Insulin and leptin receptors as possible new candidates for endocrine control in normal and disturbed human pregnancy

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ABSTRACT: Leptin and insulin are secreted into the maternal and to a lesser extent into the fetal bloodstream where they act as placental signals and nourish the fetus, making them possible candidates for the endocrine control of the placenta. We investigated differences in leptin (LR) and insulin receptors (IR) expression in normal and disturbed first trimester human pregnancy at protein level by immunohistochemistry and at mRNA level by real-time reverse transcriptase–polymerase chain reaction (TaqMan). Highest expression of LR and IR was present in villous (VT) and extravillous trophoblasts (EVT). In hydatidiform mole trophoblasts, significantly higher LR and IR expression was observed when compared with normal pregnancy. In addition, LR and IR were also expressed in glandular epithelial cells of the decidua, again to the highest extent in hydatidiform mole when compared with normal pregnancy. With regard to abortive placentas, significant differences were also present when compared with normal first trimester placenta in the expression of LR and IR in VT, EVT and in glandular epithelial cells of the decidua. Results at protein expression of LR and IR were confirmed at mRNA level. The majority of IR and LR are expressed on structures that are currently assumed to drive placental growth. LR and IR are strongly up-regulated in placentas of hydatidiform mole and abortion. Our findings may suggest IR and LR as possible new candidates for the endocrine control of human pregnancy.

Key words: abortion / first trimester pregnancy / hydatidiform mole / insulin receptor / leptin receptor

Introduction

Immunologic, endocrine, metabolic and vascular regulatory mechanisms being genetically controlled are involved in the success of human pregnancy. Disturbances of any of these regulatory processes can lead to fetal loss. However, 25–50% of reproductive-aged women experience one or more miscarriages, often due to fetal chromosomal abnormalities, especially with increasing maternal age (Toth et al., 2007a). Furthermore, 1–3% of women during child-bearing years suffer from recurrent miscarriage (RM), the occurrence of three or more consecutive spontaneous miscarriages regardless of previous live births (Regan and Rai, 2000). In nearly 50% of affected patients, the cause of RM remains unknown leading to investigations on new risk factors and possible treatment strategies (Regan and Rai, 2000). Along these new risk factors, leptin and insulin as well as their receptors seem to play a possible role in the maintenance of normal pregnancy (Toth et al., 2008).

Leptin was originally identified as an adipocyte-derived protein and is a regulator of satiety and energy homeostasis. Nearly 50% of circulating leptin is bound to plasma proteins contributing to steady-state plasma leptin levels (Toth et al., 2007b). Furthermore, leptin seems to play a functional role in the implantation process as it stimulates matrix metalloproteinase (MMP) expression in cytotrophoblasts (Sagawa et al., 2002a) and in fetal developments, as its levels in the umbilical vessels have been related with birthweight (Harigaya et al., 1997; Papageorgiou et al., 2004). It also modulates glucose metabolism by increasing insulin sensitivity (Sagawa et al., 2002b). Yamashita et al. (2001) were able to demonstrate that animals with mutations in the leptin receptor (LR)
gene develop gestational diabetes during pregnancy. In human pregnancy, LR gene expression was the only angiogenesis-related gene that was up-regulated in chorionic villi of RM patients when compared with normal pregnancy (Choi et al., 2003).

So far, little is known on the expression of the insulin receptor (IR) in disturbed pregnancies. However, there is evidence that the IR regulates choriocarcinoma cell invasion (Diaz et al., 2007). In human placenta of normal pregnancy, the IR distribution pattern is characterized by a spatio-temporal change between first trimester and term (Desoye and Hauguel-de Mouzon, 2007). Although the IR is found predominantly on the maternal side (syncytiotrophoblast and cytotrophoblast) in first trimester pregnancy, it is expressed on the fetal side (fetal vessels) at term (Desoye and Hauguel-de Mouzon, 2007).

We were able to show leptin expression in normal and disturbed pregnancy (Toth et al., 2008). Leptin expression was lowest in placentas of miscarriage patients and highest in placentas of mole pregnancies, whereas expression of leptin in glandular epithelial cells of the decidua was increased in miscarriage when compared with normal pregnancy. Leptin-expressing cells at the fetomaternal interface were identified as extravillous trophoblast (EVT) by double immunofluorescence and Cytokeratin 7 (CK7) staining.

