Ovine chorionic somatomammotrophin (oCS) production by isolated cotyledon cells from sheep in early and mid gestation: auto-regulation by recombinant oCS

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Abstract

We report the ability of sheep placental cotyledonary cells, isolated at different periods of pregnancy (40 to 90 days) to produce ovine chorionic somatomammotrophin (oCS) in *in vitro* culture conditions. This oCS production increased gradually with stage of pregnancy. Endogenous oCS net production by isolated placental cells was increased, in a dose-dependent manner, by addition of recombinant oCS (roCS). This effect was not observed after addition of recombinant ovine growth hormone. The roCS effect was more potent on cells collected during early pregnancy.

Specific immunoprecipitation of oCS revealed that roCS treatment was associated with an increased dose-dependent incorporation of [³⁵S]methionine-[³⁵S]cysteine. These findings provide evidence that oCS may act in a paracrine/autocrine manner to up-regulate its own production during early gestation. We suggest that this autoregulation may be associated with morphological and functional differentiation of the trophoblast during the growth of the placenta.

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Introduction

The ovine placenta consists of cells from both maternal and fetal origins. A hormone produced by this tissue is a 22 kDa protein known as ovine chorionic somatomammotrophin (oCS), or placental lactogen (oPL). It is produced by binucleate cells of the fetal trophoblast and was first purified by Handwerger *et al.* (1974) and Martal & Djiane (1975). Ovine chorionic somatomammotrophin shares significant structural homology with both ovine prolactin (oPRL – 49%) and growth hormone (oGH – 28%) and can serve as a bifunctional hormone by binding to GH or PRL receptors (Chan *et al.* 1978a, Servely *et al.* 1983, Colosi *et al.* 1989, Warren *et al.* 1990). Production of oCS was first detected in the ovine trophoblast as early as day 16 (d16) of gestation (Martal & Djiane 1977). It was postulated that oCS was delivered to the maternal circulation by migration of fetal binucleate cells from the chorionic epithelium across the fetal–maternal boundary into the uterine epithelium to form a syncytium (Wooding 1981, Lee *et al.* 1986), but how oCS is delivered to the fetal circulation has not been defined. Schoknecht and co-workers (1991) suggested that oCS release was controlled

differently on the fetal and maternal sides of the placenta. In the maternal circulation, oCS was detected from day 40 to day 50 of pregnancy (Handwerger *et al.* 1977, Martal & Djiane 1977) and maximum levels were observed 10 to 15 days prior to parturition (Chan *et al.* 1978b). In the ovine fetal plasma, oCS concentrations increased until mid gestation and subsequently declined to term (Gluckman *et al.* 1979, Schoknecht *et al.* 1992).

Taylor and co-workers (1980) suggested that oCS could act as a growth-promoting agent in early pregnancy. The somatogenic effects of oCS include enhancement of fetal plasma concentration of insulin-like growth factor-I, hepatic glycogen deposition during late gestation and stimulation of the growth of several vital organs in lambs (Fowlkes & Freemark 1992, Singh *et al.* 1992, Schoknecht *et al.* 1996). The conclusions from studies on oCS specific binding remain in question: a specific binding site for oCS in lamb liver has been described by some authors (Freemark *et al.* 1986, 1988, Pratt *et al.* 1995), while others have suggested that oCS binds to oGH receptors (Breier *et al.* 1994, 1994, Klempt *et al.* 1993). Ovine chorionic somatomammotrophin has been sequenced, cloned and the recombinant form expressed (Colosi *et al.* 1989).

Availability of recombinant oCS (roCS) has now made it possible to investigate the specific effects of oCS in sheep (Sakal *et al.* 1997).

