Calreticulin in human pregnancy and pre-eclampsia

V.Y. Gu1,2, M.H. Wong1,2, J.L. Stevenson1, K.E. Crawford1,2, S.P. Brennecke1,2 and N.M. Gude1,2,3

1Department of Perinatal Medicine, Pregnancy Research Centre, Royal Women’s Hospital, 132 Grattan Street, Carlton, VIC 3053, Australia; 2Department of Obstetrics and Gynaecology, University of Melbourne, Parkville, VIC, Australia
3Correspondence address. E-mail: neil.gude@rwh.org.au

Pre-eclampsia is a disorder of human pregnancy that involves pregnancy-induced maternal hypertension and proteinuria. Evidence indicates that pre-eclampsia involves widespread activation of maternal endothelial cells. Calreticulin is a ubiquitously expressed, multi-functional protein that has been shown to have both pro- and anti-inflammatory effects on cultured endothelial cells in vitro and in whole animals. In order to clarify the role of this protein in normal human pregnancy and in pre-eclampsia, this study has measured expression of calreticulin in maternal blood and in placenta in patients with pre-eclampsia and in control pregnancies. There was a significant increase (\(\sim 5\)-fold) in calreticulin in plasma in term pregnant women compared with women who were not pregnant. There was no difference, however, in calreticulin in plasma from women who were sampled at first trimester, second trimester and at term. In addition, there was a significant increase (\(\sim 50\%\)) in calreticulin in plasma from pre-eclamptic women compared to controls. Calreticulin mRNA and protein expression in placenta were not changed between pre-eclampsia and control pregnancies. These novel results indicate that calreticulin is increased in peripheral maternal blood early in pregnancy and remains elevated throughout normal gestation and that there is a further increase in calreticulin in pre-eclampsia.

Keywords: calreticulin; pre-eclampsia; pregnancy; placenta

Introduction

Pre-eclampsia is a multi-system syndrome that is exclusive to human pregnancy (Redman and Sargent, 2005). It is one of the leading causes of maternal death (WHO, 2005). It usually occurs during the latter half of gestation and is characterized by pregnancy-induced maternal hypertension and proteinuria (Higgins and Brennecke, 1998). In severe cases, maternal renal failure, elevated liver enzymes, pulmonary oedema, thrombocytopenia, diffuse intravascular coagulation, haemolysis and eclampsia may also be present (Australasian Society for the Study of Hypertension in Pregnancy, 1993). The fetus is commonly affected, with 25% of cases complicated by fetal growth restriction (FGR) (Brown, 2003). Management of pre-eclampsia involves rigorous antenatal monitoring and symptomatic treatment (Australasian Society for the Study of Hypertension in Pregnancy, 1993), with the only cure being delivery of the fetus and removal of the placenta, which can be required before the pregnancy reaches full term.

Although the pathogenesis of pre-eclampsia is not well understood, it is recognized that the presence of the placenta is a key to its development. Pre-eclampsia is thought to involve abnormally shallow invasion of placental trophoblast cells into the maternal uterine decidua and incomplete uterine spiral arteriole remodelling (Redman and Sargent, 2005). This results in vessels that retain much of their endothelium and smooth muscle and hence they remain relatively low in capacity and resistant to blood flow (Zhou et al., 1997). Owing to the consequent reduced blood flow to the placenta, trophoblast cells suffer oxidative stress and release back into the maternal circulation toxic metabolic factors that cause widespread activation of maternal endothelium (Redman and Sargent, 2005). Perturbed endothelial cells promote vasoconstriction and micro-thrombi formation, which lead to hypoperfusion of most, if not all, maternal organs, and the features of pre-eclampsia that are observed in the clinical setting (Baker et al., 1995).

Changes in the balance of vasodilators (such as nitric oxide and prostacyclin) and vasoconstrictors (such as thromboxane and endothelin) towards vasoconstriction have been described in pre-eclampsia, and are believed to affect endothelial-dependent vascular relaxation and smooth muscle contractility. Recently, evidence indicates that factors that regulate vascular growth and permeability also play a pathogenic role in pre-eclampsia. The circulating anti-angiogenic protein, soluble fms-like tyrosine kinase, was increased in placenta and blood of women with pre-eclampsia (Maynard et al., 2003; Levine et al., 2004), and circulating concentrations of vascular endothelial growth factor and placental growth factor were decreased with pre-eclampsia. Elevated serum concentrations of other angiogenesis inhibitors have also been reported in pre-eclampsia, e.g. endostatin (Hirtenlehner et al., 2003) and endoglin (Venkatesha et al., 2006). Subsequent to these observations it has been postulated that pre-eclampsia involves an imbalance of circulating angiogenic/anti-angiogenic placental factors that contribute to endothelial cell dysfunction (Levine et al., 2004). Other as yet uncharacterized factors may play similar roles in pregnancy and pre-eclampsia. One such factor with demonstrated anti-angiogenic activity that has not been well described in pregnancy is the protein calreticulin.

