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ABSTRACT

Pregnancy-associated plasma protein-A (PAPP-A) is a metzincin metalloproteinase that cleaves the insulin-like growth factor (IGF)-dependent binding protein-4 and increases in maternal serum during pregnancy. In human placenta PAPP-A is expressed both in villous cytotrophoblasts (VCT) that cover the chorionic villi and in extravillous cytotrophoblasts (EVCT) of the anchoring villi. Due to the key role of PPARγ in human trophoblast differentiation such as syncytiotrophoblast formation and EVCT invasion, we studied the effect of PPARγ activation on PAPP-A expression using our *in vitro* model of EVCT and VCT primary cultures isolated from the same first trimester chorionic villi. First, we demonstrated that invasive EVCT expressed and secreted 10 times more PAPP-A than VCT did. Then, we showed that activation of PPARγ inhibited PAPP-A gene expression and secretion in EVCT, whereas it had no effect in VCT. Since we have previously shown that PPARγ agonist inhibits EVCT invasion *in vitro*, we suggest that PPARγ-mediated inhibition of PAPP-A might decrease the amount of bioactive IGFII, a factor known to promote trophoblast invasion.

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INTRODUCTION

The human placenta is characterized by extensive invasion of cytotrophoblasts into the uterus wall allowing direct contact of cytotrophoblasts with the maternal blood (hemomonochorial placentation) and by the extent and specificity of its hormonal production [1]. After the initial phase of nidation, human cytotrophoblasts differentiate along either the villous cytotrophoblast pathway leading to the formation of the syncytiotrophoblast that secretes the majority of placental hormones, or the extravillous cytotrophoblast pathway implicated in the anchoring of chorionic villi in the uterus (Fig. 1A)[2]. The specificity of human placenta has led to the development of *in vitro* models, such as explants [3-6] or primary cell cultures [7-9] allowing the study of human cytotrophoblast differentiation. We recently described a sequential enzymatic method permitting isolation, from the same first trimester chorionic villi, of extravillous and villous cytotrophoblasts that differentiate *in vitro* into invasive cytotrophoblasts or multinucleated syncytiotrophoblast by cell-cell fusion, respectively (10).

Pregnancy-associated plasma protein-A (PAPP-A) was first isolated in the 1970s from the serum of pregnant women [11, 12]. PAPP-A levels increase in the maternal circulation until the end of pregnancy [13, 14] and decline after delivery, suggesting a placental production [14, 15]. PAPP-A is secreted as a dimer of 400 kDa but circulates in maternal blood as a disulfide bound 500 kDa 2:2 complex with the proform of eosinophil major basic protein (pro-MBP). Recently, PAPP-A has been identified in different cell systems and in human pregnancy serum as a metzincin superfamily metalloproteinase, which cleaves the insulin-like growth factor (IGF)-dependent binding protein-4 [16-19]. PAPP-A is expressed in the placenta [20, 21] and more specifically in the villous and extravillous cytotrophoblasts [22, 23, 24, 25] and in the decidua [26]. *In vitro*, PAPP-A is secreted by human endometrial

cells, decidual cells and trophoblastic cells [25]. Its production is regulated by progesterone [27, 28].

Recent studies have pointed to a key regulatory role for the peroxisome proliferator-activated receptor-γ (PPARγ) in human trophoblast differentiation and function [30-32]. PPARγ is a member of the nuclear receptor superfamily that controls the expression of a large array of genes in a ligand-dependent manner. DNA binding of PPARγ to its response element requires obligate heterodimerization with another nuclear receptor, the retinoid X receptor (RXR). We have shown *in situ* and *in vitro* that human villous and extravillous trophoblasts express both PPARγ and RXRα [30, 31]. We next demonstrated that activation of PPARγ by ligands from the thiazolidinedione family inhibits invasion of primary extravillous cytotrophoblasts isolated from first trimester placentas [30] and induces differentiation of villous cytotrophoblasts from term placentas and placental hormone gene expression as hCG, hPL and leptin [31].

Due to the key role of PPARγ in trophoblast differentiation, the aim of this study was to investigate the role of PPARγ in the regulation of PAPP-A gene expression and protein secretion in both villous and extravillous trophoblastic cells isolated from the same first trimester chorionic villi.

