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Trophoblast- and vascular smooth muscle cell-derived MMP-12 mediates elastolysis during uterine spiral artery remodelling

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Abstract

Objective - During the first trimester of pregnancy the uterine spiral arteries are remodelled, creating heavily dilated conduits lacking maternal vasomotor control. To effect permanent vasodilatation the internal elastic lamina and medial elastin fibres must be degraded. We sought to identify the elastolytic proteases involved. Methods and Results - Primary first trimester cytotrophoblasts (CTB) derived from the placenta exhibited intracellular and membrane-associated elastase activity; membrane-associated activity was primarily attributable to matrix metalloproteinases (MMP). Affymetrix microarray analysis and immunocytochemistry implicated MMP-12 (macrophage metalloelastase) as a key mediator of elastolysis. Cultured human aortic smooth muscle cells (HASMC) exhibited constitutive membrane-associated elastase activity and inducible intracellular elastase activity, and also expressed MMP-12 protein. A specific inhibitor of MMP-12 significantly reduced CTB- and HASMC-mediated elastolysis in vitro, to 31.7±10.9% and 23.3±8.7% of control activities, respectively. MMP-12 is expressed by interstitial and endovascular trophoblast in the first trimester placental bed and by vascular SMC (VSMC) in remodelling spiral arteries. Perfusion of isolated spiral artery segments with CTB-conditioned medium stimulated MMP-12 expression in medial VSMC. Conclusions - Our data support a model in which trophoblast and VSMC utilize MMP-12 cooperatively to degrade elastin during vascular remodelling in pregnancy, with the localized release of elastin peptides and CTB-derived factors amplifying elastin catabolism.
Introduction
Transformation of the uterine spiral arteries during the first twenty weeks of gestation ensures that a constant supply of blood is delivered to the developing placenta, at an optimal rate of flow. This allows the placenta to meet an increasing requirement for nutrients and oxygen, and enables the developing fetus to attain its growth potential. The remodelling process leads to vessel dilatation, loss of spirality and decreased vasoactivity, allowing a non-pulsatile, low pressure supply of blood to be delivered to placental villi at the maternofetal interface. Early alterations in arterial structure include endothelial vacuolation, hypertrophy of vascular smooth muscle cells (VSMC) and disruption of medial smooth muscle layers, which occur in the absence of fetal-derived trophoblast and correlate with perivascular accumulation of macrophages and uterine natural killer (uNK) cells. Following colonisation of the uterine decidua and myometrium by extravillous cytotrophoblast (EVT), endothelial cells and VSMC are lost from the arterial wall and replaced by trophoblast embedded in a fibrinoid matrix. Remodelling is regulated in a spatial and temporal manner, such that the successive steps of trophoblast adherence, intravasation, fibrinoid deposition and mural incorporation are effected without any loss in vessel integrity. A complex and highly orchestrated combination of vascular cell apoptosis, dedifferentiation and matrix breakdown is probably required to achieve this alteration in vessel wall structure.

Two distinct populations of EVT originate from anchoring placental villi and contribute to vessel transformation. Interstitial EVT invade the uterine wall, migrating through the decidua and myometrium to adopt a perivascular position. Endovascular EVT enter the lumen of the spiral arteries and migrating as far as the first third of the myometrium, colonising the arterial wall from within. Impaired arterial remodelling is distinguished by shallow EVT invasion, decreased numbers of EVT and the persistence of muscular, narrow-
bore arteries, and is associated with second trimester miscarriage\textsuperscript{12}, preterm labour\textsuperscript{13}, pre-eclampsia\textsuperscript{14} and fetal growth restriction\textsuperscript{15}.

To effect a permanent increase in vessel diameter it is crucial that elastin fibres within each artery are catabolised, eliminating their capacity for stretch and recoil. Myometrial segments of the spiral arteries possess an internal elastic lamina (IEL), and the musculo-elastic media of both decidual and myometrial arteries is rich in elastic fibres\textsuperscript{16,17}. During pregnancy, EVT traverse the IEL during mural incorporation\textsuperscript{18}, thus it is highly likely that they possess elastase activity: indeed, first trimester EVT synthesize and secrete the elastolytic proteases matrix metalloproteinase-2 (MMP-2), MMP-7, MMP-9, cathepsin B and cathepsin L\textsuperscript{19,20}. Although both uNK cells and macrophages produce enzymes capable of elastolysis\textsuperscript{5}, uNK cells are not abundant in myometrium\textsuperscript{21}, and elastin breakdown is associated with the presence of endovascular EVT\textsuperscript{17}, rather than macrophages\textsuperscript{22}. Previous studies have demonstrated that the availability of nitric oxide (NO) can influence protease expression and activity\textsuperscript{23-26}, and we have shown NO to be an important regulator of trophoblast function\textsuperscript{27-29}. As dysregulation of NO production has been implicated in the pathogenesis of pre-eclampsia and IUGR\textsuperscript{30-32}, NO availability may regulate the process of arterial remodelling by controlling trophoblast elastolysis.