The present study was designed to improve our understanding of the role of LR and IR in placental and fetal development. The frequency and tissue distribution patterns of LR and IR in the first trimester of normal human pregnancy, abortion and hydatidiform mole were investigated.

### Materials and Methods

Samples of paraffin wax-embedded placental tissue were randomly obtained from women with spontaneous miscarriage (n = 14), hydatidiform mole (n = 14) or termination of normal pregnancy (n = 14), each from the 6th to 12th week of pregnancy. Demographic and clinical data of the study population are summarized in Table I. The exact number of slides of each week of gestational age is shown in Table II.

Signed informed consent was obtained from all participants allowing analysis of all clinical and laboratory data mentioned in this paper. The Human Investigation Review Board of the Ludwig-Maximilians-University Munich approved the study.

Tissue storage was performed as followed: placentas were put on ice immediately after curettage and placental tissue pieces for RNA isolation were stored in RNAlater® buffer solution (Ambion, Darmstadt, Germany). Within 30 min, RNA isolation from placental tissue was started.

### Immunohistochemistry

For immunohistochemistry, paraffin sections were deparaffinized in xylol, incubated with methanol/H₂O₂ (20 min) to inhibit endogenous peroxidase activity, rehydrated in alcohol gradient to PBS and subsequently incubated with mouse serum for LR and rabbit serum for IR antibody (20 min, 22°C). Then, the slides were incubated with the primary antibodies (Table III), the LR antibody for 1 h at room temperature and the IR antibody overnight at 4°C. The Vectastain® Elite ABC-Kit (Vector Laboratories, Peterborough, UK) was used for visualization according to the manufacturer’s instructions. Finally, slides were counterstained with hemalaun (2 min) and then cover-slipped.

As positive control tissue for LR and IR, placental slides of normal and term pregnancy were used. In addition, slides of human pancreas were used for IR. For negative controls, primary antibodies were replaced by horse serum.

The intensity and distribution patterns of antigen expression were evaluated using a semi-quantitative method [immunoreactive score (IRS)] as previously described (Remmele and Stegner, 1987). Briefly, the IRS 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 stored on computer.

The SPSS/PC software package version 16.0 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.

### RNA extraction from placental tissue

A total amount of 5 × 10 mg abortive and 5 × 10 mg normal control placental tissue ranging from the 7th to 12th week of gestation was used for extraction of mRNA. Total RNA was investigated by NucleoSpin® RNAII kit (Macherey-Nagel, Düren, Germany), according to the manufacturer’s protocol. Purified RNA was quantified and evaluated for purity by UV spectrometry.

### Reverse transcription

Reverse transcription (RT) was carried out with the ‘High Capacity cDNA Reverse Transcription Kit’ (Applied Biosystems, Weiterstadt, Germany) according to the protocol in a mastercycler gradient (Eppendorf, Hamburg, Germany). RT conditions were: 10 min 25°C, 2 h 37°C, 5 s 85°C and 4°C on hold.

### Real-time RT–PCR

Real-time reverse transcriptase–polymerase chain reactions (RT–PCRs) were performed in quadruplicate in optical 96-well reaction microtiter plates covered with optical caps, in a volume of 20 µl containing 1 µl MgCl₂.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Demographic and clinical characteristics of study population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td>Normal pregnancy (n = 14)</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>27.6 ± 7.4 (16–43)</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>8.6 ± 1.8 (6–12)</td>
</tr>
<tr>
<td>Gravidity (n = 14)</td>
<td>2.6 ± 1.5 (1–6)</td>
</tr>
<tr>
<td>Party</td>
<td>0.9 ± 1.0 (0–3)</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD. The range is given in parentheses. P-values are not significant.
Table II  Number of slides used for immunohistochemical staining for each week of gestational age.

