Aberrant expression of corticotropin-releasing hormone in pre-eclampsia induces expression of FasL in maternal macrophages and extravillous trophoblast apoptosis

G. Petsas1,†, U. Jeschke2,†, D.U. Richter3, V. Minas1, A. Hammer4, S. Kalantaridou1, B. Toth5, C. Tsatsanis6, K. Friese2, and A. Makrigiannakis1,*

1IVF Unit, Department of Obstetrics and Gynecology, Medical School, University of Crete, Heraklion 71110, Greece 2Department of Obstetrics and Gynecology, Ludwig-Maximilians-University of Munich, Maistrasse 11, 80337 Munich, Germany 3Department of Obstetrics and Gynecology, University of Rostock, Rostock, Germany 4Institute of Cell Biology, Histology and Embryology, Center of Molecular Medicine, Medical University of Graz, Harrachgasse 21, 8010 Graz, Austria 5Department of Gynecological Endocrinology and Fertility Disorders, University of Heidelberg, Vossstrasse 9, 69115 Heidelberg, Germany 6Department of Clinical Chemistry-Biochemistry, Medical School, University of Crete, Heraklion, Greece

*Correspondence address. Tel: +30-2810-392333; Fax: +30-2810-392131; E-mail: makrigia@med.uoc.gr

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ABSTRACT: Corticotropin-releasing hormone (CRH) and its receptors are expressed in human placenta. Recently, the impaired function of this system has been associated with a number of complications of pregnancy, including pre-eclampsia. The aim of the study was to test the hypothesis that CRH participates in the pathophysiology of pre-eclampsia through the induction of macrophage-mediated apoptosis of extravillous trophoblasts (EVTs). We found that the expression of CRH was increased in the EVT of the placental bed biopsy specimens from pre-eclamptic pregnancies (1.8-fold increase; \( P \), 0.05). In addition, significantly larger numbers of apoptotic EVT were detected in pre-eclamptic placentas compared with normal ones (\( P \), 0.05), and only in pre-eclamptic placentas, decidual macrophages were found to be Fas ligand (FasL)-positive.

In vitro studies on the effect of CRH on human macrophages suggested that CRH induced the expression of the FasL protein in human macrophages and potentiated their ability to induce the apoptosis of a Fas-expressing EVT-based hybridoma cell line in co-cultures. These findings demonstrate a possible mechanism by which the aberrant expression of CRH in pre-eclampsia may activate the FasL-positive decidual macrophages, impair the physiological turnover of EVT and eventually disturb placentation.

Key words: CRH / FasL / macrophages / EVT / pre-eclampsia

Introduction

Pre-eclampsia is a multisystem disorder that is unique to human pregnancy. The incidence of pre-eclampsia is 2–10% depending on the population studied and definitions of pre-eclampsia (World Health Organization International Collaborative Study of Hypertensive Disorders of Pregnancy, 1988). This common disorder is associated with the highest maternal and fetal morbidity and mortality of all pregnancy complications, with >90% of the most serious outcomes occurring in developing countries (Villar et al., 2003). Despite the widespread occurrence of pre-eclampsia, the underlying cause or causes of the disease remain elusive. Accumulated evidence, however, strongly suggests that the causative agent is the placenta (Redman, 1991).

Several studies have emphasized the importance of trophoblast in the pathophysiology of pre-eclampsia. Increased apoptosis of the
extravillous trophoblast (EVT) in the placental bed has been reported in pre-eclampsia (DiFederico et al., 1999). Serum from pre-eclamptic women has been shown to reduce trophoblast viability, and this effect seems to be related to changes in trophoblast sensitivity to Fas-mediated apoptosis (Neale et al., 2003). The Fas/Fas ligand (FasL) system is one of the main apoptotic pathways in cells and tissues (Nagata, 1994). Both villous (Bamberger et al., 1997; Hammer et al., 1999) and EVT cells (Hammer et al., 1999; Kauma et al., 1999) express Fas and/or FasL. The binding of the Fas receptor by FasL results in downstream activation of a cascade of intracellular proteolytic enzymes ending in the apoptosis of the Fas-bearing cell (Aschke-nazi et al., 2002). Although a greater incidence of EVT apoptosis has been detected in placentas from pregnancies complicated by pre-eclampsia, the role of Fas/FasL system in the regulation of trophoblast apoptosis and its potential role in the pathophysiology of pre-eclampsia have not been yet fully elucidated.

