Evidence for the presence of luteinizing hormone–chorionic gonadotrophin receptors in the pig umbilical cord

G. Wasowicz¹, K. Derecka¹, A. Stepień¹, L. Pelliniemi², T. Doboszynska¹,
B. Gawronska¹ and A. J. Ziecik^{1*}

¹Division of Reproductive Endocrinology, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, 10-718 Olsztyn, Poland; and ²University of Turku, Kiinamyllynkatu 10, SF 20520 Turku, Finland

Pig umbilical cord, like that of humans, contains two arteries and a vein surrounded by Wharton's jelly with amnion covering the exterior surface. The aim of the present study was to investigate whether LH–hCG receptors are present in the pig umbilical cord, using light microscope immunohistochemistry, semiquantitative autoradiography, western blotting and reverse transcription–polymerase chain reaction. Umbilical cords were collected on days 48, 71 and 103 of fetal life ($n = 6$). Monoclonal and polyclonal anti-LH receptor antibodies were used to study receptor distribution. Immunoreactivity was observed in the umbilical blood vessels, the epithelium of umbilical amnion and cells in the Wharton's jelly. No differences in LH–hCG receptor distribution related to the sex of the fetus, period of fetal life or section of the umbilical cord were observed. Strong immunostaining was observed in umbilical vein and in umbilical arteries. However, in the arteries, the tunica media expressed weaker receptor immunostaining than did the tunica intima and tunica adventitia. No immunoactivity was detected in non-target tissue (skeletal muscle) but LH receptors were immunostained in the pig ovary. Topical autoradiography showed that vein and arteries in the umbilical cord bind ¹²⁵I-labelled hCG, which was highly diminished after co-incubation with an excess of unlabelled hCG. The binding of ¹²⁵I-labelled hCG to the Wharton's jelly and epithelial amnion was less intense than it was to vessels. Gonadotrophin binding sites were not present in the skeletal muscle. The pig umbilical arteries, vein and Wharton's jelly contained a 75 kDa immunoreactive LH–hCG receptor protein similar to that found in corpora lutea. Southern blot analysis of reverse transcription–polymerase chain reaction products, performed to enhance the sensitivity and specificity of LH receptor transcripts determination in umbilical cord tissues, revealed that the expected fragments of 740 and 470 bp were present in the arteries, vein, Wharton's jelly and corpora lutea (positive control). An additional product of 670 bp was found in the corpora lutea and arteries of umbilical cord, but not in the vein and Wharton's jelly. This is probably the first reported evidence of the presence of LH–hCG receptors in the umbilical cord of a non-human female mammal.

Introduction

The effect of LH and its agonist, hCG, on extragonadal tissues, such as myometrium, is mediated through binding to the same receptors. Uterine LH–hCG receptors are found in pigs (Ziecik *et al.*, 1986) and their presence has been also confirmed in rabbits (Jensen and Odell, 1988; Sawitzke and Odell, 1991), rats (Bonnamy *et al.*, 1990; Sawitzke and Odell, 1991), mice (Ziecik *et al.*, 1992; Mukherjee *et al.*, 1994), heifers (Freidman *et al.*, 1995) and humans (Reshef *et al.*, 1990). Detailed studies in pigs (Ziecik *et al.*, 1986) and women (Reshef *et al.*, 1990) demonstrated the presence of these receptors in both the endometrium and myometrium of the

uterus. LH–hCG receptors have also been detected in the superficial smooth muscle layer of the pig broad ligament and the ligamental blood vessels (Ziecik *et al.*, 1995). After hCG treatment, the majority of vessels supplying and draining blood from the uterus show LH–hCG binding sites, as determined by topical autoradiography and increased uterine blood flow in gilts (Ziecik *et al.*, 1996). However, human smooth muscle and the endothelial cells of the umbilical arteries and vein, the umbilical amnion and cells in the Wharton's jelly contain receptor mRNA and receptor proteins that can bind ¹²⁵I-labelled hCG (Rao *et al.*, 1993). The presence of hCG receptors in human umbilical cords indicates that hCG, which is present in cord blood and amniotic fluid, may be directly responsible for the vascular tone and other functions of human umbilical cord.

*Correspondence.

Revised manuscript received 8 March 1999.

The umbilical cord of pigs, like that of humans, contains two thick-walled arteries carrying deoxygenated fetal blood to the placenta and one thin-walled vein carrying oxygenated and nutrient-bearing blood to the fetus. Because there is no evidence of the production of CG by the pig placenta, the present study also investigated whether LH–CG receptors are present in the structures of the pig umbilical cord.