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MATERIALS AND METHODS

Isolation and purification of villous (VCT) and extravillous (EVCT) cytotrophoblasts.

Placental tissues were obtained from the Department of Obstetrics and Gynecology at Broussais Hospital (Paris, France) during the first trimester (7-12 weeks of gestation) following legal voluntary interruption of pregnancy in patients who had signed informed consent forms. The tissue was washed in Ca^{2+}-, Mg^{2+}-free HBSS supplemented with 100 UI/mL penicillin and 100 µg/mL streptomycin. Chorionic villi were dissected and rinsed for cell isolation. EVCT and VCT were isolated from the same first trimester chorionic villi by differential trypsin digestion according to Tarrade et al [10] with slight modifications.

For EVCT isolation, the tissue was incubated in HBSS (5 mL/g) containing 0.125% trypsin (Difco Laboratories, Detroit, Michigan), 4.2 mM $MgSO_4$, 25 mM Hepes, 50 U/mL DNAse type IV (Sigma, Saint-Quentin Fallavier, France) for 35 min at 37°C without agitation. After tissue sedimentation, the supernatant containing isolated EVCT was filtered (100 µm pores) and stored in the presence of 10% (v/v) fetal calf serum (FCS) to inhibit trypsin activity. The tissue was washed five to six times with warm HBSS and supernatant fractions pooled. Cells were centrifuged at 300 X g for 10 min, washed twice in HBSS supplemented with 10% FCS and filtered through a 50 µm pore nylon membrane. The cell suspension was carefully layered over a discontinuous Percoll gradient and centrifuged for 25 min at 1000 X g. The layer corresponding to 40-45% Percoll containing cytotrophoblasts was washed twice in HBSS supplemented with 10% FCS. Cells were diluted to a concentration of 2.5 x 10^5 cells/mL in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 UI/mL penicillin and 100 µg/mL streptomycin and plated on Matrigel-coated (5 mg/mL; Collaborative Biomedical Products, Le Pont de Claix, France) 35-mm Falcon culture dishes.

After 2 h of culture in 5% CO_2 at 37°C, cells were carefully washed three times to eliminate non-adherent cells. These purified primary EVCT were characterized using immunocytochemistry and real-time PCR and were shown to express *in vitro* the specific markers of human invasive EVCT *in situ* as described previously *i.e*. cytokeratin 7, human leukocyte antigen G , human placental lactogen, *c-erb*B2 and α5β1 [10].

VCT isolation was based on the methods of Kliman et al [33] and is a modified version of Tarrade et al [10]. After EVCT isolation, the same chorionic villi were incubated for 30 min at 37°C without agitation in 5 mL/g of tissue of the digestion buffer described above but containing only 0.0625% trypsin. The first 30 min trypsin digestion, containing a mix of EVCT and VCT, was discarded. The chorionic villi were then incubated in the same trypsin solution for 30 min and twice for 15 min at 37°C without agitation and finally washed with warm HBSS. Each time, the supernatant containing VCT was collected after tissue sedimentation, filtered (100 μm pores), and incubated on ice with 10% FCS (v/v) to stop trypsin activity. After Percoll gradient fractionation, cells were diluted to a concentration of 1.25 x 10^6 cells/mL in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 UI/mL penicillin and 100 μg/mL streptomycin and plated on 35-mm or 60-mm TPP (Techno Plastic Products) culture dishes. VCT were incubated overnight in 5% CO_2 at 37°C and washed three times to eliminate non-adherent cells. Purified VCT cultures were characterized by the observation of cell aggregates and syncytiotrophoblast at 48 h and 72 h, respectively.

Cells were treated with 1 μM of a PPARγ agonist, rosiglitazone (BRL49653, Cayman Chemical, Ann Arbor, MI).

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Immunocytochemistry

Cytokeratin 7 immunodetection

Cultured VCT and EVCT were fixed for 20 min in 4% paraformaldehyde. Saturation of unspecific sites was performed with 7% dunkey serum diluted in PBS for 1 h at room temperature. Primary antibody (monoclonal mouse anti-CK7, 1/200, DAKO, France) diluted in PBS containing 1% BSA was added for 2 h at room temperature and amplified with the secondary antibody (FITC-conjugated donkey anti-mouse IgG1, 1/300, Jackson Immunoresearch Labotories, West Groove, PA) diluted in PBS for 1 hour in dark at room temperature. Cells were mounted in a fluorescent Dapi mounting medium, examined and photographed on an Olympus BX60 epifluorescence microscope.

