

Increased levels of HMGB1 in trophoblastic debris may contribute to preeclampsia

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Abstract

Preeclampsia is triggered by an as yet unknown toxin from the placenta. Antiphospholipid antibodies (aPL), a strong risk factor for preeclampsia, have been shown to induce the production of toxic trophoblastic debris from the placenta. High mobility group box 1 (HMGB1) is a proinflammatory danger signal, and the expression of it has been reported to be increased in preeclampsia. This study examined whether aPL or preeclamptic sera increase the expression of HMGB1 in the syncytiotrophoblast or trophoblastic debris. Trophoblastic debris from normal placental explants that had been cultured with aPL or preeclamptic sera was exposed to endothelial cells. Endothelial cell activation was quantified by cell-surface ICAM-1 expression and U937 monocyte adhesion. The expression of HMGB1 in placental explants and trophoblastic debris that had been treated with aPL or preeclamptic sera was measured by immunohistochemistry and western blotting. The expression of the receptor for advanced glycation end products (RAGE) in endothelial cells was quantified by western blotting. Compared with controls, the expression of HMGB1 in the cytoplasm of the syncytiotrophoblast and trophoblastic debris was increased by treating placental explants with aPL or preeclamptic sera. The increased levels of HMGB1 contributed to endothelial cell activation, mediated in part by the RAGE. Preeclamptic sera and aPL both induced an increase in the cytoplasmic levels of the danger signal HMGB1 in trophoblastic debris. This increased HMGB1 in trophoblastic debris may be one of the toxic factors released from the placenta in preeclampsia.

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Introduction

Preeclampsia is a human pregnancy-specific disease, characterised by multisystem dysfunction. It is generally manifested by maternal hypertension after 20 weeks of gestation, and it is a leading cause of maternal and perinatal mortality and morbidity (Sibai *et al.* 2005). Before the onset of symptoms, women destined to develop preeclampsia exhibit endothelial cell activation and an exaggerated inflammatory response. Although the complete pathogenesis of preeclampsia is still uncertain, it seems that preeclampsia is triggered by a placental factor(s), given that preeclampsia resolves after delivery. A number of studies have reported that the inflammatory cytokines are increased in preeclampsia at presentation (Lau *et al.* 2013), suggesting that a mediator of inflammation is involved in the pathogenesis of preeclampsia (Redman & Sargent 2010).

During pregnancy, a large amount of trophoblastic debris is shed from syncytiotrophoblast into maternal circulation by an apoptotic process as early as six weeks of gestation (Covone *et al.* 1984). However, in preeclampsia, there is a well-documented increase in the amount of trophoblast debris shed into the

maternal blood (Douglas *et al.* 1959, Attwood & Park 1961, Knight *et al.* 1998). In addition, a switch from the shedding of apoptotic (safe) to necrotic (dangerous/toxic) trophoblastic debris may be one of the placental triggers of preeclampsia (Huppertz *et al.* 2003).

The intracellular protein high mobility group box 1 (HMGB1) was originally described as a DNA-binding nuclear protein and transcription factor, but more recently, has been shown to be a proinflammatory danger signal that causes sterile inflammation when released from necrotic cells or cells under stress (Lotze & Tracey 2005). HMGB1 is expressed by trophoblasts (Wang *et al.* 2011) and can be found in either the nucleus or the cytoplasm of these cells. In general, when HMGB1 is translocated to the cytoplasm or released to the extracellular environment, it acts as a danger signal (Tsung *et al.* 2014). Circulating levels of HMGB1 are increased in many inflammation-related diseases including preeclampsia (Tsung *et al.* 2014). We have recently shown that the expression of HMGB1 was increased in syncytiotrophoblast in the placenta of women with preeclampsia and was associated with the severity of preeclampsia (Chen *et al.* 2016b).

Once released from necrotic cells, HMGB1 can interact with the receptor for advanced glycation end products (RAGE) (Hori *et al.* 1995) or toll-like receptor (TLR) 2 or TLR4 (Park *et al.* 2006, Bianchi & Manfredi 2007, Campana *et al.* 2008), and HMGB1 released from cells into the extracellular environment may play an important role in the response to tissue damage and particularly necrotic cell death.

An association between antiphospholipid antibodies (aPL) and preeclampsia is well documented (Noris *et al.* 2005). Treating normal placental explants with aPL increases the amount of dangerous/necrotic trophoblastic debris shed from the explants, and this necrotic trophoblastic debris activates the endothelial cells (Duckitt & Harrington 2005, Chen *et al.* 2009b). Sera from women with preeclampsia have also been shown to induce the production of dangerous/necrotic trophoblastic debris (Chen *et al.* 2012b, 2013, Shen *et al.* 2014). However, whether there is an association between aPL or sera from preeclampsia and increased levels of HMGB1 in trophoblastic debris has not been fully investigated. Therefore, we undertook this study to investigate the role of HMGB1 in dangerous trophoblast debris from aPL-treated placentae in inducing endothelial cell activation. Our secondary objective was to examine the expression of RAGE, one of the HMGB1 receptor in endothelial cells.

Materials and methods

This investigation conforms to the principles outlined in the Declaration of Helsinki. This study was approved by the Auckland Regional Health and Disabilities Ethics Committee, New Zealand. All patient-derived tissue and blood samples were obtained with written informed consent.

Antiphospholipid antibodies (aPL)

The murine monoclonal antiphospholipid antibody, ID2 (Chamley *et al.* 2001), was produced in this laboratory and generated by hybridoma culture, and then purified using HiTrap Protein G columns (GE Healthcare). This monoclonal aPL has been extensively characterised and has, anticardiolipin, anti β_2 GPI and lupus anticoagulant activities (Pantham *et al.* 2015). A murine IgG1 antibody (Life Technologies) was used as an isotype-matched treatment control antibody in experiments involving ID2.