Materials and Methods

Tissues

Nine Large Polish White pregnant sows (gestation period 113–115 days) were slaughtered and the umbilical cords were taken as soon after death as possible: 8–10 umbilical cords were taken on days 48, 71 and 103 of fetal life. About half of the cords were collected from male and half from female fetuses. The cords were brought to the laboratory in ice cold physiological saline and then washed to remove the blood. Each cord (except those collected on day 48 of fetal life) was cut into three segments. The segment closest to the fetus was considered the fetal portion, the one closest to the placenta, the placental portion, and the remaining portion was considered the middle portion. Cords collected from 48-day-old fetuses were examined in one piece. Ovaries (pig, rat) and pig skeletal muscles were used as positive and negative control tissues, respectively.

All tissues were fixed overnight in 4% (w/v) paraformaldehyde in 0.1 mol phosphate l^{-1} buffer (PB; pH 7.4) at 4°C (for autoradiography and immunohistochemistry) and subsequently stored in 18% sucrose in PB for up to 6 days at 4°C. The tissues were cut into 12 μm thick sections with a cryostat (–25°C), transferred onto chrome alum–gelatine-coated slides and air-dried for about 20 min. Sections were then frozen at –30°C in air-tight boxes until needed for immunohistochemistry or autoradiography.

Autoradiography

Highly-purified hCG (CR 127) was labelled with ^{125}I (Amersham, Bucks) according to the chloramine method (Catt and Dufau, 1975; Greenwood *et al.*, 1963). Topical autoradiography was performed as described by Ziecik *et al.* (1995) with modification.

Before the autoradiographic procedures, the tissues were thawed and rinsed for 20 min in 50 mmol Tris–HCl l^{-1} , pH 7.4. Each slide was covered with 50–60 μl of solution (50 mmol Tris–HCl l^{-1} , 0.1 mmol bacitracin l^{-1} , 5 mmol MgCl_2 l^{-1} and 1% (w/v) BSA; pH 7.4) containing 20–30 pmol ^{125}I -labelled hCG l^{-1} and kept in a humid chamber overnight. Nonspecific binding was assessed by incubating adjacent sections with an excess of unlabelled hCG. Preliminary studies revealed that a dose of 1 μg hCG (CR127) was optimal to determine the nonspecific binding both in fetal (umbilical cords) and control (ovaries) tissues. After incubation, the sections were washed again in 50 mmol Tris–HCl l^{-1} , dried at room temperature and exposed in a tight-light cassette at 4°C

for 20 days to Hyperfilm ^3H (Amersham, Uppsala). Films were then developed in Rodinal (Agfa-Gevaert AG, Leverkusen).

Densitometry

The specific binding ^{125}I -labelled hCG in umbilical blood vessels, amnion epithelium and cells of Wharton's jelly was quantified in fetuses using HPScanJet/T scanner (Hewlett-Packard, Lorrvalis, OR) and the Micrograf Picture Publisher LE program.

For each autoradiogram, the mean absorbance of the film was measured over blood vessel walls, amnion epithelium or Wharton's jelly and then diminished for absorbance of autoradiogram exposed to an excess of non-radioactive hCG (nonspecific binding). The mean absorbance for each tissue was determined from the average of at least five measurements made from five autoradiograms containing that region in different fetuses. The values obtained were averaged and expressed as the arbitrary units of grey level. The number of observations (n) was equal to the total number of female or male fetuses from three sows per day of gestation.

After scanning and saving the initial image as a .tif file, the file was opened in PhotoStyler Version 2.0 and the scanned image was shown in the greyscale to examine its immunohistological properties (Velleman, 1995). Student's t test for independent samples was used for statistical analysis.

Immunohistochemistry

Primary antibodies. Polyclonal rabbit antibodies (PR) to synthetic fragments of rat LH receptors [anti-PCR II–(1–11)] were kindly provided by P. C. Roche (Mayo Clinic, Rochester, MN). These antibodies were used at a dilution of 1:500.

Polyclonal rabbit antibodies (HR) raised against rat LH receptor (Lakkakorpi *et al.*, 1990) were a gift from I. Huhtaniemi (Turku, Finland). They were used at a dilution of 1:100.

Monoclonal antibody (3B5) to rat LH receptors (Indrapichate *et al.*, 1992) was kindly provided by J. Wimalasena (Knoxville, TN). This antibody was used at a dilution of 1:50.

Secondary antibodies. Biotinylated goat anti-rabbit IgG (dilution 1:400 for PR or 1:500 for HR) and biotinylated horse anti-mouse IgG (dilution 1:400 for 3B5), used as second antibodies, were from a Vectastain kit (Vector Laboratories Inc., Burlingame, CA).