Besides increased EVT apoptosis, pre-eclampsia is also associated with increased infiltration of activated maternal macrophages around the non-remodeled spiral arteries (Reister et al., 1999) and changes in their distribution pattern. Normally, macrophages are located in the stroma surrounding the EVT and spiral arteries. In pre-eclamptic placentas, however, macrophages have been observed within and around the spiral arteries separating them from the trophoblast cells (Reister et al., 2001; Abrahams et al., 2004). Moreover, in pregnancies complicated by pre-eclampsia, activated macrophages around the spiral arteries have been shown to secrete tumor necrosis factor-α (TNF-α) at levels that lead to the apoptotic death of the invading EVT and therefore prevent them from invading and remodeling the vessels (Reister et al., 2001). However, the cellular interactions and timeline of events during spiral artery remodeling remain still poorly defined. Recently published data suggest that spiral artery remodeling occurs in distinct trophoblast-independent and -dependent stages and provide the first evidence of decidual macrophage involvement in trophoblast-independent stages (Smith et al., 2009).

It is suggested that abnormalities of the placental corticotropin-releasing hormone (CRH) system are implicated in the pathogenesis of pre-eclampsia (Karteris et al., 2003, 2005). The hypothalamic neuropeptide CRH is the principal mediator of the stress response in mammals. Its major role is to orchestrate the integrated stress response by coordinating a series of homeostatic mechanisms, involving the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Bale and Vale, 2004). Beyond its classical role, increasing evidence suggests a role for CRH in reproductive function. CRH and its type I and type 2 receptors (CRH-R1 and CRH-R2) are expressed at the fetoplacental interface (Hillhouse and Grammatopoulos, 2002). A number of autocrine/paracrine roles have been shown so far for placental CRH including the regulation of trophoblast invasion, the placental vasculature, myometrial contractility and onset of labor (Hillhouse and Grammatopoulos, 2002; Kalantaridou et al., 2003; Bamberger et al., 2006). Interestingly, CRH might regulate apoptosis at the fetoplacental site through the regulation of the FasL in trophoblasts and decidual lymphocytes (Makrigiannakis et al., 2001; Minas et al., 2007). In pre-eclampsia, maternal plasma and cord venous plasma CRH concentrations were found to be significantly elevated compared with normal pregnancies (Goland et al., 1993; Warren et al., 1995; Laatikainen et al., 1991). In the present study, we tested the hypothesis that aberrantly expressed CRH in pre-eclamptic placentas plays a role in the pathophysiology of excessive trophoblast apoptosis observed in pre-eclampsia via modulating the function of decidual macrophages.

### Materials and Methods

#### Placental tissues

Placental tissues were obtained from 16 women who underwent delivery at the First Department of Obstetrics and Gynaecology of the LMU Munich. Specimens were collected immediately after delivery from eight patients with pre-eclampsia (mean date of delivery: 34 ± 3.2 weeks of gestation) and eight gestational age-matched patients (mean date of delivery: 35.2 ± 3 weeks of gestation) following a normal course of pregnancy. Pre-eclampsia was diagnosed according to the following criteria, recommended by the National High Blood Pressure Education Program (NHBPEP): systolic blood pressure of ≥140 mmHg or diastolic blood pressure of ≥90 mmHg, accompanied by new-onset proteinuria, defined as ≥300 mg per 24 h (Gifford et al., 2000). Exclusion criteria for both groups included chorioamnionitis, chronic hypertension, chronic renal disease, cardiac disease, connective-tissue disease, pre-existing diabetes mellitus and gestational diabetes mellitus. The study had the approval of the local ethical committee of the LMU Munich, Germany (No. 158/00), and informed consent from the patient was obtained.

#### Cell lines

The cells used for in vitro apoptosis experiments are EVT-choriocarcinoma hybrid cells (clone AC1MB8) (hereafter hyEVT), which have been described previously (Funayama et al., 1997). The cells were kept in Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with 10% fetal calf serum, 1% L-glutamine 200 mM, 1% Penicillin–Streptomycin. HyEVT cells were kept in RPMI containing 10% fetal calf serum, 1%L-glutamine 200 mM, 1% Penicillin–Streptomycin (all from Invitrogen, Carlsbad, CA, USA) at 5% CO2 and 37°C.

