Demonstration of *in vivo* mammogenic and lactogenic effects of recombinant ovine placental lactogen and mammogenic effect of recombinant ovine GH in ewes during artificial induction of lactation

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Abstract

The present study demonstrates that ovine placental lactogen (oPL) (ovine chorionic somatotrophin) may have an important role in the mammogenesis and/or lactogenesis of the ewe. Its effects were compared with that already described for ovine growth hormone (oGH).

In the first experiment, 40 nulliparous ewes were induced to lactate by means of a 7 day (days 1–7) oestro–progestative treatment (E_2+P_4). The ewes from Group 1 ($n=12$) received no further treatment, while those of the other groups received either recombinant oGH (roGH, 28 µg/kg, i.m., twice daily, Group 2, $n=12$) or recombinant oPL (roPL, 79 µg/kg, i.m., twice daily, Group 3, $n=12$) from day 11 to 20. All ewes received 25 mg hydrocortisone acetate (HC) twice daily on days 18–20. Control Group 00 ($n=2$) received no steroid treatment at all, and the control Group 0 ($n=2$) received only the E_2+P_4 treatment. Thirteen ewes (three from each experimental group and the two of each control group) were slaughtered at the end of hormone treatments (day 21) before any milking stimulus. The 27 remaining ewes from Groups 1–3 were machine-milked and milk yields recorded daily from day 21 to 76. The E_2+P_4 treatment enhanced the plasma levels of oPRL, oGH and IGF-I between days 1 and 7 by 1.5, 2.3 and 2.6 times respectively ($P=0.002$); roGH treatment induced a highly significant enhancement of IGF-I plasma levels from day 11 to 20, whereas a similar effect appeared for roPL-treated ewes only from day 17 to 20 ($P<0.01$). Eight weeks after the last exogenous hormone injections, milk yields of both roGH- and roPL-treated groups progressively rose to twice that of

unsupplemented groups ($P<0.001$). The mammary DNA content on day 21 was higher for animals which received either oGH or oPL but, due to individual variations in so few samples ($n=3$), this difference was not significant. No β -casein was measured in mammary tissue from control ewes, whereas steroid-treated ewes (E_2+P_4+HC) had higher casein concentrations regardless of subsequent hormonal treatment on days 11–20 ($P<0.001$). β -Casein concentrations in mammary parenchyma of roGH-treated ewes did not differ from that of ewes which received only E_2+P_4+HC ; roPL supplementation clearly enhanced expression of β -casein ($P<0.001$).

IGF-I stimulation by either roGH or roPL was more precisely examined during a second experiment, in which two twice-daily i.m. doses (58 or 116 µg/kg) of either roGH or roPL were administered to four groups of six ewes that were E_2+P_4 treated as those of Experiment 1. A control group ($n=6$) received no exogenous hormone from day 11 to 13. On day 13, hourly blood samples were taken from all ewes over 11 h. Both doses of roGH significantly stimulated IGF-I in a dose-dependent manner. The 58 µg/kg dose of roPL did not significantly stimulate IGF-I, but although being somewhat less efficient than the 58 µg/kg dose of roGH, the 116 µg/kg dose of roPL significantly stimulated IGF-I secretion ($P<0.001$).

These results suggest that mammogenesis and/or lactogenesis in the ewe is in part controlled by somatotrophic hormones such as oGH and oPL and that IGF-I could be one of the mediators of these hormones.

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Introduction

Ovine placental lactogen (oPL) belongs to the prolactin (PRL)–growth hormone (GH) protein hormone family

and is secreted by the placenta at high levels into the maternal compartment during pregnancy (Djiane & Kann 1975, Gluckman *et al.* 1979). It has been purified and