Rodent models of atherosclerosis have highlighted a role for VSMC-derived cathepsins as mediators of IEL breakdown during lesion formation\textsuperscript{33}, demonstrating that the arterial wall may be a potential source of elastases. Similarly, caspase-2, -3 and -7 derived from apoptotic VSMC have been implicated as mediators of elastin breakdown\textsuperscript{34}. Thus, during the process of spiral artery transformation, resident VSMC may also be stimulated to produce elastase(s) in response to pregnancy hormones, trophoblast invasion or soluble factors released by cells.
within the placental bed. In this study we have investigated the origin and identity of the proteases involved in mediating elastin breakdown during spiral artery remodelling.
Materials and methods

Reagents

Caspase inhibitor zVAD-fmk, cathepsin inhibitor Z-Phe-Gly-NHO-Bz-pOMe, Calbiochem (San Diego, CA, USA); MMP inhibitor N-Isobutyl-N-(4-methoxyphenylsulfonyl)-glycyl hydroxamic acid (NNGH), Biomol International (Exeter, UK); uPA-STOP, American Diagnostica Inc. (Stamford, CT, USA); in situ cell death detection kit (TUNEL), Roche (Lewes, UK); CellTracker CM-Dil, Molecular Probes (distributed by Invitrogen Ltd, Paisley, UK); recombinant human TRAIL, PeproTech (London, UK); iNOS inhibitor 1400W, Axxora (Nottingham, UK); porcine pancreatic elastase, N-succinyl-(L-alanine)3-p-nitroanilide, elastin from bovine neck ligament, Congo-Red labelled elastin, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME hydrochloride), phenylmethanesulfonyl fluoride (PMSF), Sigma- Aldrich (Poole, UK); tissue culture medium, glutamine, antibiotics, Cambrex (Wokingham, UK); fetal bovine serum (FBS), Gibco (distributed by Invitrogen Ltd, Paisley, UK); OCT embedding medium, Raymond A Lamb (London, UK); Matrigel, BD Discovery Labware (Bedford, MA, USA); mouse anti-human cytokeratin-7 monoclonal antibody (clone OV-TL 12/30), mouse anti-human α-smooth muscle actin monoclonal antibody (clone 1A4), mouse anti-human CD31 monoclonal antibody, universal negative control IgG (IgG1, IgG2a, IgG2b, IgG3 and IgM; 20µg/ml of each isotype, 100µg/ml total concentration), biotinylated rabbit anti-mouse, goat anti-mouse and swine anti-rabbit secondary antibodies, streptavidin-FITC, Dako (Glostrup, Denmark); mouse anti-human MMP-12 monoclonal antibody (catalytic domain), R&D Systems (Minneapolis, MN, USA); rabbit anti-human MMP-12 monoclonal antibody (carboxyterminal; EP1261Y), mouse anti-human HLA-G monoclonal antibody, Abcam (Cambridge, UK); Vectashield mounting medium, Vector Laboratories Inc. (Burlingame, CA, USA); Elastolux® intracellular elastase activity assay, OncoImmunin (Gaithersburg, MD, USA). U133A/B GeneChips were obtained from
Affymetrix (Affymetrix Inc., Santa Clara, CA). Labelling reagents for GeneChips were purchased from Enzo Life Sciences (Enzo, Farmingdale, NY). The specific MMP-12 inhibitor 470.1 ($K_i$ 0.19nM; purity >98%) was kindly donated by Dr V Dive (Compound 1 in \textsuperscript{35}).

Cell culture

Primary CTB were isolated as previously described \textsuperscript{36} and were cultured in a 1:1 ratio DMEM : Ham’s F12 medium supplemented with FBS (10%), glutamine (2 mM), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). CTB were grown on Matrigel-coated flasks (diluted 1:10 in serum-free medium) to promote a more advanced extravillous phenotype. Cells were also seeded onto Matrigel-coated cover slips and cultured for 48h for immunostaining. After 48h in culture, 91.1 ± 4.2% (n=5) of cells were immunopositive for cytokeratin-7. Cells were also positive for HLA-G. To prepare CTB-conditioned medium, 1.5 x 10\textsuperscript{6} CTB were seeded into a Matrigel-coated T\textsubscript{25} flask and left for 3h to adhere. The medium was removed, the cells were washed with PBS and 5ml of fresh, serum-free DMEM/F12 was added to the flask. After 48h the medium was removed, centrifuged to eliminate cell debris, and stored at -20°C. The mean protein concentration of the CTB-conditioned medium was 193.45 ± 5.6 µg/ml (n=8). Conditioned medium was diluted 1:1 with unconditioned medium before use.

Human aortic SMC (HASMC) were cultured in Kaighn’s modification of Ham’s F12 medium and SGHPL-4 cells were cultured in Ham’s F10 medium. Media was supplemented with FBS (10%), glutamine (2 mM), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). To generate HASMC- and SGHPL-4-conditioned medium, 0.5 x 10\textsuperscript{6} cells were seeded into a T\textsubscript{25} flask. After 24h the medium was removed,
centrifuged to eliminate cell debris and stored at -20°C. The mean protein concentrations of the HASMC and SGHPL-4 conditioned media were 244.36 ± 10.0 µg/ml (n=8) and 261.31 ± 6.7 µg/ml (n=6), respectively. Conditioned medium was diluted 1:1 with unconditioned medium before use.

Tissue
Informed consent was obtained for all myometrial and placental tissue used in this study and local ethics committee approval was in place. First trimester placenta (n=21) and decidua (n=8; 7-12 weeks gestation) was obtained following surgical or medical termination of pregnancy for psychosocial reasons. Term decidual/myometrial biopsies (n=6) taken from non-placental bed tissue were obtained from women with normal pregnancies at elective caesarean section.

Vessel explant model
Dissection and perfusion of spiral arteries was performed as previously described. Non-placental bed decidual/myometrial biopsies were obtained at Caesarean section from healthy pregnant women at term. Unmodified spiral arteries were dissected under sterile conditions and mounted on glass cannulae in a pressure myography perfusion chamber (Living Systems Instrumentation). A subset of vessels were fixed immediately and the remaining arteries were denuded of endothelium by passing a column of air through the lumen; removal of the endothelium was confirmed by CD31 immunostaining. Denuded arteries were perfused with unconditioned CTB-medium (1:1 DMEM:Ham’s F12; control) or CTB-conditioned medium (diluted 1:1 with unconditioned DMEM:Ham’s F12). The ends of each vessel were tied off to prevent the
medium from leaking out and the arteries were cultured in 1:1 DMEM:Ham’s F12 culture medium for 24h.