#### Immunohistochemistry

Immunohistochemistry on paraffin sections (7 μm) of the different specimens was done by incubating the slides in methanol/H2O2 (30 min) to inhibit endogenous peroxidase activity, followed by washing in phosphate-buffered saline (PBS; 5 min) and treating with goat serum (20 min, 22°C) to reduce non-specific background staining. Incubation with one of the primary antibodies (CRH antibody was obtained from Phoenix Pharmaceutica, USA, cytokeratin-7 (CK7) antibody was obtained from Progen Biotechnik, Heidelberg, Germany, M30 antibody was obtained from Axxora, Gruenberg, Germany, and Fas antibody was obtained from Phar-mingen, Heidelberg, Germany) was done overnight at 4°C. Sections were then thoroughly incubated with the appropriate biotinylated secondary antibody (1 h, 22°C) and streptavidin-conjugated peroxidase [45 min, room temperature (RT)]. Between each step, sections were washed with PBS (pH 7.4). Peroxidase staining reaction was done with diaminobenzidine/H2O2 (1 mg/ml; 2 min) and stopped in tap water (10 min). Sections were counterstained in hemalaun (1 min) and then cover-slipped. Control experiments encompassed immunohistochemistry with immunonneutralization of primary antibodies by pre-incubation with the respective peptides. From each section, five digital images were obtained with a 3CCD color camera (JVC, Victor Company of Japan, Japan) and a Leitz (Wetzlar, Germany) microscope.

#### In situ nick translation

Staining of DNA fragmentation and apoptotic bodies was performed on frozen tissue slides using the in situ nick translation (ISNT) histochemical
Cytokeratin-positive EVTs were counted in randomly selected microscope sections from each tissue block. Apoptotic and total 11–50% of the cells; 3, 51–80% of the cells; 4, 81–100% of the cells. The number of apoptotic EVT was counted and plated in 24-well plates. After 2 h, non-adherent cells were discarded. The adherent fraction of peripheral blood mononuclear cells (PBMCs) consists of peripheral monocytes/macrophages. Purity of macrophages (above 90%) was confirmed by staining with an anti-CD68 marker (BD Biosciences, Heidelberg, Germany). Macrophage viability was microscopically determined by Trypan blue exclusion. Only samples with ≥90% viable macrophages were used. Cells were subsequently stimulated with CRH (Sigma) at a concentration of 10^{-8} M and 10 μg/ml of Escherichia coli-derived lipopolysaccharides (LPS) (serotype 011:B4, Sigma). For the isolation of PBMCs, we used peripheral venous blood from healthy third-trimester pregnant women not taking any drugs.

### Western blot analysis

Western blot analysis for the detection of FasL and actin was performed as described previously (Dermitzaki et al., 2002), using the antibodies anti-Fasl (Santa Cruz Biotechnologies) and anti-Actin (MAB1501, Chemicon, USA). Briefly, protein content in the lysates was measured by Bradford assay. SDS–polyacrylamide gel electrophoresis sample loading buffer was added in 10 μg of protein from each lysate electrophoresed through a 12% SDS polyacrylamide gel. Protein was transferred to nitrocellulose membranes, using an LKB electrophoresis transfer system (LKB, Bromma, Sweden). Membranes were processed according to the standard western blotting procedures. To detect protein levels, membranes were incubated with the appropriate antibodies and then exposed to Kodak X-omat AR films. A PC-based Image Analysis was used to quantify the intensity of each band (Image Analysis Inc., Ontario, Canada). To normalize for protein content, the blots were stripped in stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS, 100 mM μg-mercaptoethanol) and stained with anti-actin antibody (Chemicon). The concentration of FasL protein in each lysate was normalized versus actin. The analysis was performed three times for macrophages isolated from PBMCs as described previously, before and after stimulation with CRH or E. coli-derived LPS.

### Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated from fresh EDTA-K	extsubscript{3} anti-coagulated peripheral blood samples from healthy non-obese donors by Lymphoprep density centrifugation (Nycodema Pharma AS, Norway) as described previously (Christoforidou et al., 2004). Cells were washed twice in PBS, resuspended in DMEM (containing 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin), counted and plated in 24-well plates. After 2 h, non-adherent cells were discarded. The adherent fraction of peripheral blood mononuclear cells (PBMCs) consists of peripheral monocytes/macrophages. Purity of macrophages (above 90%) was confirmed by staining with an anti-CD68 marker (BD Biosciences, Heidelberg, Germany). Macrophage viability was microscopically determined by Trypan blue exclusion. Only samples with ≥90% viable macrophages were used. Cells were subsequently stimulated with CRH (Sigma) at a concentration of 10^{-8} M and 10 μg/ml of Escherichia coli-derived lipopolysaccharides (LPS) (serotype 011:B4, Sigma). For the isolation of PBMCs, we used peripheral venous blood from healthy third-trimester pregnant women not taking any drugs.

### Immunohistochemical evaluation

The intensity and distribution of the specific immunohistochemical staining reaction was evaluated using a semi-quantitative method [immunoreactive score (IRS)] as described previously (Remmelle and Stegner, 1987). The IRS was calculated by the multiplication of optical staining intensity (graded as: 0, no; 1, weak; 2, moderate; 3, strong staining) and the percentage of positive stained cells (0, no staining; 1, <10% of the cells; 2, 11–50% of the cells; 3, 51–80% of the cells; 4, >81% of the cells). To evaluate early apoptosis (cyto-death staining) and late (ISNT) apoptosis, 5–8 sections from each tissue block were examined. Apoptotic and total cytokeratin-positive EVTs were counted in randomly selected microscope fields at a magnification of ×400. The number of apoptotic EVT was expressed as a percentage of the total number of cytokeratin-positive EVT counted in each slide (at least 300). Each slide was independently evaluated by two investigators.

### Immunofluorescence

Double immunofluorescence was performed as described (Hammer et al., 2001). Briefly, sections were thawed, fixed once more in acetone at RT for 5 min and rehydrated in PBS. After blocking for 10 min with ultraviolet block (Dako, Vienna, Austria), the sections were incubated with the polyclonal rabbit antibody Q20 (anti-human Fas-ligand; Santa Cruz Biotechnologies, CA, USA) for 30 min at RT, followed by an incubation step with a cyanine-2 (Cy2) goat anti-rabbit IgG (Dianova, Hamburg, Germany) in a dilution of 1:400. Afterwards, the sections were incubated for 30 min with a phycoerythrin (PE) labeled anti-CD14 mAb (Pharmingen) in a dilution of 1:25. PBS was used for washing sections between different incubation steps. Sections were mounted with Moviol (Calbiochem-Novabiochem, La Jolla, CA, USA) and analyzed on a confocal laser scanning microscope (Leica SP2, Leica Lasertechnik GmbH, Heidelberg, Germany), using the 488-nm laser line for the excitation of Cy2 and the 543-nm laser line for PE. Cy2 staining is colored in green, whereas PE staining is colored in red. In the merged images, co-localization appears yellow. Control experiments encompassed immunofluorescence (i) without primary detection antibodies, (ii) with monoclonal or polyclonal non-immune antibodies as primary antibodies and (iii) without secondary antibodies.

### Apoptosis assays for in vitro experiments

The APOPercentage Apoptosis Assay (Biocolor, Newtownabbey, Northern Ireland) and fluorescence-activated cell sorting (FACS) analysis of AC1M88 cells stained with annexin and propidium iodide (BD Biosciences) were employed. Briefly, hyEVT cells were plated in 24-well plates (2 × 10^5 cells/well) and cultured for 2–3 days until confluence in DMEM/F12 medium ± 2 μg of anti-Fas blocking antibody (SM1/23, ALEXIS Bio-technologies, San Diego, CA, USA). PBMCs were seeded in co-culture inserts (2 × 10^5) (0.2 Anopore membrane, Nunc) with DMEM/F12. After 2 h of culture at 37°C, 5% CO	extsubscript{2}, supernatant and non-adherent cells were discarded and adherent cells (mostly macrophages) were carefully washed. This isolation protocol resulted in greater than 90% purity and viability of macrophages, as revealed by Giemsa staining and anti-CD68 immunocytochemistry. The macrophages were either not pretreated with any additives or pretreated for 24 h with CRH ± antiallantin (Tocris, Bristol, UK, and Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA, respectively). Co-cultures were performed by placing the co-culture inserts, containing macrophages within the 24-well plates containing hyEVT cells. The cells were co-cultured for 24 h in the presence of the additives (freshly prepared), which were used for their pretreatment, or in medium alone. Then, the macrophages were discarded and APOPercentage assay was performed according to the manufacturer’s instructions (Johnson et al., 2003) or the wells were forcefully rinsed to recover the trophoblasts for annexin-Pi stain and FACS analysis. The latter was performed as previously described (Makrigiannakis et al.,
2001) with FACScan (Becton Dickinson, Heidelberg, Germany), and the results were analyzed with the FACScan CELLQUEST software. Annexin-positive cells were considered apoptotic. The experiments were performed four times.