Numerous factors which modulate the concentration of oCS in the maternal and/or fetal circulation include: nutritional status (Brinsmead *et al.* 1981, Gluckmann & Barry 1988), serum concentrations of arginine and its derivatives (Handwerger *et al.* 1978), high density lipoproteins (Grandis *et al.* 1989), epidermal growth factor (Moore *et al.* 1984) and arachidonic acid (Huyler *et al.* 1985). Few studies exist which describe *in vitro* placental oCS production and/or regulation and generally these works have been short-term experiments (Steven *et al.* 1980, Rhodes *et al.* 1986, Rice & Thorburn 1986, Battista *et al.* 1990, Morgan *et al.* 1990). The present study was conducted to characterise oCS production *in vitro* by dispersed ovine cotyledonary cells collected from days 40 to 90 of gestation. The effects of addition of roCS and recombinant oGH (roGH) on this endogenous oCS production were investigated during early and mid gestation.

Materials and Methods

Animals

Twenty-eight ewes from the Préalpes du Sud breed were killed on days 40 to 90 of pregnancy: d40–d49 (4); d50–d59 (4); d60–d69 (7); d70–d79 (7); d80–d89 (6), according to the French recommendations for the use of experimental animals (guideline 04/19/1988). The uteri were immediately removed and the placental cotyledons collected aseptically and washed in Earle's balanced solution (pH 7.4) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml gentamycin.

Cell dispersion and culture

Placental cotyledons were separated from their attachment to maternal caruncles by gentle manual traction. Fetal cotyledons were then excised, minced and incubated for 1 h at 37 °C in 100 ml Earle's balanced saline solution containing 200 U/ml collagenase XI (Sigma, St Louis, MO, USA), 200 U/ml hyaluronidase (Sigma), 0.1% soybean trypsin inhibitor (Sigma), 0.25 mg/ml DNase I (Boehringer Mannheim, Mannheim, Germany) and 2% bovine serum albumin (IBF, Villeneuve la Garenne, France). Cells were dispersed by gentle passage through a 20/10 mm cannula and then diluted with 300 ml fresh Earle's medium and filtered through two layers of cheese cloth to remove undigested tissue. Subsequently, the cells were aliquoted into 50 ml polypropylene tubes and centrifuged (200 g, 5 min, 4 °C). Pellets were collected and washed 4 times by resuspension in 50 ml fresh Earle's medium. The lysis of red blood corpuscles in cell preparations was obtained by dispersion and incubation during

10 min in 40 ml ice-cold ammonium buffer (NH₄Cl 155 mM, KHCO₃ 10 mM, EDTA 0.01 mM). The cells were washed twice and filtered through a one-layer filter (25 µm; Polyabo, Strasbourg, France). Cell viability was determined by the Trypan blue exclusion method at the end of each period of culture. The cells were incubated at a concentration of 10⁶ viable cells/ml in Dulbecco's modified Eagle's medium (DMEM)/F12 culture medium (ATGC, Seromed, Champs sur Marne, France) supplemented with 0.5% bovine serum albumin, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml gentamycin, for 16 h at 37 °C (recovery period) under an atmosphere of 95% air, 5% CO₂. The cells were then centrifuged and resuspended in fresh medium at a concentration of 0.5 × 10⁶ cells/ml. For metabolic experiments, normal DMEM/F12 was replaced by a DMEM–methionine–cysteine-free medium (Biowhittaker, Fontenay-sous-Bois, France) supplemented with 10 µCi/ml [³⁵S]methionine–[³⁵S]cysteine (Pro-Mix, Amersham, Les Ulis, France). Partitioning of cells differed according to the experimental protocol: for RIA and metabolic assays, 2 × 10⁶ cells/well were distributed into 6- and 24-well plastic culture plates respectively (Greiner, Poitiers, France). For RNA analysis, 3 × 10⁶ cells/well were used. The cells were incubated at 37 °C under an atmosphere of 95% air, 5% CO₂ for up to 72 h without change of medium. The time at which cells were plated was designated as time zero of culture. For measurement of the amount of oCS accumulated, the media and cells were collected at time zero and at the end of each period of 8, 24, 48 or 72 h. Cells were separated from media by centrifugation (3000 g, 10 min, 4 °C). For the assay of oCS cellular content, cell pellets were submitted to lysis in 1 ml buffer (Tris 20 mM, NaCl 100 mM, EDTA 1 mM, phenylmethylsulphonyl fluoride 1 mM, Nonidet P-40 1% (Sigma), protamine sulphate 0.2 mg/ml (Merck, Darmstadt, Germany)). For oCS radioimmunoassay (RIA), all media were carefully withdrawn to avoid contamination of cell contents by conditioned medium. Both media and cellular lysates were stored at –20 °C until assayed. When we tested the effects of either roCS or roGH on oCS production, 100 or 300 ng/ml (roCS) and 10 or 50 ng/ml (roGH), kindly supplied by A Gertler (Jerusalem, Rehovot, Israel), were added in the media before cell distribution.