characterised by several groups (Martal & Djiane 1975, Chan *et al.* 1976, Hurley *et al.* 1977, Reddy & Watkins 1978, Warren *et al.* 1990, Kappes *et al.* 1992) over the past 20 years, but the physiological role of oPL in ovine physiology remains speculative. Recent results from *in vitro* binding studies and *in vivo* studies of biological activities on ovine adult or foetal tissues suggest a role(s) in foetal growth and/or mammogenesis (Chan *et al.* 1978a, Servely *et al.* 1983, Freemark & Handwerger 1986, Freemark & Comer 1989, Byatt *et al.* 1992, Anthony *et al.* 1995). Both the cDNA sequence (Colosi *et al.* 1989) and predicted amino acid sequence indicate that oPL has partial homology with both ovine PRL (oPRL) (49%) and ovine GH (oGH) (25%) and oPL binds to oGH receptors (oGH-R) or oPRL receptors (oPRL-R). oPL may elicit both PRL and GH activities when binding to its own hepatic foetal receptors (Freemark *et al.* 1987, 1988) in the lamb; however, Breier *et al.* (1994) claimed that the somatogenic action of oPL resulted only from its binding to oGH-R. *In vivo* protocols in ewes at the end of gestation (days 122–136) demonstrate that oPL may mediate trophic actions through enhancement of foetal plasma insulin-like growth factor-I (IGF-I) (Schoknecht *et al.* 1992, 1996). Recent experiments in Gluckman's group (Ogawa *et al.* 1995, Oliver *et al.* 1995, Currie *et al.* 1996, Min *et al.* 1996, 1997) used recombinant oPL (roPL) and suggested that growth-promoting actions of oPL in young lambs could result from stimulation of voluntary food intake rather than enhancement of IGF-I secretion and that administration of roPL to pregnant ewes failed to influence maternal IGF-I levels. No galactopoietic effects of oPL were detected when it was administered to lactating ewes, which differentiates it from oGH, despite known somatogenic effects of oPL (Min *et al.* 1996). Recently, we reported the large-scale preparation of biologically active oPL (Sakal *et al.* 1997). We have also demonstrated that stimulation of oGH release by human growth releasing factor (hGRF) strongly stimulates mammogenesis in ewes in artificially induced lactation (Kann 1997). The present study compares mammogenic and lactogenic activities of roPL and recombinant oGH (roGH) in ewes using the model of artificial induction of lactation, with particular attention to respective effects on IGF-I levels.

Materials and Methods

Experimental design

Two experiments were conducted, but the first part of each had a common protocol; 1-year-old nulliparous Prealpes du Sud ewes had their oestrous cycles synchronised prior to the beginning of each experiment. Fluoroprogesterone vaginal sponges (40 mg, Intervet, Angers, France) were inserted and left in place for 14 days. When sponges were removed the ewes received equine chorionic gonadotrophin (200 IU/ewe, Intervet), and 1 week

elapsed before steroid treatment, which lasted 7 days (the first day of this treatment was designated day 1 of the artificial induction of lactation protocol). Unless otherwise specified, each ewe (mean weight 50 kg) received 0.5 mg/kg oestradiol 17 beta (E_2) and 1.25 mg/kg progesterone (P_4) dissolved in 80% ethanol from day 1 to 7, as daily s.c. injections at 0900 and 1500 h. Ewes were randomly allocated to different experimental groups (see below) and received twice daily (0900 and 1500 h) 1 ml i.m. injections of either vehicle (sodium bicarbonate 10 nmol/l, pH 8.5) or different doses of either roPL or roGH dissolved in the vehicle from day 11 (3 days after the end of treatment with E_2+P_4) to day 20. Lactogenesis was triggered by i.m. administration of 25 mg hydrocortisone acetate (HC) twice daily (0900 and 1500 h) on days 18–20 to all ewes. All steroid hormones were provided by Roussel-Uclaf (Romainville, France).

Experiment 1 The experimental design is shown on Fig. 1. Ewes were allocated randomly to five groups. Group 00 was a control group that included two ewes which received no steroids for induction of lactation. Blood samples were obtained daily into evacuated tubes (Greiner, Poitiers, France) containing EDTA Na_2 (6 mg/5 ml) at 1500 h from day 1 to 20 and plasma was obtained and stored at $-20^\circ C$ until analysed for oGH, oPL, oPRL and IGF-I by RIAs. These two ewes were slaughtered on day 21 and the mammary gland carefully dissected free of external fat to select mammary parenchymal tissue. The mammary gland was separated into two halves. A small part of one half (1–2 g) was fixed in neutral buffered formaldehyde (10%) for histological studies and the remainder was stored at $-80^\circ C$ with a liver sample until analysis for oGH-R or oPRL-R and β -casein concentration. The other half of the mammary gland was weighed and stored at $-80^\circ C$ until assayed for DNA content.