**Immunocytochemistry**

Arteries were fixed (2% (v/v) paraformaldehyde in PBS, 30 min) and incubated with sucrose (0.5 M in PBS; 1 h). Tissue was embedded in OCT, frozen and cut into 10 µm transverse sections. For immunostaining of VSMC, cells were cultured on glass cover slips. Cells/tissue sections were fixed (4% (v/v) paraformaldehyde in PBS; 20 min), permeabilised (0.1% (v/v) Triton-X in PBS; 5 min) and washed in PBS. Antibodies were applied for 1 h and slides were washed (3 x 5 min; PBS) following each antibody incubation. **Working concentrations of antibodies were as follows:** cytokeratin-7 (0.9µg/ml), CD31 (5.15µg/ml), MMP-12 (catalytic domain; 10µg/ml), biotinylated goat anti-mouse (7µg/ml) and swine anti-rabbit secondary antibodies (4.05µg/ml), streptavidin-FITC (10µg/ml), control IgG (20µg/ml of each isotype, 100µg/ml final concentration). Sections and cover slips were mounted using Vectashield mounting medium containing propidium iodide or DAPI and imaged using either an Olympus IX70 inverted fluorescence microscope or a Biorad Radiance 2100 confocal microscope with a 10x or a 40x oil immersion objective lens and LaserSharp 2000 image analysis software.

**Immunohistochemistry**

Wax-embedded decidual tissue sections (5µm) were deparaffinised in Histoclear and alcohol, and microwaved for 10 minutes in sodium citrate buffer (0.01M; containing 0.05% (v/v) Tween 20, pH 6.0) to facilitate antigen unmasking. After cooling, endogenous peroxide activity was blocked by placing the slides in methanol containing 0.4% (v/v) HCl and 0.5% (v/v) hydrogen peroxide for 30 minutes. Tissue sections were washed three times in 0.05M
Tris buffered saline (TBS) and blocked with 5% (w/v) BSA in TBS for 30 minutes. Primary antibodies, diluted to working concentration with 0.05M TBS (cytokeratin-7, 0.9µg/ml; MMP-12 (carboxyterminal), 4µg/ml; HLA-G, 2µg/ml; α-smooth muscle actin, 0.18µg/ml; control IgG, (20µg/ml of each isotype, final concentration 100µg/ml) were applied to the tissue sections, which were incubated overnight at 4°C in a humidity chamber. Slides were washed (3x TBS) and the secondary antibodies, diluted in TBS (biotinylated swine anti-rabbit IgG, 4.05µg/ml; biotinylated goat anti-mouse IgG, 7µg/ml), were applied for 30 min at room temperature. Slides were washed again (3x TBS) and incubated with avidin peroxidase (5µg/ml in 0.125M TBS) for 30 min at room temperature. Slides were washed in TBS and incubated for 1-5 min with 0.05% (w/v) DAB and 0.015% (v/v) hydrogen peroxide. Slides were washed in dH2O, counterstained with hematoxylin, rehydrated in alcohol and Histoclear, and mounted in DPX mountant containing distyrene, a plasticizer, and xylene.

**Intracellular elastase activity assay**

Intracellular elastase activity was quantified using an Elastolux® assay kit. Cells were cultured alone or with protease inhibitors as described. After trypsinisation, cell pellets were incubated in culture medium containing a cell-permeable elastase substrate (9µM) and 10% FBS, for 1h at 37°C. Upon cleavage by intracellular elastases, the substrate becomes fluorescent and is retained within the cell. Cells were washed once in PBS and the percentage of fluorescent cells was quantified using a Coulter Epics Elite flow cytometer.

**Total elastase activity assay**

Cell extracts were generated by incubating CTB, SGHPL-4 or HASMC with 0.1% (v/v) Triton X100 in PBS for 30 minutes. This process induced cell lysis, release of
intracellular elastases and dissociation of membrane-associated elastases. Extracts were centrifuged to remove any cellular debris and the resulting supernatant, which contained both intracellular and membrane-associated elastases, was retained for analysis. No elastase activity was detected in the cell pellets. Supernatants or conditioned culture medium (50µl) were incubated with 200µl of N-succinyl-(L-alanine)3-p-nitroanilide (1mM) dissolved in Tris-HCl (200mM; pH 8.0) for 2h at 37°C in a 96 well plate. Upon cleavage by elastase this substrate absorbs at 405nm. After addition of 5µl of glacial acetic acid, the A405 of each sample was determined and compared to a calibration curve prepared using porcine pancreatic elastase. The protein concentration of each sample was measured using a Biorad protein assay with a standard curve prepared using bovine serum albumin. Elastase activity data was expressed as activity per mg of protein.

Time lapse microscopy

Proliferating SGHPL-4 and HASMC (0.25X10⁶ cells) were seeded into 6 well plates and left to adhere. Following addition of elastin (1mg/ml), NNGH (50µM) and L-NAME (5mM), elastin uptake was monitored by time-lapse microscopy using an Olympus IX70 inverted fluorescence microscope with a motorised stage and cooled CCD camera (Hamamatsu model C4742-95) enclosed in a humidified chamber at 37°C with 5% CO₂ in air, as described previously 37. Images were taken at 15 min intervals and time-lapse sequences were analysed using ImagePro Plus (Media Cybernetics, Silver Spring, MD, USA). Fifteen cells were scored from each field of view (wells were analysed in triplicate) and the time at which a cell took up a fragment of elastin was recorded.

RNA extraction, cRNA synthesis, and gene expression profiling
EVT outgrowths from villous explants cultured on Matrigel (obtained from six different first trimester placentas, 7-10 weeks gestation) were isolated and total RNA was extracted as previously described \(^{38, 39}\). RNA integrity was checked by agarose gel electrophoresis and with the Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Palo Alto, CA). 5µg of total RNA were then used for GeneChip analysis. Preparation of cRNA, hybridization to human U133 GeneChips, and scanning of the arrays were performed according to the manufacturer’s protocols (Affymetrix Inc., Santa Clara, CA), as previously described \(^{40}\).