Collection of placentae and blood

Fifteen first-trimester placentae were collected after elective surgical terminations of on-going pregnancies ranging from 8 to 12 weeks of gestation. In addition, blood samples from six women with preeclampsia and six gestation-matched normotensive pregnant women were collected by venepuncture into plain vacutainer tubes. The blood was allowed to clot, centrifuged at 2500g and the serum was aspirated and stored in aliquots at -80°C .

Preeclampsia was defined as maternal systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg on two occasions separated by 6 h, and proteinuria > 300 mg in a 24-h period after 20 weeks of gestation following the guidelines of the American College of Obstetricians and Gynaecologists ('Hypertension in Pregnancy' 2013).

Cell culture

All cell culture reagents including culture media, foetal bovine serum (FBS) and Cell Tracker, CMTPX (Red) were purchased from Invitrogen. The human microvascular endothelial cell line (HMEC-1) originally derived from dermal microvascular endothelial cells (Ades *et al.* 1992) was obtained from the National Centre for Infectious Diseases (USA) and grown to confluence in MCDB 131 media as described previously (Chen *et al.* 2006). The endothelial cells were seeded at a density of 10^4 cells in each well of 96-well sterile culture plates or the equivalent density (8×10^4) in the wells of a 12-well culture plate and grown to confluence. The passages of HMEC-1 used in this study were under 15. U937 cells, a human monocyte cell line, were grown in DMEM/F12 media supplemented with 10% of FBS.

Culture of placental explants and preparation of trophoblastic debris

Trophoblastic debris was collected from first-trimester placentae ($n=15$) that had been treated with the aPL ID2 (20 $\mu\text{g}/\text{mL}$) or isotype-matched control antibody (20 $\mu\text{g}/\text{mL}$) or sera, from women with preeclampsia or normotensive controls, as described previously (Abumaree *et al.* 2006, Chen *et al.* 2006). Briefly, approximately 400 mg explants were dissected from first-trimester placentae. The explants were then cultured in Netwell culture inserts, suspended in 12-well culture plates, for 24 h at 37°C in DMEM/F12-containing 10% FBS in an ambient oxygen atmosphere containing 5% CO_2 in the presence of aPL (20 $\mu\text{g}/\text{mL}$) or isotype-matched control antibody (20 $\mu\text{g}/\text{mL}$). In other experiments, the explants were treated with 10% serum from each of the six women with preeclampsia or serum from each of six normotensive women, and in addition, fifteen untreated, control explants were cultured with medium without human serum, for 24 h. The Netwell inserts (containing the explants) were then removed from the culture wells and the trophoblastic debris shed from the explants, which passes through the Netwell inserts, was aspirated from the culture wells and centrifuged at 300g for 10 min. The supernatant was discarded and washed with PBS, and the trophoblastic debris was resuspended in PBS buffer, and then depleted of contaminating CD45^+ leukocytes using magnetic beads (Dyna, Invitrogen) according to the manufacturer's instructions. Contaminating red blood cells were removed by incubation in MilliQ water for one minute, and then one volume of $10\times$ PBS was added to immediately return the trophoblastic debris to isotonic conditions. These procedures resulted in trophoblastic debris (confirmed by cytokeratin 7 and vimentin immunostaining) essentially free from contaminating cells as described previously (Abumaree *et al.* 2006, Chen *et al.* 2009a, 2010). The trophoblastic debris

from approximately 1200 mg of placental explants was pooled and added to endothelial cell cultures.

Immunohistochemistry

The expression levels of HMGB1 in trophoblastic debris or first-trimester placentae that had been treated with aPL or an isotype-matched control antibody, or preeclamptic or normotensive pregnant sera were measured by immunohistochemistry. Non-specific antibody binding was blocked by incubating with 10% normal goat serum in PBS for 10 min at room temperature. Murine anti-human HMGB1 monoclonal antibody (diluted 1:100 in 10% normal goat serum, Abcam, Sapphire Biosciences, Sydney) was added for one hour at room temperature. Sections were then washed with PBST and incubated with 10% H₂O₂ in methanol for 3 min. After washing three times with goat anti-mouse IgG, biotin-conjugated antibody (Jackson ImmunoResearch Laboratories) was then added for one hour at room temperature. After washing, streptavidin-conjugated horse radish peroxidase (HRP) was added for a further hour. After washing, the sections were stained for 15 min with 3-amino-9-ethylcarbazole (AEC) using a kit according to the manufacturers instruction (Dako, Global Science), and sections were then counter-stained for three minutes with haematoxylin. Negative staining controls were performed as mentioned previously but omitting the primary (anti-HMEC1) antibody.

Determination of endothelial cell activation by cell-surface ICAM-1 ELISA

HMEC-1 endothelial cells were grown until confluent in 96-well culture plates in MCDB 131 media. The cell cultures were incubated with trophoblastic debris from aPL-treated, isotype-matched control antibody-treated or -untreated placental explants or with human recombinant HMGB1 (0.2 µg/mL and 2 µg/mL) for 24 h. In some experiments, neutralising RAGE antibody was added to the endothelial cell cultures for 60 min before adding the HMGB1. The neutralising antibody was maintained in the cultures along with the HMGB1. After 24 h, remaining trophoblastic debris or HMGB1 was washed out from the cultures, and the cell-surface expression of ICAM-1 by the HMEC-1 monolayers was determined by cell-based ELISA as described previously (Chen *et al.* 2006).