Calreticulin was first identified in the sarcoplasmic reticulum of skeletal muscle as a 55 kDa high affinity calcium-binding protein (Ostwald and MacLennan, 1974). Subsequently, it has been found in
the endoplasmic reticulum of most tissues and cells, including placenta (Hojrup et al., 2001) and heart (Michalak et al., 2004). It has three domains, two of which bind calcium with differing capacities and affinities. It has been described as a calcium-binding molecular chaperone protein and forms part of the quality control systems of the endoplasmic reticulum for newly synthesized proteins. In a soluble form, and in association with the homologous membrane bound protein calnexin, calreticulin binds to glycoproteins, preventing aggregation and allowing the proteins to attain their correct folding conformation (Bedard et al., 2005). Calreticulin also plays an important role in maintenance of cellular calcium homeostasis (Michalak et al., 1992). By regulating the amount of free and bound calcium within the lumen of the endoplasmic reticulum, calreticulin affects many different cellular functions, including cell shape, adhesion and motility (Bedard et al., 2005). Other activities that influence the various roles that this multi-functional protein plays include its capacity to bind to zinc (Khanna et al., 1986; Baksh et al., 1995) and a number of hormone receptors (Burns et al., 1994; Dedhar et al., 1994).

The multi-functional nature of calreticulin may also be influenced by its distribution. Although primarily found in the endoplasmic reticulum, it has also been detected on the cell surface and in the nuclear envelope (Bedard et al., 2005). Further, calreticulin can also be released into the extracellular environment in some circumstances. For example, there is a 10-fold increase in calreticulin in the blood of patients with systemic lupus erythematosus (Eggleton et al., 1997). The sources and roles of extracellular calreticulin are not clear. Nevertheless, evidence indicates that extracellular as well as intracellular calreticulin can also affect many cellular functions including adhesion, migration and proliferation (Bedard et al., 2005). In particular, its effects on vascular endothelial cells may be relevant to the normal pregnancy and pre-eclampsia.

Little is known about role of this multi-functional protein in human pregnancy and its disorders. On the basis of the work of Pal et al. (2006), which demonstrated that a peptide overexpressed in pre-eclampsia, neurokinin-B (Page et al., 2000), induces expression of calreticulin, it might be expected that calreticulin is also increased in pre-eclampsia. The aim of this study was to clarify the nature of calreticulin in pregnancy and pre-eclampsia by measuring its expression in human placenta and in maternal blood, and by comparing such expressions in patients with pre-eclampsia with those from matched, normotensive, control pregnancies.

Materials and Methods

Patient recruitment and sample collection

Subjects included in this study were pregnant and non-pregnant women presenting to the Royal Women’s Hospital for antenatal and gynaecological care, respectively. The pregnant patients were assigned to two groups; those diagnosed with pre-eclampsia (n = 32) or those with normotensive control pregnancies (n = 38). Women were invited to donate a specimen of blood and/or their placenta. In some, but not all, cases both specimens were obtained from the same patient. Blood specimens were also obtained from 12 non-pregnant women who had no current, diagnosed medical condition. Pre-eclampsia was diagnosed in previously healthy women with a pregnancy-induced blood pressure ≥140 mmHg systolic or ≥90 mmHg diastolic and proteinuria defined as ≥300 mg protein in 24 h or at least ++ on a urine dipstick test. There were no pregnancies involving FGR in this study. FGR was classified as a birthweight <10th percentile for gestational age, and at least one of the following: asymmetric fetal growth, abnormal umbilical artery end diastolic flow and reduced liquor volume.

Women selected for the control group did not have pre-eclampsia or any other confounding pathology. Specific exclusion criteria for the study of both pre-eclamptic and control pregnancies included maternal history of hypertension and/or renal disease, maternal infection, smoking, chemical dependency, multiple pregnancies and fetal congenital anomalies.