**Statistics**

Statistical analyses were performed using GraphPad Prism software version 4 (GraphPad Software, San Diego, CA, USA). Non-parametric data was represented by median and range, and was analysed using a Kruskal-Wallis tests with appropriate post-hoc analysis. Data represent at least 3 independent experiments. Significance was taken as p<0.05.
Results

Elastase activity in trophoblasts is attributed to caspases and matrix metalloproteinases

To investigate the contribution of trophoblast-derived elastases to arterial remodelling, we analysed intracellular and total elastase activity of human first trimester CTB. When cultured with Congo red-labelled elastin, CTB were observed to engulf and degrade elastin fibres (Figure 1A). CTB exhibited detectable intracellular elastase activity, which was significantly reduced by a broad spectrum caspase inhibitor, but only when CTB were isolated from placentas of less than 9 weeks of gestation (P<0.05; Figure 1B). None of the broad spectrum protease inhibitors tested reduced the intracellular elastase activity of CTB at later gestations, when added individually, or in combination (Figure 1C). Cell supernatants containing both cytoplasmic and membrane-associated proteases were prepared by incubating CTB (8-10 weeks gestation) or the first trimester extravillous trophoblast cell line SGHPL-4 with 0.1% (v/v) Triton X-100. Supernatants were incubated with the elastase substrate N-succinyl-(L-alanine)3-p-nitroanilide to quantify elastase activity. Both SGHPL-4 cells and CTB supernatants demonstrated marked elastase activity, which was completely inhibited by the broad spectrum MMP inhibitor NNGH (P<0.05, Figure 1D, E). As the MMP inhibitor had no effect on intracellular elastase activity, the MMP activity in the CTB supernatants must be attributable to membrane-associated elastases. Elastase activity in CTB conditioned medium was below the level of detection of this assay (data not shown).

To investigate the kinetics of elastin breakdown, SGHPL-4 cells were incubated with elastin and monitored at 15 minute intervals for 24h by time lapse video-microscopy. Over time, cells began to engulf and degrade these fibres, retaining small pieces of elastin within their cytoplasm (Figure 2A). After 24h, approximately 85% of control cells contained one or more elastin fragments (Figure 2B). Addition of the broad spectrum MMP inhibitor zVAD-fmk to
the cultures significantly reduced the percentage of SGHPL-4 cells containing elastin to 66% of controls after 6h and 81% after 24h (P<0.05; Figure 2C). It has previously been shown that in some cell types, the availability of nitric oxide (NO) can influence the expression and activity of certain proteases 23-26, and we have previously identified MMP-9 as a potential target for S-nitrosylation in trophoblast 29. Thus, we investigated whether elastin uptake was NO-dependent, using the NOS inhibitor L-NAME. In the presence of L-NAME, the percentage of cells containing elastin fragments was significantly reduced after 6h (P<0.01), but not at any other time points, implying that NO availability is not a prerequisite for elastin uptake.

**Identification of candidate elastases in first trimester trophoblasts**

To determine the identity of candidate trophoblast elastases, a transcriptomic approach was employed. Affymetrix gene chip analysis of two separate pools of first trimester EVT mRNA (7-10 weeks gestation) demonstrated that caspase-2, MMP-2 and MMP-12 were the most highly expressed protease transcripts (Table 1). The role of MMP-2 in migration and invasion of first trimester EVT has been well characterised previously 20; however, the role of MMP-12 (macrophage metalloelastase) in trophoblast function has not been investigated.

**MMP-12 mediates elastolysis in first trimester trophoblasts**

To validate our transcriptomic data, isolated first trimester CTB were immunostained with an antibody to MMP-12, confirming protein expression (Figure 3A, B). As previously shown, pre-treatment of individual CTB supernatants (7-12 weeks gestation) with a broad spectrum MMP inhibitor abolished elastase activity (P<0.001; Figure 3C). Pre-treatment of the same supernatants with a specific inhibitor of MMP-12 (470.1) significantly reduced elastase
activity to a mean value of 31.7±10.9% (P<0.05) of control activity, implicating MMP-12 as an important membrane-associated elastase in trophoblast.

**Intracellular elastase activity of HASMC is stimulated by elastin fragments**

As VSMC-derived elastases have been shown to mediate elastin breakdown during the progression of atherosclerosis and aneurysm, we investigated whether VSMC could contribute to the elastolysis observed during arterial remodelling in pregnancy. Only a small proportion of cultured HASMC exhibited exogenous intracellular elastase activity (4.81%±1.4; n=7); this was unchanged following culture on Matrigel, a collagen IV and laminin-rich extracellular matrix preparation similar in composition to the basement membrane on which VSMC reside in vivo (Figure 4A). Similarly, HASMC cultured with CTB-conditioned medium did not exhibit increased elastase activity, suggesting that soluble factors released from trophoblast do not modulate intracellular elastase activity in vitro. As VSMC may be exposed to elastin fragments generated by trophoblast-mediated elastolysis during remodelling in vivo, HASMC were cultured with elastin fibres. This treatment significantly enhanced intracellular elastase activity (P<0.05), suggesting that the presence of elastin fragments in vivo may stimulate elastase production in the arterial wall.

**Intracellular elastase activity of HASMC is partially mediated by serine proteases**

To identify the intracellular proteases responsible for the observed elastase activity, whilst minimising the amount of elastin introduced into the flow cytometer, HASMC were cultured on elastin-coated plates for 48h and incubated with cell-permeable protease inhibitors for a further 6h. Broad spectrum caspase-, MMP- and cathepsin inhibitors had no effect on intracellular elastase activity (Figure 4B); however, the serine protease inhibitor PMSF reduced activity by approximately 35% (P<0.05; Figure 4C). Pre-treatment of HASMC with
the broad spectrum NOS inhibitor L-NAME or the specific inducible NOS (iNOS) inhibitor 1400W for 24h had no effect on intracellular elastase activity, suggesting that it was not NO-dependent.

