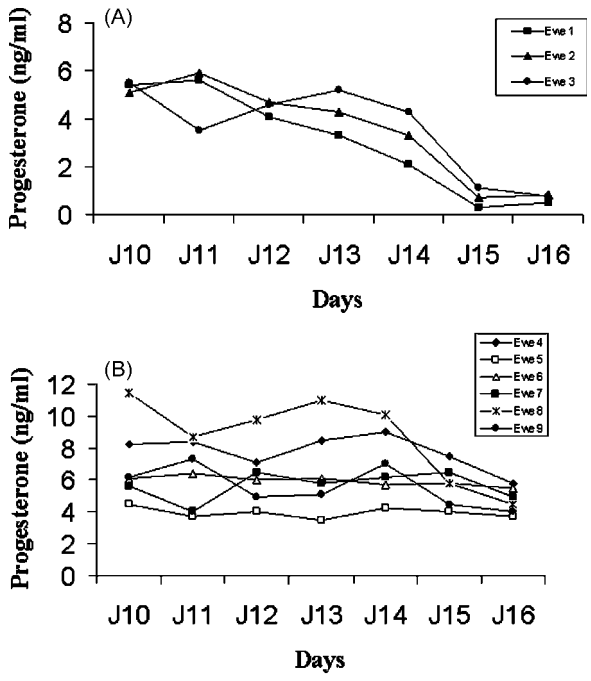

Figure 1 Progesterone concentrations (ng/ml) in peripheral serum of cyclic recipient ewes after intrauterine injection of (A) 2 mg BSA or (B) 2 mg recombinant ovine IFN-τ on day 10 of oestrus.

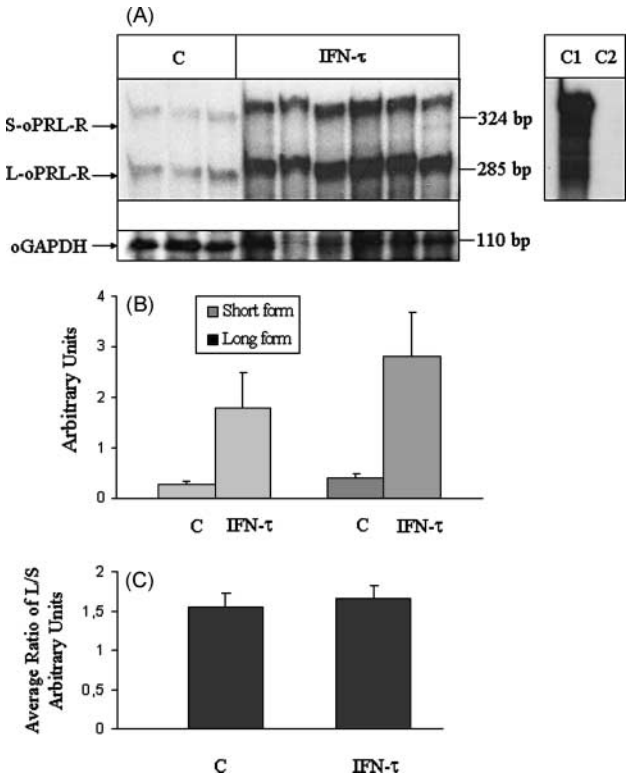

Figure 2 (A) Detection by ribonuclease protection assay of ovine (o) PRL-R mRNA in the endometrium of the BSA-treated control (C) and IFN-τ-treated (IFN-τ) ewes. The radiolabelled 324 bp PRL-R fragment, used as cRNA probe, was hybridized to 5 μg endometrial poly(A)+ RNA. Sizes of the protected fragments, calculated from a sequencing ladder, are indicated on the right. As controls, the ovine PRL-R cRNA probe was hybridized with yeast tRNA and then digested with RNases (lane C2) or not (lane C1). Each lane represents mRNA from individual ewes of each experimental group (S, short form; L, long form). (B) Normalized ratio of ovine PRL-R mRNA to oGAPDH mRNA determined after STORM quantification of the gel. Long and short forms of the PRL-R are indicated in control (C) and IFN-τ treated groups. (C) Changes in relative ratio of the long (L) to the short (S) ovine PRL-R mRNA in each experimental group. Results are expressed as means ± S.E.M. of three or six animals respectively in the control (C) and IFN-τ-treated groups.

form of PRL-R mRNA remained similar in the control and IFN-τ-injected groups, indicating that IFN-τ produces the same transcriptional enhancing effect on both forms of the PRL-R (Fig. 2C).