Radioimmunoassay

Concentrations of oCS in culture medium and cell lysates were measured by a double antibody RIA already described, using a specific anti-oCS rabbit serum (Lacroix *et al.* 1996). The sensitivity of the assay was 0.25 ng oCS/tube. Intra-assay and interassay coefficients of variation were 6.8% and 8.0% respectively. Before analysis of oCS conditioned medium data, values corresponding to the roCS concentrations added in the medium (100 or 300 ng/ml) were subtracted from the values assayed.

Immunoprecipitation and autoradiograph analysis

Samples of cellular lysates and conditioned media from metabolic experiments were pooled after 24 h and 72 h of culture respectively. Pools were concentrated and dialysed on Centriprep-10 concentrators (Amicom, Epernon, France). The protein concentrations from conditioned medium and cell lysates were determined using the method of Lowry-Peterson (Peterson 1977). Equivalent protein amounts were incubated overnight at 4 °C in 100 µl NET-gel buffer (Tris 50 mM, NaCl 150 mM, Nonidet P-40 0.1%, EDTA 1 mM (pH 8.0), gelatine 0.25%, NaN₃ 0.02%) with 5 µl rabbit anti-oCS gamma globulin obtained from the specific anti-oCS serum described in the RIA section. This concentration of antibody was tested so as to bind up to 2 µg/ml oCS. The antibody-antigen complex was precipitated after incubation with a purified ovine anti-rabbit gamma globulin (25 µl; 24 h) and centrifugation for 15 min at 17 000 g. The pellets were washed twice with 1 ml washing buffer (Tris 50 mM, NaCl 100 mM, SDS 0.05%, Nonidet P-40 0.1%, NaN₃ 0.02%). The samples and control roCS (20 ng) were analysed under denaturing and reducing conditions in the same SDS-PAGE (16% acrylamide gels). The gels were stained for 1 h with Coomassie blue to identify the control roCS band. Then, gels were incubated in a 1 M salicylate solution to increase the signal (Chamberlain 1979), dried during 2 h, and then exposed in a phosphor screen. The intensity of the oCS signal was quantified using a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA, USA) and the computer analysis program ImageQuant (Molecular Dynamics). The electrophoretic mobility of the labelled bands obtained after immunoprecipitation was compared with that of the control roCS. The specificity of the oCS immunoprecipitated band was checked by the addition of increasing amounts of unlabelled roCS to samples before immunoprecipitation and by probing blots with anti-oCS antibody.

Northern blot analysis

Total cellular RNA was isolated from cells by lysis in 4 M guanidinium isothiocyanate solution using the method of Chomczynski and Sacchi (1987) and modified by Puissant and Houdebine (1990). The amount and purity of the isolated RNA was determined by absorbency at 260 nm. Total RNA of cultured placental cells and of d60 placental tissue as a control (20 µg) were submitted to electrophoresis on 1% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes (Nytran NY 13N, Schleicher & Schuell, Equevilly, France). Blots were prehybridised in a 0.5 M sodium dihydrogenophosphate solution pH 7.2 containing 5% SDS, 1 mM EDTA, and 0.5% non-fat dry milk for 5 h and hybridised in the same buffer with an oCS cDNA probe (0634) kindly supplied

by Genentech Inc., (South San Francisco, CA, USA), labelled with [³²P]dCTP (3000 Ci/mmol; Amersham) for 18 h at 65 °C. Blots were washed twice for 5 min in 4 × SSC (NaCl 600 mM, sodium citrate 60 mM, 0.5% SDS, pH 7.0) at 65 °C. Variation in loading and quality of RNA was checked using radiolabelled 18S ribosomal cDNA probe (Raynal *et al.* 1984). The blots were exposed on a phosphor screen and the generated signal quantified using a PhosphorImager SF (Molecular Dynamics) and the computer analysis program ImageQuant (Molecular Dynamics).

