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Expression of beta hCG and alpha CG mRNA and hCG hormone in human decidual tissue in patients during tubal pregnancy

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We recently showed that endometrial tissue produces hCG during the secretory phase of the menstrual cycle. Based on these findings, we hypothesized that the decidua should also be able to secrete hCG. We examined the decidualized endometrium of patients with extrauterine pregnancies. Decidual specimens were obtained for mRNA extraction and paraffin embedding from 24 patients that were between weeks 6–11 of tubal pregnancy. Tissues were evaluated and classified into one of three groups based on the endometrial differentiation that took place prior to conception: (A) high secretory transformation, (B) diminished transformation with restricted decidualization and (C) inferior endometrial proliferation. Decidual gland hCG secretion was demonstrated immunohistochemically and by Western blotting. Serum hCG levels were higher ($P < 0.0001$) in patients from group A than group C. mRNA expression of both the β subunit (β-hCG) and α subunit (α-CG) was determined by RT–PCR. Furthermore, the specificity of β-hCG amplification was confirmed by restriction enzymes. β-LH amplification was not found. Moreover, the degree of endometrial transformation and the level of decidualization was found to correlate with hCG hormone staining and β-hCG mRNA expression. hCG protein in the decidua was present in the glands of the compact layer and in the spongy layer, and was more pronounced in previously transformed high secretory endometrium than in inferior endometrium. In conclusion, this study provides the first evidence that hCG is produced in the decidua of patients during extrauterine pregnancies and might play a possible paracrine role.

Key words: hCG/human decidua/immunohistochemistry/mRNA expression/Western blot

Introduction

hCG has been regarded as a pregnancy-specific hormone that is produced in the trophoblast. Later in the pregnancy, hCG is released in large quantities from the villous syncytio-trophoblast into maternal circulation. The glycoprotein hormone is composed of two non-covalently associated α and β subunits. The common α chorionic gonadotrophin (α-CG) subunit is encoded by a single α gene. Unlike α-CG, β-hCG is encoded by a cluster of six homologous genes localized in chromosome 19 (Fiddes and Goodman, 1979; Jameson and Hollenberg, 1993). The free α-CG and β-hCG proteins combine to form an intact biologically active hCG molecule. hCG bioactivity is dependent upon the glycoprotein side chain stucture, which changes during pregnancy (Elliott *et al*., 1997; Mock *et al*., 2000). In the previous years, authors have reported that hCG is also secreted in non-trophoblastic healthy tissues and carcimoma (Yoshimoto *et al*., 1977; Braunstein *et al*., 1979; Marcilliac *et al*., 1992). Recently, it was shown that gonadotrophin production appears to be associated with several different types of carcinomas (Dirnhofer *et al*., 1998; Hotokainen *et al*., 1999; Coleman *et al*., 2000).

We were the first group to demonstrate that the uterine glandular epithelium expresses hCG mRNA and produces hCG protein during the normal menstrual cycle (Alexander *et al*., 1998). hCG was also detected in the Fallopian tubes (Lei *et al*., 1993). Ovarian granulosa-lutein cells and testicular Leydig cells both express the hCG/LH receptor. This receptor binds hCG and relays the hormone message into the cell. Additionally, non-gonadal hCG/LH binding sites were identified in other tissues, such as the endometrium, decidua and the Fallopian tubes. Endometrial hCG/LH receptor expression is most pronounced in epithelial cells then stromal cells and finally vascular endothelial cells in the uterus (Reshef *et al*., 1990; Lei *et al*., 1992; Toth *et al*., 1994).

The maternal decidua is the transformed endometrium of a pregnant women. After conception, it develops from the pars functionalis of cyclic endometrium into two layers: the more superficial compact layer, which has glands with narrow lumen and the lower spongy layer, which has typical sawtooth-shaped papillar glands which are more dilated. The aim of our study was to examine whether the maternal decidua continues to produce the locally derived hCG, in addition to hCG produced by the trophoblast, in early pregnancy. A suitable early pregnancy model free of uterine cavity trophoblast tissue was needed for this experiment. Therefore, for our experiment we selected decidual tissue from patients with tubal pregnancy, which is free of villous or extravillous trophoblast hCG production.

RT–PCR and restriction enzyme analysis were used to demonstrate mRNA expression of α-CG and β-hCG subunits. Immunohistochemistry and the Western blotting method were used to demonstrate hCG secretion. Furthermore, we studied whether the amount of decidual hCG production depends on the degree of endometrial differentiation.

Materials and methods

Patients and decidual samples

To examine the α-CG and β-hCG expression and β-hCG protein production of uterine decidua lacking extravillous trophoblast, 24 cases of tubal pregnancies were identified for this study. Uterine decidual samples of extrauterine pregnancies serve as models of uterine mucosa during the pregnant state.

Decidua was collected by curettage during the laparoscopic extirpation of pregnancy. Informed consent was obtained from the patients. The gestational age was between weeks 6–11 of pregnancy. In each case, the ectopic pregnancy was verified by the clear localization of trophoblast in the Fallopian tube by ultrasound. Decidual specimens were only included in the study if the clinical analysis suggested the absence of other gynaecological pathologies. The collected decidual tissue samples were divided into two parts for routine paraffin embedding after they had been fixed in 4% neutral buffered formalin overnight, and for rapid processing or snap-freezing and storage at –80°C for subsequent RT–PCR analysis. Each of the decidual samples was examined histologically. Histological dating was confirmed by an experienced pathologist, who classified the decidual samples into three groups according to endometrial differentiation that took place prior to conception: (A) highly secretory transformed endometrium with distinct decidualized stromal areas, (B) diminished secretory transformed endometrium with partially decidualized stromal content, and (C) inferior and/or disturbed endometrial proliferation with minimal or no secretory transformation. Peripheral hCG concentrations were detected using the IMX-Total/β-hCG MEIA (micro particle enzyme immunoassay) from Abbott.

Isolation of mRNA and RT–PCR

mRNA was extracted from the decidua. Approximately 20 mg of fresh specimens prewashed in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco), were disrupted using an ulta-turrax. The mRNA was isolated using the Quick prep mRNA kit (Pharmacia) with mRNA binding to oligo dT-cellulose, and 50 µl of eluate was stored in small portions at –80°C.