Cellular localization of PRL-R mRNA in the endometrium of IFN-τ-treated ewes

In order to reaffirm our observation of enhanced expression of PRL-R mRNA in ovine endometrium after IFN-τ stimulation, *in situ* hybridization was carried out on both control and IFN-τ-treated ewes. In control ewes, endometrial sections hybridized with an [α-^{35}S]-labelled ovine PRL-R cRNA antisense probe revealed a weak hybridization signal localized within the cells of the glandular epithelium (Fig. 3A and B). In contrast, a greater abundance of signal was observed in ewes treated with IFN-τ (Fig. 3C, D, E and F) and localized specifically in the deepest endometrial glands. No specific hybridization signal was observed in stromal cells of the intercaruncular endometrium in any ewe. Control tissue sections hybridized with a sense probe (Fig. 3G and H) exhibited no specific signal, further demonstrating the specificity of the reaction. Thus, these data provided spatial evidence that IFN-τ was able to upregulate PRL-R mRNA expression in the glandular epithelial cells of the endometrium.

Expression of PRL-R mRNA in ovine endometrium during the peri-implantation period

In this study, the expression of the two forms of PRL-R mRNA was investigated in the endometrium of nine ewes on days 7–8 ($n = 4$) and 14–15 ($n = 5$) of pregnancy. These stages of pregnancy were chosen according to IFN-τ secretion patterns: just prior to IFN-τ production (days 7–8) and during the maternal recognition of pregnancy interval when IFN-τ secretion is maximum (days 14–15). The abundance of long and short form PRL-R mRNA were determined by ribonuclease protection assay (Fig. 4A). Using the 324 bpAflII-XbaI PRL-R cDNA fragment as a probe and after STORM analysis, data are illustrated in Fig. 4B and presented as corrected values relative to the expression of