Immunohistochemistry was performed as follows: slides were taken out of the freezer and air-dried for 10 min. The sections were incubated for 30 min in 1% (v/v) H_2O_2 in methanol to destroy endogenous peroxidases. Sections were then hydrated in PBS (pH 7.4) three times for 10 min each. All incubations were carried out in a humid chamber. Sections were blocked with 2.5% normal goat serum and 1% (w/v) BSA for antibodies PR and HR, or with 2.5% normal horse serum with 1% (w/v) BSA for antibody 3B5 in PBS, for 60 min

at room temperature to block nonspecific binding of the 'link' antiserum to the tissue. The sections were incubated overnight at room temperature with the primary antibodies at the dilutions listed above. After this incubation, the slides were washed three times for 10 min with PBS, pH 7.4, and incubated for 60 min with a biotinylated antibody (the dilution and type of the secondary antibody were matched with the primary antibody). The slides were again washed in PBS and incubated for at least 60 min with the components, avidin (A) and biotinylated peroxidase (B), of the ABC staining kit (Vectastain). Both components (A and B) were diluted 1:500 and mixed at least 30 min before application on the sections. The slides were washed again in PBS (3×10 min) and for 2 min in 50 mmol Tris-HCl l^{-1} , pH 7.6. The bound antibody appeared after the addition of 50 mg 3,3'-diaminobenzidine tetrahydrochloride l^{-1} (Sigma Chemical Co., St Louis, MO) in a Tris-HCl buffer, to which 0.03% (v/v) H_2O_2 was added. The sections were then rinsed for 25 min in running water.

Every fourth section was lightly counterstained (5 min) with Mayer's haematoxylin. Sections were then dehydrated in a series of ethanol solutions, cleared in xylene and mounted with coverslips and DPX.

The following controls were performed: (1) omission of the primary or secondary antibody during the immunostaining procedure; (2) substitution of normal rabbit serum instead of the first polyclonal antibody (PR and HR), or normal mouse serum instead of the first monoclonal antibody (3B5); (3) immunostaining of pig ovary (positive control tissue); and (4) immunostaining of pig skeletal muscle (negative control tissue).

Identical conditions were used in the processing of all specimens, including positive and negative control tissues and procedural controls.

Western blotting

Membrane fractions of umbilical vein, arteries, Wharton's jelly and corpora lutea were prepared as described by Ziecik *et al.* (1986) with the exception that 0.2 mol Tris-HCl l^{-1} , pH 7.2 buffer, containing 0.1 mol phenylmethylsulphonyl fluoride l^{-1} to inhibit the protein activity, was used. Aliquots (70 μ g) of protein were dissolved in double-strength loading buffer, consisting of 0.5 mol Tris-HCl l^{-1} (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol and 2% (v/v) 2-mercaptoethanol and separated on 10% (w/v) SDS-PAGE. The separated proteins were electroblotted onto 0.1 μ m nitrocellulose membranes for 2 h at 250 mA in a 0.025 mol Tris-HCl l^{-1} buffer (pH 8.2), 0.192 mol glycine l^{-1} , containing 0.1% (w/v) SDS. The nonspecific binding sites were blocked with 5% (w/v) non-fat dry milk in 0.1 mol Tris-HCl l^{-1} (pH 7.4), 0.15 mol NaCl l^{-1} , 0.1% (v/v) Tween-20 (TBST buffer) overnight at 4°C. The membranes were then incubated with a 1:1000 dilution of the LH receptor antibody (PR) for 1.5 h at 22°C and washed three times for 10 min each with TBST. The washed blots were reincubated for 1 h at 22°C with a 1:3000 dilution of biotinylated antirabbit IgG, washed with TBST and incubated for 1 h at 22°C with a 1:3000 dilution of avidin-biotin-horseradish peroxidase (HRPO) complex (Vectastain ABC kit, Vector Laboratories, Inc, Burlingame, CA). TBS

containing 0.01% (v/v) H_2O_2 and 0.04% (w/v) 3,3'-diaminobenzidine was added to develop the colour.

RNA isolation and reverse transcription-polymerase chain reaction

Briefly, total RNA was isolated from umbilical cord blood vessels, Wharton's jelly, corpora lutea, intestine and kidney, using the methods of Chomczynski and Sacchi (1987) and Puissant and Houdebine (1990). The purity of RNA was determined by the $A_{260}:A_{280}$ ratio in spectrophotometry and its quality by agarose-formaldehyde gel electrophoresis (Sambrook *et al.*, 1989). The isoforms of the pig LHR cDNA from Gene Bank (pLHRA, pLHRB, pLHRC and pLHRD) were aligned and sequences of splice site junctions in pig LHR cDNA were fitted to this sequence commission.

The oligonucleotides used for RT-PCR were designed using the DNASTar Primer Selection program (DNASTAR Inc. Madison, WI). The specificity of the primers was confirmed by the BLAST Program (Altschul *et al.*, 1990). The sense primer (nucleotides 738–761, exon 9) 5'-GACGCTA-ATTGCCACATCATCCTA was common to all cDNA isoforms, and antisense primer (nucleotides 1456–1475, exon 11) 5'-ATTATGCTTGGAGGGTGGCT was related to full length isoform pLHRA and variant pLHRB.