The two ewes which were allocated to Group 0 were steroid-primed ewes given E_2+P_4 on days 1–7, but received no HC on days 18–20 and were treated exactly as the two ewes in Group 00. Group 1 contained 12 ewes which received the complete induction of lactation steroid treatment, but no protein hormone injections on days 11–20 (only 1 ml vehicle i.m.); therefore, these ewes served as a control group for ewes given roGH or roPL injections. Blood samples were obtained as described for Groups 0 and 00. Three ewes were slaughtered on day 21 and mammary tissues handled as described previously. The remaining nine ewes were milked for 8 weeks beginning on day 21.

Ewes in Group 2 ($n=12$) were treated exactly as those in Group 1 except that they received twice-daily injections (0900 and 1500 h) of roGH (28 $\mu g/kg$ body weight in 1 ml vehicle) from day 11 to 20. Ewes in Group 3 ($n=12$) were treated exactly as ewes in Group 2 except that they received twice-daily injections (0900 and 1500 h) of roPL (79 $\mu g/kg$ body weight in 1 ml vehicle from day 11 to 20).

Figure 1 Diagram of procedures for Experiment 1. After synchronisation of the oestrous cycle, D1 (day 1) (1 week after removal of vaginal sponges) was the first day of a 7 day period of steroid administration for Groups 1–3 (0.5 mg/kg E_2 and 1.25 mg/kg P_4 dissolved in ethanol 80%, in twice-daily s.c. injections). Control Group 00 received only ethanol 80%. From day 11 to 20, ewes received either twice-daily i.m. injections of saline (Group 00, $n=2$; Group 0, $n=2$; Group 1, $n=12$), 56 µg/kg oGH (Group 2, $n=12$) or 158 µg/kg oPL (shown on figure as its alternative name, oCS – ovine chorionic somatotrophin) (Group 3, $n=12$). From day 18 to 20, all ewes except Group 00 received twice-daily i.m. injections of hydrocortisone acetate (Hyd). On day 21, ewes were either slaughtered (Groups 00 and 0, three ewes from Groups 1–3) or milked twice daily for 8 weeks (nine ewes from Groups 1–3).

Three ewes each from Groups 2 and 3 were slaughtered on day 21, and nine ewes each from Groups 2 and 3 were milked from day 21 to 76.

The roPL was as described by Sakal *et al.* (1997), and roGH as E Sakal, A Gertler & H Leibovitch (unpublished data).

Blood sampling and measurement of the milk yield protocols were identical for all groups.

All experiments were carried out according to the French recommendations for the use of experimental animals including animal welfare and appropriate conditions in terms of animal handling before slaughter (Guideline 19 April 1988).

Experiment 2 Experiment 2 was designed as described for the first part of Experiment 1 (days 1–13) but lasted

only 13 days. After the period of administration of E_2+P_4 (days 1–7), five groups of six ewes each were studied. Group 1 (controls) received only 1 ml vehicle i.m. from day 11 to 13. roGH was administered i.m. twice daily to ewes of Groups 2 (58 µg/kg) and 3 (116 µg/kg) from day 11 to 13. roPL was similarly administered to ewes of Groups 4 (58 µg/kg) and 5 (116 µg/kg) from day 11 to 13. Blood samples were collected hourly on day 13 from 0800 to 2300 h. All ewes returned to normal oestrous cycles within 2 months of the last injections.

RIAs

The oPRL, oGH, oPL and IGF-I double antibody RIAs have been described previously (Kann 1971, 1997, Breier *et al.* 1991, Lacroix *et al.* 1996). Ovine β -casein was

measured in mammary tissues according to the procedure of Jahn *et al.* (1987) and expressed as the concentration of β -casein/mg protein. Protein concentration was determined using the method of Lowry *et al.* (1951).

Mammary gland parenchymal DNA content

Tissue from one-half of each mammary gland was homogenised in a blender in liquid nitrogen until a homogeneous powder. The homogenate was stored at -80°C until DNA was measured for each sample in triplicate in 1 g of this powder according to the method of Labarca & Paigen 1980 (fluorescence of DNA after binding to bisbenzimidazole) and results expressed as mg/mammary gland.

