Leptin and peroxisome proliferator-activated receptors: impact on normal and disturbed first trimester human pregnancy

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Summary. Recent in vitro and in vivo studies emphasize the impact of leptin, peroxisome proliferator-activated receptors (PPAR) and PPAR coactivators (retinoic X receptor α (RXR), amplified in breast cancer-3 gene (AIB3)) on placental and fetal development. Therefore, the frequency and distribution pattern of PPAR, RXR, AIB3 and leptin expression in normal human first trimester pregnancy, miscarriage and hydatidiform mole was investigated by immunohistochemistry and double immunofluorescence staining.

Enhanced expression of PPARß/δ, RXR and AIB3 was identified in miscarried placentas. With regard to hydatidiform mole, increased expression of PPARγ and PPARß/δ was observed, whereas RXR was significantly down-regulated. Leptin expression was lowest in miscarriage and highest in mole pregnancies. In contrast to trophoblast tissue, expression of leptin in glandular epithelial cells of the decidua was increased in miscarriage. PPAR and leptin expressing cells at the feto-maternal interface were identified as extravillous trophoblast (EVT) by double immunofluorescence and CK7 staining.

In summary, significantly reduced leptin expression was accompanied by enhanced PPARß/δ, RXR and AIB3 expression in miscarried placentas. However, in mole pregnancy, up-regulation of leptin and increased expression of PPAR was detected. RXR, on the other hand, was down-regulated in mole decidua. So far, the study results implicate strong regulatory interaction of PPARs, their coactivators and leptin in human placentas. PPAR and leptin are potential targets for new treatment strategies concerning pregnancy disorders, such as miscarriage. The increasing knowledge about the role of PPARs and leptin in normal and disturbed pregnancy may help to improve pregnancy outcome.

Key words: PPAR, RXR, AIB3, Leptin, Human pregnancy, Miscarriage, Hydatidiform mole

Introduction

The success of human pregnancy depends on a variety of physiologic, immunologic and metabolic processes, and disturbance at any gestational time may induce miscarriage, affecting between 25-50% of reproductive-aged women (Rai and Regan, 2006). Beside established risk factors such as chromosomal disorders, endocrine dysfunctions, anatomical malformations, acquired and inherited thrombophilias, the underlying mechanisms remain unknown in up to 50% of patients with recurrent miscarriage (Regan and Rai, 2000). Therefore, in vitro and in vivo investigations are needed to establish further risk factors and new treatment strategies (Toth et al., 2007).

Peroxisome proliferator-activated receptors (PPAR) and Leptin are two important adipose tissue factors involved in energy metabolism regulation (Tontonoz et al., 1994; Jiang et al., 1998; Ricote et al., 1999; Paracchini et al., 2005). Leptin was originally identified as an adipocyte-derived protein and is a regulator of satiety and energy homeostasis. It is synthesized by different organs, including the placenta, especially in the
syncytiotrophoblast (ST) and extra villous trophoblast (EVT) (Castellucci et al., 2000). During normal pregnancy, leptin production is up-regulated and leptin gene expression is regulated by a variety of hormones, growth factors and cytokines, including estrogens (up-regulation) and androgens (down-regulation) (Lambrinoudaki et al., 2008). Proinflammatory cytokines, such as tumor necrosis factor alpha and interleukin-1 may also directly induce leptin gene expression (Lappas et al., 2005). Human placental leptin is identical to leptin of adipose origin, and leptin induces hCG production in trophoblast cells (Chardonnens et al., 1999).

Activation of PPARγ by natural ligands or thiazolidinediones (TZD) inhibits leptin gene expression and leptin release, both in vivo in rodents and in vitro in isolated adipocytes and cultured cell lines (Hong et al., 2004; Lee et al., 2007).

However, activation of PPAR requires heterodimerisation in the cell nucleus with another nuclear hormone receptor: retinoic X receptor α (RXR). Amplified in breast cancer 3 (AIB3) was characterized as a strong transcriptional coactivator for many nuclear receptors, including RXR and PPAR.

Studies on knock-out mice emphasize the major influence of PPARγ, PPARβ/δ, RXR and AIB3 on trophoblast differentiation and placental vascularisation (Barak et al., 1999; Wendling et al., 1999; Kuang et al., 2002; Schaff et al., 2006; Fournier et al., 2007a,b). Although recent investigations pointed out possible interactions between PPARs and leptin, detailed regulatory pathways remain unknown (Guettier et al., 2007).