RT-PCR was performed using 2 μ g total RNA per 100 μ l reaction mixture. Reverse transcription at 60°C for 1 h and denaturation at 97°C for 3 min were followed by PCR of cDNA at 40 cycles, including denaturation at 96°C for 30 s, primer annealing at 55°C for 30 s and extension at 74°C. Tth DNA polymerase, having both reverse transcriptase and DNA polymerase activities (Epicentre Technologies, Madison, WI), was used. The PCR products (20 μ l) were subjected to electrophoresis on 1% (w/v) agarose gel and photographed under UV light.

The primers selection program (DNASTar) was used to predict the annealing temperature, and the alignment of the pig LHR sequence predicted two RT-PCR products of 740 and 470 bp, respectively.

Southern hybridization

The RT-PCR products (10 μ l) were subjected to electrophoresis on 1.5% (w/v) agarose gels and transferred on nylon filter (Hybond N, Amersham) using the standard procedure. The hybridization was performed according to hybridization protocols for nylon membranes (Hybond N, Amersham) with a pig cDNA probe (fragment 900–2081 of pig LHR cDNA) labelled with [α - 32 P]dCTP, using the random prime method (Multiprimer™ DNA Labelling System, GIBCO BRL, Gaithersburg, MD), at 65°C for 12 h. The pig LH receptor cDNA was obtained from Dr H. Loosfelt (Hormones at Reproduction, Hospital de Biocentre, Kremlin Biocentre, Paris). After hybridization, the blots were washed twice with $1 \times$ SSC (0.15 mol NaCl l^{-1} , 15 mmol sodium citrate l^{-1} , pH 7.0) containing 0.1% SDS for 15 min at room temperature, and twice with $0.2 \times$ SSC–0.1% SDS at 65°C for 15 min. The washed sheets were exposed to Amersham X-ray film at –80°C.

Table 1. Summary of staining of umbilical cord and control tissues

Tissue	Day of fetal life		
	48	71	103
Arteries			
Tunica intima	++	++	++
Tunica media	+	+	+
Tunica adventitia	++	++	++
Vein			
Tunica intima	++	++	++
Tunica media	++	++	++
Tunica adventitia	++	++	++
Amnion epithelium	++	++	+++
Cells of Wharton's jelly	+	+	+
Vein incubated with NRS	ND	ND	0
Corpus luteum (positive control)		+++	
Skeletal muscle (negative control)		0	

Scoring of intensity of staining from 0 to + + + (expressed as arbitrary units of grey level; 0: 0–18; +: 19–43; ++ 44–101; +++ > 101; ND: not determined).

Results

Immunolocalization of the LH-hCG receptors in pig umbilical cord

Immunohistochemistry showed receptors in the umbilical blood vessels (Fig. 1a,b), epithelium of umbilical amnion (Fig. 1c) and cells in the Wharton's jelly (Fig. 1c,d). No differences in LH-hCG receptor distribution related to the sex of fetus, period of the fetal life or part of the umbilical cord were observed.

There was strong immunostaining in the umbilical vein (Fig. 1a) and in the umbilical arteries (Fig. 1b; Table 1). The LH-hCG receptor was detected in the tunica intima, media and adventitia of the umbilical vein (Fig. 1a). However, in the arteries, the tunica media showed weaker receptor immunostaining than did the tunica intima and adventitia (Fig. 1b; Table 1).

Receptor immunostaining was stronger in the umbilical amniotic epithelium (Fig. 1c) than it was in the Wharton's jelly (Fig. 1c,d; Table 1).

The pig corpora lutea (Fig. 1e) were immunostained with specific receptor antibodies to confirm this specific immunostaining technique. LH-hCG receptors were not detectable in pig skeletal muscles (Fig. 1f). Umbilical vein with Wharton's jelly plus amnion epithelium (103-day-old

fetus) was incubated with normal rabbit serum (Fig. 1g). There was no binding of nonspecific IgG. Identical immunostaining results were obtained with both HR and PR antibodies. However, significantly weaker staining was seen after application of 3B5 antibody and, for this reason, HR and PR antibodies were used to localize LH-hCG receptors in pig umbilical cord.