For placental collection, all deliveries were by elective Caesarean section, the indications for which were at term either the presence of pre-eclampsia or breech presentation, placenta praevia and previous Caesarean section. The indications for pre-term Caesarean section were either the presence of pre-eclampsia or placenta praevia, spontaneous rupture of membranes with fetal distress and previous Caesarean section or poor past obstetric history. No difference in the measurement of placental calreticulin expression could be attributed to the indications for either pre-term or term Caesarean section.

Blood samples were collected by clinical research midwives into EDTA tubes. Samples were centrifuged, and platelet-poor plasma was removed, aliquoted and stored at −40 °C until use. For all sets of experiments that compared control and pre-eclamptic groups, patients were matched for gestational age. The mean gestational ages are stated in the results section.

Placental dissection and protein extraction

Approximately 1 g of tissue was dissected from the maternal side of the placentas and rinsed briefly in 0.9% saline, before being snap frozen in liquid nitrogen and stored at −40 °C prior to extraction. Subsequently, 500 mg of partially thawed placental tissue was homogenized in a 50 mM glycine buffer (Bio-Rad, CA, USA) with 0.5% Triton X-100 (BDH, VIC, Australia), 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (Sigma–Aldrich, MO, USA) and 5 mM EDTA (Merck, Germany), centrifuged (10 min, 2500 × g, 4 °C) and the supernatant stored at −40 °C. Samples were assayed for protein using the Bradford assay (Bradford, 1976).

Placental perfusion

Bilateral perfusion of single placental lobules from six placentas with a modified Krebs solution was commenced within 20 min of delivery. The perfusion technique was a modification of a previously described method (Gude et al., 1993). A suitable fetal vein and artery pair on the surface of the chorionic plate leading to a peripheral lobule was cannulated and perfused via the artery using a Masterflex perfusion pump (model 7554.00 with pump head 7013; Cole-Parmer Instruments, Vernon Hills, IL, USA) at a constant flow rate of 5 ml/min. The Krebs solution, which contained the following (mmol/l): NaCl 97.0, NaHCO3 24.4, KCl 3.0, KH2PO4 1.2, CaCl2 2H2O 1.89, MgSO4·7H2O 1.0, glucose 5.5 (pH 7.4), was equilibrated with 90% nitrogen, 5% oxygen and 5% carbon dioxide. The maternal component of the lobule was also continually perfused with Krebs solution at a flow rate of 10 ml/min via two pieces of plastic tubing inserted through the basal plate into the intervillous space. The whole placenta was supported on a perspex frame within a laboratory incubator (Thermoline Scientific, NSW, Australia) and maintained at 37 °C. Fetal arterial inflow perfusion pressure was measured by a pressure transducer (Gulton-Stratham, CA, USA) and MacLab and Chart software (version 3.2, ADInstruments, NSW, Australia). At approximately 1 h after the commencement of perfusion maternal effluent from the perfused lobule was collected into a plastic tube containing AEBSF (80 μM) and stored for later measurement of calreticulin. At the end of the experiment the perfused lobule was removed by dissection and weighed. Maternal effluent samples from two placentas were subjected to high speed centrifugation (200 000 × g for 1 h) and supernatant and pellet fractions were collected. The pellet fractions were washed four times with and resuspended in phosphate-buffered saline with AEBSF (1 mM).

Western immunoblotting

Plasma samples (0.5 μl), placental extracts (0.5 μg total protein) and whole and fractionated placental effluent samples were diluted with tris-buffered saline (TBS) as required and mixed with sample loading buffer containing XT reducing agent (Bio-Rad). Samples were heated to 95 °C for 5 min and then, together with dual colour molecular weight standards and calreticulin recombinant protein (Genway, San Diego, CA, USA), were loaded onto 4–12% Bis-Tris Criterion XT Precast Gels (Bio-Rad). SDS–PAGE was performed at 100 V for 150 min using XT MOPS buffer (Bio-Rad). The resolved proteins were then electroblotted onto a polyvinylidene difluoride membrane at 50 V for 60 min. After transfer, the membrane was washed in TBS for 10 min and non-specific sites were blocked in 5% skim milk/TBS at room temperature.
for 2 h. The membrane was incubated overnight at 4°C with polyclonal rabbit anti-human calreticulin antibody (C4606, Sigma–Aldrich) at 1:2000 in 2.5% skim milk/TBS. This antibody was raised against the C-terminus of human calreticulin (amino acids 401–417). After 3 × 5 min washes in TBS, the membrane was incubated at room temperature for 1 h with HRP-conjugated swine anti-rabbit antibody (Dako, USA) at 1:1000 in 2.5% skim milk/TBS. Following another three washes in TBS, the membrane was incubated with Lumi-Light Western Blotting Substrate (Roche Diagnostic Corporation, IN, USA) for 5 min and the immunopositive bands were captured onto X-ray film (Kodak, NY, USA). Densitometric scans to quantify calreticulin-positive signal were performed with a Personal Densitometer (Molecular Dynamics, CA, USA).

