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PPARγ expression in normal human placenta, hydatidiform mole and choriocarcinoma

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Peroxisome proliferator-activated receptor (PPAR) γ belongs to a subclass of nuclear hormone receptors that execute their transcriptional functions as heterodimers with the retinoid X receptors (RXR). PPARγ plays a pivotal role in cellular differentiation. This study investigated PPARγ protein expression in normal human placentas, hydatidiform moles and choriocarcinoma, using immunohistochemical and Western blot analyses. In first trimester normal placenta, PPARγ was mainly localized in the nuclei of the villous cytotrophoblastic cells, whereas at term it was mainly localized in the nuclei of the syncytiotrophoblast. Extravillous cytotrophoblast of cell islands and cell columns also showed nuclear PPARγ immunostaining. A striking result was the altered expression patterns of PPARγ in pathological tissues; PPARγ showed a reduced immunostaining in the trophoblastic diseases. In hydatidiform moles, PPARγ was mainly localized in the nuclei of the trophoblastic collections of the pathological villi and in the extravillous trophoblastic cells, whereas in the choriocarcinoma, only a few trophoblastic cells showed weak PPARγ nuclear immunostaining. These findings suggest an involvement of PPARγ in trophoblast differentiation during normal placental development. The down-regulation of PPARγ expression in the gestational trophoblastic diseases analysed in this study provides a new insight into the progression of these pathologies.

Key words: choriocarcinoma/hydatidiform mole/placenta/PPARγ

Introduction

Peroxisome proliferator-activated receptors (PPARs) α, β (also called δ or NUC-1) and γ belong to a subclass of the nuclear hormone receptors that, like the retinoic acid, vitamin D and thyroid hormone receptors, execute their transcriptional functions as heterodimers with the retinoid X receptor (RXR) (Kliewer *et al*., 1992a,b). The human PPARγ gene is located on chromosome 3 at position 3p25, extending over >100 kb of genomic DNA and giving rise to three mRNAs, i.e. PPARγ1, PPARγ2 and PPARγ3 mRNA. These differ at their 5′-end as a consequence of alternate promoter usage and splicing (Desvergne and Wahli, 1999). The translation of PPARγ mRNAs gives rise to three proteins of 477 (PPARγ1 and γ3) and 505 amino acids (PPARγ2) respectively (Fajas *et al*., 1998; Desvergne and Whali, 1999). PPARγ is expressed at the highest level in adipose tissue and adipose cell lines, and at low levels in other non-adipose tissues such as the human epithelial surfaces. In this context, PPARγ expression has been detected in colon epithelium (Lefebvre *et al*., 1999), urinary tract (Guan *et al*., 1997) and human placental trophoblast (Schaiff *et al*., 2000). Recent reports have indicated that PPARγ is also expressed in different cancer tissues (Tontonoz *et al*., 1997; Elstner *et al*., 1998), suggesting a role for PPARγ in the induction of terminal differentiation in human solid tumours.

This study investigates the expression of PPARγ in normal human placentas and gestational trophoblastic diseases by immunohistochemical and Western blot analyses. The human placenta is characterized by a fine balance between proliferation, differentiation and invasive processes of the trophoblast. The trophoblast may be considered as a true epithelium with remarkable characteristics. During placental development, various subsets of trophoblasts originate from the trophectoderm of the blastocyst; these include the villous trophoblast, which covers the placental villi, and the extravillous cytotrophoblast of cell islands and cell columns (Benirschke and Kaufmann, 2000).

Hydatidiform moles and choriocarcinoma are two gestational trophoblastic diseases which differ in their degree of severity. Hydatidiform moles (partial and complete) are characterized by enlarged, vesicular oedematous chorionic villi accompanied by a variable amount of proliferative trophoblast. Choriocarcinoma is a malignant neoplasm characterized by masses and sheets of trophoblastic cells without chorionic villi, which invade surrounding tissues and permeate the vascular spaces (Mazur and Kurman, 1994).

Under normal conditions, most of the invasive processes of the trophoblast are performed by the extravillous cytotrophoblastic cells, whereas the villous trophoblast which covers the placental villi does not show invasive behaviour. In contrast, the gestational trophoblastic diseases show alterations in the physiological differentiation and development of the trophoblast. Because the mechanisms related to normal and pathological differentiation and invasion of the trophoblast

are not yet fully understood, we have hypothesized that molecules such as PPARγ which play an important role in terminal differentiation could be involved in such mechanisms of trophoblast behaviour.