PPARγ ligands, like oxidized lipids, induced hCG production in human trophoblasts (Schild et al., 2002). PPARγ agonists positively regulate hCG, Leptin and human placental lactogen (Tarrade et al., 2001). Furthermore, PPARγ stimulation altered the differentiation of ST (Tarrade et al., 2001). In a human in vitro model, cytotrophoblast invasion was abrogated dose-dependently by PPARγ stimulation and blocking lead to increased EVT invasion (Schaff et al., 2000; Wang et al., 2002; Rodie et al., 2005).

The aim of the present investigation was to determine the frequency and tissue distribution patterns of PPARγ, PPARβ/δ, RXR, AIB3 and leptin in the first trimester of normal human pregnancy, miscarriage and hydatidiform mole.

**Materials and methods**

**Immunohistochemistry**

Paraffin-wax embedded placental tissue samples were obtained from women with legal termination of normal pregnancy (n=16) and matched to samples of patients with miscarriage (n=16) and partial hydatidiform mole (n=16) of the same gestational age. Termination of pregnancy was performed by uterine abrasion (no extraction remove by suction) of the whole placenta (and fetus), without hormonal treatment.

Demographic and clinical data of the study population are summarized in Table 1. Placental slides of the second and third trimester and slides of breast cancer patients served as positive controls.

For immunohistochemistry, paraffin sections were deparaffinised in xylool, rehydrated in alcohol gradient to H2O and subsequently incubated with: Methanol/H2O2 (30 min), H2O, PBS, goat serum (30 min) and primary antibody (Table 2) for 16 h at 4°C.

The Vectastain® Elite ABC-Kit (Vector Laboratories, Peterborough, GB) was used for

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**Table 1. Demographic and clinical characteristics of study population.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>normal pregnancy n=14</th>
<th>miscarriage n=14</th>
<th>mole n=14</th>
<th>p value (Kruskal-Willis-Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>maternal age</td>
<td>28.1±7.4 (16-43)</td>
<td>30.9±6.6 (22-41)</td>
<td>31.1±5.2 (23-40)</td>
<td>0.35</td>
</tr>
<tr>
<td>gestational age</td>
<td>8.9±1.8 (6-12)</td>
<td>8.9±1.8 (6-12)</td>
<td>8.9±1.7 (6-12)</td>
<td>1.0</td>
</tr>
<tr>
<td>gravidity</td>
<td>2.4±1.5 (1-6)</td>
<td>2.8±1.6 (1-7)</td>
<td>1.9±1.1 (1-4)</td>
<td>0.26</td>
</tr>
<tr>
<td>parity</td>
<td>0.8±1.0 (0-3)</td>
<td>0.6±1.3 (0-5)</td>
<td>0.4±0.5 (0-1)</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Values are given as mean±SD; the range is given in parentheses (p values not significant).

**Table 2. Antibody used for immunohistochemical characterisation of decidual tissue samples.**

<table>
<thead>
<tr>
<th>antibody</th>
<th>clone</th>
<th>isotype</th>
<th>dilution</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ</td>
<td>BC004174</td>
<td>Rabbit IgG</td>
<td>1:2000</td>
<td>Biozol, Eching, Germany</td>
</tr>
<tr>
<td>PPARβ/δ</td>
<td>GC06P035418</td>
<td>Rabbit IgG</td>
<td>1:1600</td>
<td>Biozol, Eching, Germany</td>
</tr>
<tr>
<td>RXRα</td>
<td>K8508</td>
<td>Mouse IgG</td>
<td>1:4000</td>
<td>Perseus Proteomics, Inc, Tokyo Japan</td>
</tr>
<tr>
<td>AIB3</td>
<td>NB 100-1728</td>
<td>Rabbit IgG</td>
<td>1:700</td>
<td>Novus Biologicals, Inc, Littleton, CO</td>
</tr>
<tr>
<td>Leptin</td>
<td>GC07P127668</td>
<td>Rabbit IgG</td>
<td>1:500</td>
<td>Biozol, Eching, Germany</td>
</tr>
<tr>
<td>CK7</td>
<td>OV-TL12/30</td>
<td>Mouse IgG1</td>
<td>1:30</td>
<td>Novocastra, Dossenheim, Germany</td>
</tr>
</tbody>
</table>
visualization according to the manufacturers instructions.

Finally, slides were counterstained with hemalaun and cover-slipped. The intensity and distribution pattern of antigen expression were evaluated by two independent observers, neither of whom had access to the diagnosis, by using a semi quantitative method (immunoreactive score (IRS)) as previously described (Remmele and Stegner, 1987). Briefly, the IRS score was calculated by multiplication of optical staining intensity (graded as 0=none, 1=weak, 2=moderate and 3=strong staining) and the percentage of positive staining cells (0=no staining, 1≤ 10% of the cells, 2=11-50% of the cells, 3=51-80% of the cells and 4>81% of the cells).