Membrane-associated elastase activity in HASMC is MMP-dependent
To identify membrane-associated, pericellular or secreted elastases, HASMC supernatants or conditioned medium were incubated with N-succinyl-(L-alanine)₃-p-nitroanilide. HASMC cultured on plastic exhibited constitutive membrane-associated elastase activity, which was completely inhibited by a broad spectrum MMP inhibitor (P<0.01; Figure 4D). HASMC cultured on plates coated with elastin did not exhibit an enhancement of elastase activity (0.435 ± 0.16 µg elastase/mg protein (plastic) versus 0.316 ± 0.09 µg elastase/mg protein (elastin), mean±SEM; n=4) and again, activity was completely inhibited by a broad spectrum MMP inhibitor (P<0.05). The broad spectrum caspase inhibitor zVADfmk, the cathepsin inhibitor Z-FG-NHO-BzOMe and the urokinase plasminogen activator (uPA) inhibitor uPA-STOP had no effect. As the MMP inhibitor had no effect on intracellular elastase activity of HASMC, the activity detected in the cell supernatants must be attributable to membrane-associated elastases. Elastase activity present in the conditioned medium of HASMC was below the level of detection (data not shown).

Membrane-associated elastase activity in HASMC is NO-dependent
To investigate whether the activity of elastolytic MMP was NO-dependent, HASMC were pre-treated with L-NAME or the iNOS inhibitor 1400W for 24h, prior to preparation of cell supernatants. Elastase activity was significantly decreased by both inhibitors, indicating that NO availability is necessary for effective MMP function in HASMC (P<0.05; Figure 4E).
Elastin uptake is MMP- and NO-dependent

To study elastin fragment uptake over time, HASMC were incubated with elastin and monitored by time lapse video-microscopy (Figure 5A). When HASMC were cultured with elastin alone, approximately 80% of control cells contained elastin fragments after 24h (Figure 5B). Addition of NNGH or L-NAME to the cultures had a marked effect on the kinetics of elastin uptake. Uptake was significantly decreased at 6h, 12h and 24h in the presence of the MMP inhibitor (P<0.01, P<0.001), and at 6h and 12h by L-NAME (P<0.05, P<0.001; Figure 5C). These data suggest that MMP and NO are required for effective elastin uptake by HASMC.

VSMC express elastolytic MMP in vitro and in vivo

To investigate whether cultured HASMC express MMP-12, immunostaining was used to confirm expression of the active form (Figure 6A, B). Membrane-associated elastase activity of HASMC was reduced to a mean value of 23.3±8.7% of control activity following pre-treatment of HASMC supernatants with a specific inhibitor of MMP-12 (P<0.05; Figure 6G). As previously observed, the broad spectrum MMP inhibitor NNGH abolished elastase activity (P<0.001). These data implicate MMP-12 as an important mediator of HASMC elastolysis in vitro.

During pregnancy, invading EVT release soluble factors including FasL and TRAIL, which trigger VSMC apoptosis. To assess whether these factors can alter MMP-12 expression in spiral artery VSMC ex vivo, unremodelled spiral arteries were dissected from myometrial biopsies obtained from healthy pregnant women undergoing caesarean section at term. Vessels were denuded of endothelium to facilitate access of factors to the underlying VSMC, then perfused with unconditioned medium (control), CTB-conditioned medium (which
contains detectable soluble FasL but not TRAIL \(^{41, 42}\) or recombinant human TRAIL (rhTRAIL), and cultured for 24h. CD31 immunostaining confirmed removal of the endothelium, whilst morphological analysis of the arterial wall verified the integrity of the underlying VSMC layers (Figure 6C, D). No MMP-12 expression was observed in VSMC of freshly isolated arteries with intact endothelium (Figure 6E), or in denuded vessels perfused with unconditioned (control) medium (Figure 6F), indicating that removal of the endothelium does not upregulate MMP-12 expression in the underlying VSMC. Introduction of CTB-conditioned medium or rhTRAIL into the lumen of denuded arteries induced expression of active MMP-12 in VSMC, although the extent of expression was heterogeneous, with only 30% of tissue sections examined containing MMP-12-positive cells (Figure 6G, H). These data suggest that factors produced by EVT during colonisation and remodelling of the spiral arteries have the ability to influence MMP-12 expression in a proportion of mural VSMC.

**MMP-12 is expressed by trophoblast and VSMC in the first trimester of pregnancy**

Sections of first trimester placenta and decidua (8-12 weeks gestation) were stained with an antibody to MMP-12; villous CTB, trophoblast cell columns and EVT were immunopositive (Figure 7A, B, D, E). A subset of villous stromal cells, most likely placental macrophages (Hofbauer cells), also expressed MMP-12 (Figure 7D). Medial VSMC in unremodelled arteries did not express MMP-12, consistent with our ex vivo observations; however, decidual stromal cells were weakly positive for MMP-12 and strong expression was noted in the vascular endothelium (EC; Figure 7C, F). MMP-12 expression was also observed in endovascular EVT (eEVT; identified by HLA-G immunostaining) and heterogeneously in disrupted VSMC (identified by \(\alpha\)-smooth muscle actin immunostaining) in remodelling spiral arteries (Figure 7G-L).
Discussion

Remodelling of uterine spiral arteries during pregnancy involves not only replacement of vascular endothelial cells and VSMC by invading trophoblast, but also structural and functional transformation of the vascular extracellular matrix. To allow a permanent increase in luminal diameter, the internal elastic lamina and medial elastin fibres must be degraded. Matrix breakdown is mediated in part by the actions of trophoblast-derived proteases, and we now provide evidence that spiral artery VSMC-derived proteases add their own contribution to elastin catabolism.