Real-time PCR
Total RNA (2 μg) was reverse-transcribed into cDNA for 22 RNA samples extracted from placental tissue using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Australia) according to the manufacturer’s protocol. Quantification of calreticulin mRNA expression was performed in an ABI Prism 7700 (Applied Biosystems, CA, USA) using the Taq Man Gene Expression Assays containing 2 unlabelled CALR PCR primers (900 nM each) and Taq Man FAM labelled MGB probe (250 nM, Assay No. Hs00189032_m1, Applied Biosystems). Gene expression quantification for the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was performed using the ABI Prism 7700 (Applied Biosystems, CA, USA) using the Taq Man Gene Expression Assays containing 2 unlabelled CALR PCR primers (900 nM each) and Taq Man FAM labelled MGB probe (250 nM, Assay No. Hs00189032_m1, Applied Biosystems). Gene expression quantification for the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was performed in the same reaction. The GAPDH primers (5' TGCTTAGCA3' and 5' GTCTTCTGGTGCGAGTGATG3') and Taq Man probe (5'VIC-TGGTGAAGGACTCATGACCACAGTCC-TAMRA3') were designed using the Primer Express 1.5 Software (Applied Biosystems). Reaction volumes of 20 μl were prepared in 96 well MicroAmp optical plates with optical caps. Each reaction contained Taq Man Universal PCR master mix (Applied Biosystems), placental cDNA (10 ng), the GAPDH forward and reverse primers, probe (0.2 μM) and Taq Man Gene Expression Assay mix. Reactions were made up to 20 μl using RNase free water. Amplification was for 40 cycles including denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. All samples were run in triplicate. Relative quantification of calreticulin expression normalized to GAPDH was calculated according to the 2^(-ΔΔCt) method using the mean Ct for the gestation-matched controls (GMCs) as standard.

Statistical analysis
Student’s t-test was used to analyse any difference in calreticulin expression between the two groups for both western blot and RT-PCR analyses. Linear regression analysis and ANOVA tests were also used as specified. A value of P < 0.05 was considered as significant. All results and clinical characteristics are expressed as mean ± SEM unless otherwise stated.

Results
Western blotting of placental extract and maternal plasma
Samples of placental extract and whole maternal plasma both gave a single band on the western immunoblot of ~50 kDa (Fig. 1). Recombinant full-length calreticulin also gave a single band at a slightly higher molecular weight probably due to the presence of a His–tag on the N-terminus. Fig. 1 shows that the increasing amounts of the calreticulin standard gave a linear increase in the density of the bands over the expression range for the placenta and plasma samples that were applied to the gel. An ELISA was performed that successfully measured the recombinant calreticulin standard. Under the conditions of the ELISA, however, factors in plasma were found to block the antibody–protein interaction. As calreticulin in maternal blood during normotensive pregnancy
A western blot was undertaken to compare the concentration of calreticulin in the blood of women who were not pregnant (n = 12) with that from women with normal pregnancies at term (n = 12). The GAPDH primers (5' GCACCACCAAC TGCTTAGCA3' and 5' GTCTTCTGGTGCGAGTGATG3') and Taq Man probe (5'VIC-TGGTGAAGGACTCATGACCACAGTCC-TAMRA3') were designed using the Primer Express 1.5 Software (Applied Biosystems). Reaction volumes of 20 μl were prepared in 96 well MicroAmp optical plates with optical caps. Each reaction contained Taq Man Universal PCR master mix (Applied Biosystems), placental cDNA (10 ng), the GAPDH forward and reverse primers, probe (0.2 μM) and Taq Man Gene Expression Assay mix. Reactions were made up to 20 μl using RNase free water. Amplification was for 40 cycles including denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. All samples were run in triplicate. Relative quantification of calreticulin expression normalized to GAPDH was calculated according to the 2^(-ΔΔCt) method using the mean Ct for the gestation-matched controls (GMCs) as standard.