Reverse transcription

mRNA was reverse transcribed using standard methods. A 2.5 µl aliquot of mRNA solution was heated at 65°C for 5 min, followed by cooling at room temperature. A 7.5 µl aliquot of cDNA mixture was added to give a final concentration of 10 mmol/l Tris–HCl, pH 8.3, 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l each of dATP, dCTP, dGTP and dTTP, 20 IU/10 µl RNase inhibitor, 5 IU/10 µl AMV revertase, and 0.4 µg/10 µl oligo-p(dT)15 from Roche. The mixture was incubated for 10 min at 25°C and for 60 min at 42°C followed by a 5 min step for destroying revertase activity, all carried out in a thermocycler from Perkin Elmer.

PCR

The PCR procedure was continued immediately in the reverse transcription tube, by adding 40 µl of PCR mixture to a final concentration of 10 mmol/l Tris–HCl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 2.5 U/50 µl *Taq* DNA polymerase and 20 pmol/50 µl of each primer pair of different β-hCG-, α-CG- and β-actin-oligonucleotides. Alternatively, for 300 bp products, 0.5% (v/v) dimethylsulphoxide (DMSO) was included in the total PCR mixture volume to raise the efficiency, as well as 10 mmol/l $NaHCO_3$, and 125 µg/50 µl transferrin to protect DNA polymerase activity. All PCR amplifications started with a denaturation step of 2 min at 95°C and finished with an elongation step of 5 min at 72°C.

Amplifications of cDNA were performed using four different primer pairs specific for β-hCG gene 3, 5, 7 and 8 products respectively (all forward then reverse primer): β-hCG1 5′-TCGGGTCACGGCCTCCT-3′ and 5′-CCGGCAGGACCCCCTGCAGCA-3′ (562 bp product) with 1 min denaturation at 95°C, 2 min annealing at 60°C and 3 min extension at 74°C for 35 cycles (Bo *et al*., 1992); β-hCG2 5′-TTCCTACACCCTACTCCCTGT-3′ and 5′-CCGGCAGGACCCCCTGCAGCA-3′ (511 bp) with 60 s at 95°C, 40 s at 61°C and 45 s at 72°C for 35 cycles (Jameson and Hollenberg, 1993; Dirnhofer *et al*., 1996); β-hCG3 5′-TCACTTCACCGTGGTCTCCG-3′ and 5′-TGCAGCACG-CGGGTCATGGT-3′ (423 bp) with 30 s at 95°C, 30 s at 60°C and 30 s at 72°C for 35 cycles (Miller-Lindholm *et al*., 1997); β-hCG4 5′-TGGCTGTGGA-GAAGGAGGGCTGC-3′ and 5′-GGAAGCGGGGGTCATCACAGGTC-3′ (300 bp) with 30 s at 95°C, 30 s at 65°C and 60 s at 72°C for 35 cycles (Talmadge *et al*., 1984; Krichevsky *et al*., 1995). In addition, the primer α-CG of 5′-TGCAGGATTGCCCAGAATGC-3′ and 5′-CCGTGTGGTTCTCCA-CTTTG-3′ (233 bp) with 30 s at 94°C, 30 s at 56°C and 60 s at 72°C for 35 cycles (Fiddes and Goodman, 1979) and β-actin 5′-GTGGGGCGCC-CCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′ (547 bp) with 30 s at 95°C, 30 s at 65°C and 60 s at 72°C for 35 cycles (Stewart-Akers *et al*., 1998) were used for PCR amplification. All the oligonucleotide primer pairs were synthesized by Applied Biosystems. Aliquots of 9 µl PCR products were run on a 2.0% agarose gel in 0.05 mol/l Tris-buffered 0.15 mol/l saline (TBS) buffer, pH 7.4, to prove the efficiency and fidelity of the β-hCG- and α-CG-DNA fragment production, using 0.01% ethidium bromide for UV identification and photographic documentation.

Restriction enzyme analysis

The identity of the 300 bp PCR product was verified by restriction enzyme digestion. Several β-hCG-specific restriction enzymes (*Sty*I, *Bsp*1286, *Hae*III and *Ava*II from Roche) were included in cleavage experiments to confirm the PCR-derived DNA sequence for β-hCG as opposed to β-LH. The enzyme concentration and optimal buffer conditions for DNA digestion were chosen according to the manufacturer's instructions. The RT–PCR products were separated electrophoretically on an agarose gel and the 300 bp β-hCG DNA amplificate was extracted using a DNA purification kit (Biozym). DNA digestion fragments resulting after overnight incubation at 37°C were demonstrated on a 2% agarose gel. The patterns of cleavage products obtained from digestion of the 300 bp amplificate with *Sty*I (300 bp), *Bsp*1286 (175/125 bp), *Hae*III (300 bp) and *Ava*II (94/87/43/43/33 bp) would be consistent with the β-LH DNA sequence.

Immunohistochemistry

The divided parts of fresh decidua collected from tubal pregnancies were used in parallel for PCR studies as well as histological embedding in paraffin. The tissue blocks of decidua, and as a control, paraffin blocks of early pregnancy placenta, were cut at 5 µm serial tissue sections, mounted on superfrost slides, deparaffinized, cleared in xylene and a gradient of ethanol, and rehydrated and incubated for 10 min in 0.05 mol/l TBS with 0.1% Tween-20, pH 7.6 (TBST). After rehydration, the specimens were incubated with 0.3% hydrogen peroxide in methanol (30 min) to block endogenous peroxidase activity. Triplicate sections were stained for all 24 samples and controls.