Primary first trimester CTB can phagocytose elastin in vitro, and exhibit both intracellular and membrane-associated elastase activity. Intracellular activity is partially attributable to caspases in early gestation, but we were unable to identify candidate enzymes beyond 9 weeks, possibly due to functional redundancy of the enzymes involved. Approximately 60-70% of membrane-associated elastase activity was attributable to MMP-12 (macrophage metalloelastase), which is known to act in association with the cell surface; it is likely that the remaining proportion is mediated by a combination of MMP-2, -7 and -9, which are capable of elastolysis and are expressed by first trimester trophoblast. MMP-12 has previously been implicated as a potential regulator of trophoblast adhesion and migration; here we identify MMP-12 as a candidate elastase which is expressed by both interstitial and endovascular trophoblasts in close proximity to spiral arteries in first trimester. Although NO bioavailability is an important regulator of trophoblast function, including MMP-9 activity at the leading edge of migrating cells, phagocytosis of elastin by trophoblast was not reduced in the presence of a NOS inhibitor.
Elastin degradation appears to be a cooperative process, because VSMC also have the capacity to engulf and degrade small particles of elastin using intracellular and membrane-associated enzymes. *In vitro*, HASMC expressed low endogenous levels of intracellular elastase; however, this was significantly increased following culture with elastin, and was mediated by serine proteases independently of NO bioavailability. In contrast, HASMC exhibited constitutive, membrane-associated elastase activity, which was attributable to MMP and caspases, was significantly reduced by broad spectrum- and iNOS-specific inhibitors, and was not significantly increased following culture with elastin. Phagocytosis of elastin fragments was reduced in the presence of inhibitors of either MMP or NOS. A major fraction of membrane-associated elastase activity in VSMC was attributable to MMP-12; moreover, expression of MMP-12 was induced in the mural VSMC of excised spiral arteries that had been denuded of endothelium and perfused with CTB-conditioned medium. We therefore hypothesise that soluble factors secreted by endovascular EVT upregulate MMP expression in spiral artery VSMC *in vivo*, which contributes to, or is a consequence of, the alteration in VSMC phenotype observed in actively remodelling vessels. Indeed, the hypertrophy, dedifferentiation, and disruption of medial smooth muscle layers observed *in vivo* reflects transition from a stable, contractile phenotype towards a more synthetic, proliferative state, a change that has previously been linked to enhanced MMP expression. Furthermore, cultured HASMC constitutively expressed MMP-12 *in vitro*, whereas medial VSMC in excised spiral arteries did not, and CTB-conditioned medium induced MMP-12 expression in spiral artery VSMC in situ but did not enhance elastase activity of undifferentiated HASMC *in vitro*. These observations suggest that induction of MMP-12 expression in VSMC may be coincident with dedifferentiation and loss of contractile phenotype. Our finding that MMP-12 is expressed in layers of disrupted VSMC within remodelling arteries, but is absent from the organised VSMC layers of unremodelled vessels, supports this hypothesis.
Breakdown of elastin and formation of elastin-derived peptides (EDP) plays an important role in the regulation of tissue remodelling. Macrophage-derived MMP-12 is crucial for the development of emphysema in a murine model: release of chemotactic EDP recruits monocytes to the lung, which differentiate into macrophages, generate more EDP and perpetuate the condition. EDP also stimulate chemotaxis and chemokinesis of leukocytes into the walls of damaged arteries, where they contribute to matrix breakdown and repair. It is therefore interesting to note that leukocytes adopt a perivascular location around untransformed vessels in the first trimester placental bed, and mediate the initial stages of vascular transformation. EDP can also stimulate quiescent arterial VSMC to adopt a more proliferative phenotype, and promote MMP expression in cancer cell lines. In addition, EDP may regulate NOS activity, as elastin peptides stimulated NO production in coronary artery endothelial cells and cardiomyocytes. Our data support a model whereby trophoblast-mediated breakdown of the elastin within the spiral arteries liberates EDP that promote MMP-12 expression in medial VSMC. EDP may also increase NO production and MMP activity in trophoblasts, establishing a positive feedback mechanism by which EDP production and MMP-12 activity are maintained.

Elastase activity in HASMC, but not CTB, was dependent on NO bioavailability. This is in contrast to previous work showing that NO donors stimulated MMP expression and activity in term CTB, whereas NOS inhibitors reduced it. These conflicting data may be explained by the later gestational age of the cells, or because we were studying elastolysis, rather than collagenolysis, which is primarily mediated by MMP-2 and -9. Nitration of a tyrosine residue in active site of MMP-2 by peroxynitrite or nitrosylation of a critical cysteine residue in MMP-9 by NO and other reactive nitrogen oxides has been shown to promote enzymatic
activity. However, cysteine residues in the zinc-tetrathiolate cluster of endothelial NOS (eNOS) and iNOS are susceptible to NO-induced thiol modification, leading to impaired stability of NOS dimers \(^{61-63}\). Hence, high concentrations of NO resulting from forced expression of iNOS may reduce NOS activity, and therefore MMP activity. Cellular localisation could hold the key to successful MMP regulation by NO: we and others have observed concentrated expression of eNOS \(^{64}\) and iNOS \(^{29}\) in membrane ruffles at the leading edge of migrating, but not static cells. Thus, localization of NO production to the site of MMP activation may be important in regulating enzyme activity, matrix breakdown and cell migration.