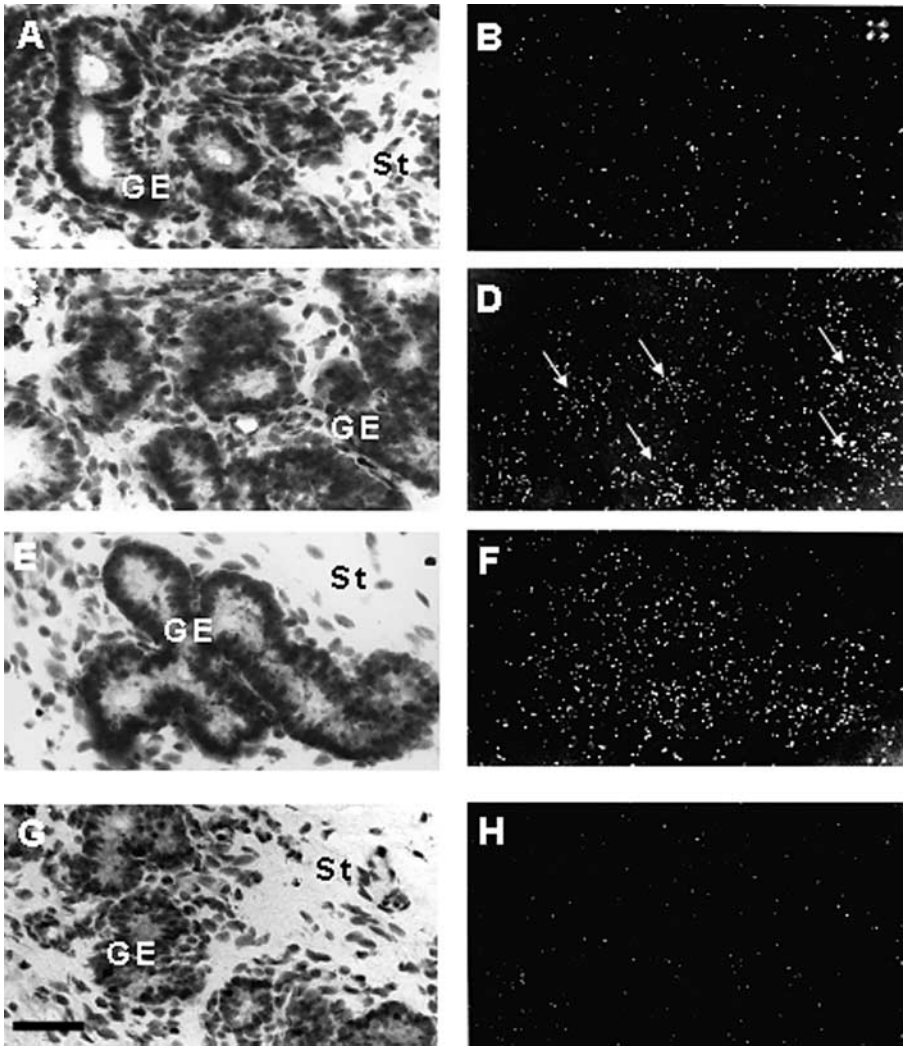

Figure 3 *In situ* hybridization analysis of ovine PRL-R gene expression in ovine endometrium of (A and B) BSA-treated controls and (C, D, E and F) IFN-τ-treated ewes. A section of endometrium was hybridized with the sense probe and used as a control of specificity (G and H). Left and right columns correspond respectively to brightfield and epi-illuminated micrographs. The glandular epithelium (GE) and the stroma (St) are illustrated. Bar = 50 μm.

