Invasive Trophoblasts Stimulate Vascular Smooth Muscle Cell Apoptosis by a Fas Ligand-Dependent Mechanism

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During pregnancy, trophoblasts migrate from the placenta into uterine spiral arteries, transforming them into wide channels that lack vasoconstrictive properties. In pathological pregnancies, this process is incomplete. To define the fundamental events involved in spiral artery remodeling, we have studied the effect of trophoblasts on vascular smooth muscle cells (SMCs). Here we demonstrate for the first time that apoptosis of SMCs can be initiated by invading trophoblasts. When trophoblasts isolated from normal placenta (primary trophoblasts) or conditioned medium was perfused into spiral or umbilical artery segments, apoptosis of SMCs resulted. Culture of human aortic SMCs (HASMCs) with primary trophoblasts, primary trophoblast-conditioned medium, or a trophoblast-derived cell line (SGHPL-4) also significantly increased SMC apoptosis. Fas is expressed by spiral artery SMCs, and a Fas-activating antibody triggered HASMC apoptosis. Furthermore, a Fas ligand (FasL)-blocking antibody significantly inhibited HASMC apoptosis induced by primary trophoblasts, SGHPL-4, or trophoblast-conditioned medium. Depleting primary trophoblast-conditioned medium of FasL also abrogated SMC apoptosis in vessels *in situ*. These results suggest that apoptosis triggered by the release of soluble FasL from invading trophoblasts contributes to the loss of smooth muscle from the walls of spiral arteries during pregnancy. (*Am J Pathol* 2006, 169:1863–1874; DOI: 10.2353/ajpath.2006.060265)

During pregnancy, the uterine spiral arteries are remodeled to form high-flow, low-resistance vessels. This transformation, known as physiological change, results in an increased arterial diameter and loss of maternal vasomotor control,¹ which is necessary to meet the increasing demands of the fetus for blood and oxygen. Impaired arterial remodeling is associated with various pregnancy complications,^{2–5} yet the molecular mechanisms controlling this process remain poorly understood.

Remodeling of the spiral arteries mainly occurs during the first 20 weeks of gestation¹ and is mediated by the fetal-derived trophoblasts.^{6,7} The trophoblast, which originates from the trophectoderm of the developing blastocyst, is essential for initiating implantation and maintenance of a successful pregnancy. Trophoblast gives rise to two major cell lineages, the syncytiotrophoblasts and the invasive cytotrophoblasts (CTBs), while maintaining a progenitor cell population of villous CTBs. Early in pregnancy, proliferating CTBs detach from the tips of anchoring placental villi and form cell columns from which invasive CTBs are derived.⁸ Two distinct populations of infiltrating cells are observed: interstitial CTBs, which migrate through the uterine stroma, and endovascular CTBs, which migrate into the lumen of the spiral arteries. Arterial wall remodeling occurs apparently as a result of the combined action of these two cell populations.^{6,7} Migration occurs along spiral arteries as far as the first third of the myometrium, with partial loss of endothelium, disappearance of medial smooth muscle, and replacement of the musculo-elastic media of the vessels with an amorphous fibrinoid material in which the CTBs are embedded.^{7,9}

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Importantly, impaired arterial remodeling is associated with pre-eclampsia, miscarriage, and fetal growth restriction. Indeed, shallow trophoblast invasion, decreased numbers of invasive trophoblasts,^{2,5} persistence of vascular smooth muscle, and the absence of endovascular CTBs from the myometrial segments of spiral arteries have all been observed in pre-eclampsia.⁴ In addition, spiral artery remodeling is reduced or absent in hypertensive diseases of pregnancy³ and in the maternal uterus of small-for-gestational-age infants.²