<table>
<thead>
<tr>
<th>Gestational age</th>
<th>Number of slides</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>6th week</td>
<td>2</td>
</tr>
<tr>
<td>7th week</td>
<td>1</td>
</tr>
<tr>
<td>8th week</td>
<td>3</td>
</tr>
<tr>
<td>9th week</td>
<td>3</td>
</tr>
<tr>
<td>10th week</td>
<td>2</td>
</tr>
<tr>
<td>11th week</td>
<td>2</td>
</tr>
<tr>
<td>12th week</td>
<td>1</td>
</tr>
</tbody>
</table>

n = 14

Table III  Antibodies used for immunohistochemical characterization of decidual tissue samples

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin receptor</td>
<td>Mouse IgG</td>
<td>MAB867</td>
<td>1:2000</td>
<td>R&amp;D Systems, Minneapolis, USA</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>Rabbit IgG</td>
<td>AHP 1216</td>
<td>1:20</td>
<td>AbD Serotec, Oxford, UK</td>
</tr>
<tr>
<td>CK7 for insulin receptor</td>
<td>Mouse IgG</td>
<td>OV-TL 12/30</td>
<td>1:30</td>
<td>Novocastra, Berlin, Germany</td>
</tr>
<tr>
<td>CK7 for leptin receptor</td>
<td>Rabbit IgG</td>
<td>Polyclonal</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>Prolactin for insulin receptor</td>
<td>Rabbit IgG</td>
<td>Polyclonal</td>
<td>1:500</td>
<td>Dianova, Hamburg, Germany</td>
</tr>
<tr>
<td>Prolactin for leptin receptor</td>
<td>Rabbit IgG</td>
<td>Polyclonal</td>
<td>1:500</td>
<td>Dianova, Hamburg, Germany</td>
</tr>
<tr>
<td>Goat-anti-mouse Cy3</td>
<td>Goat IgG</td>
<td>Polyclonal</td>
<td>1:100</td>
<td>Dianova, Hamburg, Germany</td>
</tr>
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<td>Goat-anti-rabbit Cy2</td>
<td>Goat IgG</td>
<td>Polyclonal</td>
<td>1:10</td>
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</tr>
<tr>
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<td>Goat IgG</td>
<td>Polyclonal</td>
<td>1:100</td>
<td>Dianova, Hamburg, Germany</td>
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<tr>
<td>Goat-anti-rabbit Cy3</td>
<td>Goat IgG</td>
<td>Polyclonal</td>
<td>1:300</td>
<td>Dianova, Hamburg, Germany</td>
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</table>

Results

Leptin receptor
We identified the expression of LR in the cytoplasm and apical cell membrane of villous trophoblasts (VTs), EVTs and glandular epithelial cells of the decidua in first trimester normal and abortive placentas as well as in hydatidiform mole.

LR expression was up-regulated in the VT of hydatiform mole when compared with normal pregnancy (P < 0.001) (Fig. 1a). With regard to abortion, the expression of LR in the VT was also increased, but without reaching significance (Fig. 1a).

In abortive tissue (P = 0.007) as well as in hydatidiform mole (P < 0.001), LR expression in the EVT was significantly up-regulated in comparison to normal pregnancy (Fig. 1b–e).

Both mole pregnancy and abortion were accompanied by a significant up-regulation of LR (P = 0.001 and P = 0.005) in glandular epithelial cells of the decidua when compared with normal pregnancy (Fig. 1f).

Insulin receptor
Expression of IR was seen in the cytoplasm and apical membrane of VT, EVT and glandular epithelial cells of the decidua in first trimester normal and abortive placentas as well as in hydatidiform mole. Fetal blood vessels, decidual stromal cells and decidual maternal blood vessels
were also examined. IR expression in the VT was significantly increased in abortive tissue ($P = 0.003$) and also in hydatidiform mole ($P = 0.001$) compared with normal first trimester pregnancy (Fig. 2a–d).

Both mole pregnancy and abortion were accompanied by an increase of IR expression in EVT compared with normal pregnancy (both $P < 0.001$) (Fig. 2e).