Materials and methods

Tissues

A total of 15 human placentas aged 8 (n = 3), 10 (n = 1), 12 (n = 3), 38 (n = 5) and 40 (n = 3) weeks post menstruation (pm) were collected from clinically normal pregnancies, either interrupted by curettage (aspiration technique) for psychosocial and medical reasons that were unlikely to affect placental structure and function, or terminated by Caesarean sections or normal vaginal deliveries. Specimens of eight partial moles (15–33 weeks pm), seven complete moles (10–13 weeks pm) and five choriocarcinomas were collected from the pathology files of the Institute of Pathology, University of Ancona, Italy. All the cases were documented with clinical and histopathological data.

Specimens from normal and pathological tissues were fixed for 24 h at 4°C in 4% buffered formalin and routinely processed for paraffin embedding at 56°C as previously described (Mühlhauser *et al*., 1993). In addition, from each normal placenta some specimens were frozen in liquid nitrogen for biochemical analysis. Brown adipose tissue from adult rats was also frozen and used as a positive control for the Western blot (see below) (Tai *et al*., 1996).

Western blot analysis

Tissue lysates were obtained after complete potter homogenization in extraction buffer (0.25 mol/l sucrose, 10 mmol/l Tris–HCl pH 7.4, 1 mmol/l EDTA) and 16 000 *g* centrifugation at 4°C for 15 min. Protein concentrations were assessed with the Lowry protein assay. Equal amounts of proteins (100 µg), were mixed with an equal volume of 5×loading buffer (Laemmli, 1970), boiled for 5 min and fractionated on SDS–PAGE. Proteins were electrophoretically transferred (Trans-blot-cell, BioRad Laboratories Inc., Richmond, USA) to polyvinyldifluoride membranes (Roche Diagnostics SpA, Milano, Italy) and subjected to Western blot analysis. Non-specific protein binding was blocked with 5% (w/v) non-fat dry milk (BioRad) in phosphate-buffered saline (PBS)–0.05% Tween. Blots were incubated with the mouse monoclonal anti-PPARγ (sc-7273; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:1500 v/v. After washing, blots were incubated with biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, USA) diluted 1:6000 v/v, and with streptavidin horse-radish peroxidase conjugate (Amersham SRL, Milano, Italy) diluted 1:3000 v/v. Detection of bound antibody was performed with the enhanced chemiluminescence Western blotting detection system (Amersham) according to the manufacturer's instructions.

In additional experiments, some blots were incubated with the anti-PPARγ sc-7273 monoclonal antibody and immunodetected by ^{125}I-labelled antimouse IgG (0.2 µCi/ml; Amersham) in PBS containing 0.5% of bovine serum albumin. The ^{125}I content of PPARγ bands was quantified by densitometric scanning of the autoradiograms using the Personal Molecular Imager FX System (BioRad). Because of the variability of PPARγ expression, a statistical analysis was performed using the Wilcoxon Mann–Whitney test. A value of $P < 0.06$ was considered significant.

Specificity tests were performed by incubating the primary antibody overnight with a 100-fold excess homologous peptide (sc-7273P; Santa Cruz Biotech.).

Immunohistochemical analysis

Paraffin serial sections (3 µm) were cut and stretched at 45°C, allowed to dry and stored at 4°C until use. Sections were deparaffinized and rehydrated via xylene and a graded series of ethyl alcohol and then incubated for 10 min with 10% hydrogen peroxide in distilled water to inhibit endogenous peroxidases. To unmask PPARγ and cytokeratin antigens, sections were pre-treated in a microwave oven (Cattoretti *et al*., 1992) or with 0.1% Trypsin (Sigma Chemical Co., St Louis, MO, USA) (Mühlhauser *et al*., 1995) respectively.

Sections were then incubated for 20 min at room temperature with normal horse serum diluted 1:75 v/v. Afterwards, the sections were incubated overnight at 4°C with the following primary monoclonal antibodies: PPARγ (sc-7273; Santa Cruz Biotech.) diluted 1:50 v/v, or cytokeratin 7 (OV-TL 12/30; Dako, Glostrup, Denmark) (Haigh *et al*., 1999) diluted 1:50 v/v.