PAPP-A immunodetection

Cultured VCT were fixed for 8 min in methanol at –20°C. Saturation of unspecific sites was performed as described above. Primary antibody (APS13: monoclonal mouse anti-PAPP-A, 1/100, Cisbio international France) diluted in PBS containing 1% BSA was added for 2 h at room temperature and amplified as described above. Negative control was obtained by substituting the primary antibody by a mouse isotypic IgG1 (Jackson Immunoresearch Labotories, West Groove, PA). Cells were mounted and examined as described above.

For cultured EVCT, human IgG (1:200, Jackson Immunoresearch Labotories, West Groove, PA) were added to the donkey serum solution and saturation was performed overnight at 4°C. Primary and secondary antibodies were diluted in this saturation solution and immunostaining was performed as described above.

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DNA quantification

Cell pellets were lysed in 5 M GuSCN, 0.1 M EDTA, pH 7 and stored at –80°C until use. DNA was quantified by fluorometry using the fluorochrome Hœchst 33258 as described by Labarca and Paigen [34].

Quantification of specific transcripts by real-time RT-PCR

Total RNA was extracted from 48 h-cultured primary EVCT and 24, 48 and 72 h-cultured VCT, using TRIzol®Reagent. cDNA synthesis and PCR amplification were performed as described previously [35]. PAPP-A transcripts were amplified using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) and the Syber Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems). The PPIA gene coding the peptidyl prolyl isomerase A was used as the endogenous RNA control and each sample was normalized on the basis of its PPIA content. (PAPP-A primers: (+) CAGAATGCACTGTTACCTGGA, (-) GCTGATCCCAATTCTCTTTCA; PPIA primers: (+) GTCAACCCCACCGTGTTCTT, (-) CTGCTGTCTTTGGGACCTTGT)

PAPP-A assay

Each 24 hours, culture media were removed and stored, and fresh medium was added. PAPP-A concentrations in culture media of 24h-, 48h- and 72h-cultured VCT and EVCT were determined using TRACE (time-resolved amplified cryptate emission) technology on the KRYPTOR automated immunofluorescent analyzer (Brahms, Berlin, Germany) as described elsewhere [25]. The PAPP-A assay was performed with mouse monoclonal antibodies (APS 20 and APS 30) against the circulating complex PAPP-A/ProMBP [36]. This assay was standardized against World Health Organization (WHO) IRP 78/610 reference

material. The detection limit is 4 mU/L, and the within- and between-run coefficients of variation are 0.6% and 1.4%, respectively.

Statistical analysis

Values represent the mean ± SEM of 3 to 7 separate cultures obtained from different placentas. The Mann and Whitney U test was used for comparison between groups and results were considered significant for $p < 0.05$.

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RESULTS

Immunodetection of PAPP-A in villous and extravillous cytotrophoblast *in vitro*.

We investigated the *in vitro* expression of PAPP-A in culture of villous and extravillous cytotrophoblasts isolated from first trimester chorionic villi. Figure 2A shows immunodetection of PAPP-A in cultured-VCT. Mononucleated VCT isolated from early placentas adhere to plastic dish, aggregate and fuse *in vitro* within 72h to form a syncytiotrophoblast (Fig. 1B, upper panel), as described previously [33]. We detected PAPP-A *in vitro* in the cytoplasm of isolated, aggregated villous cytotrophoblasts (data not shown) and syncytiotrophoblast (Fig. 2A, left panel). Extravillous cytotrophoblasts need extracellular matrix-coated dishes for adhesion and culture. They display an invasive phenotype and emit pseudopodia after 48 h of culture on Matrigel (Fig. 1B, lower panel). They express specific markers and are able to invade Matrigel in Boyden chambers [10, 30]. PAPP-A was also detected in the cytoplasm of these cells (Fig. 2B, left panel).