Immunolocalization of hCG

For hCG staining using polyclonal antibody, after brief washes in TBS, the tissue sections were placed in a humidified chamber and then sequentially overlaid and incubated with 100 µl of the following reagents: TBS with 0.2% Triton X-100 as antigen demasking procedure for 10 mins; the biotin and then avidin blocking solution (Dako) for 10 min each for endogenous biotin suppression; 10% normal goat serum (NGS) in TBS for 30 min to block the non-specific staining; after removing excess NGS blocking serum, the anti-β-hCG rabbit A0231 (Dako) primary antibody (diluted 1:500 in TBST/10% NGS) was left overnight at 4°C; Vector Elite ABC kit (Vector) with biotinylated goat anti-rabbit IgG as second antibody diluted 1:200 in TBST/NGS for 30 min; POD-conjugated avidin–biotin complex (Vectostain ABC) for 30 min; finally, diaminobenzidine (DAB) from Vector for 5 min to develop a brown reaction product. Negative controls were performed by omitting the use of primary hCG or other primary antibodies. Initially, the specimens were mounted on aqueous-based histogel and later dehydrated, cleared in xylene and mounted with a non-aqueous permanent mounting medium.

For hCG staining in endometrial sections using mouse monoclonal anti-β-hCG antibody (INN2 or INN22, Serotec) diluted 1:5000 or 1:200 in TBST/normal rabbit serum (NRS), the Catalyzed Signal Amplification (CSA) system of Dako was required and was used according to manufacturers' instructions. After POD blocking of sections with hydrogen peroxide in methanol, biotin/avidin blocking and blocking non-specific antibody binding with TBST/NRS as described, incubation with the primary antibody was continued at 4°C overnight, followed by the secondary biotinylated rabbit anti-mouse IgG (Vector) antibody diluted 1:200 in TBST/NRS and the POD-conjugated avidin–biotin complex (Dako), each for 30 min. In the CSA system a supplementary amplification step was included to increase biotin signals localized at the antibody-binding site. Finally, the incubation with the POD–avidin conjugate allowed the amplified DAB staining reaction. The

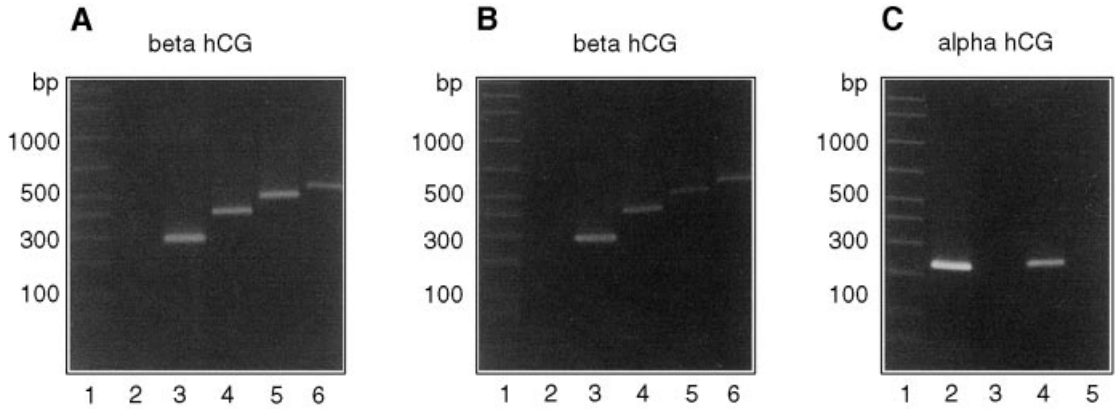

Figure 1. Expression of β-hCG and α-CG mRNA analysed by RT–PCR from decidua of tubal pregnancy. Ethidium-stained agarose gels showing representative products amplified from cDNA derived from early pregnancy placenta and decidua specimens. (**A**) Placental cDNA was amplified with primer pairs for 300 (lane 3), 423 (lane 4), 511 (lane 5) and 562 bp (lane 6) showing predicted β-hCG DNA products as positive control. (**B**) Amplification of decidual cDNA of group A patients with high secretory transformation showing obvious β-hCG fragment products. (**C**) mRNA of α-CG is expressed in decidua (lane 4) of tubal pregnancy compared to placental expression (lane 2) as control (**A**, lane 2; **B**, lane 2; **C**, lane 3 without mRNA; **C**, lane 5 without primer).

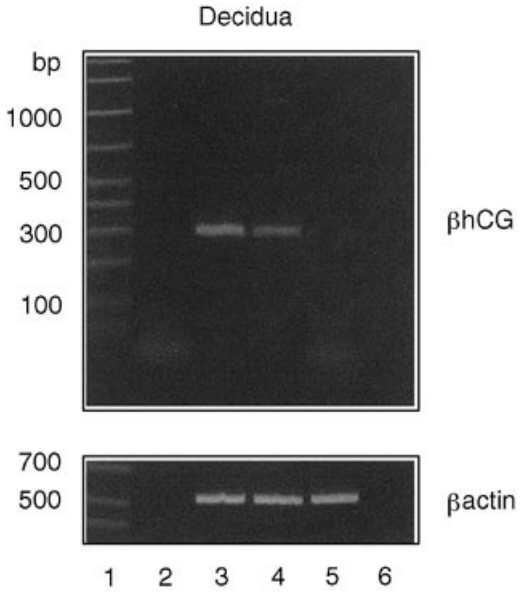

Figure 2. Expression of decidual β-hCG mRNA in patients with different degrees of secretory endometrial differentiation prior to conception. A representative PCR demonstrates the decidual β-hCG mRNA expression for specimens in patient groups A, B and C, shown in Table I. The β-hCG expression is related to β-actin expression in the same tissues. Lane 3: β-hCG mRNA is expressed at the highest level in tissue of the high secretory transformation group A. Lane 4: Expression is lower in patients with diminished secretory transformation group B. Lane 5: The expression is lacking in tissue with inferior proliferation and without secretory transformation group C. Lane 2: Controls without mRNA. Lane 6: Without hCG primers.

intensity of hCG staining scaled semi-quantitatively from 3-fold positive to negative compared with controls.

Trophoblast immunolocalization

In order to exclude the presence of extravillous trophoblast cells we examined the decidua specimens with the monoclonal human cytokeratin antibody (MNF116, Dako). It is specific for cytokeratin 5, 6, 7, 17 and probably 19, and binds to epithelial cells and all trophoblast cells. Before staining, the paraffin-embedded sections were treated as described.

