Localization of type 1 17β-hydroxysteroid dehydrogenase mRNA and protein in syncytiotrophoblasts and invasive cytotrophoblasts in the human term villi

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Abstract

The 17β-hydroxysteroid dehydrogenases (17β-HSDs) play a key role in the synthesis of sex steroids. The hallmark of this family of enzymes is the interconversion, through their oxydoreductive reactivity at position C17, of 17-keto- and 17β-hydroxy-steroids. Because this reaction essentially transforms steroids having low binding activity for the steroid receptor to their more potent 17β-hydroxysteroids isoforms, it is crucial to the control of the physiological activities of both estrogens and androgens. The human placenta produces large amounts of progesterone and estrogens throughout pregnancy. The placental type 1 17β-HSD enzyme (E17β-HSD) catalyzes the reduction of the low activity estrogen, estrone, into the potent estrogen, estradiol. We studied the cell-specific expression of type 1 17β-HSD in human term placental villous tissue by combining *in situ* hybridization to localize type 1 17β-HSD mRNA with immunohistochemistry using an antibody against human placental lactogen, a trophoblast marker. Immunolocalization of E17β-HSD was also performed. To ascertain whether other steroidogenic enzymes are present in the same cell type, cytochrome P450 cholesterol side-chain cleavage (P450scc), P450 aromatase, and type 1 3β-hydroxysteroid dehydrogenase (3β-HSD) were also localized by immunostaining. Our results showed that the syncytium is the major steroidogenic unit of the fetal term villi. In fact, type 1 17β-HSD mRNA and protein, as well as P450scc, P450 aromatase, and 3β-HSD immunoreactivities were found in these cells. In addition, our results revealed undoubtedly that extravillous cytotrophoblasts (CTBs), e.g. those from which cell columns of anchoring villous originate, also express the type 1 17β-HSD gene. However, CTBs lying beneath the syncytial layer, e.g. those from which syncytiotrophoblasts develop, contained barely detectable amounts of type 1 17β-HSD mRNA as determined by *in situ* hybridization. These findings, along with those from other laboratories confirm the primordial role of the syncytium in the synthesis of steroids during pregnancy. In addition, our results indicate for the first time that CTBs differentiating along the invasive pathway contain type 1 17β-HSD mRNA.

Journal of Endocrinology (2000) **165,** 217–222

Introduction

During normal pregnancy, the human placenta actively synthesizes high amounts of progesterone and estrogens *in situ*. It plays important roles in the control of essential maternal endocrine functions (Simpson & McDonald 1981, Ringler & Strauss 1990), and contributes to the normal differentiation and maturation of many fetal organ systems (Pepe & Albrecht 1995). Progesterone synthesis depends on maternal lipoprotein-cholesterol delivery, cytochrome P450 cholesterol side-chain cleavage (P450scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD) (Albrecht & Pepe 1990). The synthesis of estradiol from C19 steroid precursors of maternal and fetal origins depends upon different enzymatic systems that involve 3β-HSD, P450 aromatase, and 17β-hydroxysteroid dehydrogenase (17β-HSD). However, the physiological activity of estrogens is exclusively dependent upon reductive or oxidative reactions at position C17. These reactions are catalyzed by the 17β-HSDs. To date, eight different isoforms of 17β-HSD have been cloned and chronologically numbered as types 1–8 (for a review see Peltoketo *et al.* 1999). These enzymes interconvert steroids with low steroid receptor binding activity, namely dehydroepiandrosterone, estrone, androstenedione, and androstanedione, into their more active steroid derivatives, androstenediol, estradiol, testosterone, and dihydrotestosterone respectively. In humans, type 1 17β-HSD is exclusively estrogenic (Tremblay *et al.* 1989). Traditionally, it has been named estradiol-17β-HSD (E17β-HSD)

0022–0795/00/0165–217 *Printed in Great Britain*

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(Jarabak 1969). We showed by Northern blot analysis that cytotrophoblasts (CTBs) freshly isolated from human term villi expressed moderate amounts of type 1 17β-HSD mRNA that increased with syncytialization *in vitro* (Beaudoin *et al.* 1995). In addition to type 1 17β-HSD, the synthesis of estradiol (E2) also requires 3β-HSD and cytochrome P450 aromatase. E17β-HSD was the first 17β-HSD to be purified (Jarabak 1969) and cloned (Luu-The *et al.* 1989*a*) and its gene expression and regulation in the placenta became the focus of a large number of studies.