Production of PAPP-A in human cytotrophoblasts *in vitro*

We next analysed the expression and the secretion of PAPP-A in VCT and EVCT cytotrophoblasts. As shown in figure 2A, PAPP-A levels in VCT culture medium increased significantly during the 72 h-culture period ($P<0.05$ at 48 h vs 24 h and $P<0.01$ at 72 h vs 24 h), even though the increase in mRNA levels was not significant. PAPP-A transcripts were largely expressed in 48 h-cultured EVCT while PAPP-A secretion increased in the same time as the cells acquire their invasive phenotype, with a 12-fold increase between 24 and 72 hours of culture ($P<0.01$, Fig. 2B).

We then compared PAPP-A expression in VCT and EVCT. PAPP-A mRNA content was significantly higher in EVCT than VCT: 11 fold increase at 48 h (Fig. 2B, middle panel; $P<0.01$), and 13 and 5 fold increase at 24 h ($P<0.01$) and 72 h ($P<0.05$) respectively (data not shown). Concomitantly, PAPP-A levels in cell culture medium were higher in EVCT than in VCT (Fig.2B, right panel). At 48 h and 72 h of culture EVCT secreted 8 ($P<0.001$) and 9 ($P<0.01$) fold more PAPP-A than aggregated VCT and syncytiotrophoblast, respectively.

PPARγ modulates PAPP-A expression and secretion in human cytotrophoblasts *in vitro*.

We previously showed that PPARγ regulates VCT and EVCT functions, i.e. endocrine function and cell invasion, respectively. In the present work, we investigated the role of PPARγ in the regulation of PAPP-A gene expression. VCT primary cells were incubated for 24 h, 48 h and 72h with 1 μM of the PPARγ activator rosiglitazone and EVCT only for 48 h, and PAPP-A transcript levels and protein secretions were analyzed (Fig. 3). In VCT, neither PAPP-A transcript levels nor PAPP-A secretion were significantly affected by PPARγ agonist treatment irrespective of the time of culture (Fig. 3A). By contrast, after 48 h of culture, PAPP-A transcripts were significantly decreased by 70% in EVCT after PPARγ activation (Figure 3B, left panel; $P<0.01$). Concomitantly, the PPARγ agonist significantly decreased PAPP-A secretion by 35% in EVCT culture supernatant (Figure 3A, right panel; $P<0.05$).

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DISCUSSION

In this study, using specific tools and *in vitro* models, we showed that PAPP-A was expressed and secreted by well-characterized trophoblastic cells populations [10, 37, 38] i.e. both villous and extravillous cytotrophoblasts isolated from first trimester human chorionic villi. Indeed, human placenta, but not mouse placenta [39], contains high levels of PAPP-A mRNA [21] as compared with other tissues. However, the precise sources of PAPP-A during pregnancy are controversial. Published data vary according to the sensitivity of the assay (e.g. *in situ* hybridization versus RT-PCR). The specificity of the antibodies used is also of importance because the antibodies may recognize either PAPP-A or ProMBP and, in some cases, other ligands of ProMBP such as C3 and haptoglobin [40, 21]. Monoclonal antibodies targeting either PAPP-A or proMBP have recently been developed [36, 41], but little *in vitro* data have been published to date. Using these specific tools, we recently showed that PAPP-A expression increased with *in vitro* differentiation of term villous cytotrophoblasts [25]. In this study, using the same tools, we showed that expression and secretion of PAPP-A were about ten times higher in extravillous cytotrophoblasts than in villous cytotrophoblasts isolated from the same first trimester placentas.

Considering the different functions of villous and extravillous cytotrophoblasts during pregnancy, our results suggest that placental PAPP-A from villous or extravillous origin might play different roles. Indeed, PAPP-A physiological functions are still under investigation. PAPP-A is a protease able to cleave the insulin-like growth factor (IGF)-dependent binding protein-4. PAPP-A cleavage of IGFBP-4 occurs in the presence of IGFs whereas the cleavage of IGFBP-5 is independent of IGFs [42]. This PAPP-A enzymatic activity is inhibited by proMBP [18]. Produced by the syncytiotrophoblast, PAPP-A is therefore secreted directly into the maternal blood due to the position (bathing in maternal

blood after 12 weeks of pregnancy) and the major endocrine functions of the syncytiotrophoblast. Thus, PAPP-A might be involved in the decrease of IGFBP-4 levels in maternal serum during human pregnancy [43, 44]. The high levels of PAPP-A secretion and expression by extravillous cytotrophoblasts suggest a possible important role for this protease at the implantation site by controlling the trophoblast invasion process through a paracrine or autocrine pathway. Indeed as suggested, PAPP-A might be an important regulator of local IGF bioavailability and cell growth [29]. IGFBP-4, highly expressed in placental bed [45], is a modulator of IGF activity [46] as proteolysis of IGFBP-4 enhances IGF bioactivity [19]. IGFII mRNA were reported to be expressed by the extravillous trophoblasts *in situ* [45] and was shown to stimulate trophoblast invasion *in vitro* [47].