**Statistical analysis**

The SPSS/PC software package, version 11.01 (SPSS, Munich, Germany), 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-values of <0.05 were considered statistically significant.

**Results**

**Expression of CRH protein is up-regulated in pre-eclamptic placentas**

Immunohistochemical evaluation of CRH protein expression in the placental bed biopsies from pre-eclamptic (n = 8) and normal (n = 8) placentas showed that the majority of EVT in pre-eclamptic placentas expressed CRH (Fig. 1C), whereas fewer EVT stained for CRH protein in control placentas (Fig. 1A). EVTs were identified as such by labeling of serial sections with anti-CK7 antibody (Fig. 1B and D). IRS scoring revealed a 1.8-fold increase in CRH staining intensity in cases of pre-eclamptic (3 ± 0.4) compared with control (1.7 ± 0.5) placentas (Fig. 1E and F; P < 0.05).

**Pre-eclampsia is associated with elevated rates of EVT apoptosis**

The rate of early apoptosis evaluated by M30 staining in normal EVT cells was below 10% (6.6 ± 0.5%) in all control cases (n = 8) investigated (Fig. 2A). In pre-eclampsia (n = 8), EVT early apoptosis was elevated up to 25% (25.4 ± 11.8%; Fig. 2C). These results are summarized in Fig. 2E and F (P < 0.05). The rate of late apoptosis indicated by ISNT in normal (n = 8) EVT cells ranged between 1 and 5% (Fig. 2G). In pre-eclampsia (n = 8), EVT late apoptosis was elevated up to 15% (Fig. 2I). These results are summarized in Fig. 2K (P < 0.05). To identify EVT cells, CK7 staining was performed on parallel sections (Fig. 2B, D, H and J).

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**Figure 1** Immunohistochemical expression of CRH in normal and pre-eclamptic placentas. Representative photos of CRH (A and C) and CK7 (B and D) expression in serial sections in normal (A and B) and pre-eclamptic placenta (C and D). Please note that only a few EVTs were stained for CRH in normal placenta, whereas most of EVTs were strongly positive for CRH in pre-eclamptic placenta (×100). (E) Staining intensity was determined by the semi-quantitative immunohistochemical IRS. There was a statistically significant increase in CRH expression between normal and pre-eclamptic placentas. Data represent the mean ± standard error (*P < 0.05). (F) Scattergram of CRH staining intensities in EVT of normal and pre-eclamptic placentas.
Pre-eclampsia is associated with expression of FasL in decidual macrophages

In normal placentas ($n=8$), decidual macrophages, identified by CD14 staining, did not express FasL protein (Fig. 3A–C). Contrarily, in pre-eclampsia ($n=8$), deciduas were rich in FasL-positive macrophages (Fig. 3D–F). Furthermore, in pre-eclampsia, T cells and natural killer cells did not express FasL as shown by double staining for FasL and CD3 or CD56 (data not shown).

Detection of Fas in EVT of normal and pre-eclamptic placentas

To investigate the expression of Fas in EVT, immunohistochemistry was performed. In both normal ($n=8$) and pre-eclamptic ($n=8$) placentas, EVT expressed the Fas peptide (Fig. 4A and C, respectively), with no significant difference in staining intensities. EVTs were identified as such by labeling of serial sections with anti-CK7 antibody (Fig. 4B and D).
Figure 3  Expression of FasL in normal and pre-eclamptic placentas. (A–F) Representative photos of double immunofluorescence for FasL (green) (A and D) and CD14 (red) (B and E) in normal (A–C) and pre-eclamptic (D–F) placenta. Yellow colored cells representing decidual macrophages expressing FasL were detected only in pre-eclamptic placentas (F) (×400).