To explore the hypothesis that human villous trophoblasts represent an important site of E2 formation, immunohistochemical and *in situ* hybridization studies and *in vitro* studies with isolated CTBs were performed. In a study with placental tissues from the first trimester of pregnancy, Fournet-Dulguerov *et al.* (1987) observed immunoreactive E17β-HSD only in the syncytiotrophoblasts. In that study, they reported an absence of immunoreactivity in CTBs. Immunoreactivity for E17β-HSD was also reported exclusively in syncytiotrophoblasts very early in pregnancy (in the placenta of the 7th and 9th weeks of gestation) and after the 25th week of gestation, but CTBs were also found to be positive in the placenta of the 10th and 14th weeks of gestation (Dupont *et al.* 1991). It was reported in another study performed with tissues obtained at the 8th and the 20th weeks of human gestation, that E17β-HSD immunoreactivity was found exclusively in syncytiotrophoblasts (Takeyama *et al.* 1998). In term placental tissue, type 1 17β-HSD mRNA was detected in the syncytiotrophoblasts (Mustonen *et al.* 1998). In addition, we have reported that nearly pure cultures of CTBs isolated from normal term villi contained type 1 17β-HSD mRNA detectable by Northern blots at the stage of mononuclear cells in culture. As expected, levels of type 1 17β-HSD mRNA increased in parallel with *in vitro*-formed syncytium. Accordingly, each type of culture, CTBs and syncytiotrophoblasts, showed E17β-HSD activity, but the reductase activity with estrone (E1) (synthesis of E2) was 10-fold higher than the corresponding dehydrogenase activity with E2 (synthesis of E1). Hence, a net production of E2 was favored in these two types of cultured cells (Beaudoin *et al.* 1995). Although observations suggest that the syncytium is the principal steroidogenic unit of the human placenta, the presence of the type 1 17β-HSD mRNA in CTBs remains to be confirmed.

To determine which cell types express the type 1 17β-HSD gene *in vivo* and to gain better knowledge of the role of that enzyme, we used *in situ* hybridization and immunohistochemistry in tandem to reveal the presence of type 1 17β-HSD mRNA and protein in human term villi. To compare the cellular distribution of the type 1 17β-HSD with the other enzymes involved in placental steroidogenesis, the immunohistochemical localization of P450scc, 3β-HSD-1, and P450 aromatase was also examined in the same series of experiments.

Materials and Methods

Antisera and nucleotides

Rabbit antiserum to human placental lactogen (hPL) was obtained from BioGenex (San Ramon, CA, USA). The other antibodies were also raised in rabbits against either a synthetic peptide for P450 aromatase (Fournet-Dulguerov *et al.* 1987) or purified proteins for P450scc (Black *et al.* 1993), 3β-HSD-1 (Luu-The *et al.* 1989*b*), and E17β-HSD (Luu-The *et al.* 1989*a*). Each antiserum was previously described and used in immunohistochemical or Western blots and no cross-reactivity with other known steroid-converting enzymes has been reported. Each antiserum was used at a concentration of 1:5000, except for aromatase antiserum that was diluted 1:1000. Radiolabeled ^{35}S-UTP (1250 Ci/mmol) was obtained from New England Nuclear (Boston, MA, USA).