In summary, we have identified a novel role for MMP-12 as a mediator of uterine vascular remodelling during pregnancy. Our data support a model whereby trophoblast and vascular VSMC utilize MMP-12 to cooperatively degrade mural elastin during spiral artery remodelling, with the localized release of elastin peptides and CTB-derived factors amplifying elastin catabolism. The role of leukocytes in this scheme of events has yet to be addressed; however, cytokines or chemokines released by decidual macrophages and uNK cells may influence MMP-12 expression in VSMC and EVT \(^{44}\). Breakdown of elastin in decidual vessels may be initiated by resident VSMC prior to the arrival of the trophoblast, facilitating the entry of both leukocytes and EVT into the arterial wall. Macrophages and uNK cells may also contribute to elastolysis in decidual arteries, via the release of MMP-7 and MMP-9 \(^{5}\). However, MMP-12 expression and elastolysis may not occur efficiently in the deeper myometrial vessel segments until trophoblasts reside in close proximity to the medial VSMC. Interestingly, elastin catabolism is impaired in the myometrial spiral arteries of women with pre-eclampsia \(^{17}\), and a dramatic reduction in expression of MMP-12 mRNA has been reported in the first trimester placentas of women.
who subsequently develop pre-eclampsia \textsuperscript{65}, implicating MMP-12 as a potential therapeutic target. As MMP-12 is a multifunctional enzyme that is capable of degrading not only elastin, but collagen IV, laminin, fibronectin, heparan sulphate and vitronectin \textsuperscript{66}, there may be serious consequences if MMP-12 expression is suboptimal. Pre-eclampsia is also characterised by increased circulating concentrations of the endogenous inhibitor of NOS asymmetric dimethyl arginine (ADMA) \textsuperscript{30, 32}. It is therefore possible that this may limit NO production, reduce elastase activity and impair arterial vasodilatation. However, further work is needed to elucidate the complex mechanisms controlling matrix breakdown and vascular transformation, and to investigate the spatiotemporal interplay between medial VSMC, invasive trophoblast and other cell types present within the placental bed.
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References


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Figure legends

Figure 1: Elastase activity in first trimester cytotrophoblast

[A] First trimester cytotrophoblasts (CTB) were cultured with Congo Red-labelled elastin (1 mg/ml) for 48h and stained with an antibody to cytokeratin-7 (green). Serial images at 2µm intervals along the z axis were captured by confocal microscopy; inset depicts intracellular elastin fragments. Scale bars represent 50µm, (n=3). [B] CTB were isolated from placentae of less than 9 weeks gestation, or greater than 9 weeks gestation. Cells were cultured with a vehicle control (DMSO), the broad spectrum caspase inhibitor zVADfmk (50µM), the broad spectrum MMP inhibitor NNGH (50µM) or the broad spectrum cathepsin inhibitor Z-FG-NHO-BzOMe (10µM) or [C] with all three inhibitors for 18h. [B, C] Intracellular elastase activity was determined using a cell-permeable elastase substrate. Median + range, (n=3). [B] *p<0.05; Kruskal-Wallis test. [D, E] Membrane-associated elastase activity in [D] SGHPL4 and [E] first trimester CTB was assessed using the substrate N-succinyl-(L-alanine)$_3$-p-nitroanilide. Cell supernatants were assayed for elastase activity in the presence of a vehicle control (DMSO), zVADfmk (50µM), NNGH (50µM), Z-FG-NHO-BzOMe (10µM) or the uPA inhibitor uPA-STOP (10µM). Median, (n=3); *p<0.05; Kruskal-Wallis test.

Figure 2: Elastin uptake and MMP expression in first trimester trophoblast

SGHPL-4 were cultured with elastin fragments (1mg/ml) (♦) in the presence of a vehicle control (DMSO), the broad spectrum MMP inhibitor NNGH (50µM; ▲) or L-NAME (5mM; □) for 24h. Cultures were monitored by time lapse video-microscopy and the time point at which an elastin fragment appeared within a cell was recorded; 45 cells were scored for each treatment. [A1-6] Selected images taken at hourly intervals showing accumulation of elastin fragments within a cell. Arrows denote elastin fragments. Scale bar 10µm. [B] Time course of elastin uptake. Mean ± SEM, (n=4). [C] Percent of SGHPL-4 containing
elastin fragments at 6h, 12h and 24h. Black bars represent control cells, grey bars represent NNGH-treated cells, white bars represent L-NAME-treated cells. Median + range, (n=4); *p<0.05, **p<0.01; 2-way ANOVA.

Figure 3: Membrane-associated MMP-12 mediates elastolysis in first trimester CTB

First trimester CTB cultured for 48h on Matrigel-coated cover slips were immunostained with [A] control IgG or [B] an antibody to MMP-12 (catalytic domain; green); nuclei were counterstained with propidium iodide (red). Scale bar 50µm. Pictures are representative of 3 independent CTB isolations. [C] Membrane-associated elastase activity in first trimester CTB was assessed using the substrate N-succinyl-(L-alanine)₃-p-nitroanilide. Cell supernatants were assayed for elastase activity in the presence of a vehicle control (DMSO), the broad spectrum MMP inhibitor NNGH (50µM), or the specific MMP-12 inhibitor 470.1 (100µM). Median, (n=6); *p<0.05; ***P<0.001; Kruskal-Wallis test.

Figure 4: HASMC exhibit elastase activity attributable to serine proteases and MMP

[A] HASMC were cultured on uncoated plastic dishes (control), Matrigel-coated dishes, or with first trimester CTB-conditioned medium (50% (v/v)), or elastin fibres (1 mg/ml) for 48h. Intracellular elastase activity was quantified using a cell-permeable elastase substrate and flow cytometry. Median, (n=3); *p<0.05; Kruskal-Wallis test. [B] HASMC were cultured on plates coated with elastin fragments for 48h, and were incubated with a vehicle control (DMSO), the broad spectrum caspase inhibitor zVADfmk (50µM), the MMP inhibitor NNGH (50µM) or the cathepsin inhibitor Z-FG-NHO-BzOMe (10µM) for a further 6h. Intracellular elastase activity was determined as above. Median, (n=4). [C] HASMC were cultured on elastin coated flasks for 48h, then were incubated with a vehicle control (DMSO), the serine protease inhibitor PMSF (10mg/ml; 6h), the NOS inhibitor L-NAME (5mM; 24h) or the
iNOS inhibitor 1400W (5µM; 24h). Intracellular elastase activity was determined as above. Median, (n=4); *p<0.05; Kruskal-Wallis test. [D, E] **Membrane-associated elastase activity in HASMC was assessed using the substrate N-succinyl-(L-alanine)3-p-nitroanilide.** Cell supernatants were assayed for elastase activity in the presence of a vehicle control (DMSO), the caspase inhibitor zVADfmk (50µM), the MMP inhibitor NNGH (50µM), the uPA inhibitor uPA-STOP (10µM) or the cathepsin inhibitor Z-FG-NHO-BzOMe (10µM).