Figure 4  Expression of Fas in normal and pre-eclamptic placentas. Immunohistochemistry using antibodies against Fas (A and C) and CK7 (B and D) in serial sections of normal (A and B) and pre-eclamptic (C and D) placentas showed Fas expression in EVT in both cases. No statistically significant difference in IRS was observed between normal and pre-eclampsia (data not shown) (×250).
CRH up-regulates the expression of FasL protein in human macrophages

To investigate the effect of CRH on FasL expression in human macrophages, western blot was performed. Incubation of human macrophages with $10^{-8}$ M CRH for 6 h, increased FasL by 1.5-fold compared with control ($n = 3$, $P < 0.05$). The effect of CRH was comparable with that of E. coli-derived LPS at $10^{-m}$g/ml (Fig. 5A). The results of the FasL expression of three independent experiments are presented in a scattergram (Fig. 5B).

CRH potentiates the ability of macrophages to induce Fas-mediated apoptosis of hyEVT

All additives were initially tested for their toxic effect on hyEVT cultured alone for 24 h. FACS analysis of annexin-Pi stained cells revealed no toxic effect for any of the additives that were subsequently used in co-cultures of hyEVT with macrophages ($n = 4$; Fig. 6A).

Macrophages pretreated for 24 h with 100 nM CRH and then co-cultured with hyEVT for 24 h in the presence of 100 nM CRH, showed increased apoptotic activity against hyEVT compared with untreated macrophages (25 ± 0.9 and 4.3 ± 1.8% apoptotic hyEVT, respectively). This effect was specifically mediated through CRH-R1 since the addition of antalarmin completely reversed it. Blocking of Fas receptor in hyEVT also significantly reduced the levels of hyEVT apoptosis in co-cultures with macrophages. Our findings underlie the potential role of the ‘CRH-macrophage-Fas/FasL triad’ in the pathogenesis of pre-eclampsia.

In recent years, CRH has been found to serve a number of functions outside the classic neuroendocrine domain. Its roles in female reproductive physiology and pathophysiology are gradually being clarified (Makrigiannakis et al., 2006; Kalantaridou et al., 2007). Abnormally increased maternal plasma CRH has been reported by various groups in pregnancies complicated by pre-eclampsia (Petraglia et al., 1996; Leung et al., 2000). Additionally, in patients with pre-eclampsia, the mean umbilical cord plasma CRH and the concentration of circulating CRH mRNA were shown to be significantly higher (Goland et al., 1993; Ng et al., 2003). The vasodilatory response of CRH in the human fetal-placental circulation (Clifton et al., 1994) led Florio et al. (2002) to suggest that in pre-eclampsia, the placenta takes part in a stress syndrome by releasing CRH, which may help to influence uterine perfusion, thereby protecting the fetus from a hostile environment. The compensatory increase in CRH secretion from human placental tissues in maternal pathologic stress conditions is reinforced by McLean et al. These authors suggest that placental CRH activates a number of potentially useful adaptive responses for the stressed fetus. These include the acceleration of fetal organ maturation, maximization of placental blood flow and the early initiation of labor when fetal survival is threatened (McLean and Smith, 1999).

Discussion

In this study, we have found that pre-eclampsia is associated with the up-regulation of CRH in EVT from affected pregnancies, a significant increase in FasL-positive maternal macrophages, the expression of Fas peptide in EVT and the increased apoptosis of EVT in situ. Furthermore, we examined the possible pathway by which CRH accomplishes its role in the pathogenesis of pre-eclampsia. Our data show that CRH stimulated the expression of FasL in human macrophages, thereby potentiating their ability to induce the apoptosis of the Fas-expressing EVT-based hybridoma cell line. This effect of CRH appeared to be specifically mediated through its type I receptor (CRH-R1), since the addition of the appropriate antagonist, antalarmin, completely reversed it. Blocking of Fas receptor in hyEVT also significantly reduced the levels of hyEVT apoptosis in co-cultures with macrophages. Our findings underlie the potential role of the ‘CRH-macrophage-Fas/FasL triad’ in the pathogenesis of pre-eclampsia.
Contrarily to the above findings, we propose here a possible detrimental role for CRH in the pathogenesis of pre-eclampsia. Our in vitro data show that CRH can induce the expression of FasL in human macrophages which in turn induce the apoptosis of EVT. That suggests that in pre-eclamptic placentas, the heightened sensitivity of trophoblast cells to apoptosis may be due in part to the CRH-induced alterations in the Fas–FasL system. Our results also show that EVT might be responsible themselves for the increased expression of placental CRH in pre-eclampsia. These findings raise the question whether this deranged secretion of CRH is part of the primary pathophysiology of pre-eclampsia, i.e. whether it has a detrimental effect in placentation which occurs in first and mid-trimester or occurs as a secondary response to the increased vascular resistance later on. Our placental tissues where obtained at around 34 weeks of gestation. It would be impossible to perform similar experiments in 18-week placentas, the time when spiral artery remodeling completes, as we would not be able to follow-up their fate, in terms of whether the patients developed pre-eclampsia or not. However, considering the data of Leung et al. (2000), who showed elevated plasma CRH in mid-trimester in patients who went on to develop pre-eclampsia later, we suggest that there might be a potential causative link between a deranged CRH system and pre-eclampsia as a result of defected placentation.