Sections were examined using a Leitz (Wetzlar, Germany) photomicroscope. Digital images were obtained with a digital camera system (JVC, Japan) and were saved on computer.

Immunofluorescence

Cryosections were examined to characterize PPAR- or leptin-expressing cells in placental tissue. All samples were fixed in 5% buffered formalin. Slides were incubated with primary antibodies (Table 2) overnight at 4°C. After washing, Cy2-labeled goat anti-mouse IgG and Cy3-labelled goat anti-rabbit IgG (both Dianova, Hamburg, Germany), were diluted 1:200, and served as secondary antibodies. The slides were finally embedded in mounting buffer containing 4’,6-diamino-2-phenylindole, resulting in blue staining of the nucleus. Slides were examined with a Zeiss (Jena, Germany) photomicroscope. Digital images were obtained with a digital-camera system (Axiocam, Zeiss, Jena, Germany) and saved on computer.

Statistics

The SPSS/PC software package version 15.0 (SPSS, Chicago, USA) was used for collection, processing, and statistical data analysis. Statistical analysis was performed using the non-parametrical Mann-Whitney-U signed rank test for comparison of the means. p<0.05 values were considered statistically significant. In some cases, Wilcoxon test was used for comparison of paired samples in matched pair analysis. In addition, non-parametric spearman correlation coefficient was used for estimating correlations between PPARs, AIB3 and RXR.

Results

PPARs

We identified expression of PPARγ and PPARβ/δ in the nuclei of ST in normal, miscarried and mole placentas (Figs. 1a-c, 2a-c). The expression of PPARγ and PPARβ/δ in normal and disturbed pregnancy did not change significantly between the 6th and 12th week of pregnancy.

Additionally, mean values of PPARγ and PPARβ/δ expression in normal and disturbed pregnancy were analyzed in the 6th-12th week of gestation. In ST of miscarried placentas, the expression of PPARγ was not altered compared to normal pregnancy and accompanied by a significant rise in PPARβ/δ expression (p=0.03). Whereas in mole pregnancy, expression of both PPARs was significantly increased in the ST (PPARγ: p=0.006; PPARβ/δ: p<0.001, respectively). PPARγ and PPARβ/δ were also expressed in the nuclei of EVT (Figure 1d-f, 2d-f). In miscarriage patients, PPARγ and PPARβ/δ expression in the EVT was significantly elevated compared to normal pregnancy in the 6th-12th week of gestation (p<0.001 and p=0.021, respectively).

This significant increase in the expression of both PPARs was also observed in mole pregnancy in the time period of 6th-12th weeks of gestation (p=0.006 and p=0.001, respectively). A summary of staining results is given in Figure 1g and 2g.

RXR and AIB3

Expression of RXR and AIB3 was identified in the nuclei of the ST and EVT in normal, miscarried and mole placentas (Figs. 3a-g, 4a-g). Significant differences were seen by comparing median values of RXR expression in normal first trimester pregnancy and miscarriage, both in the ST and EVT (p=0.001, in both cases).

This was also true when normal pregnancies were compared to mole pregnancies (p=0.001, in both cases). A positive correlation between RXR and AIB3 expression was seen in normal first trimester pregnancy (p=0.034; r=0.512), whereas in miscarried tissue, RXR and PPARβ/δ expression correlated significantly (p=0.043; r=0.512). In mole pregnancies, a positive correlation between AIB3 and PPARγ expression occurred (p=0.003; r=0.695).

With further regard to AIB3, increased expression was seen in the ST and EVT of miscarried tissue compared to normal pregnancy. Significance was reached when AIB3 expression in ST was analyzed by matched-pair analysis (p=0.036). In mole pregnancies, AIB3 expression was not altered in the ST and in the EVT in comparison to normal pregnancy and miscarriage.

Leptin

Leptin expression was identified in the cytoplasm of ST and EVT in normal and disturbed first trimester human pregnancy (Figure 5a-k). The highest expression of leptin was seen in the ST of mole pregnancies (p<0.001) and lowest expression in the ST of miscarried placentas (p=0.001). However, in EVT, no differences were seen in the expression of leptin in normal compared to disturbed pregnancies. In addition, leptin
expression was also present in glandular epithelial cells of the decidua, and was significantly up-regulated in miscarried tissue as compared to normal pregnancy (p=0.003).