Localization of hCG-binding sites with autoradiography

The tissue localization of LH-hCG receptors was assessed on the sections that had been used to produce the autoradiograms (Fig. 2). Analysis of autoradiograms revealed that LH-hCG receptors are situated in both arteries and vein as well as in the Wharton's jelly of umbilical cord and the epithelial cells of umbilical amnion (Fig. 2b). Incubation of sections with non-radioactive hormone significantly displaced ^{125}I -labelled hCG binding (Fig. 2d). In addition to the above control for umbilical cord, other negative and positive tissues were also tested: pig skeletal muscle (negative control) did not show the presence of gonadotrophin binding sites (Fig. 2c); and pig ovary from the luteal phase (positive control) showed strong binding of ^{125}I -labelled hCG in follicles and corpora lutea (Fig. 2a). A computer-generated image of autoradiograms of umbilical

Fig. 1. Immunohistochemistry of LH-hCG receptors in pig umbilical cords. (a) Umbilical vein of 71-day-old male fetus (middle part of the umbilical cord); (b) umbilical artery of 71-day-old female fetus (periplacental part of the umbilical cord); (c) epithelium of umbilical amnion and Wharton's jelly of 103-day-old male fetus (perifetal part of the umbilical cord); (d) cells of Wharton's jelly of 103-day-old female fetus (middle part of the umbilical cord); (e) pig corpus luteum; (f) pig skeletal muscle (negative control tissue); (g) umbilical amnion, Wharton's jelly and umbilical vein of 103-day-old female fetus (periplacental part of umbilical cord); (h) umbilical artery of 103-day-old female fetus (middle part of the umbilical cord). Plates show sections stained with polyclonal rabbit antibodies, except (g) and (h), in which normal rabbit serum was substituted for primary antibody. (a,b,d,f,g,h) Nuclei counterstained with Mayer's haematoxylin. Photographs were made using a light microscope (Axioskop, Zeiss). Photographs (c,d,e,f) were made using Nomarsky optics. Arrow: cell of Wharton's jelly; arrowhead: tunica intima; A: tunica adventitia; AM: amniotic epithelium; M: tunica media; WJ: Wharton's jelly. Scale bars represent (a) 200 μm , (b,g,h) 100 μm , (c,e,f) 25 μm and (d) 10 μm .

Table 2. A computer base image of autoradiograms (expressed as the arbitrary units of grey level) of umbilical cord

Tissue of umbilical cord	Day of fetal life											
	71						103					
	Portion of umbilical cord						Portion of umbilical cord					
	Placental		Middle		Fetal		Placental		Middle		Fetal	
	Male (n = 10)	Female (n = 12)	Male (n = 10)	Female (n = 12)	Male (n = 10)	Female (n = 12)	Male (n = 11)	Female (n = 12)	Male (n = 11)	Female (n = 12)	Male (n = 11)	Female (n = 12)
Vein	114.2 ± 1.5 ^a	104.0 ± 1.9 ^a	105.3 ± 2.2 ^a	123.5 ± 6.0 ^a	109.3 ± 3.5	108.8 ± 0.3 ^{ab}	112.6 ± 2.9 ^a	143.5 ± 1.5 ^a	106.5 ± 3.0 ^{ab}	113.6 ± 1.2 ^a	117.1 ± 0.4 ^{ab}	103.5 ± 1.3 ^a
Arteries	117.3 ± 1.0 ^a	113.1 ± 1.1 ^a	127.5 ± 1.0 ^b	138.3 ± 0.6 ^a	115.2 ± 2.2	124.7 ± 1.0 ^{bc}	109.8 ± 4.1 ^a	136.9 ± 1.9 ^a	106.0 ± 0.5 ^{ab}	104.5 ± 0.5 ^{ac}	123.3 ± 0.4 ^b	84.9 ± 1.4 ^b
Epithelium												
amnion	85.7 ± 0.0 ^c	92.9 ± 7.4 ^a	97.5 ± 9.4 ^a	105.7 ± 6.5 ^a	97.7 ± 5.5	111.7 ± 4.7 ^{ab}	105.5 ± 0.0 ^{ab}	99.7 ± 3.9 ^b	116.5 ± 2.0 ^a	103.0 ± 2.1 ^{ac}	114.3 ± 2.6 ^{ab}	118.4 ± 1.9 ^c
Wharton's jelly	57.1 ± 3.6 ^b	67.5 ± 2.5 ^b	67.5 ± 6.5 ^c	68.2 ± 5.5 ^b	89.2 ± 2.9	92.5 ± 1.1 ^{bd}	91.0 ± 1.1 ^b	80.3 ± 3.5 ^c	95.0 ± 1.5 ^b	98.4 ± 1.5 ^{bc}	110.3 ± 3.5 ^a	89.5 ± 2.5 ^b

All values presented as mean $\pm$ SEM.

^{a,b,c,d} Means within columns with different superscripts differ significantly ($P < 0.05$).