Determination of endothelial cell activation by monocyte adhesion assay

HMEC-1 endothelial cells were grown and exposed to trophoblastic debris or recombinant HMGB1 as for the ICAM-1 ELISAs mentioned previously. After exposure to trophoblastic debris or HMGB1 for 24 h, the remaining HMGB1 or trophoblastic debris was washed off, and then the U937 cells that had been labelled with cell tracker, CMTPX (Red) were added, and the incubation was continued for three hours at 37°C. The cultures were then washed to remove non-adherent U937 cells, the adherent U937 cells were quantified using a fluorescence plate reader (Synergie 2, BioTek). Data were expressed as the fold increase of adhesion of U937 cells relative to the control, untreated endothelial cells.

Determination of the expression of HMGB1 in trophoblastic debris by western blotting

The relative levels of HMGB1 in trophoblastic debris from first-trimester placental explants that had been treated with aPL or isotype-matched control antibody were measured by western blotting. Trophoblastic debris was homogenised in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Nonidet P40 substitute, protease inhibitor, 1 mM phenylmethanesulfonylfluoride). All samples (20 µg of total protein) were loaded on 14% SDS-PAGE gels and electrophoresed, and then transferred to nitrocellulose membranes. Non-specific binding was blocked by incubating membranes in the blocking solution (10% non-fat milk in PBST) for 1 h at room temperature, and then membranes were incubated with mouse anti-human HMGB1 antibody (1:250) in blocking solution for 2 h at room temperature. After washing with PBST three times, the membranes were incubated with HRP-conjugated goat anti-mouse antibody (1:2000, Jackson ImmunoResearch Laboratories, Pennsylvania) for 1 h at room temperature. After washing with PBST, the membranes were incubated with Amersham ECL Prime Western blotting detection reagent. Chemiluminescence from the membranes was detected using an Image Quant LAS3000 (Thermo Fisher). Protein levels of HMGB1 were analysed relative to the β-actin loading control. β-Actin was detected on the membranes as follows. Membranes were stripped with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS and 100 mM 2ME) at 50°C for 30 min, and then non-specific binding was blocked by incubation in blocking solution for one hour. Then the membranes were incubated with mouse anti-human β-actin (1:5000, Abcam, Sapphire Biosciences, Sydney) for 2 h at room temperature. After washing three times with PBST, the membranes were incubated with HRP-conjugated goat anti-mouse antibody (1:5000, Jackson ImmunoResearch Laboratories, Pennsylvania) for 1 h at room temperature. After washing with PBST, the membranes were incubated with Amersham™ ECL™ Prime Western blotting detection reagent. Chemiluminescence from the membranes was detected using an Image Quant LAS3000 (Thermo Fisher).

Determination of the expression of HMGB1 in the cytoplasm of placental explants by western blotting

Placental explants were treated with aPL or isotype-matched control antibody ($n=4$) or preeclamptic or normotensive sera ($n=6$). The explants were washed three times with PBS, and then incubated in 500 µL RIPA buffer on ice for 6 h or 24 h without agitation. Samples were then centrifuged at 13,000 g for 10 min, and the supernatants were collected for western blotting. All samples (20 µg of total protein) were loaded on SDS-PAGE gels and electrophoresed, and then transferred to nitrocellulose membranes, which were probed for HMGB1 as mentioned previously. After detecting HMGB1, the membranes were washed extensively and incubated with rabbit anti-human Lamin B1 antibody (1:750, Abcam, Sapphire Biosciences, Sydney) in blocking solution for 2 h at room temperature. After washing with PBST three

times, the membranes were incubated with HRP-conjugated goat anti-rabbit antibody (1:2000, Jackson ImmunoResearch Laboratories, Pennsylvania) for 1 h at room temperature. After washing with PBST, the membranes were incubated with Amersham ECL Prime Western blotting detection reagent. Chemiluminescence from the membranes was detected using an Image Quant LAS3000 (Thermo Fisher).

Determination of the expression of RAGE in endothelial cells by western blotting

The relative levels of RAGE in endothelial cells that had been treated with trophoblastic debris shed from first-trimester placental explants that had been treated with aPL or isotype-matched-control antibody or sera from preeclamptic or normotensive pregnant women were measured by western blotting. Endothelial cells were homogenised in RIPA buffer. All samples (20 µg) were loaded on 14% SDS-PAGE gels and electrophoresed, and then transferred to nitrocellulose membranes. Non-specific binding was blocked by incubating membranes in blocking solution for 1 h, and then membranes were incubated with mouse anti-human RAGE antibody (1:500, Sigma) in blocking solution for 2 h at room temperature. After washing with PBST, the membranes were incubated with biotin-conjugated goat anti-mouse antibody (1:2000) for 1 h at room temperature. After washing with PBST, the membranes were then incubated with streptavidin-conjugated HRP (1:3000, Jackson ImmunoResearch Laboratories, Pennsylvania) for 1 h at room temperature. After washing with PBST, the membranes were incubated with Amersham ECL Prime Western blotting detection reagent. Chemiluminescence from the membranes was detected using an Image Quant LAS3000 (Thermo Fisher). Protein levels of RAGE were analysed relative to the β-actin loading control as mentioned previously.

Determination of soluble levels of HMGB1 in conditioned medium

The levels of HMGB1 in conditioned medium from placental explants that had been treated with aPL, isotype-matched control antibody or sera from preeclamptic or normotensive pregnant women were measured by ELISA according to the manufacturer's instructions (Lifespan BioSciences, Seattle, WA, USA).

Statistical analysis

Data for measuring ICAM-1 expression by endothelial cells and U937 adhesion to endothelial cells were conducted in quadruplicate and repeated at least three times. Data are presented as the median and 5th and 95th percentiles of the fold change relative to untreated controls. Data for the levels of soluble HMGB1 in conditioned medium are presented as the mean and s.d. The statistical significance of the results was assessed by a Mann–Whitney *U* test using the Prism software package. Semi-quantification of western blotting was analysed by a Mann–Whitney *U* test using Prism software package. $P < 0.05$ was considered as statistically significant.