Human tissue preparation

Four placentas taken after normal spontaneous vaginal delivery and one taken after uncomplicated elective cesarean section were used for this study and were obtained with informed consent from patients after approbation by the ethical committee from our institution. These full-term placental tissues (38 to 42 weeks) with attached fetal membranes, decidua, and umbilical cords were collected on ice and brought to the laboratory within 1 h after delivery. Soft villous tissue free of membranes and decidua was mechanically dissected, cut into 0·5 cm^3 pieces, rinsed in 0·9% saline until the tissue turned pink, and embedded with the cryoprotector, Tissue-Tek OCT compound (Miles, Elkhart, IN, USA). The cups were quickly frozen on dry ice, wrapped in aluminum foil and kept at −70 °C. Best results were obtained with serial sections of 7- to 10-μm thick (Kryostat 1720 model, Ernst Leitz Wetzlar, Germany). The tissue sections were placed on Superfrost Plus glass microslides (Fisher Scientific, Nepean, ON, Canada), and kept at −70 °C until used.

Riboprobes

We used a type 1 17β-HSD EcoRI/SacI cDNA fragment of 964 bp (Tremblay & Beaudoin 1993) subcloned in the expression vector pSV-SPORT-1 (Tremblay *et al.* 1994). The recombinant plasmid was linearized with EcoRI (antisense RNA probe) or with SacI (sense RNA probe; negative control). RNA probes were synthesized from 800 ng DNA template using the Riboprobe Combination System kit (Promega, Madison, WI, USA). Probes with less than $2{\cdot}5 \times 10^7$ d.p.m. were discarded.

Combination of immunohistochemistry (IHC) and in situ *hybridization (ISH)*

Embedded placental samples (two tissue sections per slide) were thawed and washed 3 × 15 min in phosphate buffer

(22 mM K_2HPO_4, 3 mM KH_2PO_4, 140 mM NaCl; KPBS). Human PL antiserum was diluted in KPBS containing 0·4% Triton-X100, and 2·5 mg/ml heparin as a blocking agent (176 USP units/mg, Sigma Chemicals Co., St Louis, MO, USA), applied onto slides (100 µl/slide), and left at 4 °C overnight. All slides were treated as described above, except in the negative control where hPL was omitted. Immunostaining was detected with an avidin-biotin peroxidase method (Hsu *et al.* 1981) using the ABC Vectastain kit (Vector Lab., Burlingame, CA, USA) with diaminobenzidine (60 µg/ml, Sigma) as the chromagen. After IHC, slides were dried for 30 min under vacuum and immediately processed for ISH. Slides were fixed for 20 min in 4% paraformaldehyde (Sigma) and rinsed in KPBS. Proteinase K (Sigma, 10 µg/ml prepared in 100 mM Tris pH 8·0 and 50 mM EDTA) was added for 25 min at 37 °C. Next, sections were acetylated in 37·5 mM triethanolamine (Sigma) and 0·25% (v/v) anhydric acid (Sigma) for 10 min. Slides were dehydrated in graded alcohol and air-dried. Hybridization (5×10^3 d.p.m./µl; 100 µl/slide) was in 50% formamide, 0·3 M NaCl, 10 mM Tris pH 8·0, 1 mM EDTA, 1 × Denhart's solution, 1% dextran sulfate, 10 mM dithiothreitol (DTT) and 500 µg/ml tRNA overnight at 60 °C. Then, slides were incubated at 37 °C for 30 min in 0·5 M NaCl, 10 mM Tris pH 8·0, 1 mM EDTA and 20 µg/ml RNase A (Sigma). Slides were washed at high stringency in 0·1 × SSC (standard saline citrate) and 1 mM DTT at 60 °C for 30 min. After defatting, tissues were coated with NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY, USA), and exposed for 10 days. Slides were developed with D-19 developing solution (Eastman Kodak), fixed, washed under running water for 1 h, counterstained with 0·25% (w/v) thionine for 30 s, dehydrated, and mounted with DPX (Electron Microscopy Sciences, Washington, WA, USA).

Immunohistochemistry

When no ISH was performed, we followed a similar protocol for IHC except that the heparin was replaced by a preincubation of the tissue sections with 4% normal goat serum (NGS) for 30 min. Secondly, NGS was added to each enzyme antiserum at 1% final dilution (v/v). Endogenous peroxidase activity was reduced by preincubation of slide sections with 0·03% H_2O_2 for 10 min.