[D] **HASMC** were cultured in uncoated flasks (white bars) or in flasks coated with elastin fragments (black bars) for 48h. Median + range, (n=4); *p<0.05, **p<0.01; Kruskal-Wallis test. [E] **HASMC** were cultured on elastin coated flasks, in the absence or presence of the NOS inhibitor L-NAME (5mM; 24h) or the iNOS inhibitor 1400W (5µM; 24h). Control supernatants were analysed in the presence of a vehicle control (DMSO) or the serine protease inhibitor PMSF (10mg/ml). Median, (n=4); *p<0.05; Kruskal-Wallis test.

**Figure 5: Elastin uptake in HASMC is regulated by MMP and NO**

**HASMC** were cultured with elastin fragments (1mg/ml) (♦) in the presence of a vehicle control (DMSO), the broad spectrum MMP inhibitor NNGH (50µM; ▲) or L-NAME (5mM; □) for 24h. Cultures were monitored by time lapse video-microscopy and the time point at which an elastin fragment appeared within a cell was recorded. Forty five cells were scored for each treatment. [A1-4] **Selected images taken at hourly intervals showing accumulation of elastin fragments within a cell.** Arrows denote elastin fragments. Scale bar 10µm. [B] **Time course of elastin uptake.** Mean ± SEM, (n=4). [C] **Percent of HASMC containing elastin fragments at 6h, 12h and 24h.** Black bars represent control cells, grey bars represent NNGH-treated cells, white bars represent L-NAME-treated cells. Median + range, (n=4); *p<0.05, **p<0.01, ***p< 0.001; 2 way ANOVA.
Figure 6: MMP-12 expression in cultured HASMC and excised human spiral arteries

[A, B] Cultured HASMC were immunostained with [A] control IgG or [B] an antibody to the catalytic domain of MMP-12 (green). Nuclei were counterstained with propidium iodide (red). Scale bar represents 50µm. [C] Intact human spiral arteries and [D] arteries denuded of endothelium were fixed immediately and stained with an antibody to CD31 (green). Nuclei were counterstained with propidium iodide (red). Scale bar 100µm. [C] Arrows denote endothelial cells. Note autofluorescence of elastin. [E-H] Spiral arteries were denuded of endothelium and were [E] fixed immediately or were perfused with [F] unconditioned first trimester CTB medium (1:1 DMEM:Ham’s F12; control), [G] CTB-conditioned medium (diluted 1:1 with DMEM:Ham’s F12) or [H] rhTRAIL (0.5 µg/ml in DMEM:Ham’s F12) and cultured for 24h. Sections were stained with an antibody to the catalytic domain of MMP-12 (green). Scale bar 100µm. Nuclei were counterstained with propidium iodide (red). Images are representative of 6 independent experiments; however, MMP-12-positive VSMC were only observed in 7 or 8 (~30%) of the 24 serial tissue sections examined from each vessel. [I] HASMC supernatants were assayed for membrane-associated elastase activity in the presence of a vehicle control (DMSO), the broad spectrum MMP inhibitor NNGH (50µM), or the specific MMP-12 inhibitor 470.1 (100µM). Median, (n=6); *p<0.05; ***p<0.001; Kruskal-Wallis test.

Figure 7: MMP-12 expression in cultured VSMC and excised human spiral arteries

Serial sections of first trimester placenta (n=5) and decidua basalis (n=8) stained were immunostained with [A] control IgG, [B, C] an antibody to cytokeratin-7 (CK-7) or [I] HLA-G detect trophoblast, [G, H] an antibody to α-smooth muscle actin (α-SMA) to detect VSMC or [D-F, J-L] an antibody to MMP-12 (carboxyterminal). Sections were counterstained with hematoxylin. Scale bar 50µm. Serial sections: [A, D], [B, E], [C, F], [G, J], [H, K], [I, L].

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EC = endothelial cells, vCTB = villous cytotrophoblast, VSMC = vascular smooth muscle, eEVT = endovascular extravillous trophoblast.
Figure 2

A

B

C

* * *

% cells containing elastin

Time (hours)

% cells containing elastin

Time (hours)

* * *

*
Figure 3

A

B

C

% control elastase activity

CTB control  CTB + NNGH  CTB + 470.1

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Figure 4

A

% control intracellular elastase activity

SMC, SMC + Matrigel, SMC + CTB medium, SMC + elastin

B

% control intracellular elastase activity

SMC, SMC + zVAD, SMC + NNGH, SMC + BzOMe

C

% control intracellular elastase activity

SMC, SMC + PMSF, SMC + L-NAME, SMC + 1400W

D

% control elastase activity

SMC, SMC + zVAD, SMC + NNGH, SMC + uPASSTOP, SMC + BzOMe

E

% control elastase activity

SMC, SMC + PMSF, SMC + L-NAME, SMC + 1400W
Figure 7

[A] Negative

[B] CK-7

[C] CK-7

[D] MMP-12

[E] MMP-12

[F] MMP-12

[G] α-SMA

[H] α-SMA

[I] HLA-G

[J] MMP-12

[K] MMP-12

[L] MMP-12

Cell column

EVT

EC

VSMC