Results

The expression of HMGB1 was increased by treating placental explants with aPL

To investigate whether aPL changed the expression of HMGB1, first-trimester placental explants were treated with aPL or an isotype-matched control antibody, and the expression of HMGB1 was measured in both trophoblastic debris and the placental explants by immunohistochemistry. In control-antibody-treated or -untreated explants, the expression of HMGB1 was limited predominantly to the nuclei of cells of the mesenchymal core with rare staining of nuclei in the syncytiotrophoblast (Fig. 1A: untreated and B: isotype matched control antibody treated). There was very low cytoplasmic staining for HMGB1 in any cell type in control explants. In explants treated with aPL, the nuclear staining for HMGB1 in mesenchymal cells was retained, but there was strong cytoplasmic staining for HMGB1 in the syncytiotrophoblast and cytotrophoblasts (Fig. 1C).

Syncytial nuclear aggregates (SNAs) extruded from explants treated with aPL had strong cytoplasmic staining for HMGB1 (Fig. 1D). In contrast, syncytial nuclear aggregates (SNAs) shed from untreated or

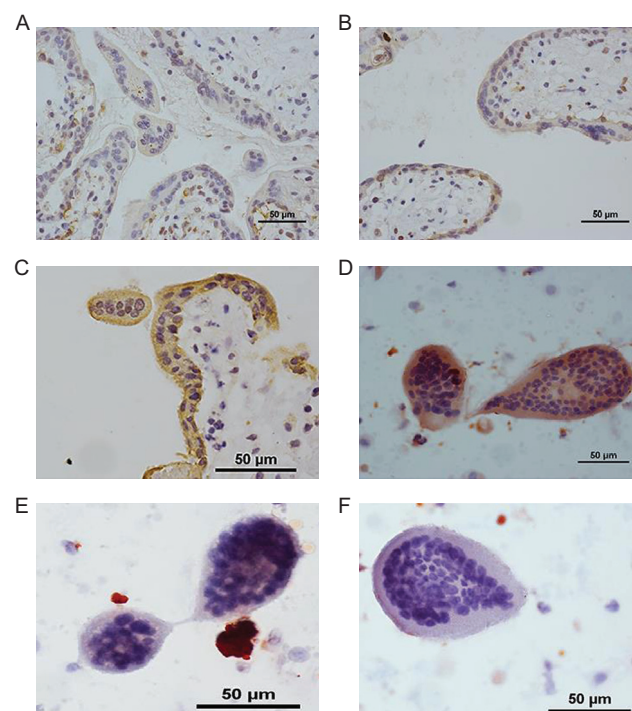


Figure 1 Photomicrographs demonstrating immunohistochemical staining for HMGB1 of first-trimester placental explants that had been (A) untreated, (B) treated with an isotype-matched control antibody or (C) had been treated with aPL for 24 h. Photomicrographs demonstrating the immunohistochemical staining of HMGB1 in trophoblastic debris from placental explants that had been (D) treated with aPL or (E) treated with an isotype-matched control antibody (F) or from explants that were untreated for 24 h.

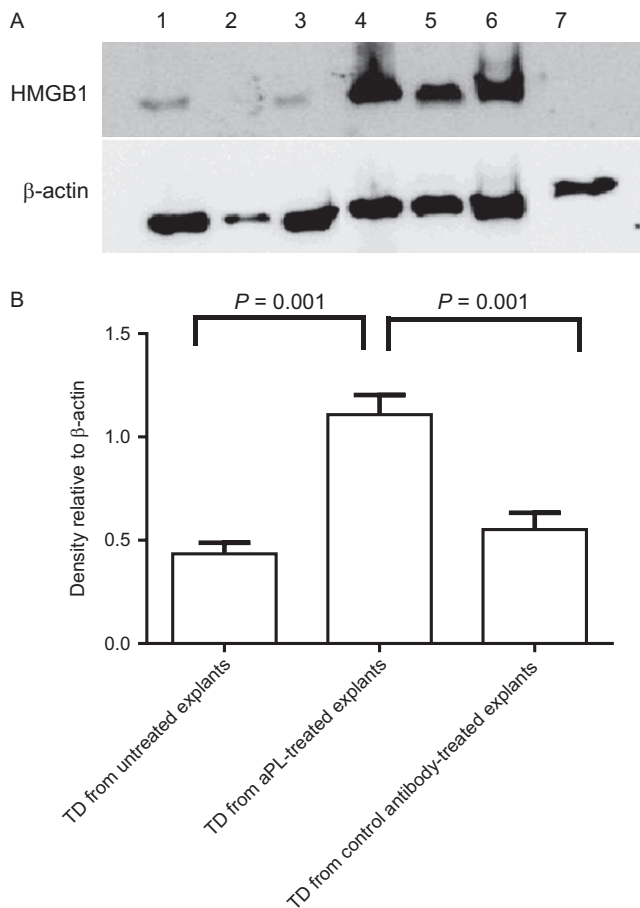


Figure 2 Representative western blot (A) demonstrating that the levels of HMGB1 were increased in trophoblastic debris (TD) from placental explants that had been treated with aPL ($n=16$) (lanes 4–6) compared with trophoblastic debris from placental explants that had been treated for 24 h with isotype-matched control antibody ($n=6$) (lane 7) or explants that were untreated ($n=16$) (lanes 1–3). (B) Semi-quantitative analysis showed that when normalised to levels of β -actin, the levels of HMGB1 were significantly increased in trophoblastic debris from placental explants that had been treated with aPL for 24 h.

isotype-matched control antibody-treated explants did not stain for HMGB1 (Fig. 1E and F). The fact that the increase in levels of HMGB1 in trophoblastic debris (SNAs) from placental explants that had been treated with aPL was significant was confirmed by western blot (Fig. 2).