However, cytokeratin antigen staining required demasking pretreatment with target retrieval citrate solution, pH 6.2 (Dako), for 30 min at 95°C in a water bath followed by 20 min of cooling. The hydrogen peroxide and biotin/avidin-blocked and NRS-preincubated slides were incubated with cytokeratin antibody diluted 1:1000 overnight at 4°C, followed by biotinylated secondary rabbit anti-mouse IgG antibody (Dako). The immunohistochemical staining procedure was performed according to the CSA method as given above, visualized by application of VIP chromogen (Vector).

Western blotting

Approximately 50 mg of fresh decidual tissue specimens were cut with scissors into small pieces and washed repeatedly with RPMI 1640 containing 50 IE/ml penicillin and 50 μg/ml streptomycin. The separated tissue was taken up in 2 ml of 50 mmol/l Tris–HCl reducing lysis buffer containing 150 mmol/l NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 1 Complete Protease Inhibitor tablet (to 1 mmol/l EDTA)/10 ml (Roche), 1 μmol/l pepstatin, inhibiting acid proteases, and disrupted by the ultra turrax method at 4°C. Clarified lysate supernatant was prepared by centrifugation at 12 000 *g* for 5 min at 4°C. To reduce non-specific binding to the nitrocellulose membrane, the lysate supernatant was precleared using protein A–agarose incubation at 4°C overnight (Roche). The lysate proteins and a low molecular weight protein standard mixture (Pharmacia) were size separated by reducing 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose by electroblotting. Resulting filters were incubated with polyclonal primary rabbit anti-β-hCG antibody (A0231, Dako) at a dilution of 1:500 or monoclonal anti-β-hCG antibody (INN22, Serotec) at a dilution of 1:100 at 4°C overnight followed by incubation with biotinylated secondary goat anti-rabbit (1:500) or anti-mouse (1:500) antibodies respectively, for 1 h at room temperature and then reaction with ABC complex (Vector). The molecular forms of hCG were detected by visualization with DAB staining using the same conditions as immunohistochemistry and correlated to molecular weight markers. Dimeric hCG products were obtained from Sigma, Biotrend and Serono. The staining of the biotinylated secondary antibodies without any hCG binding could be localized by omission of the first hCG antibody.

Statistical analysis

All hCG data were expressed as the mean ± SEM. Statistical analyses were performed using the SPSS statistical software program. The data were checked for normal distribution (Kolmogorov–Smirnov test) and are distributed normally. The two-tailed *t*-test reflects a significant difference at a value of $P < 0.050$.

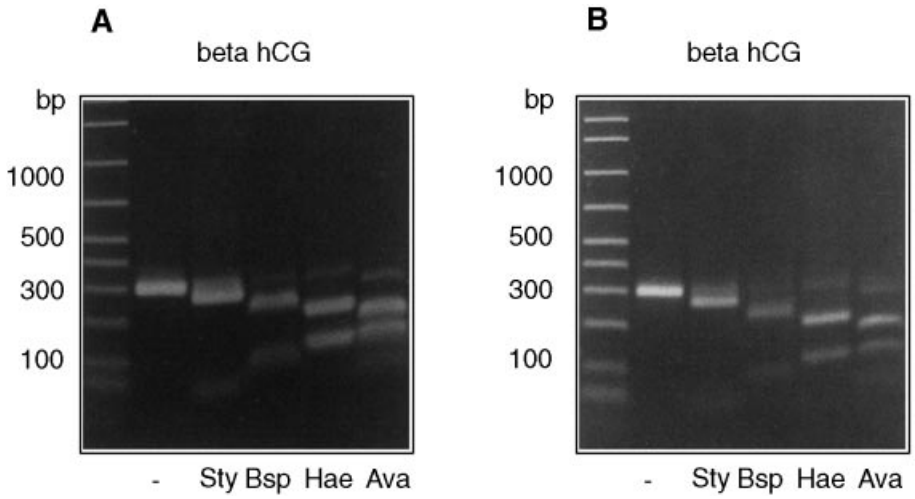

Figure 3. Cleavage of DNA amplificates of placental and decidual β-hCG by restriction enzymes. (**A**) Placental β-hCG amplificate of 300 bp without restriction enzyme (lane 2) and after digestion with enzymes *Sty*I, *Bsp*1286, *Hae*III and *Ava*II in lanes 3–6. (**B**) Decidual β-hCG amplificate was cleaved to identical fragments as the placenta specimen by *Sty*I (261/39 bp), *Bsp*1286 (226/74 bp), *Hae*III (196/104 bp) and *Ava*II (163/94/35/8 bp).

Results

We examined mRNA transcription and corresponding hCG production in decidual samples from patients during tubal pregnancy. β-hCG and α-CG mRNAs were detected with the RT–PCR technique (Figure 1) and β-hCG quantified with respect to β-actin (Figure 2). β-LH expression was excluded with restriction enzyme analysis (Figure 3). hCG production in the decidua was determined in decreasing stages of differentiation (Figure 4) and with different hCG antibodies (Figure 5) by immunohistochemical staining. The absence of extravillous trophoblast cells in the decidua was verified by cytokeratin staining (Figure 5). The molecular weights of hCG subunit proteins were determined using SDS–PAGE and Western blotting (Figure 6).

The immunohistochemical and clinical findings of the decidual samples were classified into three groups according to the morphological developmental stage as described above (Table I). The peripheral hCG levels of patients were recorded immediately before termination of the tubal pregnancy. We found a significant difference between groups A and C ($P < 0.001$). In group A (high secretory transformation and decidualization), the mean serum hCG was 1829 ± 292 mU hCG/ml (mean ± SEM), in group B (diminished secretory transformation and partial decidualization), 1072 ± 354 mU hCG/ml, and in group C (inferior or disturbed proliferation and minimal or no secretory transformation), 349 ± 98.7 mU hCG/ml. Uterine bleeding, indicative of steroid hormone deficiency, occurred mainly in group C with poor proliferation. Haematosalpinx (haemorrhagia into the tubal lumen) was found in all three groups. A likely positive correlation between secretory transformation and immunospecific glandular hCG formation in both the decidual compact and non-decidualized spongy layers was demonstrated by the strength of the glandular immunohistochemical hCG staining (from 3-fold positive to negative).