**Figure 1** (a) Summary of staining results (IRs) of immunohistochemical localization of LR in human VT cells in normal first trimester pregnancy, abortion and hydatidiform mole (mean + SEM). (b) Expression of LR in normal human EVT cells, ×10 lens. Expression of LR is significantly enhanced in (c) abortive EVT cells ($P = 0.007$) and (d) hydatidiform mole EVT cells ($P < 0.001$), ×10 lens. Summary of staining results (IRs) of immunohistochemical localization of LR in (e) human EVT cells and (f) human glandular epithelial cells of the decidua in normal first trimester pregnancy, abortion and hydatidiform mole (mean + SEM).
IR expression in glandular epithelial cells of the decidua was significantly up-regulated in abortive tissue ($P < 0.001$) as well as in hydatidiform mole ($P < 0.001$) compared with normal first trimester pregnancy (Fig. 2f).

With regard to IR expression in villi fetal blood vessels, we found an increased expression in both abortive tissue ($P = 0.028$) and tissue of hydatidiform mole ($P < 0.001$) (Fig. 2g).

Figure 2 (a) Expression of IR in normal human EVT cells, $\times 25$ lens. Expression of IR is significantly enhanced in (b) abortive EVT cells ($P = 0.030$) and (c) hydatidiform mole EVT cells ($P = 0.001$), $\times 25$ lens. Summary of staining results (IRSs) of immunohistochemical localization of IR in (d) human VT cells, (e) human EVT cells, (f) human glandular epithelial cells of the decidua, (g) villi fetal blood vessels, (h) decidual stromal cells and (i) decidual maternal blood vessels in normal first trimester pregnancy, abortion and hydatidiform mole (mean $\pm$ SEM).
In abortive tissue \((P = 0.003)\) as well as in hydatidiform mole \((P = 0.001)\), IR expression in decidual stromal cells was significantly up-regulated in comparison to normal pregnancy (Fig. 2h).

Both mole pregnancy and abortion were accompanied by an increase of IR expression in decidual maternal blood vessels compared with normal pregnancy \((P = 0.007 \text{ and } P < 0.001)\) (Fig. 2i).

**Reverse transcriptase–polymerase chain reaction**

LR and IR mRNA expression were analyzed in abortive and normal control placental tissue by quantitative RT–PCR. LR mRNA expression was increased 4.8-fold for LR exon boundary 19–20 or 2.2-fold for LR exon boundary 6–7, respectively, compared with normal controls \((P = 0.003)\). A LR overlapping transcript and LR overlapping transcript-1 showed 1.15-fold up-regulation in abortive material. IR mRNA was 1.3-fold higher in abortive placental tissue compared with normal controls \((P = 0.013)\) (Fig. 3).

**Double immunofluorescence staining**

CK7 serves as a specific marker for EVT. EVT cells were identified as LR- and IR-expressing cells after co-incubation with CK7 (Fig. 4). We also examined glandular epithelial cells of the decidua where prolactin
Expression of IR and LR in pregnancy

Figure 3 Expression of LR mRNA (exon boundaries 19–20 and 6–7), LR overlapping transcript mRNA + LR overlapping transcript-1 mRNA, and in addition IR mRNA in abortive placental tissue compared with normal controls.

Figure 4 (a–c) LR (a) and CK7 (b) are expressed in EVT. Triple filter excitation shows expression of LR and CK7 in the same type of cells (c), all pictures ×20 lens. (d–f) IR (d) and CK7 (e) are expressed in EVT. Triple filter excitation shows expression of IR and CK7 in the same type of cells (f), all pictures ×20 lens.
serves as a specific marker. These cells were also identified as LR- and IR-expressing cells (Fig. 5).

**Discussion**

Within the present study, numerous differences in the expression of LR and IR in normal and disturbed pregnancy occurred. Highest expression of LR and IR was present in VT, EVT and glandular epithelial cells of the decidua of hydatidiform mole, which were significantly higher when compared with normal pregnancy. This was also true with regard to LR and IR expression in VT, EVT and glandular epithelial cells of the decidua of abortive compared with normal first trimester placenta.