To confirm the increase in HMGB1 in the cytoplasm of the syncytiotrophoblast, we isolated a fraction enriched for syncytiotrophoblast cytoplasm from explants and confirmed a significant ($P < 0.05$) increase in cytoplasmic HMGB1 in response to treatment with aPL compared with an isotype-matched control antibody (Fig. 3). Western blotting for the nuclear protein Lamin B1 confirmed that this fraction was enriched for cytoplasmic material (data not shown).

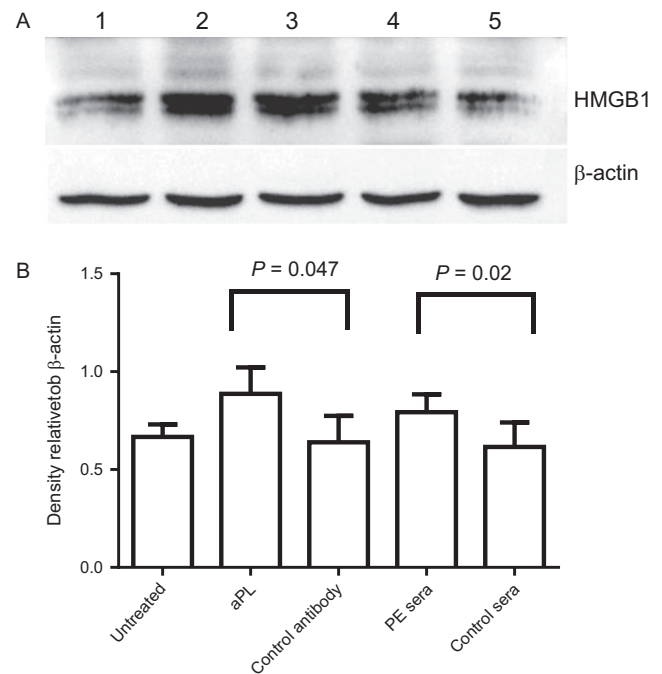


Figure 3 Representative western blot demonstrated that the levels of HMGB1 were increased in the cytoplasmic fraction of the syncytiotrophoblast from explants that had been treated with aPL (lane 2) or preeclamptic sera (lane 4) compared with untreated explants (lane 1) or explants that had been treated with isotype-matched control antibody (lane 3) or normotensive control sera (lane 5) (A). Semi-quantitative analysis showed that when normalised to levels of β -actin, the levels of HMGB1 were significantly increased in explants that had been treated with aPL or preeclamptic sera (B).

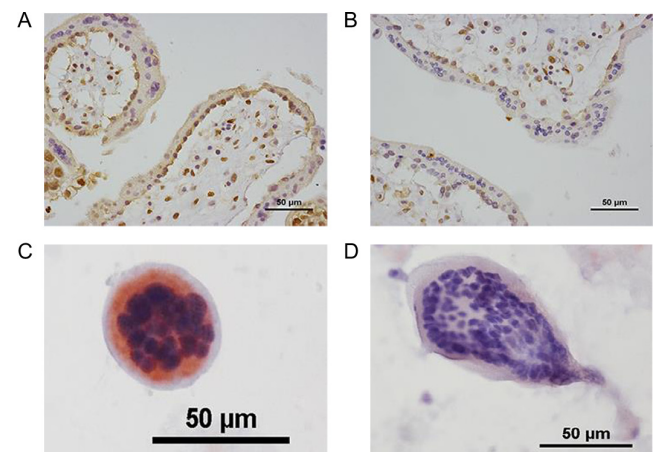


Figure 4 Representative photomicrographs from an immunohistochemical analysis of first trimester placental explants that had been treated with (A) 10% preeclamptic sera or (B) 10% normotensive control sera for 24 h or trophoblastic debris from placental explants that had been treated with (C) preeclamptic sera or (D) control normotensive sera for 24 h. The samples were probed with an anti-HMGB1 antibody.

The expression of HMGB1 was increased by treating placental explants with preeclamptic sera

Immunohistochemical and western blotting analyses indicated that the expression of HMGB1, particularly in the cytoplasm of the syncytiotrophoblast, was increased in placentae after treatment with preeclamptic sera (Figs 3 and 4) compared with explants treated with sera from normotensive pregnant women (Figs 3 and 4). Likewise, the level of HMGB1 in the cytoplasm of trophoblastic debris extruded from explants that had been treated with preeclamptic sera (Fig. 4C) was increased compared with debris from explants treated with sera from normotensive pregnant women (Fig. 4D).

The level of HMGB1 in conditioned medium from placenta treated with aPL or preeclamptic sera was increased

As our data mentioned previously showed that the expression of HMGB1 is increased in the cytoplasm of the syncytiotrophoblast as well as, in trophoblastic debris, we also quantified the levels of HMGB1 in conditioned medium from placental explants culture that had been treated with aPL or preeclamptic sera, as well as the relevant controls. The levels of HMGB1 were significantly increased in conditioned medium from explants that had been treated with aPL compared with those from explants that were untreated or treated with the isotype-matched control antibody (Fig. 5). However, there was no significant increase in the levels of HMGB1 in conditioned medium from explants that had been treated with preeclamptic compared with normotensive control sera (data not shown).

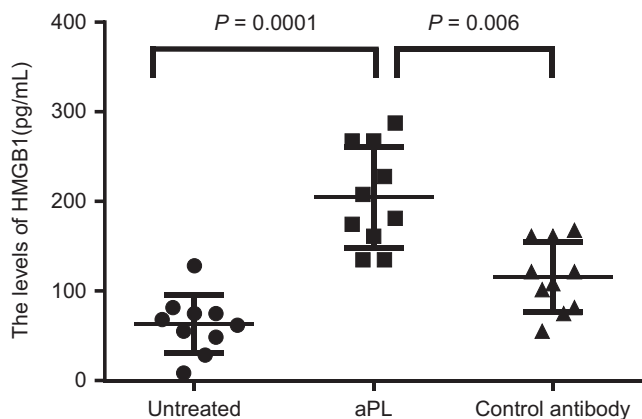


Figure 5 The levels of HMGB1 in conditioned medium from placental explants that had been treated with aPL for 24 h was significantly increased compared with untreated placental explants ($P=0.0001$) or conditioned medium from explants that had been treated with an isotype-matched control antibody ($P=0.006$) as measured by a commercial ELISA kit.