Using specific oligonucleotide primer pairs in the PCR, which have been described previously (Bo and Boime, 1992; Dirnhofer *et al*., 1996; Miller-Lindholm *et al*., 1997), we found the expected β-hCG cDNA amplification products of 423, 511 and 562 bp. This confirmed decidual β-hCG gene expression. Early pregnancy placenta specimens were used as a positive control (Figure 1A, B). While the above-mentioned primer pairs cover the full-length exon 1 to exon 3 cDNA, a further primer pair (Krichevsky *et al*., 1995) produced a 300 bp amplification of exon 2 to exon 3. The expression of α-CG mRNA was found in decidual tissue from the patients with tubal pregnancy as well as the placental control (Figure 1C). We also compared the extent of β-hCG mRNA expession in the samples with varying decidual differentiation.

We were able to semi-quantitatively demonstrate that the 300 bp β-hCG nucleotide products are expressed in varying amounts relative to the constitutive β-actin mRNA expression (Figure 2).

β-hCG messenger RNA is lacking or poorly expressed in decidual tissue that is only poorly proliferated or has not undergone secretory transformation. To verify the identity of β-hCG amplificates, DNA cleavage experiments were performed. Digestion of the 300 bp β-hCG cDNA with different restriction enzymes (*Sty*I, *Bsp*1286, *Hae*III, *Ava*II) resulted in the anticipated smaller fragments, which characterize the β-hCG cDNA origin and proved that β-LH mRNA was not present (Figure 3A, B).

Serial decidual tissue sections were evaluated immunohistochemically for hCG and correlated to decidual immune cells to identify competent decidual cells for hCG expression and release (data not shown). The immunolocalization indicates that glandular epithelial cells seem to be the major site of uterine hCG expression, in both the non-decidualized spongy layer and the more decidualized compact layer of decidua that is closed to the luminal epithelium. However, it is likely that immunostaining of glandular hCG in decidualized compact areas and non-decidualized spongy layer is more prominent in the high secretory transformation group (Figure 4A, B) than in the reduced secretory group, where only singular stained cells in the spongy glands can be found (Figure 4C, D). In contrast, hCG protein expression could not be detected in inferior and disturbed secretory endometrium sections using the immunohistochemical hCG staining method (Figure 4E, F). We used placenta specimens from early pregnancy as a positive control for hCG, and decidua sections that were not treated with hCG antibody as a negative control (Figure 5C, D). In order to exclude the presence of interstitial extravillous trophoblast cells in the studied decidua specimens of tubal pregnancy, serial tissue sections were stained using trophoblast-specific cytokeratin antibody.

Extravillous trophoblast cells in the decidual stromal or spiral artery regions, which could have been responsible for the β-hCG production, were not found in any of the investigated tissue (Figure 5A, B). In contrast to intrauterine pregnancies, decidual extravillous trophoblast cells should not occur.