Results and Discussion

Term villi sections from normal placenta were first subjected to immunohistochemical study for hPL, followed by *in situ* hybridization with type 1 17β-HSD RNA probes. As expected, IHC with anti-hPL IgG produced the dark yellow immunoreactive pattern typical of hPL staining of the syncytial cells (Sciarra *et al.* 1963, Watkins 1978, Hoshina *et al.* 1982, Petraglia *et al.* 1990). From the ISH study, we found that the distribution of type 1 17β-HSD mRNA signals colocalized with hPL-positive cells in term villi (Fig. 1). These experiments clearly identified the syncytiotrophoblasts that compose the syncytial layer at the edge of the villi (arrows) as the major cell type containing type 1 17β-HSD mRNA. No differences in the distribution of the type 1 17β-HSD mRNA were noted between specimens obtained after vaginal delivery (Fig. 1A, B, and C) or cesarean section (Fig. 1D and E). These conclusions were confirmed by the use of a sense riboprobe as a negative control (Fig. 1F). Immunostaining with antibodies against P450scc (Fig. 2A), type 1 3β-HSD (Fig. 2B), and P450 aromatase (Fig. 2C) also produces positive signals specific to the syncytiotrophoblasts. In addition, cells that were immunoreactive for type 1 17β-HSD antiserum (Fig. 2D) were also localized at the edge of the villi and corresponded exactly to the positive cells identified by ISH for the presence of type 1 17β-HSD mRNA and to the cells detected by IHC using antisera against the other steroidogenic enzymes.

Table 1 Number of silver grains per cell

	Silver grains/cell		Total number of cells
	≤ 3*	4 ≤	
Cell type			
Villous cytotrophoblast	41	6	47
Extravillous Cytotrophoblast	25	25	50
Syncytiotrophoblast	0	84	84
Villous tissue	84	0	84

*Equivalent surface area to cytotrophoblasts was used to calculate the background. After counting 84 different areas in the villous core tissue, the background level was fixed to ≤ 3 silver grains per cell.

Because freshly isolated CTBs express detectable type 1 17β-HSD mRNA (Beaudoin *et al.* 1995), we looked at whether similar expression occurred in placental tissue sections. Cells were identified as CTBs based on their localization in the tissue. Cells that were directly underneath the syncytial layer were counted as villous CTBs (e.g. CTBs that remain in the fetal compartment and fuse, and from which multinucleate syncytiotrophoblasts develop). As reported, occasional CTBs can react positively for hPL, but only weakly. Although the reaction may be the result of artifact from adjacent syncytiotrophoblasts, one cannot exclude the possibility that hPL may also be produced by CTBs (Nadji & Morales 1986). We counted 47 villous CTBs including occasional CTBs that stained positive for hPL. From these, only six contained detectable amounts of type 1 17β-HSD mRNA (Table 1). In contrast, we identified a group of about 50 extravillous CTBs grouped into a cell column. Early in gestation, these CTBs invade the uterine wall (interstitial migration) and its blood vessels (endovascular invasion). Anchoring villi