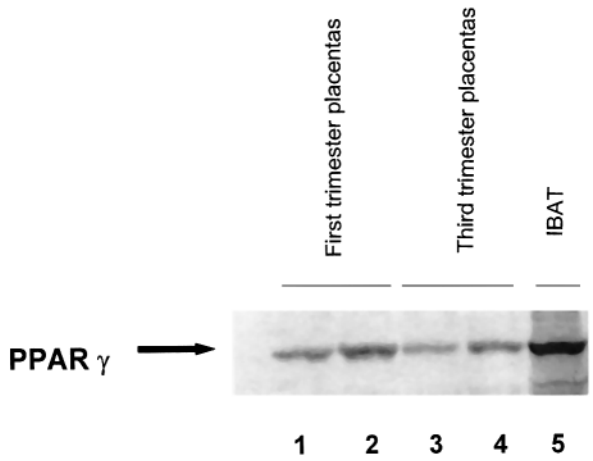

Figure 1. Immunoblotting of PPARγ protein in first and third trimester human placentas. PPARγ protein expression was detected in first trimester (lanes 1 and 2) and third trimester (lanes 3 and 4) placentas. Inter-scapular brown adipose tissue (IBAT) from adult rats (lane 5) was used as a positive control.

After washing with PBS, the bound antibody was visualized by the peroxidase ABC method (Hsu and Raine, 1981) using a biotinylated horse anti-mouse IgG (Vector) as secondary antibody and 3′,3′diaminobenzidine hydrochloride (Sigma) as the chromogen. The sections were rinsed with distilled water, counterstained with Mayer's haematoxylin and mounted in Eukitt (Kindler GmbH and Co., Freiburg, Germany). For the above immunohistochemical procedures, controls were performed by replacing the primary antibody by PBS or non-immune serum. Further controls were performed by omitting the secondary antibody. The specificity test of primary antibody was performed by pre-incubating the anti-PPARγ antibody for 30 min at room temperature with a blocking peptide (sc-7273P; Santa Cruz Biotech.) specific for the anti-PPARγ antibody, at ~100-fold excess relative to the antigen-binding sites of the antibody.

Eight placentas (38–40 weeks gestation) were analysed by statistical analysis considering 20 villi for each placenta. Each placental tissue section was examined at ×63 magnification using light microscopy (Carl Zeiss, Germany). Calculations for the percentage of PPARγ-positive cytotrophoblastic cells for each villus analysed were made.

Results

Western blotting

To evaluate the expression of PPARγ protein in the human placenta, Western blot analysis of first and third trimester placental samples was performed (Figure 1). We found that PPARγ protein was expressed in first and third trimester placentas. A band (potentially representing a doublet) with a molecular mass of ~60 kDa consistent with the predicted mass of PPARγ1 and γ2 (Fajas *et al*., 1997) was detected. However, the signal detected in Figure 1 may also partly represent a phosphorylated form of the protein, as previously suggested (Adams *et al*., 1997).

To clarify the variability of PPARγ expression in normal placental tissues, we performed a quantitative analysis of seven first trimester and eight third trimester placentas by densitometric scanning. We found that the quantitative difference in PPARγ expression of first trimester compared with third trimester placentas was not significant ($P > 0.06$) (Figure 2).

Specificity tests performed by incubating the primary antibody overnight with 100-fold excess homologous peptide (sc-7273P; Santa Cruz Biotech.) showed negative results.

Immunohistochemistry

The immunohistochemical results for normal and pathological tissues are summarized in Table I.

Table I. PPARγ expression in normal and pathological placental tissues. Summary of the immunohistochemical results

	NT first trimester $n = 7$	NT third trimester $n = 8$	Partial moles $n = 8$	Complete moles $n = 7$	Choriocarcinomas[a] $n = 5$
Syncytiotrophoblast	–	++	+/–	+/–	–
Villous cytotrophoblast	++	+/–	+/–	+/–	
Villous stroma	–	–	–	–	
Fetal vessel endothelium	+/–	–	–	–	
Extravillous cytotrophoblastic cells	++	+/–	+/–	+/–	–

[a]Villous cytotrophoblast and villous stroma are not components of choriocarcinoma.
NT = normal tissues; ++ = evident positive staining; +/– = irregular staining; – = negative staining.