Fig. 2. Autoradiography of LH-hCG receptors in pig umbilical cords, positive and negative controls. ^{125}I -labelled hCG was present in (a–c) and unlabelled hCG was present in (d). (a) Pig ovary from luteal phase; (b,d) umbilical vein and umbilical arteries (103-day-old fetus); (c) pig skeletal muscle. a: artery; AM: amniotic epithelium; c: corpora lutea; f: follicle; v: vein; WJ: Wharton's jelly. Scale bars represent (a) 5 mm and (b–d) 2 mm.

cord in 48-day-old fetuses showed that the umbilical arteries and vein contained relatively large amounts of grey (arbitrary units: mean $\pm$ SEM) both in males (130.5 ± 0.5 and 134.4 ± 0.5 ; $n = 13$) and females (142.3 ± 0.5 and 149.2 ± 0.7 ; $n = 14$, respectively) when compared with male (60.4 ± 8.9 ; $P < 0.05$) and female (118.6 ± 2.0) amniotic epithelium or male and female Wharton's jelly (56.3 ± 3.5 and 68.5 ± 0.5 , $P < 0.05$, respectively).

The results of densitometry of autoradiograms for different portions of umbilical cords collected from fetuses on days 71 and 103 of gestation are shown (Table 2). As was the case in 48-day-old fetuses, the umbilical cord arteries and vein of 71- and 103-day-old fetuses showed greater increases in the concentrations of LH-hCG receptors than did the Wharton's jelly in the placental and middle portion of umbilical cord ($P > 0.05$) in both sexes. However, there was little difference in the fetal portion of umbilical cord (Table 2). In general, the epithelial amnion showed a concentration of binding sites that was a little lower than that of blood vessels and a little higher than that of the Wharton's jelly of the umbilical cord.

LH-hCG receptor protein

Western immunoblotting with a polyclonal LH-hCG receptor antibody showed that umbilical arteries and vein contained a 75 kDa protein (Fig. 3; lines 3–5). There was also

Fig. 3. Western immunoblotting for pig LH-hCG receptors. Line 1: pig corpora lutea; line 2: Wharton's jelly; line 3: umbilical vein; lines 4 and 5: umbilical arteries of 103-day-old fetus. Forty milligrams of protein of each tissue was used.

a weak receptor protein band in Wharton's jelly (line 2). Corpora lutea contained two receptor proteins of 75 and 48 kDa (line 1). The skeletal muscle did not show receptor protein (data not shown).

LH-hCG receptor mRNA

A Southern blot of RT-PCR products was performed to enhance the specificity of determinations of LH receptor transcripts in umbilical cord tissues (Fig. 4) and revealed that the expected fragments of 740 and 470 bp were present in the vein and arteries and Wharton's jelly of the umbilical cord as well as in corpora lutea (positive control). However, the signals for Wharton's jelly were very weak. An additional product of 670 bp was found in the corpora lutea and arteries

Fig. 4. Southern blot of RT-PCR products of pig LH receptor in umbilical cord tissues. a: artery; c: corpora lutea (positive control); i: intestine (negative control); -RNA: reaction control without RNA; v: vein; Wj: Wharton's jelly. After hybridization, the membrane was exposed to Amersham X-ray film 3 days for umbilical cord and negative control tissues, respectively, and for 2 h for corpora lutea.

of the umbilical cord, but not in the vein and Wharton's jelly. The negative control tissue (for example, intestine and kidney tissue; data not shown) and the control of RT-PCR reaction (tube without RNA) did not show the presence of any RT-PCR products.

Discussion

The presence of functional LH-hCG receptors in human umbilical cords (Rao *et al.*, 1993) and the discovery of similar receptors in the uterine broad ligamental blood vessels in pigs (Ziecik *et al.*, 1995) raised the question of the presence of LH-hCG receptors in pig umbilical cord and their biological significance.

The results of the present study showed the presence of LH-hCG receptors in pig umbilical cord. Two methods were used to visualize LH receptors in the umbilical cord: immunostaining and autoradiography. Both methods gave consistent results and showed the highest intensity of LH receptor immunostaining or 125 I-labelled hCG binding in umbilical cord vessels and the lowest in cells of the Wharton's jelly. A small divergence of results between both methods was found only in umbilical veins and arteries, since immunostaining was usually stronger in veins than in arteries. However, autoradiograms in most cases did not show significant differences. A relatively high concentration of LH receptors was found in the amniotic epithelium using both immunostaining and autoradiography. The distribution of gonadotrophin receptors in pig cords was similar to that in humans and there were no consistent differences in receptor expression between cords from male and female pig fetuses. In the present study, in pigs, the higher receptor expression in the blood vessels of the fetal portion of the cord than in the placental portion as reported in humans (Rao *et al.*, 1993) was not observed. The various portions (fetal, middle and

placental) of the pig umbilical cord and cells in Wharton's jelly with the amniotic epithelium showed a similar distribution of LH-hCG receptor immunostaining. The autoradiographical method of receptor identification did not have as strong a reaction as did the immunohistochemical reaction in epithelial cells of umbilical amnion, particularly on day 103 of fetal life.