Calreticulin concentration in maternal blood during normotensive pregnancy
The maternal effluent was collected from six placentas during perfusion of single placental lobules. A western immunoblot was performed to measure calreticulin concentration in these samples and in paired maternal plasma samples (i.e. from the same pregnancies). A calreticulin standard curve was generated by including varying concentrations of recombinant calreticulin protein in the immunoblot, similar to that shown in Fig. 1. Comparison of the density of bands for the two types of samples with the standards determined that the mean (± SEM) concentration of calreticulin was 0.84 ± 0.12 μg/ml for effluent and 1.80 ± 0.33 μg/ml for plasma. Furthermore, the effluents from two placentas were subjected to high speed centrifugation (200 000 × g, 1 h) and separated into supernatant and pellet fractions. Calreticulin was measured in these fractions and compared to the whole placental effluent. In both cases greater than 90% of the calreticulin was found in the supernatant (soluble) fractions.

Calreticulin in the maternal effluent of perfused placental lobules
The maternal effluent was collected from six placentas during perfusion of single placental lobules. A western immunoblot was performed to measure calreticulin concentration in these samples and in paired maternal plasma samples (i.e. from the same pregnancies). A calreticulin standard curve was generated by including varying concentrations of recombinant calreticulin protein in the immunoblot, similar to that shown in Fig. 1. Comparison of the density of bands for the two types of samples with the standards determined that the mean (± SEM) concentration of calreticulin was 0.84 ± 0.12 μg/ml for effluent and 1.80 ± 0.33 μg/ml for plasma. Furthermore, the effluents from two placentas were subjected to high speed centrifugation (200 000 × g, 1 h) and separated into supernatant and pellet fractions. Calreticulin was measured in these fractions and compared to the whole placental effluent. In both cases greater than 90% of the calreticulin was found in the supernatant (soluble) fractions.

Calreticulin concentration in maternal blood with pre-eclampsia
Two separate western immunoblots were preformed to compare the concentration of calreticulin in maternal blood between pre-eclamptic and GMC pregnancies. The first compared pregnancies in which pre-eclampsia was diagnosed at a range of gestational ages between 26 and 39 weeks with control pregnancies sampled at the appropriate...
gestations. Mean gestational ages for the two groups were: GMC, 32.8 ± 1.7 weeks (n = 12) and pre-eclampsia, 33.2 ± 1.4 weeks (n = 10). The second blot compared the calreticulin concentration in the blood of women whose pre-eclampsia was first diagnosed before 32 weeks gestation with another group of control pregnancies sampled at the appropriate gestations. In this case, the mean gestational ages for the two groups were: GMC, 28.3 ± 0.7 weeks (n = 12) and pre-eclampsia, 28.7 ± 0.5 weeks (n = 12). Fig. 3 shows that both analyses found a significant increase of approximately 50% in calreticulin concentration in maternal blood with pre-eclampsia (t-test, P < 0.05).

**Calreticulin mRNA and protein expression in placenta with pre-eclampsia**

Calreticulin mRNA was measured by RT-PCR in extracts of placentas from pre-eclamptic pregnancies (mean gestation, 30.1 ± 1.1 weeks, n = 9) and compared with extracts from GMC pregnancies (mean gestation, 30.9 ± 0.7 weeks, n = 13). The data were expressed relative to the housekeeping gene GAPDH, which was not significantly different between the two groups. Fig. 4A shows that no significant change in expression of placental calreticulin mRNA was measured in the pre-eclamptic group compared with controls.

A western immunoblot was also performed on placental extracts to compare calreticulin protein expression between pre-eclamptic and GMC pregnancies. Fig. 4B shows that there was also no significant change in calreticulin protein expression between the two groups.