Characterization of PPAR- and leptin-expressing cells as EVT

To verify the EVT origin of PPARγ, PPARβ/δ and leptin expressing cells at the feto-maternal interphase, fluorescence double labelling with a trophoblast marker (cytokeratin-7) and PPARδ, PPARβ/δ and leptin antibodies were performed. EVT were stained with both antibodies (Fig. 6).

Discussion

In our study, we were able to demonstrate that nuclear hormonal receptors, like PPARγ, PPARβ/δ, RXR and AIB3, as well as leptin, are expressed in normal first trimester human pregnancy, as well as disturbed pregnancies, such as miscarriage and hydatidiform mole. PPAR and leptin expressing cells at the feto-maternal interphase were identified as EVT by double immunofluorescence and CK7 staining. Expression of PPARβ/δ, RXR and AIB3 was significantly elevated in ST and EVT in miscarried placentas. In hydatidiform mole, the highest expression of PPARs was seen in ST and EVT, whereas RXR expression, was down-
regulated. Expression of leptin was highest in the ST of mole pregnancies and lowest in the ST of miscarried placentas. Additionally, leptin expression in glandular epithelial cells was significantly increased in miscarried tissue, as compared to normal human first trimester pregnancy.

Dunn-Albanese et al. (2004) described reciprocal expression of PPARγ and cyclooxygenase-2 in human term placentas, predominantly within the ST and EVT. Furthermore, increased expression of PPARγ and PPARδ in the decidua during labor was paralleled with significantly decreased PPARγ expression in fetal membranes once labor commenced (Berry et al., 2003; Dunn-Albanese et al., 2004).

Our investigations showed no significant changes in PPARγ and PPARδ expression during the first trimester of normal human pregnancy (6th-12th week of gestation). These data were reached by comparing PPARγ and PPARδ expression with gestational age in two groups (6 cases: 6-8 weeks and 8 cases: 9-12 weeks of gestation). With regard to the whole study population also no significant differences occurred. Fournier et al. (Fournier et al., 2002) investigated the effect of PPAR agonists and antagonists on cultured EVT isolated from

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**Fig. 2.** a. Immunohistochemical labelling of PPARδ in normal human villous trophoblast cell nuclei. b. Expression of PPARδ in miscarried villous trophoblast cell nuclei. c. Expression of PPARδ in villous trophoblast cell nuclei of hydatidiform mole. d. Immunohistochemical labelling of PPARδ in normal human extravillous trophoblast cell nuclei. e. Expression of PPARδ in miscarried extravillous trophoblast cell nuclei. f. Expression of PPARδ in extravillous trophoblast cell nuclei of hydatidiform mole. g. Summary of staining results (IRS scores) of immunohistochemical localisation of PPARδ in normal first trimester pregnancy, miscarriage and hydatidiform mole (mean+SEM). x 25
human first-trimester placentas. They were able to show that PPARγ agonists inhibit invasion of EVT, whereas antagonists promoted EVT invasion. Castellucci et al. (2000) suggested that leptin and leptin-R may have a role in the invasive processes of the EVT by modulating the expression of matrix metalloproteinase. We speculate that expression of PPAR, RXR, AIB3 and leptin in the EVT may be linked to trophoblast proliferation or invasion, and therefore are involved in the pathophysiology of miscarriage.

Capparuccia et al. (2002) investigated protein expression in normal human placentas, hydatidiform moles and choriocarcinomas, using immunohistochemical and Western blot analyses. In first trimester placentas of normal pregnancies, PPARγ was mainly localized in villous cytotrophoblastic cells, whereas at term it was mainly localized in the ST. Furthermore, PPARγ expression was reduced in trophoblastic diseases: in hydatidiform mole, PPARγ was mainly localized in the trophoblastic collections of the pathological villi and