The cloning and sequencing of pig LH-hCG receptor messenger RNAs revealed the presence of a full-length receptor and shorter variants, lacking either the transmembrane and the intracellular domains or only the transmembrane domain. Immunoblotting of testicular membrane extracts detected 85, 68 and 45–48 kDa proteins reacting with anti-receptor antibodies (Vu Hai-Luu Thi *et al.*, 1992). The main full-length receptor species (85 kDa) present in the pig testis contains complex oligosaccharides. The present study showed that pig corpora lutea and umbilical cord vessels contain mainly the 75 kDa receptor species, which is in agreement with the molecular mass for LH receptor in pigs calculated by Loosfelt *et al.* (1989). This protein contains an extracellular domain of 333 amino acids proceeding a 266 amino acid transmembrane region (with seven transmembrane spans) and a 70 amino acid intracellular domain. However, it is hypothesized that the 75 kDa protein found in the present study is the glycosated 68 kDa form described by Vu Hai-Luu Thi *et al.* (1992) in pig testis. The 48 kDa receptor variant detected in pig corpora lutea is apparently a form lacking transmembrane or intracellular domains. Western immunoblotting showed receptor protein in Wharton's jelly and Southern blot analysis revealed the presence of mRNA transcripts. The low LH immunostaining and the relatively low binding of 125 I-labelled hCG indicated that LH receptors in Wharton's jelly are very dispersed. The pig luteotrophin receptor has four splice variants (Loosfelt *et al.*, 1989), and the three shorter variants are caused by alternative splicing of exon 10 with exon 11. These shorter isoforms were observed in the testes and two variants

encoded proteins lacking both transmembrane and intracellular domains and the third encoded only the transmembrane domain. Although there are no data to confirm that some isoforms of LH receptors differ in their binding affinity, the presence of LH receptor variants in nongonadal reproductive tissues may explain, in part, the variance in the results from the immunocytochemical and autoradiographical analyses in the present study.

The umbilical cord provides a vascular connection between fetus and mother via the placenta. The umbilical vessels are surrounded and protected by the Wharton's jelly, which contains modified fibroblasts known as myofibroblasts (Takechi *et al.*, 1993). These cells contribute to the elasticity of the Wharton's jelly by synthesizing collagen fibres and may play a role in the regulation of umbilical blood flow. LH-hCG umbilical cord receptors in humans are functionally coupled to the regulation of the eicosanoid biosynthetic pathway (Rao *et al.*, 1993) and may mediate hCG action on the relaxation of umbilical blood vessels. Such an action would increase the blood flow between fetus and placenta and play an important role in fetal growth and development. Since it has been established that human cord blood (Crosignani *et al.*, 1972) and amniotic fluid contain hCG (Clements *et al.*, 1976; Ozturk *et al.*, 1988), the physiological importance of LH-hCG receptors is clear. The biological significance discovered in the present study on LH-hCG receptors in pig umbilical cord is more difficult to explain because the presence of chorionic gonadotrophin in pig placenta has yet to be proved. However, Saunders *et al.* (1980) reported the presence of a gonadotrophin-like substance in fetal membranes and allantoic fluid on days 52 and 66 of gestation and in placenta at term. The gonadotrophin-like material in the trophoblast was shown to be distinct from pig LH by subjecting the same extracts to specific radioimmunoassay for LH; <8% of the material that crossreacted in radioreceptor assays could be accounted for by pLH. Whether LH-hCG receptors in the pig umbilical cord are functional and able to bind unknown gonadotrophin-like hormones, or are merely a phylogenetical dead end, remains to be elucidated.

The authors are indebted to K. Wasowicz for his help with the immunohistochemical experiments and to M. Jensen for editing the manuscript. They would like to express gratitude to J. Murawska-Kempa for typing this manuscript. Some of the results were communicated in a preliminary form at the 13th International Congress on Animal Reproduction in Sydney, Australia, June 30–July 4, 1996. The studies were supported by the State Committee for Scientific Research in Poland (Grant 5 P06K 039 09).