Statistical analysis

Results from the oCS RIA are given as means ± s.e.m. and were analysed using the MIXED procedure of SAS software (SAS 1997). The model included fixed effects for stage of gestation (STAGE), treatment (TREAT), time (TIME) and their interactions, and random effects for animal (ANIMAL) and its interactions with TREAT and TIME. All these random effects were tested. As they are significant they were kept in the model and used to test fixed effects. The model used for this experiment was: $Y_{ijk(i)} = \mu + \alpha_i + \beta_j + a_{k(i)} + (\alpha\beta)_{ij} + (\beta a)_{jk(i)} + \gamma_t + (\gamma a)_{tk(i)} + (\alpha\gamma)_{it} + (\beta\gamma)_{jt} + (\alpha\beta\gamma)_{ijt} + e_{ijk(i)t}$, where μ , general mean; α_i , STAGE effect; β_j , TREAT effect; $a_{k(i)}$, ANIMAL effect nested in STAGE; $(\alpha\beta)_{ij}$, STAGE*TREAT interaction; $(\beta a)_{jk(i)}$, TREAT*ANIMAL(STAGE) interaction; γ_t , TIME effect; $(\gamma a)_{tk(i)}$, TIME*ANIMAL(STAGE) interaction; $(\alpha\gamma)_{it}$, STAGE*TIME interaction; $(\beta\gamma)_{jt}$, TREAT*TIME interaction; $(\alpha\beta\gamma)_{ijt}$, STAGE*TREAT*TIME interaction and $e_{ijk(i)t}$, residual error. STAGE effects were tested against the variability of ANIMAL(STAGE). TREAT and STAGE*TREAT were tested against the variability of TREAT*ANIMAL(STAGE). TIME and STAGE*TIME were tested against the variability of TIME*ANIMAL(STAGE). STAGE*TREAT*TIME was tested against the residual. A square root transformation of the response variable was used to control heterogeneity of variances. Tests with a *P* value <0.05 were considered statistically significant.

Results

Characterisation of net oCS production by isolated cotyledonary cells

Evaluation of oCS accumulated in the culture medium and cellular content The amounts of oCS that accumulated in the medium increased gradually with duration of culture and with the stage of gestation from which the cells were collected (Table 1). Due to individual variation, global analysis showed that this increase was significant only among tissues collected between days 40–49 and days 80–89 of gestation (*P*=0.017; *n*=28). The

Table 1 Evolution of *in vitro* oCS net production by ovine cotyledonary cells collected at different stages of gestation. oCS accumulated in the medium and the cellular content were measured at time 0, 8 h, 24 h, 48 h and 72 h of culture. Values are the means ± S.E.M. and are expressed as ng/mg/10⁶ viable cells. The experiments were conducted in sextuplicates