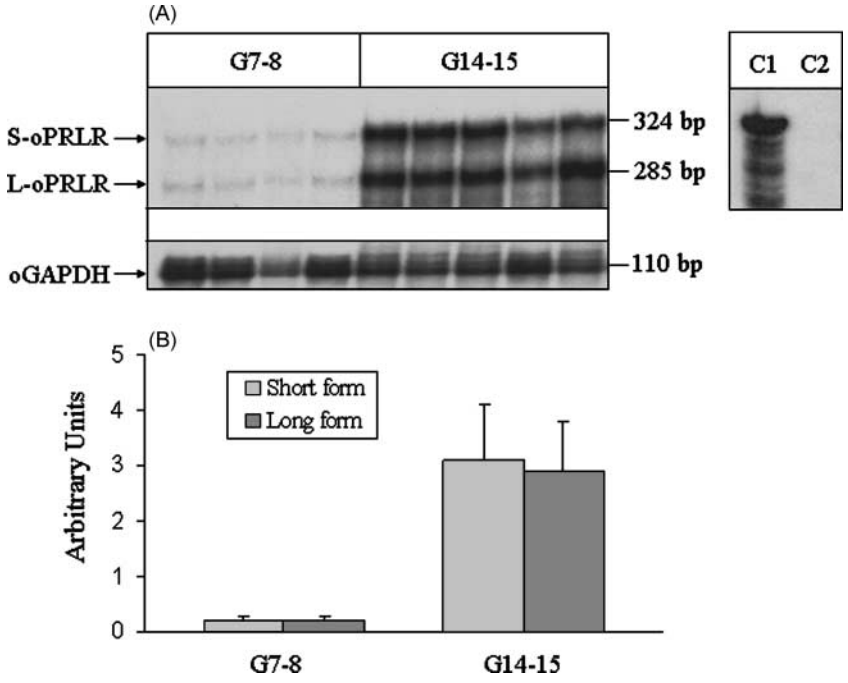

Figure 4 (A) Autoradiograph of ribonuclease protection assay products separated on a 6% polyacrylamide gel. tRNA (20 μg) extracted from individual endometrial tissue on days 7–8 (G7-8, $n = 4$) and 14–15 (G14-15, $n = 5$) of pregnancy were hybridized with radiolabelled 324 bp antisense cRNA probe. Yeast tRNA was hybridized with the radiolabelled probe and subjected to the RNases (lane C2) or not (lane C1). Each lane represents mRNA from individual ewes of each experimental group. (B) Quantification was performed by STORM scanning of the protected fragments of the gel and are presented as the normalized ratio of ovine PRL-R mRNA to oGAPDH mRNA. Results are expressed as means ± S.E.M. of four or five animals respectively in groups G7-8 and G14-15.

the housekeeping gene, GAPDH. Both long and short forms of PRL-R mRNA increased ~15-fold between days 7–8 and 14–15 of pregnancy. Thus, it seems likely that IFN-τ, a major embryonic factor secreted during this period, could play the role of a potent modulator of PRL-R transcription in ovine endometrium.

Discussion

The objective of this study was to investigate the levels of lactogenic hormone receptor mRNA during the peri-implantation period in ovine endometrium and investigate putative inducers of lactogenic receptor expression. In previously published work (Cassy *et al.* 1999), we reported that the glandular epithelium of ovine endometrium was a potential target of PRL action during pregnancy. In our current work, herein, we report that transcription of the long and short forms of PRL-R were greater on days 14–15 than on days 7–8 in the pregnant uterine endometrium of the ewe. This result is consistent with the observations of Stewart *et al.* (2000) who determined, by slot blot analysis, an increase of endometrial PRL-R mRNA levels during pregnancy.

IFN-τ is the major embryonic cytokine required for pregnancy recognition and maintenance in ruminants, and it is highly, locally and transiently expressed in ewes during the peri-implantation period (from day 12 to day 21 of pregnancy). Moreover, IFN-τ is known to mediate various paracrine effects through type I IFN receptors expressed in endometrial cells (Knickerbocker & Niswender 1989, Kaluz *et al.* 1996). Therefore, we hypothesized that IFN-τ may play the role of a putative regulator of PRL-R mRNA expression in endometrium. Using ribonuclease protection assay methods, we demonstrated that IFN-τ treatment increased the level of both the long and short forms of PRL-R mRNA. However, the ratio of the long to the short form of the PRL-R receptor is not altered in IFN-τ-treated endometrium (the long form is preferentially expressed). These observations were reaffirmed by *in situ* hybridization analysis, which provided evidence that treatment of ewes with IFN-τ had a stimulatory effect on glandular endometrial PRL-R gene expression. Moreover, the abundance of endometrial PRL-R mRNA present in ewes injected with IFN-τ was comparable with that previously documented in uterine endometrial tissue collected from day-14 pregnant animals (Cassy *et al.* 1999).

Collectively, these data support the hypothesis that IFN-τ plays a critical role in inducing lactogenic hormone receptor mRNA expression in endometrium. Furthermore, the secretion of PL, which is the main lactogenic hormone expressed during pregnancy in the ewe, occurs when IFN-τ is most abundantly secreted (days 14–15 of pregnancy). We suggest that the temporal relationship between the pattern of secretion of IFN-τ by the conceptus and the expression of PRL-R in the endometrium (especially the glandular endometrium) is paramount to the process of maternal receptivity to the conceptus. Mechanistically, IFN-τ is necessary for the expression of uterine milk protein (UTMP) and osteopontin (OPN): two endometrial proteins induced by PL in ovariectomized ewes (Spencer *et al.* 1999, Stewart *et al.* 2000). Thus, it appears that upregulation of uterine PRL-R mRNA is the link between IFN-τ secretion and lactogenic hormone target gene expression.