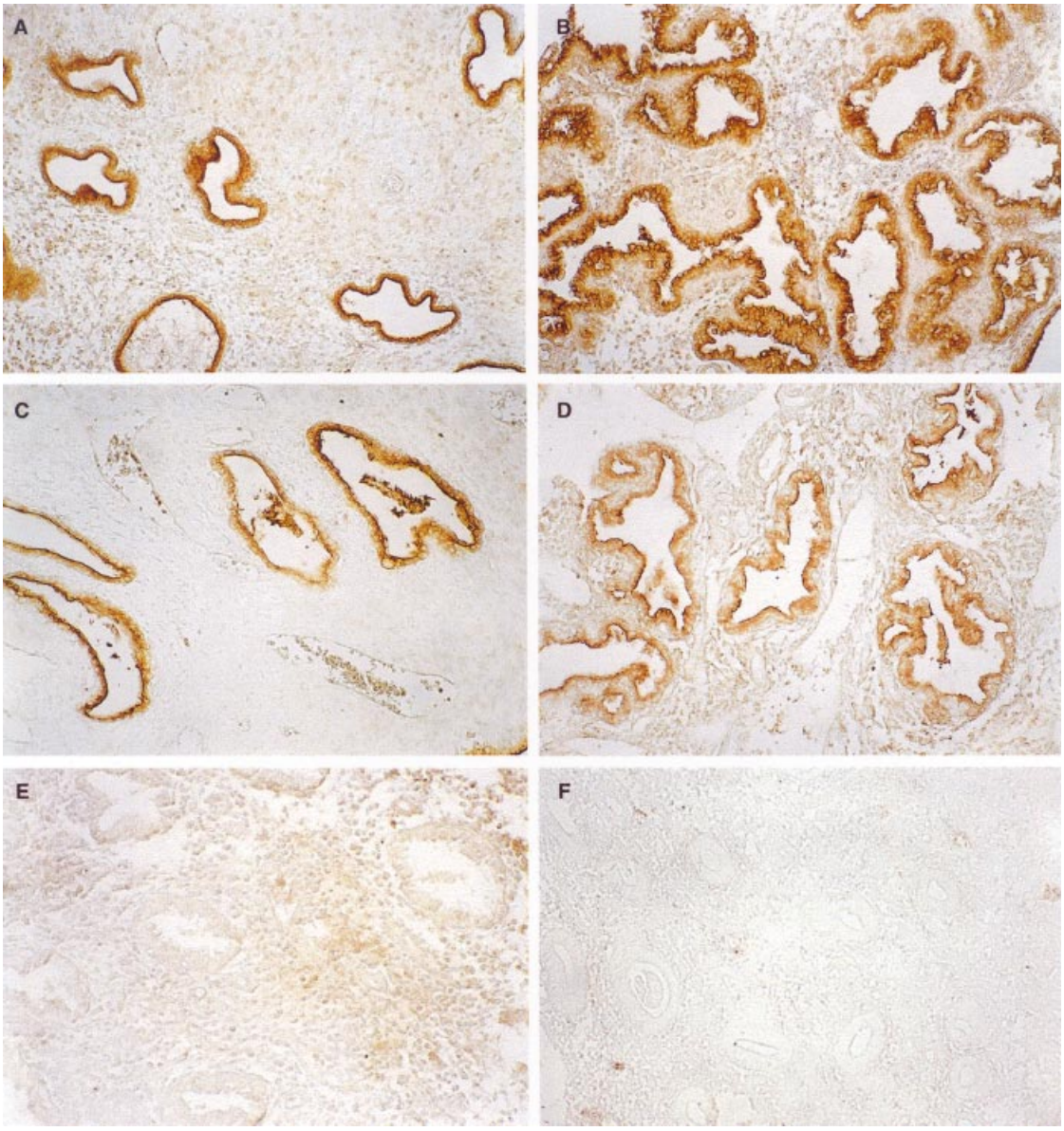

Figure 4. Immunolocalization of hCG in decidualized compact layer and non-decidualized spongy layer of decidua in patients with tubal pregnancy. The hCG staining is localized in glandular epithelium of the decidualized compact layer showing high (**A**) and diminished (**C**) secretory transformation of the decidua. In the spongy layer of tubal pregnancy, the epithelial hCG was detected decreasing from high (**B**) to diminished (**D**) secretory transformation, and was not visible in spongy layer sections of decidual tissue in inferior proliferation with failing decidualization (**E**) and secretory transformation (**F**) (original magnification ×200).

Epithelial hCG immunostaining of decidual glands was performed with a polyclonal β-hCG antibody as well as monoclonal β-hCG antibodies INN2 or INN22, characterizing the β-hCG chain epitope of cluster B I or B II from dimeric hCG respectively, as shown in Figure 5E–G (Lund and Delves, 1998).

To assign the detected immunohistochemical hCG to a molecular state, we examined the tissue extract fluid of decidua specimens and compared it with placental tissue extract from early pregnancy and pure dimeric hCG products. Western blots were obtained using polyclonal and monoclonal β-hCG antibodies, demonstrated in Figure 6A–D. The immunostained main band of decidual hCG appeared at ~30 kDa next to a faint band of 33 kDa, which was sometimes present using the polyclonal antibody. The bands were correlated to the expression profiles of *N*-glycosylated β-hCG molecular isoforms in placenta specimens or trophoblast cells (Elliot *et al*., 1997; Singh and Merz, 2000).

Discussion

The present study demonstrated that the β-hCG gene and α-CG gene are transcribed into mRNA in the uterine decidua of patients during tubal pregnancy. hCG is generally regarded as a trophoblast hormone. However, recently we identified hCG in glandular cells of the endometrium during the secretory phase of the menstrual cycle (Wolkersdörfer *et al*., 1998). In the present study, we investigated the role of decidua in hCG secretion. To our knowledge, this is the first demonstration of expression of α-CG and β-hCG subunit mRNAs and production of hCG protein in the decidua of patients during early pregnancy. We used tubal pregnancies, in contrast to normal intrauterine pregnancy, as a model that is free of extravillous trophoblast, which penetrates the decidua. We ruled out the presence of stromal or endovascular trophoblast cells by using cytokeratin antibody MNF116 for immunohistochemical trophoblast staining.

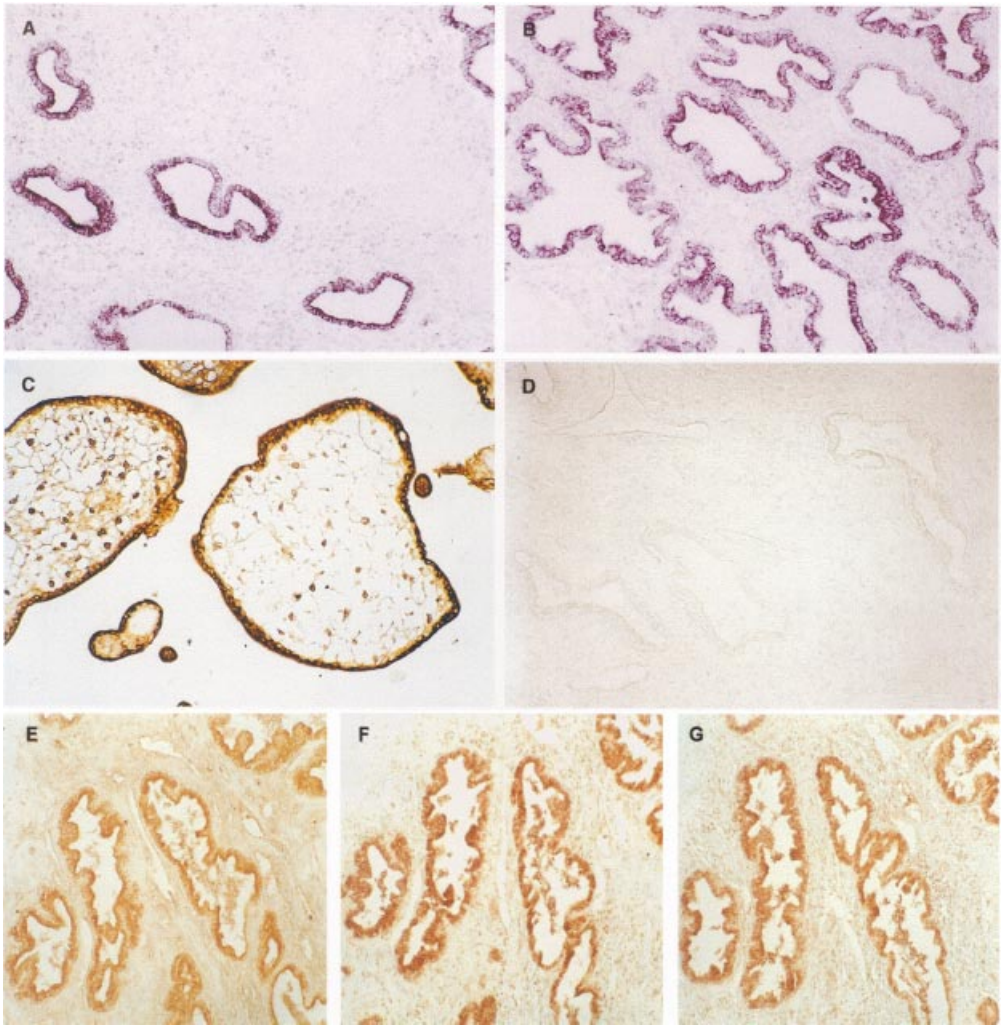

Figure 5. Lack of interstitial extravillous trophoblast cells in decidua of patients with tubal pregnancy and immunohistochemical controls. Using the cytokeratin antibody MNF116-staining no stromal or endovascular trophoblast cells have been found in the decidualized compact layer (**A**) or non-decidualized spongy layer (**B**). The positive control of placental hCG staining (**C**) and the negative control with no hCG antibody (**D**) are shown. The hCG staining of the glands could be demonstrated using of different antibodies: (**E**) Ab hCG A0231, 1:500; (**F**) Mab β-hCG INN2, 1:5000; (**G**) Mab β-hCG INN22, 1:200 (original magnification ×200).