HMGB1 induced endothelial cell activation

Our previous studies showed that after exposure to trophoblastic debris extruded from placental explants treated with aPL or preeclamptic sera, endothelial cells became activated increasing their expression of cell-surface ICAM-1 and increasing monocyte adhesion (Chen *et al.* 2006, 2009a). To investigate whether increased levels of HMGB1 in trophoblastic debris could be one of the causes of this endothelial cell activation, we treated endothelial cells with

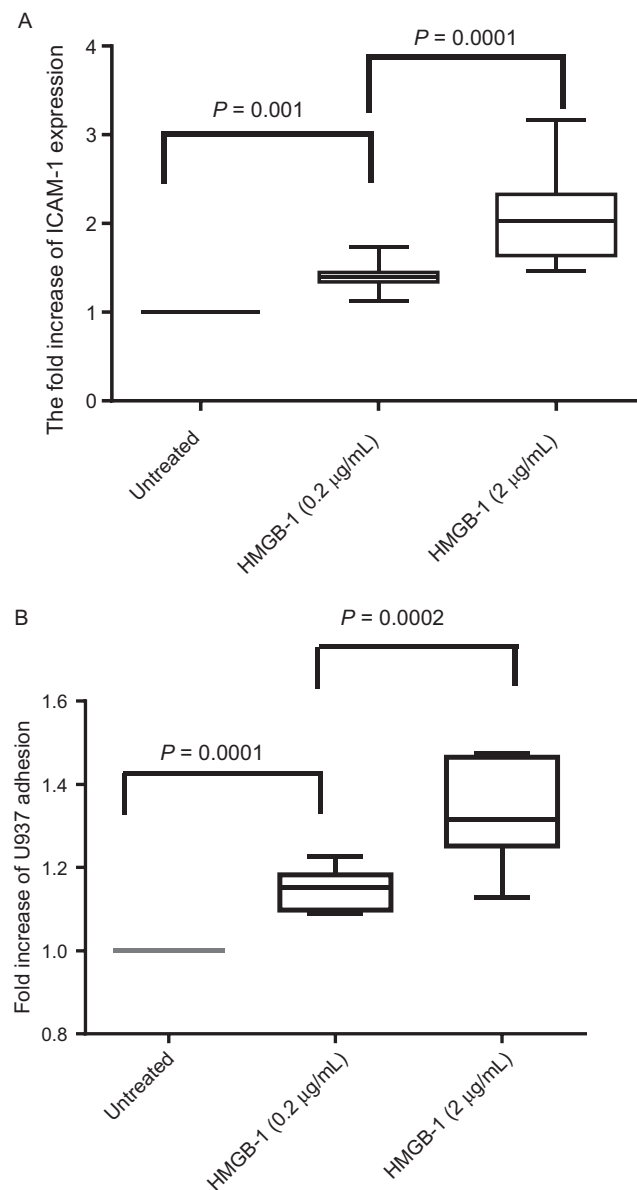


Figure 6 The level of, (A) expression of cell-surface ICAM-1 by monolayers of HMEC-1 cells determined by ELISA or (B) of U937 monocyte adhesion to HMEC-1 cells that were exposed to human recombinant HMGB1 (either 0.2 µg or 2 µg) for 24 h was significantly increased ($P=0.0001$, ANOVA). Quadruplicate wells of endothelial cells were exposed to each treatment during each of the three separate experiments. Graphs show median and 5–95th percentiles.