Binding experiments

Binding to ovine liver and mammary gland microsomal fractions was carried out as described previously (Shiu *et al.* 1973, Emane *et al.* 1986).

Histology

Fixed mammary parenchymal tissue was prepared by standard methods and sections ($5\text{ }\mu\text{m}$) stained with hematein-phloxine. Tissue was examined for lobulo-alveolar development.

Milk production

Milk yields were recorded daily by measuring weights of machine-collected milk at 0830 and 1530 h and these values were then expressed as weekly mean values for milk production.

Statistical analysis

Blood and tissue data were analysed by ANOVA using the general linear models procedure of the SAS (1985). Normality of the data was tested according to Stephens (1974) using SAS procedures. Heterogeneity of variance for blood concentrations of oGH, oPL and IGF-I was corrected by square root transformation prior to analysis. All values are expressed as mean $\pm$ S.E.M. and *n* represents the number of ewes contributing data that were submitted to the analysis.

Results

Blood hormonal patterns

Experiment 1 The daily hormonal profiles for the 36 ewes allocated to Groups 1–3 for induction of lactation in Experiment 1, are presented for oPRL, oGH, oPL and

IGF-I in Fig. 2. The modifications in concentrations of oGH, oPRL and IGF-I during the first phase of steroid treatment (days 1–7) support our earlier results (Head *et al.* 1982, Kann 1997).

During the E_2+P_4 administration period, oGH values of all groups (Fig. 2a) slowly rose from 2.5 ± 0.4 ng/ml on day 0 to 9.9 ± 1.3 ng/ml on day 10. On day 11 at 1500 h, i.e. 6 h after the first i.m. injection of roGH, plasma concentrations of oGH in ewes from Group 2 increased (14.8 ± 2.6 ng/ml) above values for ewes of Group 3 (9.5 ± 1.3 ng/ml) ($P < 0.01$). Variation in oGH levels during the period of roGH administration declined between days 12 and 14 (8.8 ± 1.0 ng/ml on day 14), but then increased to 15.0 ± 2.2 ng/ml on day 20. Plasma concentrations of oGH for ewes of Group 3 (oPL injected) were comparable to those of control ewes. During the first days of E_2+P_4 , oPRL values initially dropped from levels of 80 ± 8 ng/ml on day 1 to 24 ± 4 ng/ml on day 4, then continuously rose to 95 ± 10 ng/ml on days 10–11 (Fig. 2b). Thereafter, oPRL values for Groups 2 and 3 fluctuated between 40 ± 10 and 80 ± 9 ng/ml between days 11 and 20 and were unaffected by either roGH or roPL compared with values for ewes in Group 1 (control steroid-treated ewes).

The levels of oPL (Fig. 2c) were first detectable on day 12 or 30 h after the first injection of oPL to Group 3 ewes (119 ± 13 ng/ml). Although relatively stable during the first 6 days of oPL injections, oPL concentrations increased from 126 ± 15 ng/ml on day 17 to 249 ± 54 ng/ml on day 20 ($P < 0.001$).

Concentrations of IGF-I in plasma (Fig. 2d) increased steadily ($P < 0.001$) from day 1 (87 ± 22 ng/ml) to day 7 (220 ± 20 ng/ml) when steroid injections ceased; after day 11, when vehicle, roGH or roPL was administered, values differed according to the hormonal environment: on day 11 IGF-I values were 214 ± 28 , 240 ± 31 and 219 ± 28 ng/ml respectively for Groups 1, 2 and 3 but were enhanced on day 20 at the end of the period of hormonal stimulation to 594 ± 70 , 1050 ± 50 and 789 ± 71 ng/ml ($P < 0.001$) respectively. Comparison of IGF-I values between Group 1 (control) and Groups 2 and 3 indicated effects of roGH ($P < 0.001$) from day 12 to 20, whereas effects ($P < 0.01$) of roPL were only observed from day 17 to 20.

Experiment 2 The mean hormonal patterns for ewes from which blood samples were collected hourly during 15 h, on day 13 in Experiment 2 (five groups of six ewes) are presented in Fig. 3 for oGH, oPL and IGF-I.