This study pointed out for the first time the role of PPARγ in the modulation of PAPP-A expression. Modulation of the PAPP-A gene expression is not well characterized. PAPP-A expression is stimulated in ovaries by hCG [48] and its placental production is regulated by progesterone [27-29]. In human cytotrophoblasts, we observed a different effect of PPARγ stimulation depending upon villous or extravillous origin. In villous trophoblastic cells, PPARγ stimulation had no effect on PAPP-A mRNA levels and protein secretion. By contrast, the PPARγ agonist significantly inhibits PAPP-A gene expression and secretion by extravillous trophoblastic cells. Inhibition of PAPP-A expression and secretion by PPARγ ligands might play a role in IGF bioactivity, particularly by decreasing the amount of bioactive IGFII, which has been described to promote trophoblast invasion. We previously showed that PPARγ/RXRα heterodimers control the invasive properties of human EVCT. We suggest that PPARγ–mediated inhibition of trophoblast invasion involves the PAPP-A/IGF II cascade.

In conclusion this study shows that, depending upon the cell type, i.e., villous or extravillous cytotrophoblasts, activation of PPARγ might have different effects on the

expression of the same gene (PAPP-A), and points to the complex mechanisms involved in human trophoblast differentiation and functions. Our results underscore a new mechanism by which PPARγ might control human trophoblastic invasion.

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FIGURE LEGENDS

Fig. 1: *in vitro* models of human villous and extravillous cytotrophoblasts.

A, Structure of human implantation site. Sc : stromal core; vct : villous cytotrophoblast; st : syncytiotrophoblast; fv : floating villi; is : intervillous space; av : anchoring villi; evct : extravillous cytotrophoblast; p :proliferative evct; i : invasive evct; e : endovascular evct; gc : giant cells; d : decidual cells; usa : uterine spiral artery. **B**, Immunodetection of cytokeratin 7 in human villous and extravillous cytotrophoblast primary cultures. Upper panels – Villous cytotrophoblasts. Lower panel – Extravillous cytotrophoblasts on Matrigel. Scale bar: 20 μm.

Fig. 2: PAPP-A expression in human cultured VCT and EVCT.

A, PAPP-A expression in villous cytotrophoblasts. Immunocytochemistry at 72 h (left panel : PAPP-A detection and isotype-matched control), Scale bar: 20 μm; transcript levels (middle panel) and secretion of PAPP-A (right panel) in VCT. **B**, Comparative PAPP-A expression in VCT and EVCT. Immunocytochemistry in 48 h-cultured EVCT (left panel : PAPP-A detection and isotype-matched control), transcript levels at 48 h (middle panel) and secretion of PAPP-A (right panel) in VCT and EVCT.

Transcripts coding for PAPP-A were quantified by RT PCR. Values were normalized to PPIA transcript levels. Values represent the mean ± SEM of 3 to 5 independent cultures obtained from different placentas. Supernatants from 24, 48 and 72 h-cultured VCT and EVCT were collected and PAPP-A measured as described in Materials and Methods. Protein secretions were expressed as UI/L and normalized to DNA content for comparison between VCT and EVCT. Values represent the mean ± SEM of three independent cultures obtained from three different placentas. Mann and Whitney U test was used for statistical analysis (*$P < 0.05$ and **,$$ $P < 0.01$).

Fig. 3: Effect of PPARγ agonist on PAPP-A expression in VCT and EVCT.

PAPP-A transcripts and secreted proteins were measured in VCT (**A**) and in EVCT (**B**) treated or not with 1 μM of the PPARγ agonist rosiglitazone. Values were expressed relative to untreated cells and represent the mean ± SEM. For transcript determination, five independent cultures from five different placentas were run in duplicate. For protein determination, six independent cultures from six different placentas were run in duplicate.

Treated vs control: * $P< 0.05$; ** $P<0.01$.

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