	Stage				
	d40–49 (n=4)	d50–59 (n=4)	d60–69 (n=7)	d70–79 (n=7)	d80–89 (n=6)
Time (h)					
Medium					
0	2.20 ± 1.00	4.10 ± 0.90	5.00 ± 1.00	5.20 ± 1.50	9.30 ± 2.10
8	27.00 ± 10.90	23.70 ± 9.40	9.60 ± 2.20 ^b	33.00 ± 16.80	44.80 ± 8.40
24	21.60 ± 10.90 ^{ab}	48.40 ± 12.30 ^b	47.90 ± 17.20 ^b	72.00 ± 24.80	110.40 ± 18.90
48	33.00 ± 18.80 ^{ab}	62.90 ± 12.20 ^b	64.80 ± 22.10 ^b	101.70 ± 34.00	153.10 ± 27.10
72	34.50 ± 18.84 ^{ab}	73.40 ± 8.30 ^b	86.10 ± 29.60 ^b	129.50 ± 36.40	192.20 ± 37.30
Cellular content					
0	18.60 ± 10.60 ^b	34.20 ± 11.90 ^b	33.40 ± 10.70 ^b	43.90 ± 10.20	66.40 ± 6.70
8	69.50 ± 10.60	43.80 ± 3.50 ^b	31.90 ± 8.10 ^b	77.20 ± 25.90	83.70 ± 23.00
24	13.70 ± 6.00 ^{ab}	23.00 ± 7.50 ^{ab}	23.00 ± 8.10 ^{ab}	53.50 ± 15.90	59.90 ± 12.60
48	8.80 ± 3.70 ^b	12.10 ± 5.40 ^b	13.40 ± 4.90 ^b	34.20 ± 11.90	42.20 ± 9.60
72	6.00 ± 2.80 ^b	9.20 ± 5.50 ^b	11.30 ± 3.40 ^b	24.10 ± 8.70	37.90 ± 9.70

^{a,b}*P* < 0.05 compared with d70–79 and d80–89 respectively.

oCS accumulation in the medium was maximal at 72 h of culture. However, we observed that 59% of this amount was already released after the first 24 h of culture.

Cellular content of oCS assayed at time 0 increased significantly between d40 and d90 of gestation (18.60 ± 10.60 (*n*=4) vs 66.40 ± 6.70 (*n*=6) ng/ml/10⁶ viable cells respectively; Fig. 1A). Global analysis (*n*=28) showed that the cellular content of oCS at days 80–89 was significantly higher than that found in the first three stages studied: d40–49 (*P*=0.007), d50–59 (*P*=0.02) and d60–69 (*P*=0.008). The oCS cellular content pattern was similar during culture (0 to 72 h) whatever the pregnancy stage considered. The greatest cellular content of oCS was recorded after 8 h of culture (Table 1). It decreased progressively from 8 h up to 72 h for all stages of pregnancy. However, this decrease was less pronounced for later stages.

Total net oCS production by dispersed cells was estimated as the amount the oCS released into the medium and the cellular content at 72 h. In spite of animals' individual variations, oCS production by dispersed cells increased gradually from d40–49 up to d80–89 (Fig. 1B).

Analysis of oCS mRNA during cell culture The expression of oCS mRNA by cotyledonary cells collected from placentae between d54 and d69 of gestation was studied by Northern blot at 30 min, 8 h, 24 h and 48 h of culture. Cotyledonary cells in culture contained detectable oCS mRNA of the expected size (Fig. 2A). As the time of culture progressed, oCS mRNAs signals decreased (Fig. 2B). However, a simultaneous decrease in the 18S mRNA signal was also observed (Fig. 2C).

Incorporation of [³⁵S]methionine-[³⁵S]cysteine in the oCS by cotyledonary cells Incorporation of [³⁵S]methionine-[³⁵S]cysteine in the oCS was estimated in the medium at 72 h (maximal accumulation) and in the cellular content at 24 h (oCS cellular content and mRNA signal were important at this time). The analysis by SDS-PAGE of labelled oCS in the cellular content and media, obtained by specific immunoprecipitation, showed radiolabelled bands which had the same electrophoretic mobility as the control roCS band (Fig. 3; lanes 1 and 4). Specificity of these bands was assessed by addition of increasing amounts of unlabelled roCS to the conditioned medium before immunoprecipitation, which reduced signal intensity and by Western blot detection with an oCS antibody (not shown).