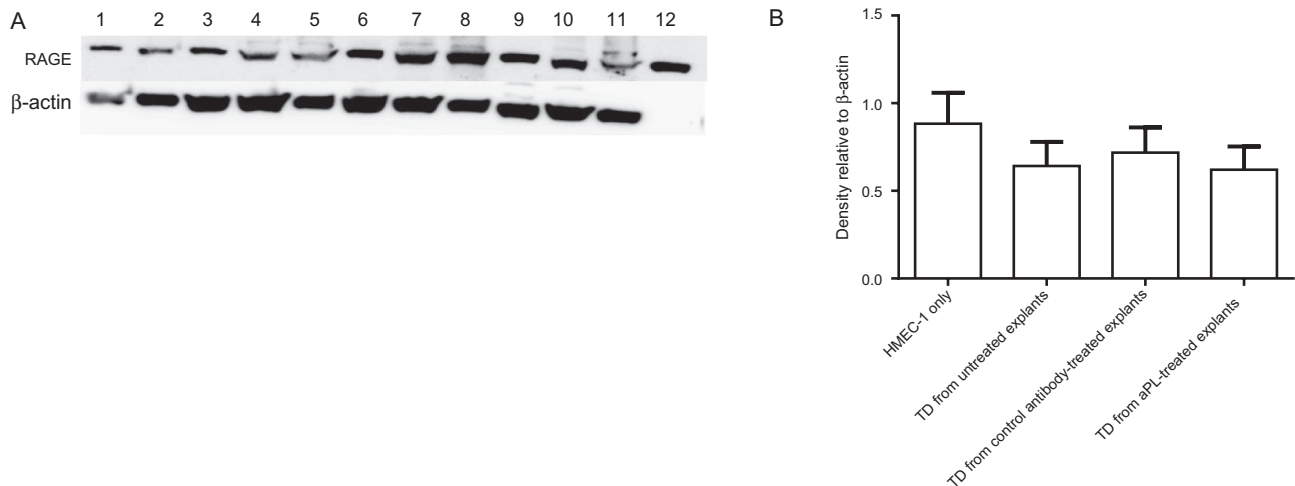


Figure 7 Representative western blots demonstrating (A) the levels of RAGE expressed by untreated HMEC-1s (lanes 1 and 2) or HMEC-1 cells exposed to trophoblastic debris (TD) from untreated placental explants (lanes 3–5) or HMEC-1 cells exposed to trophoblastic debris from placental explants that had been treated with an isotype-matched control antibody (lanes 6–8) for 24 h or HMEC-1s exposed to trophoblastic debris from placental explants that had been treated with aPL for 24 h (lanes 9–11) or normal plasma (positive control, lane 12). Levels of RAGE in the samples were normalised to β -actin (B), and there was no statistical difference among the groups. Western blots were repeated three times.

increasing concentrations of recombinant HMGB1, which resulted in a concentration-dependent, significant increase in cell-surface ICAM-1 expression and adhesion of U937 monocytes to the endothelial cells (Fig. 6, $P=0.0001$ ANOVA).

RAGE was expressed in endothelial cells

HMGB1 instigates many of its proinflammatory actions via upregulation of its receptor RAGE. Western blotting identified that RAGE was expressed by the HMEC1 endothelial cells, but RAGE was not upregulated by the endothelial cells in response to trophoblastic debris from explants that had been treated with aPL (Fig. 7). We then investigated whether blocking RAGE could block the effect of trophoblastic debris extruded from placental explants treated with aPL on endothelial cells. The increased levels of ICAM-1 induced by trophoblastic debris extruded from placental explants treated with aPL were partially reversed by treating the endothelial cells with a RAGE-neutralising antibody (Fig. 8).

Discussion

During normal pregnancy, trophoblastic debris is produced and shed into the maternal blood in large quantities where it is cleared partly by maternal endothelial cells. In normal pregnancies, the trophoblastic debris is 'safe' and has been shown to prevent endothelial cells from becoming activated (Chen *et al.* 2012a). In contrast, when placentae are exposed to preeclamptic sera or to aPL (a major risk factor for preeclampsia), the resultant trophoblastic debris is dangerous/toxic and activates endothelial cells. It is not clear why exposing placental explants to

aPL or preeclamptic serum induces the trophoblastic debris shed from the explants to become dangerous and activate endothelial cells. However, previous studies have shown that the danger signal, HMGB1, is present in the syncytiotrophoblast of preeclamptic pregnancies at increased levels (Zhu *et al.* 2015).

In this study, we have demonstrated that one change which occurs in placentae exposed to aPL or preeclamptic sera is an increase in the amount of cytoplasmic HMGB1 in the syncytiotrophoblast. This increase in cytoplasmic HMGB1 is specific to the syncytiotrophoblast (from which trophoblastic debris is derived) because a similar increase in HMGB1 did not occur in the mesenchymal cells, which also express basal levels of HMGB1, primarily in their nuclei. HMGB1 was originally described as a DNA-binding nuclear protein, and it is expressed by many cells, including trophoblasts (Wang *et al.* 2011). However, under conditions of stress or cell death, HMGB1 can translocate to the cytoplasm where it can regulate processes such as autophagy and apoptosis (Tsung *et al.* 2014). HMGB1 can also be secreted into the extracellular environment, but like some other proinflammatory proteins (e.g. IL-1 β), HMGB1 lacks a leader peptide and thus is not secreted through the cytoplasmic membrane via the classical protein secretion pathway. Instead in activated macrophages/monocytes, it is secreted via endolysosomal vesicles that are found in the extracellular environment as microvesicles that have budded from the plasma membrane (Gardella *et al.* 2002, Chen *et al.* 2016a). In this study, we quantified the level of expression of HMGB1 in cytoplasm, and we further found that placental explants that were treated with aPL or preeclamptic sera increased the levels of HMGB1 in the

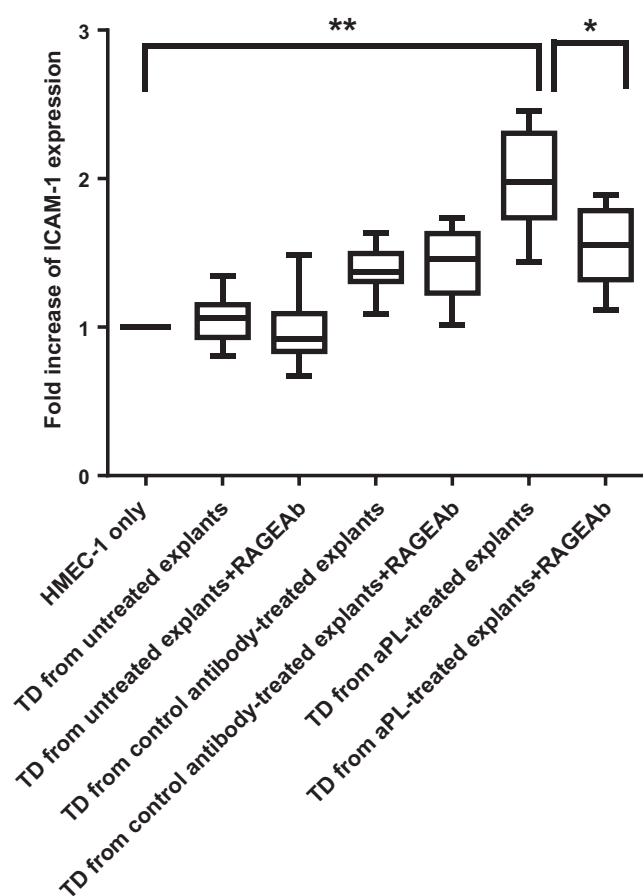


Figure 8 Cell-surface ELISA was used to determine the levels of expression of cell-surface ICAM-1 by monolayers of HMEC-1 that were exposed to trophoblastic debris (TD) extruded from placental explants that were untreated or treated with an isotype-matched control antibody or with aPL for 24 h in the presence or absence of a RAGE neutralising antibody (RAGEAb). Trophoblastic debris from aPL-treated placental explants significantly increased the ICAM-1 expression (** $P=0.0001$); however, this increased ICAM-1 expression was significantly reduced by adding the RAGE-neutralising antibody (* $P=0.0001$). Quadruplicate wells of endothelial cells were exposed to each treatment during each of three separate experiments. Graphs show median and 5–95th percentiles. (RAGEAb, RAGE neutralising antibody; TD, trophoblastic debris.)