Preliminary investigations revealed a possible role of leptin in normal and disturbed human pregnancy (Toth et al., 2008). 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. With regard to LR expression, again placentas of mole pregnancies and of miscarriages showed higher expression than placentas of normal pregnancies. This was also true for IR expression. Both mole pregnancy and miscarriage share a failure in placental (and fetal) development. This is accompanied by incomplete vascularization, endocrine dysfunction and hypoxia. A possible involvement of LR expression in RM was already indicated by Choi et al. (2003) showing increased expression of LR genes in chorionic villi of placentas from RM patients when compared with normal pregnancy. They were also able to demonstrate LR expression by RT–PCR chorionic villi, however, they did not further localize LR expression. Within our study, detailed localization of LR expression was possible at mRNA and protein level showing expression of LR in VT, EVT and glandular epithelial cells. In addition, we performed quantitative RT–PCR on different LR overlapping transcripts and LR exon boundaries. LR overlapping transcript gene shares the first and second exons with the LR (Kurokawa et al., 2008). Largest differences in LR expression were obtained with primer and probes designed between exon boundaries 19–20 (4.8-fold up-regulation of LR mRNA in abortive tissue). This assay detects the intact form of the LR. Only 2.2-fold up-regulation of abortive LR mRNA was obtained with primer and probes designed between exon boundaries 6–7. This assay also detects short or truncated forms of the LR (Uotani et al., 2006; Gao et al., 2008). Marginal up-regulation was found in abortive material with primer and probes for LR overlapping transcript genes. These genes share only the first and second exons with the LR. Therefore, we may conclude that abortive trophoblast cells express the long form of LR mRNA 4.8-fold compared with normal control trophoblasts.

Our work confirmed earlier work of Li et al. (2004) as the authors also showed significantly higher expression of leptin and LR in partial and complete mole pregnancies by immunohistochemistry. Although our results obtained by immunohistochemistry show semi-quantitative results and, therefore, are limited in giving exact numbers, immunohistochemistry is able to show exact location of protein expression.

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**Figure 5** (a–c) LR (a) and prolactin (b) are expressed in glandular epithelial cells of the decidua. Triple filter excitation shows expression of LR and prolactin in the same type of cells (c), all pictures ×20 lens. (d–f) IR (d) and prolactin (e) are expressed in glandular epithelial cells of the decidua. Triple filter excitation shows expression of IR and prolactin in the same type of cells (f), all pictures ×20 lens.
We performed real-time RT–PCR that is able to quantify gene expression in different tissues and we were able to confirm our immunohistochemistry results at mRNA level.

Although we focused on placental tissue from the first trimester of human pregnancy, Henson et al. (1998) investigated the expression of leptin and LR by RT–PCR in human placental villous tissue from term and earlier gestation (7–14 weeks). Leptin mRNA declined from earlier to late gestation, suggesting an ontogenetic decline in leptin mRNA with advancing gestation.

Castellucci et al. (2000) were able to demonstrate a strong expression of the LR in the distal extravillous cytotrophoblastic cells of cell columns invading the basal plate, whereas leptin expression was homogeneously expressed in all the cellular components of cell columns. Leptin was able to increase the secretion of immunoreactive MMP-2 and fetal fibronectin in a dose-dependent manner and enhanced the activity of MMP-9 in cultured cytotrophoblastic cells. They concluded that leptin and LR could play a role in the invasive processes of the extravillous cytotrophoblastic cells by modulating the expression of MMPs. A possible role of IR in the invasion process was also demonstrated by Diaz et al. (2007) showing that the IR regulates choriocarcinoma cell invasion.

Within our study, LR and IR expression is up-regulated in both mole and abortive placental tissue. In hydatidiform mole pregnancy, invasion of the trophoblast is disorganized. In abortive placentas, the EVT is recognized by the maternal immune system and, therefore, invasion may also be disregulated. Therefore, up-regulation of LR and IR in abortive EVT may be a compensation for this effect.

Identification of LR and IR expression in syncytiotrophoblasts, cells also responsible for the production of hormones vital to pregnancy maintenance, suggest a potential for autocrine or paracrine interactions within this tissue. The majority of IR and LR are expressed on structures that are currently assumed to drive placental growth, i.e. syncytiotrophoblastic sprouts and mesenchymal villi in first trimester placentas and fetal endothelium at term. Therefore, we hypothesize a growth promoting function, among others, of LR and IR in the placenta.

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