Effects of addition of roCS and roGH on endogenous oCS net production by ovine dispersed cotyledonary cells

Effects of roCS addition on cotyledonary cells in culture Addition of roCS in the culture medium increased significantly the endogenous oCS release in a dose-dependent manner (*P*=0.0001; *n*=28). This increase was always observed for 300 ng/ml roCS dose, but it was less important on cells collected at the later stages of pregnancy. The effect of 100 ng/ml roCS dose was not observed beyond d50–59 (Fig. 4). The cellular content of oCS was dose-related to roCS treatment but to a lesser extent than for conditioned medium. Important variability was observed between animals at the same period of gestation (Fig. 5); however, global analysis showed significant effects of roCS treatment (*P*=0.001; *n*=28). The total endogenous oCS production (medium

Figure 1 oCS net production by ovine cotyledonary cells collected at different periods of gestation: d40–49 ($n=4$), d50–59 ($n=4$), d60–69 ($n=7$), d70–79 ($n=7$) and d80–89 ($n=6$). (A) oCS cell contents at time zero. The cells were cultured as described in Materials and Methods. (B) oCS net production (addition of media and cellular content) at 72 h of culture. Values represent the means $\pm$ S.E.M. of oCS production and are expressed as ng/ml/10⁶ viable cells. (□) and (*) represent a significant difference ($P<0.05$) compared with d70–79 and d80–89 respectively.

and cellular content) was significantly stimulated by the addition of either 100 or 300 ng/ml roCS ($P=0.0001$; $n=28$).

Immunoprecipitation of radiolabelled oCS ([³⁵S]methionine-[³⁵S]cysteine incorporation) in media (72 h) and cellular content (24 h) after addition of either 100 or 300 ng/ml roCS analysed by SDS-PAGE showed a dose-dependent increase in the intensity of radiolabelled oCS bands (Fig. 3). However, statistical analysis of the quantified signals ($n=3$) showed significant differences only in media ($P=0.025$).

Effects of roCS on oCS mRNA Since roCS stimulated endogenous oCS net production, we investigated whether this stimulation occurred at the level of oCS mRNA. This study was carried out between d54 and d69, corresponding

Figure 2 (A) Representative Northern blot showing oCS mRNA in ovine cotyledonary cells collected from a placenta on day 54 of gestation. Total RNA (20 μ g per lane) of cells cultured during 30 min, 8 h, 24 h and 48 h was hybridised with oCS cDNA (time of exposure=36 h) and 18S ribosomal cDNA probes (time of exposure=6 h). (B) Quantification of hybridisation signals for oCS mRNA at 30 min, 8 h, 24 h and 48 h of culture, in the ovine cotyledonary cells collected from placentae between d54 and d69 of gestation ($n=7$). The relative levels of oCS mRNA/18S RNA were determined from STORM scanning of Northern blot membranes. (C) Evolution of 18S RNA during culture time. Values (means $\pm$ S.E.M.) were obtained from STORM scanning of Northern blot membranes ($n=7$) and are expressed as the percentage of the 18S RNA levels at 30 min of culture.

Figure 3 Incorporation of [^{35}S]methionine-[^{35}S]cysteine in the oCS by ovine cotyledonary cells collected from a placenta on day 75 of gestation: effect of addition of roCS (100 and 300 ng/ml). (A) The figure shows a representative autoradiograph of labelled oCS from medium (72 h of culture; control, lane 1; oCS 100 ng/ml, lane 2; oCS 300 ng/ml, lane 3) and cellular content (24 h of culture; control, lane 4; oCS 100 ng/ml, lane 5; oCS 300 ng/ml, lane 6). Labelled oCS was immunoprecipitated with a specific anti-oCS gamma globulin. Control unlabelled roCS stained with Coomassie blue (not shown) migrated as radiolabelled immunoprecipitated oCS in a 16% SDS-PAGE. (B) Quantification of signals was obtained from STORM scanning of the gels. Values represent the means $\pm$ S.E.M. of 3 experiments on day 75 of gestation. The symbols represent a significant difference ($P < 0.05$) between: (□) 300 ng/ml roCS-treated cultures vs controls; (*) 100 ng/ml vs 300 ng/ml roCS-treated cultures.

to the period of pregnancy when the roCS effect was maximal. A high individual variability between animals at a similar stage was observed in oCS mRNA accumulation. The levels of oCS mRNA presented a trend of being enhanced at 30 min (for 300 ng/ml) or 8 h of culture compared with controls by d54–d56 of gestation for both roCS doses used. However, it was not significant and no difference at all was observed after this gestational stage.