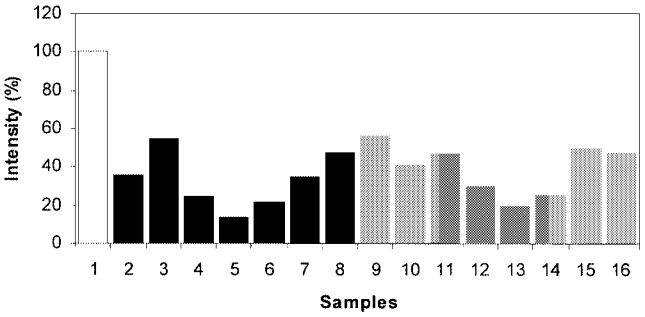

Figure 2. Expression of PPARγ protein in first and third trimester human placentas. Total cellular proteins obtained from first trimester (black bars) and third trimester placentas (grey bars) were analysed by immunoblotting with anti-PPARγ sc-7273 monoclonal antibody and revealed by ^{125}I anti-mouse IgG. The amount of ^{125}I was quantified by densitometric scanning of the autoradiograms and the results are expressed as percentage of positive control (100 μg of IBAT extract; white bar). The quantitative difference in PPARγ expression of first trimester compared with third trimester placentas was not significant ($P > 0.06$).

Normal samples

In first trimester placental tissues, the large majority of the nuclei of villous cytotrophoblastic cells were immunostained for PPARγ, while the syncytiotrophoblast was mainly negative (Figure 3a). In the villous stroma only the endothelial cells of some vessels were stained for PPARγ. Extravillous cytotrophoblastic cells of cell islands and cell columns of first trimester placentas also showed nuclear PPARγ immunostaining (Figure 3b).

In third trimester placental specimens, nuclei of the syncytiotrophoblastic layer were frequently positive for PPARγ. Only 35% of villous cytotrophoblastic cells were positive for PPARγ (Figure 3c). Fetal vessels and other villous stroma components were negative in the third trimester. The basal plate of third trimester placentas showed nuclear PPARγ expression in some extravillous cytotrophoblastic cells identified by their positivity for cytokeratin (data not shown).

Pathological samples

In the partial moles, nuclei of the villous trophoblast were positive for PPARγ, both in the cytotrophoblast and syncytiotrophoblast (Figure 3d). However, PPARγ expression was extremely irregular because nuclei were highly positive in some areas of the trophoblastic layer, while others were totally negative. The trophoblastic collections of the pathological villi showed a positive nuclear immunostaining for PPARγ (Figure 3d). The villous stroma was totally negative.

The complete moles showed an irregular staining of the trophoblast (Figure 3e). Indeed, the reaction product for PPARγ in the trophoblast was heterogeneous among the villi, i.e. positive in some villi and negative in others. Moreover, the large majority of positive villi showed a discontinuous immunostaining of the trophoblastic nuclei, showing a reaction product only in some areas of the trophoblastic layer. The villous stroma was totally negative.

In the choriocarcinoma, only some trophoblastic cells showed a weak expression of PPARγ, whereas the large majority of these cells were negative for this antigen (Figure 3f).

The specificity of the staining was confirmed by control tissue sections in which the immunostaining procedure was performed in the presence of 100-fold excess PPARγ blocking peptide. The control sections were always negative (data not shown). The immunostaining was also absent in normal and pathological tissue sections in which the primary or secondary antibody was omitted (Figure 3g).

Discussion

The villous trophoblast is composed of two layers: a continuous multinucleated highly differentiated layer (syncytiotrophoblast) and an underlying undifferentiated mononucleated layer (cytotrophoblast) (Benirschke and Kaufmann, 2000). The syncytium derives from the fusion of the cytotrophoblastic cells which represent the proliferative stem cells. Therefore, the syncytium is a terminally differentiated epithelial structure responsible for most exchange and secretory functions of the placenta (Benirschke and Kaufmann, 2000).

In this study, we have shown by Western blot analysis that the overall quantitative differences in PPARγ expression in first compared with third trimester normal placentas are not significant. In addition, we have shown by immunohistochemistry that PPARγ is mainly localized in the cytotrophoblast in the first trimester of gestation, whereas at term, PPARγ is mainly localized in the syncytiotrophoblast. These results are in agreement with and extend previous results on PPARγ expression in term placentas (Schaiff *et al*., 2000; Waite *et al*., 2000).