**Fig. 3.** a. Immunohistochemical labelling of RXR in normal human villous trophoblast cell nuclei. b. Expression of RXR in miscarried villous trophoblast cell nuclei. c. Expression of RXR in villous trophoblastic cell nuclei of hydatidiform mole. d. Immunohistochemical labelling of RXR in normal human extravillous trophoblast cell nuclei. e. Expression of RXR in miscarried extravillous trophoblast cell nuclei. f. Expression of RXR in extravillous trophoblast cell nuclei of hydatidiform mole. g. Summary of staining results (IRS scores) of immunohistochemical localisation of RXR in normal first trimester pregnancy, miscarriage and hydatidiform mole (mean±SEM).
in the EVT, whereas in the choriocarcinomas, only a few trophoblastic cells showed weak PPARγ nuclear staining. Our results were obtained from non-invasive mole pregnancies and implicate that in these mole pregnancies both PPARs investigated showed increased expression in villous trophoblast, whereas in miscarriage, expression of PPARs was increased in EVT. As mole pregnancies can further develop to choriocarcinomas, comparison of our and Capparuccias data might be affected from grading and differentiation of the tissue investigated. Mole pregnancy is characterized by increased proliferation of villous trophoblasts and miscarriage by induction of immune responses at the feto-maternal interface. Therefore, we speculate that PPARs could be involved in increased trophoblast activation or proliferation. On the other hand, PPARγ agonists do also have the capacity to downregulate pro-inflammatory mediators such as cytokines (Lappas et al., 2005, 2006) and nitric oxide (Jawerbaum et al., 2004).

In preeclampsia, increased placental synthesis of leptin was seen, although only low umbilical
PPAR and Leptin in human first trimester pregnancy

Fig. 5. a. Immunohistochemical labelling of leptin in normal human villous trophoblast cell plasma. b. Expression of leptin in miscarried villous trophoblast cell plasma. c. Expression of leptin in villous trophoblast cell plasma of hydatidiform mole. d. Immunohistochemical labelling of leptin in normal human extravillous trophoblast cell plasma. e. Expression of leptin in miscarried extravillous trophoblast cell plasma. f. Expression of leptin in extravillous trophoblast cell plasma of hydatidiform mole. g. Immunohistochemical labelling of leptin in normal human glandular cell nuclei. h. Expression of leptin in miscarried glandular cell nuclei. i. Expression of leptin in glandular cell nuclei of hydatidiform mole. j. Summary of staining results (IRS scores) of immunohistochemical localisation of leptin in normal first trimester pregnancy, miscarriage and hydatidiform mole (mean±SEM). k. Summary of staining results (IRS scores) of immunohistochemical localisation of Leptin in glandular epithelial cells of normal first trimester pregnancy, miscarriage and hydatidiform mole (mean±SEM). x 25
concentrations occurred, whereas in mole pregnancies an increase in maternal leptin levels and placental leptin synthesis was detected (Hauguel-de Mouzon et al., 2006). We were able to show that in disturbed first trimester pregnancy there is altered expression of leptin. This could be triggered by nuclear hormone receptors, like PPARs and their coactivators. However, conflicting data on the capacity of PPARγ agonists to regulate leptin expression do exist (Tarrade et al., 2001; Lappas et al., 2005).

Muhlhausler et al. (2007) were able to demonstrate that increased maternal nutrition stimulates PPARγ, adiponectin and leptin mRNA in adipose tissue before birth. Poor nutrition status, as measured by low maternal pre-pregnancy weight and low hematocrit, has been associated with adverse pregnancy outcome (El-Bastawissi et al., 2007). Therefore, obesity gene regulation and expression could be accompanied with disturbed or successful pregnancy.

There are conflicting results concerning leptin plasma levels in patients with (recurrent) miscarriage (Lage et al., 1999; Laird et al., 2001; Lepercq and Hauguel De Mouzo, 2002; Tommaselli et al., 2006). However, in our study, significant differences in the expression of leptin in ST and glandular epithelial cells compared to normal pregnancy occurred. Leptin expression was decreased in ST and increased in glandular epithelial cells. Differences in the expression of leptin in placental tissue may explain variations in the serum concentrations of leptin. Additionally, leptin expression was significantly increased in trophoblast cells of hydatidiform mole, confirming former results (Li

![Fig. 6. PPARγ is expressed in EVT nuclei (a). PPARγ expressing cells were also positive for CK7 (b), triple filter excitation (c). PPARβ/δ is also expressed in EVT nuclei (d) and PPARβ/δ expressing cells were positive for CK7 (e), triple filter excitation (f). Leptin is expressed in EVT cytosol (g). Leptin expressing cells were also positive for CK7 (h), triple filter excitation (i). x 40](image)
et al., 2004).

Within our study, insights into the expression of leptin and PPARs in normal and disturbed pregnancy were achieved. PPAR agonists are widely used in gynaecologic disorders, such as Polycystic Ovary Syndrom.

Their impact on pregnancy disorders, such as miscarriage, hydatidiform mole or preeclampsia, needs to be further elucidated. However, the mechanism regulating obesity genes, like leptin in placental and adipose tissue, and the input of PPARs and leptin in the success of human pregnancy is still incompletely understood, and warrants further in vivo and in vitro studies.

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