Further detrimental role of CRH in pre-eclampsia has been suggested by Karteris et al. (2003), who proposed that the dampening of CRH-induced vasodilation in pre-eclamptic pregnancies could be attributable to the reduced expression of CRH receptors (CRH-Rs) probably seen due to chronic exposure to elevated levels of placental CRH. Furthermore, according to Karteris et al. in the pre-eclampsia, down-regulation of CRH-Rs can lead to diminished regulation of the nitric oxide–cyclic guanosine 3,5-monophosphate (NO/cGMP) pathway, which appears to be one of the signaling pathways mediating CRH actions during pregnancy. The impaired NO/cGMP pathway may contribute in the disturbance of the balance controlling vascular tone in feto-placental circulation toward vasoconstriction and the clinical sequelae of pre-eclampsia (Karteris et al., 2005).

Regarding the pathogenesis of pre-eclampsia an old hypothesis claims that this pregnancy-specific disorder is associated with a generalized impairment of trophoblast invasion. According to DiFederico et al. (1999), widespread apoptosis of EVT might explain the insufficient/shallow interstitial and endovascular trophoblast invasion
observed in pre-eclamptic pregnancies. Contrarily, a study by Kadyrov et al. (2003) suggests that the reduced trophoblast invasiveness in pre-eclampsia cannot be explained by higher rates of apoptosis as the apoptosis index found in pre-eclampsia is even lower than in normal pregnancy with normal invasiveness. However, the same group in a more recent study reported an increased frequency of apoptosis of intramural and endovascular trophoblast in pre-eclamptic placentas. They proposed that this may account for the reduced invasion of spiral arteries from pregnancies complicated by early-onset pre-eclampsia and intrauterine growth restriction (Kadyrov et al., 2006).

As no consensus exists at present in the literature, our current study establishes that pre-eclampsia is associated with elevated levels of early and late apoptosis evaluated by M30 immunohistochemistry and ISNT, respectively. By confirming elevated rates of apoptotic death in pre-eclamptic placental tissues with M30 immunohistochemistry, we postulate that the overestimation of apoptotic events has been avoided as necrosis leading to DNA breakdown and false-positive ISNT staining does not result in CK18 cleavage and M30 positivity (Sakagami et al., 1999).

In pre-eclampsia, the concept of excessive apoptosis as a mechanism to limit trophoblast invasion into the walls of spiral arteries raises the possibility of apoptosis-promoting signals derived from maternal cells. Reister et al. described activated maternal macrophages in close vicinity to spiral arteries of the placental bed that are capable of inducing trophoblast apoptosis. Induction of apoptosis was achieved by the concerted action of the secretion of TNF-α that binds to the trophoblastic TNF type-I receptor and the secretion of indolamine 2,3-dioxygenase that catabolizes and depletes the local levels of tryptophan (Reister et al., 2001). However, Renaud et al. (2005) recently suggested that during pre-eclampsia, the abnormal increase in macrophage infiltration around spiral arteries hampers trophoblast invasion and vascular remodeling through a non-apoptotic mechanism. According to this group, the aberrant presence of activated macrophages around uterine vessels may contribute to inadequate trophoblast invasion and remodeling of the uterine spiral arteries by inducing the secretion of plasminogen activator inhibitor 1 and inhibiting the pro-urokinase plasminogen activator-associated caseinolytic activity through TNF-α secretion (Renaud et al., 2005). Interestingly, our results show for the first time that in pre-eclampsia, decidual macrophages become strongly positive for FasL, a ligand which is known to induce apoptosis in Fas-expressing EVT (Minas et al., 2007).