At the time of the first hourly sampling on the second day of exogenous protein hormone administration (0800 h, i.e. 17 h after the last hormone injection) concentrations of oGH and oPL were different: while oGH levels were similarly low (5 ng/ml) for all groups of ewes (Fig. 3a), oPL levels were relatively high and differed according to the dose administered (32 ± 5 ng/ml for ewes injected with

Figure 2 Daily plasma concentrations (mean $\pm$ S.E.M.) of oGH (a), oPRL (b), oPL (c) and IGF-I (d) for ewes in Experiment 1 ($n=12$ ewes/group). All ewes were subjected to the same steroid treatment regimen (E_2+P_4) from day 1 to 7 and HC on days 18–20. Control ewes received saline only from day 10 to 20. Bars indicate the time (days 11–20) during which ewes received twice-daily (0900 h and 1500 h) saline (control, Group 1), roGH (28 μ g/kg, Group 2) or roPL (79 μ g/kg, Group 3). Results are from a single blood sample taken at 1500 h, before the administration of the second daily dose of either vehicle or protein hormones.

58 μ g/kg oPL vs 58 $\pm$ 9 ng/ml for ewes receiving the 116 μ g/kg dose ($P<0.01$) (Fig. 3c)). Twice-daily i.m. injections of oGH and oPL (0900 h and 1500 h) increased plasma concentrations of the respective hormones ($P<0.001$) and the 6 h interval between these two injections allowed values to return to pre-injection concentrations. As a consequence, the second injection at 1500 h resulted in higher plasma values, which did not return to the morning pre-injection values by 2300 h for the 116 μ g/kg dose of either hormone. Analysis of the data from the 15 h blood sampling indicated that mean hormonal values for oGH and oPL were doubled

when the amount of exogenous hormone injected was doubled.

The IGF-I values differed according to exogenous hormonal administration. Ewes injected with roGH had higher values at 0800 h than control ewes ($P<0.001$, Fig. 3b), and the 116 μ g/kg dose had a greater effect than the 58 μ g/kg dose ($P<0.001$, Fig. 3b). Ewes injected with roPL had higher IGF-1 values only after receiving the 116 μ g/kg dose (Fig. 3d, $P<0.01$). These values after 116 μ g/kg dose administration of roPL were lower ($P<0.01$) than those recorded for both the 58 and 116 μ g/kg doses of roGH. When plasma IGF-I values were

Figure 3 Concentrations of oGH, oPL and IGF-I (mean $\pm$ S.E.M.) in Experiment 2 in plasma from blood samples collected hourly from 0700 to 2300 h on day 13, i.e. 2 days after beginning twice-daily i.m. injections of saline, roGH or roPL (58 or 116 μ g/kg) at 0900 and 1500 h ($\blacktriangle$ on abscissae) according to treatment (six ewes/group). All ewes received the same steroid treatment (E_2+P_4) on days 1–7. (a) and (b) respectively show oGH and IGF-I values for ewes receiving oGH and saline; (c) and (d) show data from ewes receiving oPL.

enhanced after oGH or oPL exogenous administration, the IGF-I pattern appeared biphasic, but not superimposed on changes in concentrations of oGH or oPL, since maximum increases in IGF-I values were delayed by 9 h.

Values for oPRL (data not shown) were not affected by either oGH or oPL administration.

Mammary parenchyma DNA content

Estimates of variation in total DNA (mg DNA/gland) within and among treatment groups were very important, and the number of observations per treatment group (three) was insufficient to detect significant differences. However, ewes not treated with HC (Groups 00 and 0)

Figure 4 Mammary (a) and hepatic (b) receptors for oPRL (white columns) and oGH (black columns) expressed as percentage specific binding (%SB $\pm$ S.E.M.) of 125 I-hGH after competition with 1 μ g of either oPRL or oGH for tissues collected on day 21. Control and E₂+P₄ groups, $n=2$; other groups $n=3$. H=hydrocortisone.

had less mammary parenchyma DNA content (189 ± 93 mg, $n=2$ and 194 ± 73 mg, $n=2$ respectively) than ewes that received HC in Group 1 (323 ± 91 mg, $n=3$). Although it was found not significantly different, the DNA content of the mammary parenchyma of ewes from either Group 2 (treated with roGH) or Group 3 treated (with roPL) was slightly enhanced when compared with that of Group 1, which received no roGH or roPL (respectively 354 ± 25 mg, $n=3$ for Group 2; 367 ± 45 mg, $n=3$ for Group 3, $n=3$; and 323 ± 91 mg, $n=3$ for Group 1).