The immunohistochemical modifications of PPARγ expression during pregnancy could be related to the role that PPARγ plays in tissue differentiation. Previous reports on the differentiation of colon epithelial cells (Lefebvre *et al*., 1999), adipose tissue (Rosen *et al*., 1999) and urothelium (Guan *et al*., 1997) support this hypothesis. It has been demonstrated that PPARγ expression increases upon differentiation in Caco2 and HT-29 human adenocarcinoma cells in culture (Lefebvre *et al*., 1999). PPARγ executes its transcriptional function as a heterodimer with RXR (Kliewer *et al*., 1992a,b) and the villous cytotrophoblast and syncytiotrophoblast from human placenta strongly express these receptors (Tarrade, 2001). Moreover, in-vitro experiments have shown that specific ligands for either RXRs or PPARγ are involved not only in the transcription, but also in the synthesis of HCG (Tarrade, 2001). Therefore, in the human placenta, PPARγ/RXRα heterodimers could be considered to have a functional role in the in-vitro differentiation of cytotrophoblast into syncytiotrophoblast and in the maintenance of the differentiated syncytiotro-

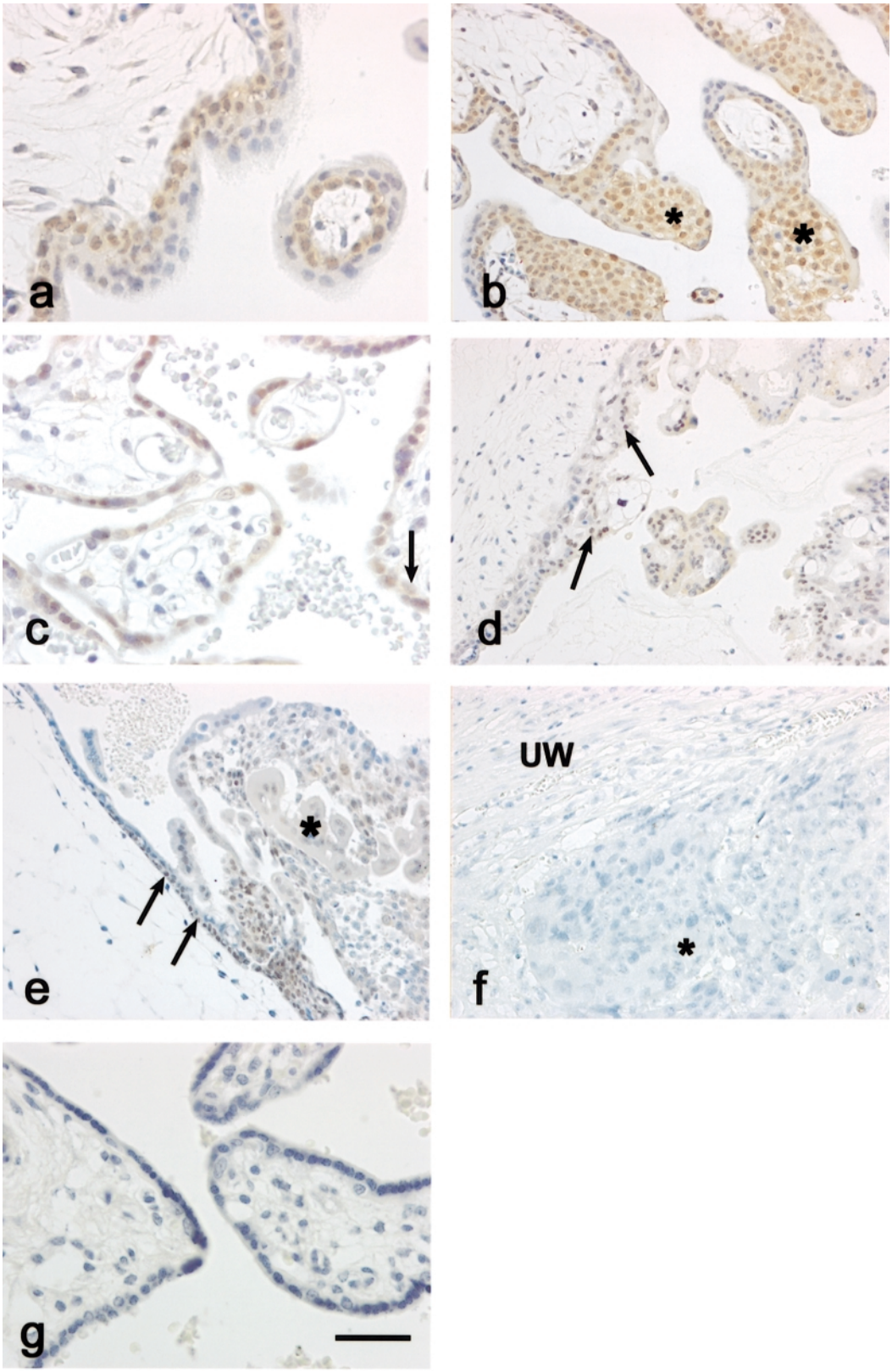

Figure 3. Immunolocalization of PPARγ in human normal and pathological placental tissues. (**a**) First trimester human placenta: the villous cytotrophoblastic cells show positive nuclear immunostaining for PPARγ. Note the absence of reaction product of the nuclei of the syncytiotrophoblast. (**b**) First trimester placenta: the nuclei of extravillous cytotrophoblastic cells of cell columns (*) are positive for PPARγ. (**c**) Third trimester placenta: nuclei of the syncytiotrophoblast show a positive staining pattern for PPARγ. Only 35% of villous cytotrophoblastic cells (arrow) are immunostained for PPARγ. (**d**) Partial mole: a positive reaction product for PPARγ is observable in the nuclei of the cytotrophoblast and of the syncytiotrophoblastic collections (arrows). (**e**) Complete mole: PPARγ is heterogeneously expressed, i.e. present in some trophoblastic nuclei (arrows) and absent in others. Syncytiotrophoblastic collections (*) usually show a more homogeneous expression of this antigen. (**f**) Choriocarcinoma: the invading choriocarcinoma (*) is mainly negative for PPARγ. UW = uterine wall. (**g**) Control section of third trimester placenta: no immunostaining is observable either in the trophoblast or in the stroma of the villi. The negative control was performed by replacing the primary antibody with PBS. **a**,**c**,**g**: bar = 28 μm; **b**,**d**,**e**,**f**: bar = 54 μm.

phoblast in the third trimester placenta (Tarrade, 2001). Our hypothesis on the involvement of PPARγ in trophoblast differentiation is also supported by data on the trophoblast of PPARγ-null mice (Barak *et al*., 1999). Interestingly, it has been shown that this trophoblast fails to undergo terminal differentiation (Barak *et al*., 1999).