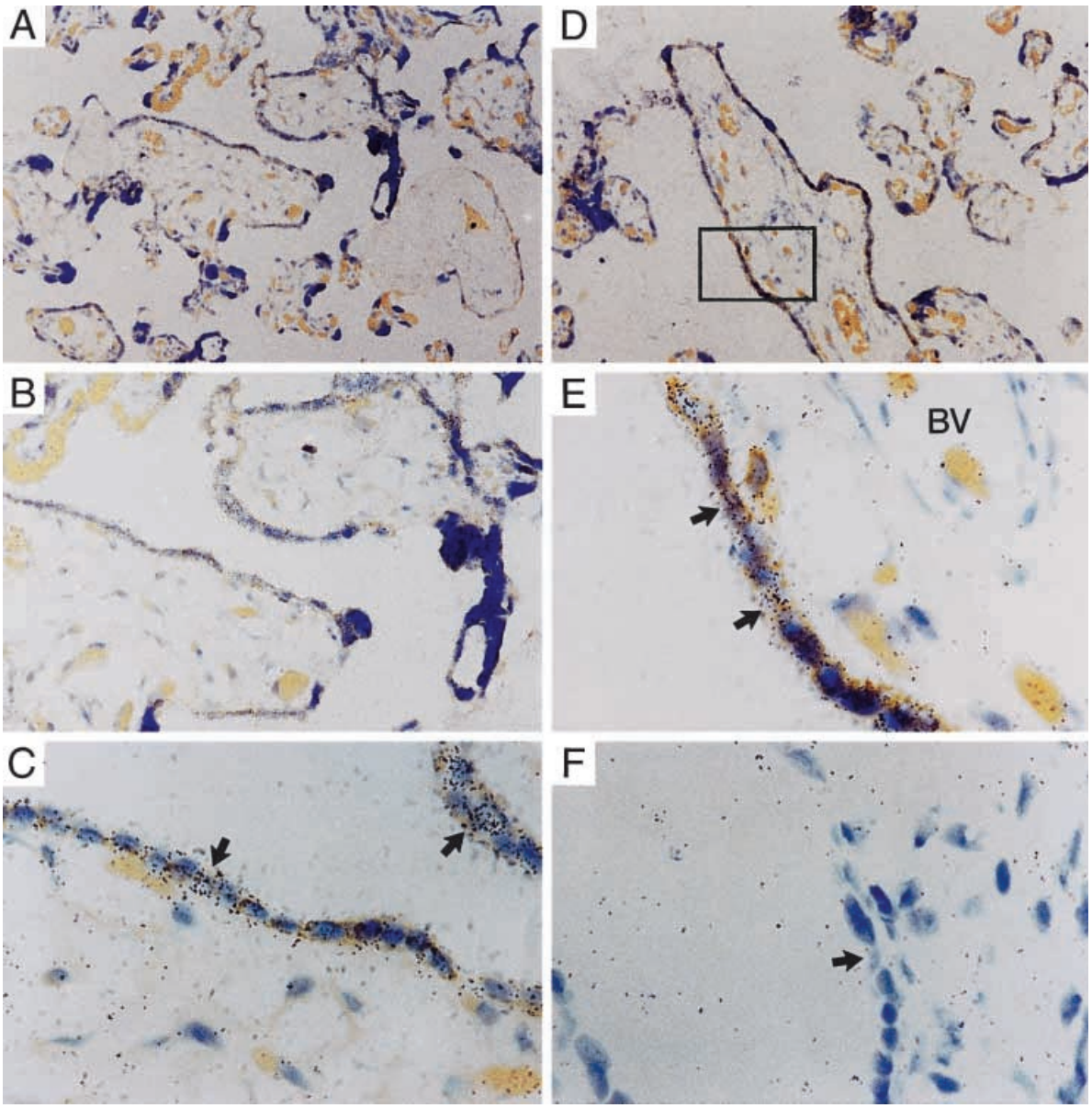

Figure 1 Detection of type 1 17β-HSD in human placenta visualized by brightfield microscope images. Human term villi sections were hybridized *in situ* with an antisense (A to E) or a sense (F) ^{35}S-labeled 17β-HSD-1 RNA probe. Experiments performed with tissues obtained from normal vaginal delivery correspond to A, B, and C (one specimen is shown) and those from cesarean section are shown in D, E, and F. Tissue sections (except for panel F) were first stained with an antiserum to hPL (yellow color). Weak background staining with the antiserum was caused by endogenous peroxidase. Tissue sections were counterstained with thionine (blue color). Human PL staining was concentrated along the edge of the villi where the syncytium lies (clearly visible on panels C and E). Silver grains corresponding to type 1 17β-HSD mRNA colocalized with hPL positive cells, e.g. in syncytiotrophoblasts (indicated by arrows on panels C and E). Arrows on panels C, E, and F point to the syncytial layer. BV, blood vessel. Original magnification: panels A and D, × 50 (the square on panel D represents the area that was magnified in E); panel B, × 100; panels C, E, and F, × 250.