**Discussion**

Calreticulin is a ubiquitously expressed, multi-functional protein that is highly expressed in the endoplasmic reticulum of the cell. Under certain conditions, however, it is released from the cell and is found...
Calreticulin in pregnancy and pre-eclampsia

Figure 4: Human placental calreticulin mRNA and protein expression with pre-eclampsia.
(A) There was no significant change in placental calreticulin mRNA between control (GMC, mean gestation, 30.9 ± 0.7 weeks, n = 13) and pre-eclamptic (PE, mean gestation, 30.1 ± 1.1 weeks, n = 9) pregnancies. The data are expressed as mean ± SEM 2−ΔΔCt with GAPDH as housekeeping gene and the mean Ct for GMC as standard. (B) There was no significant change in placental calreticulin protein expression between control (GMC, mean gestation, 32.9 ± 1.2, n = 12) and pre-eclamptic (PE, mean gestation, 32.8 ± 1.0, n = 12) pregnancies. Data are expressed as mean ± SEM arbitrary density per microgram total placental protein.

in peripheral blood. This study has determined for the first time that human pregnancy is one such condition in that there is a high concentration of calreticulin circulating in the peripheral blood of pregnant women. Calreticulin was detected in the blood of non-pregnant women at similar concentrations to that described by other studies (~0.4 μg/ml) (Eggleton et al., 1997). Increased calreticulin (~1.8 μg/ml) was detected in all pregnant samples measured; the earliest having been collected at 7 weeks gestation. As there was no difference in calreticulin in eight women who were sampled longitudinally, our results indicate that calreticulin increases in maternal blood early in, and remains elevated throughout, human pregnancy.

Our evidence suggests that the placenta may be a major source of the circulating calreticulin in pregnancy. We measured similar concentrations of calreticulin in the effluent of perfused placentas and in maternal blood. Owing to the many factors that are different between the two situations, it is not possible to make direct comparison between fluids obtained during in vitro perfusion and those sampled in vivo. For example, in our experiments, the perfusion fluid was not re-circulated and was sampled after a single passage through the intervillous space. More importantly, the maternal component of the circulation, which is obviously not present during in vitro conditions, is likely to have a major influence on the amount of calreticulin, including its metabolism and contribution from sources other than the placenta. Nevertheless, we believe the observation that calreticulin is released from the placenta in vitro at similar concentrations to that in blood supports the role of the placenta as a major source of calreticulin in the circulation in pregnancy.

The mechanism by which calreticulin is released from cells is not well understood. It is primarily localized to the endoplasmic reticulum within the cell where it performs its chaperone and calcium-binding functions. However, although it contains the endoplasmic reticulum-retrieval sequence KDEL (lysine, aspartate, glutamate, leucine) at its C-terminus, it has also been identified in the cytoplasm and the nucleus, as well as on the surface of some cells. Several theories have been proposed to explain how calreticulin might overcome its endoplasmic reticulum-retention properties and move to the cell surface (Johnson et al., 2001). For example, calreticulin may be expressed in different isoforms that do not contain the endoplasmic reticulum-retrieval sequence, the KDEL receptor may be transported to the surface in complex with calreticulin or endoplasmic reticulum-lumenal proteases might proteolytically truncate the molecule so that the KDEL sequence is absent. In addition, saturation of the endoplasmic reticulum-retention machinery, as well as calreticulin glycosylation, may also play a role in its movement outside the endoplasmic reticulum. Interestingly, the work of Hojrup et al. (2001) found no evidence of glycosylation or phosphorylation of human placental calreticulin, but did find evidence of the existence of a C-terminal hexapeptide truncation that had lost the KDEL signal. One explanation for this result is that there is cleavage by an unknown protease of high specificity. If this cleavage occurred within the endoplasmic reticulum, it would certainly facilitate translocation of placental calreticulin to other cellular compartments and possibly also secretion outside the cell.

Another possible mechanism for the extracellular release of components of the endoplasmic reticulum, including calreticulin, is via cell lysis and death. Villous trophoblast cells continually undergo differentiation and fusion with the overlying syncytiotrophoblast throughout pregnancy. Renewal of this outer epithelial layer also involves apoptotic-mediated cell fragmentation and deportation of both cellular and subcellular debris into the maternal circulation (Huppertz et al., 1998). Calreticulin present in the syncytiotrophoblast may be released into maternal blood by this process. Subcellular, syncytiotrophoblast microparticles (STBM) can be efficiently harvested by high speed centrifugation of the maternal effluent obtained during in vitro placental perfusion (Gupta et al., 2005). Comparison of STBM fractions obtained in this way with the corresponding supernatant and whole effluent fractions determined that less than 10% of the calreticulin released during in vitro perfusion is associated with STBM. Thus, our result indicates that calreticulin is released from the placenta independently of cell fragmentation and deportation processes.