References

- Altschul S, Gish W, Miller W, Myers E and Lipman D (1990) Basic local alignment search tool *Journal of Molecular Biology* **215** 403–410
- Bonnamy PJ, Benhaim A and Leymarie P (1990) Estrous cycle-related changes of high affinity luteinizing hormone/human chorionic gonadotropin binding site in the rat uterus *Endocrinology* **126** 1264–1269
- Catt KJ and Dufau ML (1975) Gonadal receptors for luteinizing hormone and chorionic gonadotrophin *Methods in Enzymology* **37** 167–193
- Chomczynski P and Sacchi N (1987) Single-step method RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction *Analytical Biochemistry* **162** 156–159
- Clements JA, Reyes FI, Winter JSD and Faiman C (1976) Studies of human sexual development III. Fetal pituitary and serum, and amniotic fluid concentrations of LH, CG, and FSH *Journal of Clinical Endocrinology and Metabolism* **42** 9–19
- Crosignani PG, Nencioni T and Brambati B (1972) Concentration of chorionic gonadotrophin and chorionic somatomammotrophin in maternal serum, amniotic fluid and cord blood serum at term *Journal of Obstetrics and Gynaecology* **79** 122–126
- Freidman S, Gurevich M and Shemesh M (1995) Bovine cyclic endometrium contains high-affinity luteinizing hormone/human chorionic gonadotropin binding sites *Biology of Reproduction* **52** 1020–1026
- Greenwood FC, Hunter WH and Glover JS (1963) The preparation of ¹³¹I-labelled human growth hormone of high specific radioactivity *Biochemical Journal* **89** 114–123
- Indrapichate K, Meehan D, Lane TA, Chu SY, Rao ChV, Johnson D, Chen TT and Wimalasena J (1992) Biological actions of monoclonal luteinizing hormone/human chorionic gonadotropin receptor antibodies *Biology of Reproduction* **46** 265–278
- Jensen JD and Odell WD (1988) Identification of LH/hCG receptors in rabbit uterus *Proceedings of Society of Experimental Biology and Medicine* **189** 28–30
- Lakkakorpi J, Keinänen KP and Rajaniemi H (1990) Production and characterization of polyclonal antiserum to rat luteinizing hormone/chorionic gonadotropin receptor and its immunohistochemical application for studying receptor location and downregulation *Endocrinology* **127** 513–522
- Loosfelt H, Misrahi M, Atger M, Salesse R, Vu Hai-Luu Thi MT, Jolivet A, Guiochon-Mantel A, Sar S, Jallat B, Garnier J and Milgrom E (1989) Cloning and sequencing of porcine LH-hCG receptor cDNA: variants lacking transmembrane domain *Science* **245** 525–528
- Mukherjee D, Manna PR and Bhattacharya S (1994) Functional relevance of luteinizing hormone receptor in mouse uterus *European Journal of Endocrinology* **131** 103–108
- Ozturk M, Brown N, Milunsky A and Wands J (1988) Physiological studies of human chorionic gonadotropin and free subunits in the amniotic fluid compartment compared to those in maternal serum *Journal of Clinical Endocrinology and Metabolism* **67** 1117–1121
- Puissant C and Houdebain LM (1990) An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction *BioTechniques* **8** 148–149
- Rao ChV, Li X, Toth P, Lei ZM and Cook VD (1993) Novel expression of functional human chorionic gonadotropin/luteinizing hormone receptor gene in human umbilical cords *Journal of Clinical Endocrinology and Metabolism* **77** 1706–1714
- Reshef E, Lei ZM, Rao ChV, Fridham DD, Chegini N and Luborsky JL (1990) The presence of gonadotropin receptors in nonpregnant human uterus, human placenta, fetal membranes, and decidua *Journal of Clinical Endocrinology and Metabolism* **70** 421–430
- Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning: a laboratory manual *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*
- Saunders PTK, Ziecik AJ and Flint APF (1980) Gonadotrophin-like substance in pig placenta and embryonic membranes *Journal of Endocrinology* **85** 25P
- Sawitzke AL and Odell WD (1991) Uterine binding sites for LH/hCG can be modulated by hormonal status in rabbits and rats *Acta Endocrinologica (Copenhagen)* **124** 322–330
- Takechi K, Kuwabara Y and Mizuno M (1993) Ultrastructural and immunocytochemical studies of Wharton's jelly umbilical cord cells *Placenta* **14** 235–245
- Velleman SG (1995) Quantifying immunoblots with a digital scanner *BioTechniques* **18** 1056–1058
- Vu Hai-Luu Thi MT, Misrahi M, Houllier A, Jolivet A and Milgrom E (1992) Variant forms of the pig lutropin/choriogonadotropin receptor *Biochemistry* **31** 8377–8383
- Ziecik AJ, Stanchev PD and Tilton JE (1986) Evidence for the presence of luteinizing hormone/human chorionic gonadotropin-binding sites in the porcine uterus *Endocrinology* **119** 1159–1163
- Ziecik AJ, Derecka-Reszka K and Rzucidlo JS (1992) Extragonadal gonadotropin receptors, their distribution and function *Journal of Physiology and Pharmacology* **43** 33–49
- Ziecik AJ, Ostrowska G, Kisieleska J and Zezula-Szpyra A (1995) Distribution and cycle phase dependency of gonadotropin receptors in musculature and blood vessels of the porcine broad ligament *Experimental and Clinical Endocrinology and Diabetes* **103** 44–51
- Ziecik AJ, Golba G and Kisieleska J (1996) Effect of human chorionic gonadotropin on uterine blood flow in intact and ovariectomized gilts *Experimental and Clinical Endocrinology and Diabetes* **104** 158–163