Binding of hepatic and mammary tissues to lactogenic hormones

Results of specific binding assays with 125 I-human GH (hGH) as a tracer and either oPRL (NIAMDDK I 1) or oGH (NIAMDDK I 4) as unlabelled competitor, indicated (Fig. 4a) that the E₂+P₄ treatment from day 1 to 7 increased ($P<0.001$) oPRL receptors in mammary membranes when compared with those from untreated

ewes and binding was greater for ewes treated with HC on days 18–20. Treatment with oGH enhanced ($P<0.01$) mammary GH receptors when compared with all other treatment groups, including ewes treated with oPL. Similar results were obtained for binding of oGH to hepatic cell membranes. Due to a high variability among animals, the differences in oPRL binding to hepatic membranes were not statistically significant (Fig. 4b). Nevertheless, specific binding to oPRL-R tended to be higher in oGH-treated ewes.

β -Casein content of mammary parenchyma

The data were not recorded for Group 1 (samples were lost), but results from other treatment groups are presented in Fig. 5. There was no β -casein in tissue from control ewes (Groups 0 and 00), whereas E₂+P₄+HC-treated ewes had higher β -casein concentrations regardless of subsequent hormonal treatment on days 11–20 ($P<0.001$). β -Casein concentrations in mammary parenchyma of roGH-treated ewes did not differ from those in control

Figure 5 β -Casein concentrations (mean $\pm$ S.E.M.) in mammary parenchyma on day 20 after hormone treatments, expressed as ng β -casein/mg tissue protein. All groups included three ewes, except the control group which had two. H=hydrocortisone.

ewes; however, roPL clearly enhanced expression of β -casein ($P < 0.01$).

Histology

Photomicrographs representative of all treatment groups in Experiment 1, including one control ewe which received the E₂+P₄ treatment from day 1 to 7, are presented in Fig. 6. The mammary gland of the control ewe (Fig. 6A) was completely undifferentiated with mammary parenchyma consisting of nearly completely closed alveoli organised in lobular structures surrounded by a thin layer of stromal tissue in the fat pad. The 7 days of treatment with E₂+P₄ induced (Fig. 6B) development of lobular structures at the expense of the fat pad, and opening of the alveoli with some containing small amounts of secretion. HC administration on days 18–20 (Fig. 6C) resulted in further expansion of alveoli and all contained light coloured secretions, appearance of a well-developed ductal system, dense stroma and almost complete loss of the fat pad. Histology of mammary glands from ewes treated with steroids plus roGH on days 1–20 revealed (Fig. 6D) further multiplication of alveolar and ductal structures which were enlarged and full of a secreted material containing lipid droplets. Ewes that received roPL instead of oGH were characterised by further development and dilatation of the alveoli and more pronounced accumulation of lipid droplets (Fig. 6E).

Milk yields

Milk production by ewes in Groups 1–3 (nine ewes/group milked daily from day 21 to 77) was individually recorded twice daily and is summarised as weekly means in Fig. 7.

Control ewes treated with E₂+P₄+HC had a lactation curve which peaked at the 6th week around 1 kg daily milk production. Additional treatment with roPL or roGH from day 11 to 20 dramatically increased subsequent milk yields to a mean 2 kg daily milk production ($P < 0.001$).

Discussion

In this study, 10 days of oPL treatment of maiden ewes after a steroid priming of the udder, as defined by Head *et al.* (1980, 1982), resulted 8 weeks after the last injection of exogenous hormone in milk production comparable to normal milk yields at the end of pregnancy (Fig. 7). Control ewes which received no hormonal supplementation other than corticoids during the 10 days preceding the milking period produced approximately half as much milk as oPL-treated ewes. This strengthens previous conclusions that E₂+P₄+HC treatment is not sufficient to trigger normal mammatogenesis and lactogenesis comparable to that induced during the course of a normal pregnancy (Head *et al.* 1980). Injections of oGH and oPL resulted in similar lactation curves, which indicates an essential role of somatotrophic hormones probably together with oPRL in the processes of mammatogenesis and lactogenesis (Kann 1997). Normal pregnancies in ewes are associated with elevated plasma levels of oPL (Djiane & Kann 1975, Chan *et al.* 1978b, Gluckman *et al.* 1979, Taylor *et al.* 1980) from day 100 to term and increases in oGH plasma levels during the month before lambing (Convey 1974, G Kann unpublished results), which suggests involvement of these two hormones in mammatogenesis and lactogenesis.