cytoplasm of syncytiotrophoblast or cytotrophoblast. This suggests that these treatments (aPL or preeclamptic sera) are in some way stressing the syncytiotrophoblast resulting in the translocation of HMGB1 from the nucleus to the cytoplasm. Indeed, we have previously shown that aPL can enter the syncytiotrophoblast and disrupt mitochondrial function, which is highly likely to induce stress in the syncytiotrophoblast (Viall *et al.* 2013). Although the exact origin of trophoblastic debris is unclear, multinucleated syncytial nuclear aggregates, which are part of the debris, are membrane enclosed structures that possibly bud from the syncytiotrophoblast membrane and thus inclusion of HMGB1 in SNAs may be equivalent to the secretion of HMGB1 via microvesicles from mononuclear cells. When released

from the cytoplasm of cells, HMGB1 has been shown to act as a danger-associated molecular pattern (DAMP) or 'danger signal'. As cytoplasmic HMGB1 has the potential to be a danger signal, the apparent specific increase in cytoplasmic HMGB1 suggests that it is likely to be able to act as a danger signal if released from the syncytiotrophoblast. Although we did not quantify it, there was also an apparent general increase in the expression of HMGB1 in response to treatment of the explants with aPL. This increase may simply reflect an attempt to correct the loss of HMGB1 from the nucleus where it is the most abundant non-histone DNA-binding protein and performs important functions such as regulating DNA structure, replication and repair and is a transcription cofactor (Tsung *et al.* 2014). Our demonstration that debris released from aPL or preeclamptic sera-treated placentae contained increased amounts of cytoplasmic HMGB1 suggests that this danger signal in trophoblastic debris has the potential to activate endothelial cells that phagocytose trophoblastic debris. Our data also showed that extracellular levels of HMGB1 were significantly increased by treatment with aPL. In vivo, this secreted HMGB1 would be released into the maternal blood during pregnancy and may, along with the HMGB1 in trophoblastic debris, contribute to maternal endothelial cell activation in women with aPL. Therefore, to test this possibility, we confirmed that stimulation of endothelial cells with human recombinant HMGB1 resulted in the activation of endothelial cells as shown by an increase in the expression of ICAM-1 and U937 adhesion to endothelial cells. Consistent with other studies (Lee *et al.* 2015), our result suggests that extracellular HMGB1 acts as a danger signal to mediate endothelial cell activation and inflammation.

HMGB1 has several potential receptors (including TLRs 2, 4 and 9) (reviewed in Harris *et al.* 2012). RAGE is expressed by endothelial cells and is thought to be the key receptor in HMGB1-mediated endothelial cell activation (Luo *et al.* 2013). RAGE is a cell-surface receptor of the immunoglobulin superfamily expressed in many cells including endothelial cells. Our data confirm that the RAGE is expressed by endothelial cells and show that inhibiting RAGE with a function-blocking antibody partly reduced the activation of endothelial cells in response to trophoblastic debris from aPL-treated explants. This suggests that the increased levels of HMGB1 in this debris are partially responsible for inducing endothelial cell activation and that this signalling is, at least in part, via RAGE.

Other studies have shown that the expression of RAGE is increased when its ligands accumulate (Schmidt *et al.* 2001) or when endothelial cells are exposed to HMGB1 (Huang *et al.* 2012). However, we did not see a similar increase in the level of RAGE by endothelial cells exposed to trophoblastic debris from aPL or preeclamptic sera-treated explants. We do not know the reason for

this difference. However, this discrepancy may relate to the manner in which HMGB1 in trophoblast debris is exposed to the endothelial cell RAGE. For example, we do not know whether the HMGB1 from trophoblastic debris is released before phagocytosis of the debris by endothelial cells, thus being available to bind to cell-surface RAGE or at some later stage during/after the phagocytosis of the debris by the endothelial cells.

Although this study specifically focused on the role of HMGB1 in dangerous trophoblastic debris, it is unlikely that a single molecule is the only signal that renders trophoblastic debris dangerous. We have previously reported that trophoblastic debris from preeclamptic placenta contains elevated levels of the proinflammatory cytokine IL-1 β , which is in part responsible for activating endothelial cells. We believe that it is likely that debris from preeclamptic placenta contains several, if not numerous, molecules that together contribute to the dangerous nature of such debris, and which contribute to the pathogenesis of preeclampsia.

In conclusion, our study clearly demonstrates that treating normal placental explants with either antiphospholipid antibodies or preeclamptic sera increases the level of the danger signal HMGB1 in the cytoplasm of the syncytiotrophoblast. Moreover, the trophoblastic debris extruded from these treated explants also contains increased amounts of cytoplasmic HMGB1, which seems able to interact with RAGE (and possibly other receptors) on endothelial cells. This interaction in part contributes to the activation of these endothelial cells in response to this debris. Thus HMGB1, contained in trophoblastic debris, is likely to be one of the toxic factors released from the placenta that contribute to the pathogenesis of preeclampsia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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