Other aspects of trophoblast differentiation are present in extravillous trophoblastic cells of cell islands and cell columns (Benirschke and Kaufmann, 2000). These are two epithelial structures composed of stratified/multinucleated extravillous cytotrophoblastic cells (Benirschke and Kaufmann, 2000). Cell islands are free-ending in the intervillous space (Benirschke and Kaufmann, 2000). Cell columns are responsible for the attachment of placental villi to the basal plate and are interposed between fetal (villous) and maternal connective tissue (basal plate). The balance between proliferation and differentiation of extravillous cytotrophoblastic cells determines the structure and function of the developing placenta and, in the case of cell columns, is also related to the ability of the extravillous cytotrophoblastic cells to invade the uterine wall (Benirschke and Kaufmann, 2000).

In this study, we have reported that PPARγ is highly expressed in cytotrophoblastic cells of cell islands and cell columns. Since these cells show a temporary invasive behaviour, it is our hypothesis that PPARγ could be involved in the modulation of trophoblast invasiveness and that its expression could be modified in gestational trophoblastic diseases, i.e. hydatidiform mole and choriocarcinoma. These pathologies are the consequence of an altered balance between proliferation, differentiation and invasiveness (Mazur and Kurman, 1994).

One of the most interesting findings of our study was that PPARγ shows a more restricted and irregular expression in hydatidiform moles than in normal tissues, whereas in choriocarcinoma, only a few trophoblastic cells show a weak immunostaining for PPARγ and the large majority of these cells are negative for this antigen. This reduced immunostaining of PPARγ in pathological samples is associated not only with the increased invasive behaviour of cytotrophoblastic cells, but also with the different severity of these two pathologies. These data are consistent with previous findings on human colon cancer (Sarraf *et al*., 1999). One allele of the PPARγ gene in four of 55 sporadic colorectal cancers has been found to be mutated, suggesting that PPARγ may function as a tumour suppressor for colon cancer (Park *et al*., 2001). In addition, it has been demonstrated that PPARγ agonists exhibit anti-tumoural and apoptosis-inducing activities in a broad range of human malignancies, including JEG3 choriocarcinoma cell line cultures, breast cancer, prostate cancer and liposarcoma (Tontonoz *et al*., 1997; Brockman *et al*., 1998; Elstner *et al*., 1998; Kubota *et al*., 1998; Demetri *et al*., 1999; Keelan *et al*., 1999; Chang and Szabo, 2000; Marvin *et al*., 2000).

In conclusion, PPARγ may play an important role in trophoblastic differentiation and invasiveness and the down-regulation of PPARγ may contribute to trophoblastic diseases such as hydatidiform moles and choriocarcinoma.

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