Effects of roGH addition on cotyledonary cells in culture Incubation of cotyledonary cells collected from placentae between d54 and d86 of gestation ($n=6$), in the presence of 10 or 50 ng/ml roGH for up to 72 h, had no effect on oCS concentrations within the media and cellular content (not shown).

Discussion

The results of the present study demonstrate that cotyledonary cells isolated from sheep placentae between

40 to 90 days of gestation, and incubated *in vitro* during 72 h, exhibited a capacity to produce oCS that increased gradually with pregnancy stage. The levels of this production at each stage studied were related to the oCS cellular content at the time when cultures were started. The oCS accumulation in conditioned medium increased during a long culture period. However, the more important production rate was observed at the end of the first 24 h of culture, probably due to a better cellular differentiation at this time than later on. In spite of a great variability in hormone production observed between placental cell preparations even at the same period of gestation, a similar pattern of oCS production was observed for all stages studied in these culture conditions. Although cellular adhesion and multiplication were not observed in our culture system, the capacity of cells to produce oCS was supported by several pieces of evidence: levels of the cellular content of oCS were more elevated at 8 h of culture compared with time zero; both incorporation of [^{35}S]methionine-[^{35}S]cysteine in the oCS protein and oCS mRNA expression were detected during the culture period.

The capacity of cotyledonary cells to produce oCS increased gradually with the stage of pregnancy. The present technique used in our laboratory did not allow us to estimate the percentage of binucleate cells in the preparation, but we observed in our experiments that, for the same number of viable cells, the cellular content of oCS at time zero of culture increased with stage of gestation. This observation could be the consequence of an increase in the number of binucleate cells late in gestation, as suggested by Kappes and coworkers (1992). Moreover, the contribution of oCS cellular content at time zero to total oCS production measured at 72 h (addition of medium and cellular content at this time) was evaluated at each stage studied. In early stages (d40–49; d50–59) this contribution accounted for 40% of total oCS production, while it accounted for only 29% in later stages. These two observations suggest that the individual cellular capacity to produce oCS was increased during the late stages of pregnancy.

Kappes and coworkers (1992) showed a gradual increase in oCS mRNA expression by ovine cotyledonary tissues from placentae collected between d60 and d120 of gestation. In our experiments we analysed oCS mRNA levels in cotyledonary cells 24 h after dispersion. These levels varied considerably among cell preparations and no significant difference was observed between placentae from d54 to d70 of gestation in spite of increased oCS production. This difference between both studies may result from a relative degradation of mRNA in our culture conditions compared with those observed when mRNA is directly extracted from fresh tissue. Equilibrium between RNases and their inhibitors plays a role in the regulated turnover of mRNA and protein synthesis in the human placenta (Gileadi *et al.* 1984). It is possible that this

Figure 4 Effect of addition of roCS (100 and 300 ng/ml) to culture medium on oCS release by ovine cotyledonary cells collected from placentae of different periods of gestation: (A) d40–49 ($n=4$), (B) d50–59 ($n=4$), (C) d60–69 ($n=7$), (D) d70–79 ($n=7$) and (E) d80–89 ($n=6$). The cells were cultured as described in Materials and Methods. Values represent the means $\pm$ S.E.M. of oCS concentrations (ng/ml/10⁶ viable cells) accumulated in the medium at different times of culture. The symbols represent a significant difference ($P<0.05$) between: (●) 100 ng/ml roCS-treated cultures vs controls; (□) 300 ng/ml roCS-treated cultures vs controls; (*) 100 ng/ml vs 300 ng/ml roCS-treated cultures.