This study found that there is an increased calreticulin in maternal blood of pre-eclamptic women compared to gestation-matched, normotensive pregnancies. The source of this additional calreticulin with pre-eclampsia is not clear. Although there was no difference in calreticulin mRNA or protein expression in placentas from pre-eclamptic and control pregnancies, the placenta cannot be ruled out as a possible source. The rate of turnover and release of placental calreticulin may be increased with pre-eclampsia, even though net expression was unchanged. In addition, increased concentration of calreticulin in blood may result from decreased rate of removal, rather than altered production. Nevertheless, calreticulin is a ubiquitously expressed protein; any one of a number of different organs
that are affected by pre-eclampsia, such as the heart, kidney or liver, may be induced to release additional calreticulin into the circulation during that condition. Vascular and blood cells may also be the additional sources of calreticulin. Neutrophils, e.g. become activated during pre-eclampsia (Mellombakkene et al., 2001), and it has been reported that activation causes them to release calreticulin (Kishore et al., 1997). It has not been reported that endothelial cells release calreticulin either basally or during activation. Nevertheless, as calreticulin is present on the surface of endothelial cells (Goicoechea et al., 2000), activation during pre-eclampsia may cause its release from them into maternal blood. It is also interesting to note that neurokinin-B, a factor known to be increased in pre-eclampsia (Page et al., 2000), stimulates increased expression of calreticulin in endothelial cells (Pal et al., 2006).

The role of calreticulin in maternal blood in pregnancy and pre-eclampsia is not known. Although its chaperone and calcium-binding activities in the endoplasmic reticulum have been well characterized, its functions on the cell surface and in secreted forms are less clear. Calreticulin is raised in the circulation of patients with a number of autoimmune conditions, including systemic lupus erythematosus (Eggleton et al., 1997). It has been postulated to contribute to the progression of autoimmune disease by preventing immune complex clearance via the binding to the first component of the classical complement pathway, C1q (Johnson et al., 2001). Calreticulin has been reported to have both pro- and anti-inflammatory effects on vascular. Calreticulin had anti-thrombotic activity in a canine coronary artery thrombosis model and caused dose-dependent increases in nitric oxide production in cultured bovine aortic endothelial cells (Kuwabara et al., 1995). In contrast, the full-length protein, as well as the 120–180 amino acid fragment (referred to as vasostatin), inhibited fetal bovine heart endothelial cell proliferation in vitro, and showed anti-angiogenic and -tumorigenic activities in mice in vivo (Pike et al., 1999). Calreticulin was shown to bind to endothelial cells following intravascular infusion in whole animals, as well as under in vitro culture conditions (Kuwabara et al., 1995). The net effect of calreticulin on vascular endothelial cells may differ according to the particular vascular bed and the local concentration achieved. Binding of calreticulin to other blood proteins may also affect its activity. Thus, circulating calreticulin might facilitate reductions in maternal systemic vascular resistance that accompany early pregnancy via increased endothelial nitric oxide production; whereas further increases in calreticulin with pre-eclampsia may contribute to the mix of anti-angiogenic factors that induce the changes in endothelial cell functions that are characteristic of that condition.

Increased cellular expression of calreticulin increases the calcium storage capacity of the endoplasmic reticulum via modulation of store-operated Ca\(^{2+}\) influx and altered Ca\(^{2+}\) transport by the sarcoplasmic endoplasmic reticulum (SERCA) ATPase (Michalak et al., 1999). It is not known whether changes in extracellular expression of calreticulin also affect calcium homeostasis. The plasma calcium concentration in women with pre-eclampsia is decreased compared to normal pregnant women (Kisters et al., 2000). In addition, calcium supplementation during pregnancy was reported to reduce the severity of pre-eclampsia in women with low calcium intake (Villar et al., 2006). Thus, the increased concentration of calreticulin in the blood of pre-eclamptic women may contribute to the disturbed calcium homeostasis observed in pre-eclampsia.

In conclusion, this work has produced novel evidence that the placenta is a major source of the calreticulin that is present in maternal blood throughout pregnancy. An increase in calreticulin in maternal blood was measured with the pregnancy disorder of pre-eclampsia. Clarification of the roles that this multi-functional protein might play in pregnancy and pre-eclampsia await further investigation.

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