We demonstrated that the mRNA gene expression of α-CG and β-hCG is actually occurring in decidual mucosa. Moreover, different PCR primer pairs spanning exon 1 to exon 3 of the β-hCG DNA sequence established decidual, as opposed to trophoblast, expression. Additionally, the application of DNA restriction enzymes for subunit-specific digestion confirmed the expected β-hCG restriction sites. In our three patient groups, decidual β-hCG mRNA expression was related to the level of endometrial differentiation. This appears to be dependent on the degree of endometrial secretory transformation and stromal decidualization that took place before conception.

Furthermore, the immunohistochemical staining of the decidua demonstrated that in early pregnancy, hCG is produced in the epithelium but probably not in the stromal cells. This hCG production seems to correlate with the level of differentiation of the epithelium. The most intensive staining was found in the mono-layered glands with rather small-sized epithelial cells surrounded by decidual cells of a superficial compact layer. Also, the common papillar indented glands in the deeper spongy layer with their round to oval cell nuclei of epithelium were readily stained. hCG staining of glands near to the basal layer, which were not as differentiated with rather oval to longish nuclei, showed only slight staining that was restricted to single cells.

Haematoxylin counterstaining of the serial tissue sections was not shown. It seems that hCG protein is either not produced at all or only in small quantities in stratified epithelium of decidual glands with cylindrical to spindle-shaped nuclei (i.e. the hCG stain disappears in the epithelial structures of this tissue with inferior proliferation without secretory transformation). Therefore, we conclude that the glandular hCG production depends on the degree of the epithelial cell differentiation in early pregnancy.

hCG is primarily considered a specific placental hormone. However, numerous authors reported that hCG is present in normal and tumour tissues (Braunstein *et al*., 1979; Marcillac *et al*., 1992; Dirnhofer *et al.*, 1996, 1998; Yokotani *et al*., 1997; Coleman *et al*., 2000). We showed for the first time that normal cyclical secretory endometrium expresses subunit mRNA and produces hCG (Alexander *et al*., 1998) which was later confirmed by Reimer *et al*. (2000). The decidua, as a highly differentiated endometrium found during pregnancy, can also produce hCG. hCG effects appear to be triggered by binding of hCG to known non-gonadal hCG/LH receptors (Reshef *et al*., 1990).

The question arises whether hCG production in the decidua has a biological function or if it is a epiphenomenon. We think that the decidual hCG functions as an autocrine and paracrine hormone that

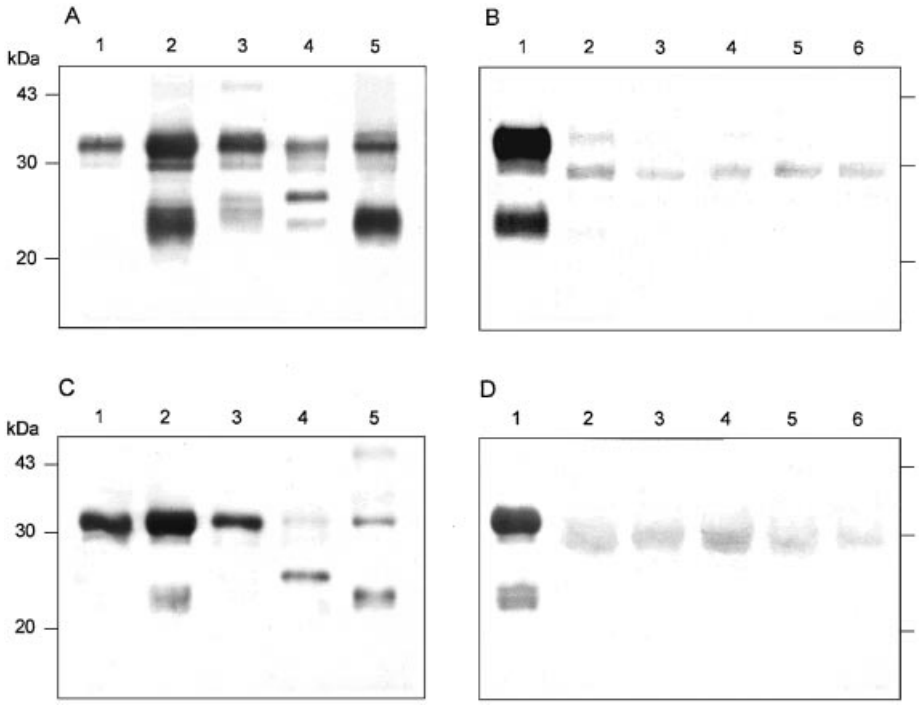

Figure 6. SDS–PAGE of hCG and Western blotting. The blots were visualized using polyclonal (**A**, **B**) and monoclonal (**C**, **D**) hCG primary antibody for hCG controls and decidual specimens. Molecular weight markers (not demonstrated) and hCG control products (lane 1, left, Serono; lane 2, left, and lane 1, right, Sigma; lane 3, left, Biotrend; lane 5, left, own preparation) were run together with lysates of placental (lane 4, left) and several patient specimens obtained from tubal pregnancy decidua (lanes 2–6, right). The bands in **A** and **B** were stained with a polyclonal antibody (A0231, Dako) and in **C** and **D** with monoclonal antibody (INN22, Serotec), followed by biotinylated second antibody, ABC complex and the DAB procedure according to the described method. The decidual β-HCG shows a predominant band of ~30 kDa with a faint 33 kDa band in comparison to the 33 kDa main product of hCG controls.