Figure 5 Effect of addition of roCS (100 and 300 ng/ml) on oCS cellular content in ovine cotyledonary cells. The figure shows 4 representative individual experiments: (A) d50–59 ($n=2$) and (B) d80–89 ($n=2$) of gestation. The effects of roCS treatment on the early stage were observed in spite of the great variability between animals in the oCS cellular content of control cells. The cells were cultured as described in Materials and Methods and values represent the means $\pm$ S.E.M. of oCS cellular content concentrations (ng/ml/ 10^6 viable cells) at different times of culture. The symbols represent a significant difference ($P<0.05$) between: (●) 100 ng/ml roCS-treated cultures vs controls; (◻X) 300 ng/ml roCS-treated cultures vs controls; (*) 100 ng/ml vs 300 ng/ml roCS-treated cultures.

equilibrium was altered when ovine cotyledonary cells were isolated. We cannot exclude the possibility that mRNA degradation was a consequence of cellular death. However, the slope of the decrease in mRNA is steeper than that of viability of cells (not shown).

The addition of roCS in the culture medium increased the endogenous oCS net production in a dose-dependent manner. This effect was more important in early stages than in later stages. The content of oCS in cells at the beginning of the cultures seemed to be, in part, a limiting factor to roCS stimulation: when this content was low (in the early stages of gestation) the enhancement of oCS production by the addition of roCS was more efficient than when it was high (d70 to d90). The 100 ng/ml dose of recombinant hormone was not effective in stimulating oCS production of cells collected after d60. However, the 300 ng/ml dose was always efficient, but less potent on

cells collected during the late periods of gestation studied. The cells collected during these late stages of gestation could already be in a stimulated state due to the elevated endogenous levels of oCS in this endocrine environment. Moreover, the enhancement of incorporation of [35 S]methionine-cysteine after addition of roCS is in favour of stimulation of neosynthesis.

These results allowed us to suggest that oCS up-regulates its own production via an autocrine and/or paracrine mechanism. Such mechanisms of autoregulation have been observed in many hormonal systems. Although these autoregulatory responses are often relevant from the perspective of down-regulation (Wei *et al.* 1988, Sanchez-Pacheco *et al.* 1995), hormone-mediated up-regulation has also been documented (Jia & Hsueh 1984, Bagnato *et al.* 1991, Peng *et al.* 1994). A system of autoregulation has been identified in human placenta for

human chorionic gonadotrophin (Licht *et al.* 1993). This up-regulated oCS system may contribute to an adequate growth and development of ovine placenta. It could be, in part, responsible for the morphological and/or functional differentiation in trophoblast cells during placental growth.

In early pregnancy (d54–56), regulation of the oCS production was concomitant with a slight although not significant increase in oCS mRNA accumulation. We impute this lack of significance to the high variability of oCS mRNA measurement between animals at these stages of pregnancy. This trend could result from a transcriptional effect of roCS or from a less important degradation of oCS mRNA in treated cells in the presence of recombinant hormone at this stage. However, more studies need to be conducted to validate definitively these hypotheses. Furthermore, we suggest that roCS autoregulation also involves posttranscriptional mechanisms, since the roCS effect was observed without a difference in oCS mRNA accumulation in placental cells collected between d60 and d69 of gestation.

The effect of addition of roCS on endogenous oCS production by cotyledonary cells *in vitro*, modulated by stage of gestation, is concomitant with the period of maximum development and growth of placenta (Reynolds *et al.* 1996). Therefore, we suggest that oCS plays an important role in cotyledonary cell differentiation which occurs *in vivo* during the same period.

The interaction of oCS and oGH with a common receptor has been suggested (Klempt *et al.* 1993, Breier *et al.* 1994). Furthermore, GH receptor mRNA has been described in the ovine placenta (Klempt *et al.* 1993). However, our results showed that autoregulation of oCS production could not be mimicked by the addition of roGH to cells collected from placentae between d54 and d86 of gestation. Binding of oCS to a specific receptor has been proposed (Freemark *et al.* 1986, 1987, 1988, Freemark & Comer 1989, Pratt *et al.* 1995) and an oCS receptor has been identified in ovine placenta (Chan *et al.* 1978a). Our results suggest the presence of a specific receptor for oCS in ovine placenta.

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