thus attach the fetus to the uterus and establish the flow of oxygenated maternal blood to the intervillous space. Previous studies indicated that cell columns can be divided into different segments according to the phenotype of CTBs. Indeed, depending on the localization of CTBs in the column, they will express distinct patterns of adhesion receptor (Damsky *et al.* 1992, Zhou *et al.* 1997). A positive signal from the 17β-HSD antisense probe was

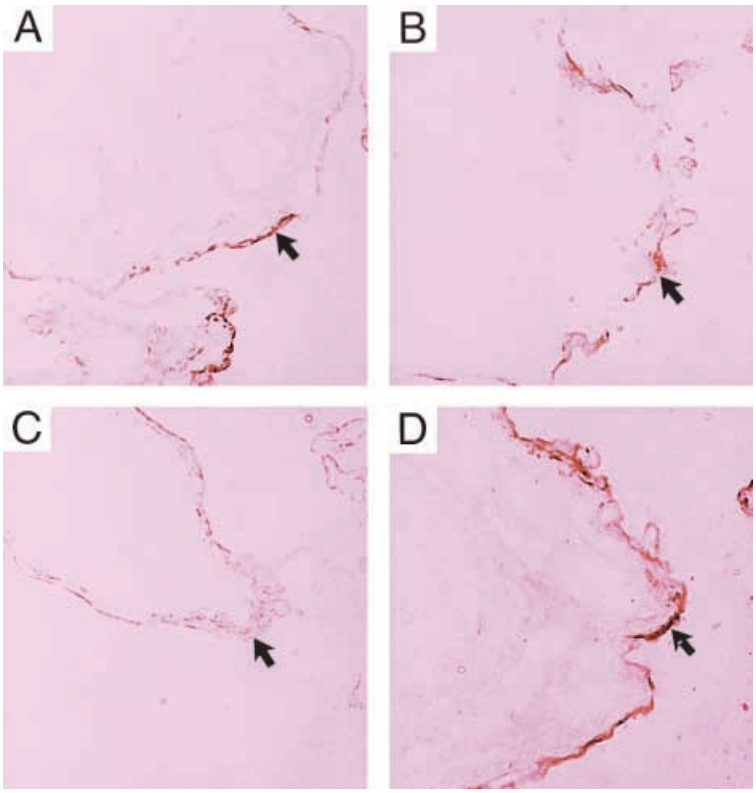

Figure 2 Immunoreactivity of cytochrome P450 scc (A), type 1 3β-HSD (B), cytochrome P450 aromatase (C), and E17β-HSD (D) enzymes in human term villi is presented. Immunostaining is exclusively associated with the syncytial layer of the villi. Arrows point to syncytiotrophoblasts. Magnification, × 100.

unambiguously observed in 50% of these CTBs (Table 1). In comparison, all syncytiotrophoblasts observed on the same tissue section produced detectable signals with this probe. No other types of cell were positive for the type 1 17β-HSD mRNA in the villous core.

Our results confirm that the expression of type 1 17β-HSD gene *in vivo* is a specific feature of trophoblastic cells, particularly of syncytiotrophoblasts. We have also ascertained that placental steroidogenic enzymes involved in estradiol and progesterone synthesis are all coexpressed in syncytiotrophoblasts *in vivo*. We also present for the first time evidence that extravillous CTBs differentiating along the invasive pathway express the type 1 17β-HSD gene.

Acknowledgements

The authors thank Dr Serge Rivest and Dr Georges Pelletier for helpful discussions, Dr Evan R Simpson for P450 aromatase antiserum, Dr Walter L Miller for P450scc antiserum, and Dr Van Luu-The for 3β-HSD-1 and E17β-HSD antisera. This work was supported by grant MT-14365 from the Medical Research Council of Canada. Y T received a scholarship senior award from the Fonds de la Recherche en Santé du Québec.

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Received 1 December 1999
Accepted 30 December 1999