Table I. Immunohistochemical and clinical data of patients with tubal pregnancy

Patients	Proliferation	Secretory transformation	Stromal differentiation	Immunohistochemical hCG		Haemato-salpinx	Uterine bleeding	Gestation week	Serum hCG (mU/ml)
				Compact layer	Spongy layer				
Group A: 1		High	Predecidual	+++	+++			11.1	989
2		High	Decidual	+++	+++	+	+	6.5	2610
3		High	Decidual	+++	+++	+	+	6.5	1840
4		High	Decidual	+++	++	+		7.3	3430
5		High	Predecidual	+++	++	+		6.1	1170
6		High	Decidual	++	+	+		5.5	1350
7		High	Decidual	++	+			7.1	1890
8		High	Decidual	++	+			8.5	1350
Group B: 1		Diminished	Decidual	+++	++	+		5.6	439
2		Diminished	Decidual	+++	++			7.5	985
3		Diminished	Decidual	++	++		+	9.5	2820
4		Diminished	Predecidual	++	++			7.0	2080
5		Diminished	Decidual	++	++	+	+	6.3	154
6		Diminished	Decidual	++	+			7.3	1620
7		Diminished	Decidual	+	+	+	+	10.5	203
8		Diminished	Predecidual	+	+		+	5.6	275
Group C: 1	Inferior	Early		+	+		+	7.4	107
2	Inferior	Diminished	Decidual	+	+		+	6.1	482
3	Inferior	Diminished	Decidual	+	–	+	+	5.1	320
4	Inferior	Early		–	+		+	7.1	138
5	Inferior	High		+	–	+	+	5.1	117
6	Inferior	Early		–	–		+	6.3	514
7	Inferior	Without		–	–			5.1	199
8	Inferior	Without		–	–		+	5.1	915

may play an important role in implantation and early pregnancy. The human decidua forms other proteins and cytokines, which supply prerequisites for adequate morphological and functional transformation during implantation and early pregnancy, and for innate immunodefence or embryonic allogene immunomodulation (Krasnow *et al*., 1996; Rier and Yeaman, 1997; Hammer *et al*., 1999; King *et al*., 1999). It was also demonstrated, using the in-vivo uterine flushing method, that patients with an adequate luteal phase

endometrium demonstrate secretion of hCG into the uterine cavity (Alexander *et al*., 1998; Licht *et al*., 1998). It seems that during pregnancy the endometrium continues to secrete hCG. The local function of hCG in this tissue is not yet clear, but it can be assumed that hCG stimulates specific hCG/LH receptors in epithelial, stromal and endothelial cells of endometrium and decidua (Eta *et al*., 1994). Therefore, hCG could locally prepare the endometrium for implantation and prime the decidua for an ongoing pregnancy via paracrine action. Local hCG effects on the induction or inhibition of uterine cytokine formation were described by several authors. hCG is known to amplify the interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα) secretion in the endometrial cells (Schäfer *et al*., 1992; Uzumcu *et al*., 1998). In-vivo administration of small quantities of hCG into the uterine cavity raises vascular endothelial growth factor (VEGF) and insulin-like growth factor binding protein 1 (IGF-BP1) and reduces the macrophage colony-stimulating factor (M-CSF) secretion of tissue into the uterine perfusate (Licht *et al*., 1998). Further, hCG-stimulated prostaglandin E_2 (PGE_2) induces a switch from the pregnancy-threatening endometrial TH1 cytokine environment characterized by IL-12 to an IL-10-dominated and pregnancy-promoting TH2 cytokine profile (Kraan *et al*., 1995; Kelly *et al*., 1997). Interestingly, hCG may be characterized as a chemo-attractant for peripheral mononuclear cells (Reinisch *et al*., 1994). Moreover, hCG directly restrains activation of local sessile macrophages capable of expressing inflammatory cytokines (Song *et al*., 2000). Additionally, hCG shows potent vasodilatory characteristics in the vascular endothelium and vascular smooth muscle of the uterine spiral arteries (Hermsteiner *et al*., 1999). hCG also supports maintenance of uterine quiescence caused by suppression of oxytocin-induced myometrial contraction (Kurtzman *et al*., 1999). Thus, hCG seems to be involved in important immunomodulating cytokine effects on decidual differentiation.

In addition to hCG's cytokine-inducing function, it was previously reported that the hCG-induced PGE_2 release by the up-regulation of cyclooxygenase-2 (COX-2) enzyme gene expression in stromal fibroblast cells promotes morphological and functional differentiation into decidual cells (Han *et al*., 1996). In contrast to humans, non-human primate endometrium stromal decidualization begins only after implantation and embryonic choriogonadotrophin secretion. However, it has been recently shown that the transformation of baboon endometrial stromal cells to epitheloid-like decidual cells can also take place *in vivo* if hCG is infused into the normal cyclic uterus (Fazleabas *et al*., 1999; Han *et al*., 1999). The physiological regulation of decidualization includes similar or comparable metabolic pathways. Both human and non-human primate endometrial transformation are connected with the disruption of the actin skeleton and the formation of decidual α-smooth muscle actin, directly induced through hCG and terminated by rising decidual IGF-BP1 levels (Kim *et al*., 1998, 1999). hCG could be regarded as a growth hormone-like protein when compared with other cysteine-knot hormones (Lapthorn *et al*., 1994).

Furthermore, it was reported that hCG causes the up-regulation of glandular epithelial glycodelin secretion, rising in early pregnancy (Hausermann *et al*., 1998). Therefore, we assume that the human spontaneous predecidualization of secretory endometrium could be supported by local hCG. The ectopic implantation of the conceptus in the tubal pregnancies studied led us to speculate that a certain local paracrine hCG effect could also exist in the decidual mucosa.

In conclusion, to our knowledge these results demonstrate for the first time decidual β-hCG and α-CG mRNA expression and hCG hormone production during extrauterine pregnancy. hCG expression appears to be associated with the degree of endometrial proliferation, the extent of epithelial secretory transformation and stromal decidualization that took place before conception.

The local glandular epithelial production of hCG in the uterine tissue lacking trophoblast cells allows for speculation on a possible paracrine contribution to the mechanism of implantation and stromal cell decidualization, however further studies are necessary to elucidate decidual hCG's biological function.

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