Previous studies of our group have shown a possible direct role for CRH in the pathophysiology of clinical conditions associated with defective placentation (Bamberger et al., 2006; Minas et al., 2007). Our present data enlighten its possible role in the pathogenesis of pre-eclampsia. In vitro, CRH stimulated the expression of FasL in macrophages, thereby potentiating their ability to induce the apoptosis of Fas-bearing EVT cells. Relevance of these data needs very cautious interpretation as peripheral blood-derived macrophages may be significantly different from their uterine counterparts. However, most macrophage functions, including cell-surface marker expression, were shown to be unchanged when decidual and peripheral blood-derived macrophages were co-cultured with trophoblasts (Rozner et al., 2011). Recently published data suggest that the Fas/FasL pathway can be employed by macrophages to induce vascular smooth muscle cell apoptosis (Imanishi et al., 2002). Furthermore, during normal pregnancy, macrophages together with trophoblast cells may induce Fas-mediated apoptosis in endothelial and vascular smooth muscle cells to promote spiral artery transformation (Keogh et al., 2004; Ashton et al., 2005). In pregnancies complicated with pre-eclampsia, however, Mor and Abrahams (2003) proposed that activated macrophages secrete pro-inflammatory cytokines, such as TNF-α and interferon-γ which induce the apoptosis of EVT. Studies of this group indicate that enhanced levels of pro-inflammatory macrophage products increase Fas expression and enhance trophoblast sensitivity to Fas-mediated apoptosis (Aschkenazi et al., 2002). This suggests a differential role for macrophages in trophoblast invasion and spiral artery transformation according to their activation status. Whereas, in normal pregnancies, macrophages function as support cells by facilitating transformation of the spiral arteries, in pre-eclampsia, macrophages act as barrier to trophoblast invasion by inducing trophoblast apoptosis, thereby preventing spiral artery transformation (Mor and Abrahams, 2003). Finally, we tried to confirm our in situ findings with a functional mechanism in vitro. We, therefore, used a co-culture system to test the hypothesis that CRH-induced expression of FasL in macrophages potentiates the ability of these cells to induce the apoptosis of EVT. Co-cultures of macrophages with a Fas-expressing EVT-based hybridoma cell line, revealed higher rates of apoptosis in hyEVT when co-cultures were performed in the continuous presence of CRH. Our data suggest that in pre-eclampsia, CRH exerts immunomodulatory effects acting on macrophages at the fetomaternal interface. The ability of CRH to modulate the function of immune cells has been reported previously (Angioni et al., 1993; Tsatsanis et al., 2006). It is suggested that in contrast to its systemic indirect immunosuppressive effects through the HPA axis, CRH produced locally at inflammation sites acts as a potent autocrine and/or paracrine pro-inflammatory cytokine (Karalis et al., 1991). Recently, Agelaki et al. (2002) suggested that CRH signals play a crucial role in augmenting pro-inflammatory cytokine production by macrophages. In accord with these observations, we propose here that in pre-eclampsia, CRH potentiates the cytotoxic effect of macrophages both directly through the up-regulation of FasL and indirectly through the production of macrophage-derived pro-inflammatory cytokines, suggesting that CRH controls a complex crosstalk between macrophages and EVT facilitating both cell-to-cell interaction and paracrine actions.

Taken together our findings of CRH up-regulation in pre-eclamptic placentas in situ, the presence of FasL-positive decidual macrophages in pre-eclampsia and the ability of CRH to induce the expression of FasL in human macrophages suggest that the immunomodulatory role of peripheral CRH may disrupt trophoblast invasion and remodeling of spiral arteries predisposing to placental dysfunction associated with later appearance of pre-eclampsia. Determining the mechanisms that control early placentation may have implications for therapeutic intervention in pregnancy complications associated with defective placentation such as pre-eclampsia.

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**Authors’ roles**

G.P. and U.J. participated in the study design, did the data analysis and interpretation and wrote the manuscript. D.U.R. and A.H. processed the placental bed biopsy specimens and performed the immunohistochemical evaluation. V.M., S.K. and B.T. contributed to the review of the manuscript. C.T. performed the isolation of PBMCs and western blot analysis. K.F. and A.M. designed the study and reviewed the intellectual content of the manuscript.

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**Conflict of interest**

None declared.

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