Results of the present study are the first to provide direct support for the hypothesis that oPL has

Figure 6 Photomicrographs of mammary parenchyma tissue from ewes in Experiment 1. (A) Control Group 00 (no treatment); (B) Group 0 (steroid primed from day 1 to 7 with E_2+P_4 ; (C) Group 1 (steroid primed from day 1 to 7 with E_2+P_4 , and with HC on days 18–20); (D) Group 2 (steroid primed from day 1 to 7 with E_2+P_4 , oGH on days 11–20 and HC on days 18–20); (E) Group 2 (steroid primed from day 1 to 7 with E_2+P_4 , oPL on days 11–20 and HC on days 18–20). Note the progressive loss of the fat pad ('FP') in (A–E), with simultaneous progressive opening of the alveoli ('A') and ducts ('D'), and appearance of dense material ('*') in the lumen of alveoli and ducts. Arrows in (E) indicate lipid droplets in secretions. (s) corresponds to stroma. The scale bars represent 50 μm . For detailed description of the pictures see text (Histology).

mammogenic and lactogenic effects in ewes. Before this study, this hypothesis was supported either by analogy to results of Byatt *et al.* (1994, 1997), who showed that

bovine PL (bPL) had a remarkable *in vivo* effect on bovine mammogenesis, or by indirect *in vivo* observations of positive correlations between litter size of pregnant ewes

Figure 7 Mean daily production ($\pm$ S.E.M.) of milk during 8 weeks for steroid-primed ewes induced to lactate after (or not for control group) a previous 10 day treatment with either oGH ($2 \times 56 \mu\text{g/kg}$ per day i.m.) or oPL ($2 \times 158 \mu\text{g/kg}$ per day). The last injection of oGH or oPL was on the last day of week 0 (day 20) and ewes were not submitted to any pre-milking stimulus before day 21.

(plurithocous ewes produce more oPL than monothocous) and the milk yield after parturition (Butler *et al.* 1981). Normal mammogenesis in ewes induced to lactate with a steroid regimen alone or with an inhibitor of PRL through bromocriptine administration (Schams *et al.* 1984) also favours a mammotrophic role for oPL.

The stimulation of the milk yield by both roGH and roPL could result from stimulation of either mammo-genesis or, in the case of oPL, both mammo-genesis and lactogenesis. The duration of both hormones' actions (at least 8 weeks after the last hormone injection) was completely different from that of the galactopoietic effect of administration of oGH in established lactation (Hart *et al.* 1985). Those authors reported that the positive effect induced by oGH or hGRF was directly dependent on sustained hormone administration, and that milk yields returned immediately to previous values when oGH was not administered or stimulated, indicating that the galactopoietic effect was metabolic and not related to a multiplication of mammary cells that ought to result in a more prolonged effect.

On the contrary, the observations made in the present study are in favour of a durable mammo-genic effect for both oGH and oPL. An enhancement of the number of epithelial mammary cells can by itself be responsible for a better lactation curve.

Mammogenesis is characterised by an increase in DNA content of the mammary epithelium and/or in mammary cell number. In the present study, the measurement of DNA and the histology were both recorded on the day after the last roGH or roPL injection, i.e. on day 21. We

have not observed at this time a clear enhancement of mammary DNA in the mammary gland but we must remember that mammary epithelial cells which are stimulated during mammo-genesis are only a part of the mammary parenchyma, most cells belonging to the stroma. Therefore when we consider DNA content of the mammary parenchyma, an important stimulation of mitosis of only the epithelial part of the mammary gland could be partly hidden by the non-stimulation of stroma cells, which form 80–90% of the mammary gland of a nulliparous ewe.

Similarly, in a situation which is very comparable to the present experiment, we have already demonstrated that after administration of hGRF to ewes artificially induced to lactate (stimulation of oGH and IGF-I) the differences between measurement of DNA contents of mammary gland parenchyma of two groups of eight ewes submitted or not to hGRF treatment were hardly significant ($P < 0.01$) in spite of an important progressive enhancement of milk yields after cessation of the treatment (Kann 1997).