IFN-τ plays a key role in the maintenance of progesterone secretion by the corpus luteum during the process of maternal recognition of pregnancy in ruminants. It has

been previously reported in the rabbit that a cross-interaction between PRL and progesterone occurs in the endometrium to regulate their own receptors, as well as the expression of other uterine genes such as uteroglobin (Chilton *et al.* 1988). Whether the effect of IFN-τ on PRL-R gene expression is mediated directly or influenced by progesterone is open to discussion. Indeed, in the ewe, progesterone receptors (PR) are undetectable in glandular epithelium cells from days 13 to 19 of pregnancy (Spencer *et al.* 1995, Stewart *et al.* 2000). The loss of PR gene expression appears to be required for glandular remodelling and differentiation in ovine endometrium (Spencer & Bazer 2002). Therefore, it seems more likely that the increase in endometrial PRL-R gene expression after IFN-τ treatment results from the direct action of IFN-τ rather than indirectly in conjunction with the influence of continued luteal secretion of progesterone. Nevertheless, in our experiments, serum progesterone levels were high in all IFN-τ-injected animals. Moreover, Spencer *et al.* (1999) suggested that the combined presence of progesterone and IFN-τ is essential for ovine PL stimulation of endometrial UTMP and OPN gene expression. Taken together, these arguments do not show a direct or progesterone-mediated effect of IFN-τ on PRL-R gene expression.

The precise function(s) of the lactogenic hormones (PL/PRL) during pregnancy have not been elucidated. The hypothesis that lactogenic hormones could play a crucial role in maintaining gestation and implantation was supported by the work of Ormandy *et al.* (1997) using PRL-R knockout mice. Furthermore, enhancing the bioactivity of ovine PL results in increased birth weight of lambs and milk production by the ewes (Leibovitch *et al.* 2000). It is now established that ruminant PLs have the ability to bind both PRL-R and GH receptor (GH-R) (Herman *et al.* 2000). Several studies provide evidence that a transient homodimerization of PRL-R occurs in response to ruminant PL (Gertler *et al.* 1996, Sakal *et al.* 1997) and, more recently, the functional heterodimerization of PRL-R and GH-R by PL has been demonstrated *in vitro* (Herman *et al.* 2000, Biener *et al.* 2003) and in the ovine endometrium (Noel *et al.* 2003). Although it has been established that GH-R was expressed throughout pregnancy in the endometrium (Lacroix *et al.* 1999), no precise quantitative study has been reported for GH-R mRNA expression during the peri-implantation period. Studies performed by Spencer *et al.* (1999) reported that the effects of GH on endometrial function require IFN-τ. Thus, it seems likely that IFN-τ could also regulate endometrial GH-R gene expression during the peri-implantation period.

Glandular epithelium undergoes intense proliferation and differentiation during pregnancy (Gray *et al.* 2001*a*), which results in an abundance of secretory activity. Even partially characterized, glandular secretions are now considered to play major roles not only in maintaining gestation but also in nutrition, growth and immunoprotection of the foetus. The uterine gland knockout phenotype supports the hypothesis that endometrial glands are required for successful pregnancy, since uterine gland knockout ewes are unable to complete the normal gestational process (Gray *et al.* 2001*b*).

In summary, it is now established that numerous cytokines (IFN-τ, PL, ovine PRL and placental GH) and sex steroids act sequentially on the ovine endometrium and are involved in growth, differentiation and secretory function of endometrial glands during gestation and foetal development. Among them, IFN-τ plays a pivotal role in the establishment of pregnancy, not only as the major signal for maternal recognition of pregnancy in ruminants, but also as the inducer of several endometrial proteins that may be critical for survival of the developing embryo. According to our findings, the PRL-R is upregulated by IFN-τ during early pregnancy, thus preparing the endometrium for the actions of lactogenic hormones, which have yet to be elucidated.

Acknowledgements

The authors are deeply grateful to Dr D H Keisler for critical discussion and revision of the manuscript and to N Charpentier for technical assistance.

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Received 31 December 2003
First decision 25 February 2004
Accepted 30 April 2004