Differentiation of the mammary gland is known to be triggered by the 3 days of HC treatment on days 18–20; this differentiation is necessary to induce lactogenesis (Head *et al.* 1980) and we can suggest that this effect could partially inhibit a simultaneous multiplication of epithelial cells measured on day 21. This could explain why milk yields appear to be significantly different only at the end of the first week of milking; we suggest that the interval of growing milk production which occurred between control and treated ewes as the milking period progressed (from

week 1 to 6) might be in part due to a progressive and non-explosive multiplication of the udder epithelial cells of treated ewes.

It is known that mammogenesis is not completely finished at the end of a normal pregnancy (Sheffield & Anderson 1985), and from the observation of normal lactation curves a role for the milking stimulus in achieving the mammogenesis is apparent (maximum milk yields are only reached after 5 or 6 weeks of lactation). Analysis of the parenchymal DNA content of mammary glands after a few days of milking might have provided better evidence for relative mammogenic potencies of roGH and roPL.

Histological evidence from the present study clearly showed enhanced development of the lobulo-alveolar system indicating mammogenic effects, for both oGH and oPL (Fig. 6). Comparable results in cattle also suggested a mammogenic role for both bovine GH and bPL (Collier *et al.* 1993).

Measurements of β -casein concentrations in the mammary tissue at the end of the induction of lactation indicated lactogenic activity enhancement only for oPL (Fig. 5) when compared with E_2+P_4+HC -treated ewes. This confirmed previous reports that oPL stimulated accumulation of β -casein mRNA in mammary explants from pregnant ewes, although oPL was considerably less potent than oPRL (Servely *et al.* 1983), and these results are comparable to lactogenic effects of bPL in the heifer with artificially induced lactation (Byatt *et al.* 1994).

Milk production in mammals is a consequence of a very complex succession of events. The external gross aspect of the mammary gland at the end of pregnancy is indicative of the extent of lobulo-alveolar development. The degree of development of the mammary glands is a good predictor of milk production for several months. In our experimental model, long-acting effects of oGH and oPL on milk yield after a short treatment period (10 days) may be due mainly to their ability to induce lobulo-alveolar development comparable to that of a normal pregnancy when more milk is produced from better developed mammary glands (under similar hormonal and nutrition conditions during the galactopoiesis period).

oGH clearly stimulated IGF-I systemic production, probably through effects on hepatic GH receptors (Figs 2–4), whereas systemic IGF-I increase in oPL-stimulated ewes was lower (Figs 2 and 3). However, when measured hourly on the second day of stimulation with two doses of each hormone, IGF-I patterns were clearly correlated with the doses of roGH or roPL administered (Fig. 3b and d). Thus, these results indicate that, in contrast to conclusions of Currie *et al.* (1996) and Min *et al.* (1997), oPL stimulates IGF-I in ewes even after 2 days of treatment, which is comparable to responses in cows (Byatt *et al.* 1994) to bPL injections. Since IGF-I stimulates DNA synthesis in ovine mammary epithelial cells (Winder *et al.* 1989) and bovine tissue (Shamay *et al.* 1988, McGrath *et al.* 1991, Collier *et al.* 1993), we speculate that both oPL and oGH effects

on IGF-I levels could account for part of their mammary effects. Secretion of IGF-I by bovine mammary tissue (Campbell *et al.* 1991) also suggests that ovine tissue may secrete IGF-I as well, and that the mammogenic effect of oGH and oPL could be partially a consequence of a local stimulation of this secretion through their receptors in mammary tissues.

Results of the present study indicate that oPL may not act through oGH hepatic receptors to stimulate IGF-I, since both different temporal and dose effects were apparent in the responses. It should also be noted that neither oGH nor oPRL secretion was modified during the 10 days of treatment with high doses of oPL; however, secretion of these two hormones is known to be self-downregulated through a retrocontrol mechanism at the hypothalamic level. If oPL acts *in vivo* on oGH-R and oPRL-R at the hypothalamic level, the secretory pattern of these hormones should have been modified. Further experiments are needed to understand the mechanisms of action of oPL and oGH on the mammary gland and to gain better knowledge of putative oPL-Rs.

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