Apoptosis¹⁰ occurs in various tissues throughout gestation, playing an important role in the development of the fetus and the placenta.¹¹ We hypothesize that medial smooth muscle cells (SMCs) within the spiral arteries undergo apoptosis in response to trophoblast-derived signals during the process of arterial remodeling. One mechanism by which apoptosis may be initiated is via ligation of death receptors of the tumor necrosis factor receptor (TNF-R) family, namely Fas/CD95, TNF-R1, death R-3/TRAMP, TNF- α -related apoptosis-inducing ligand (TRAIL)-R1, and TRAIL-R2. Crosslinking of Fas by FasL leads to receptor trimerization, recruitment of adaptor molecules RIP and FADD, initiation of a signaling cascade through the death domain of Fas, caspase activation, and apoptosis.¹² However, susceptibility to apoptosis is not solely determined by levels of Fas receptor expression because many cells are resistant to challenge by FasL, despite expressing Fas. Increased protein expression of FLICE-like inhibitory protein (FLIP),^{13,14} Bcl-X(L), and Bcl-2¹⁵ have all been shown to confer protection against Fas-mediated apoptosis.

In this study, we have investigated the mechanisms used by endovascular trophoblasts to remodel the spiral arteries in normal pregnancy, focusing on a possible role for apoptosis in this process. We demonstrate that trophoblast-induced medial SMC death occurs by apoptosis, that induction of SMC apoptosis can occur without direct cell-cell contact and, for the first time, that SMC death is, in part, mediated by Fas/FasL interactions.

Materials and Methods

Reagents

Mouse anti-human CD95 (Fas) monoclonal antibody and rabbit anti-human active caspase-3 polyclonal antibody were purchased from R&D Systems (Minneapolis, MN); mouse anti-human FasL monoclonal antibody (clone NOK-2) was purchased from BD Pharmingen (San Diego, CA); mouse IgG2a κ (clone UPC-10), horseradish peroxidase-conjugated goat anti-rabbit IgG, and horseradish peroxidase-conjugated goat anti-mouse IgG were purchased from Sigma-Aldrich (Poole, Dorset, UK); rabbit anti-human cleaved polyADP-ribose polymerase (PARP, p85 fragment) polyclonal antibody was purchased from Promega Corporation (Madison, WI); mouse anti-human cytokeratin-7 monoclonal antibody (clone OV-TL 12/30), fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG, and FITC-conjugated swine anti-rabbit IgG

were purchased from DakoCytomation A/S (Glostrup, Denmark); and mouse anti-human CD34 monoclonal antibody was purchased from Serotec (Oxford, UK). For assessing apoptosis, the *in situ* cell death detection kit (TUNEL) was obtained from Roche (Lewes, UK), the annexin V-FITC apoptosis detection kit was obtained from BD Pharmingen (Oxford, UK), and etoposide was purchased from Sigma-Aldrich. Caspase inhibitor 1 (zVAD-fmk) was purchased from Merck Biosciences Ltd. (Nottingham, UK), Vectashield mounting medium was purchased from Vector Laboratories Inc. (Burlingame, CA) and OCT embedding medium was purchased from Raymond A. Lamb (London, UK). Tissue culture medium and fetal bovine serum were purchased from Sigma-Aldrich, Matrigel was purchased from BD Discovery Labware (Bedford, MA), CellTracker Orange was purchased from Invitrogen Corp. (Carlsbad, CA), and transwell cell culture inserts (pore size, 0.4 μ m) were purchased from Millipore Corp. (Billerica, MA). Rainbow molecular weight markers, Hybond-P polyvinylidene difluoride membrane and ECL Plus Western blotting detection reagents were all obtained from Amersham Biosciences UK Ltd. (Chalfont St. Giles, UK). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich and were of AnalR grade.

Tissue

Informed consent was obtained for all myometrial and placental tissue used in this study, and local ethical committee approval was in place. Normal first trimester placenta (8 to 12 weeks) was obtained at elective termination of pregnancy (surgical or medical). Umbilical cords were obtained from normal term placentas within 30 minutes of caesarean section or vaginal delivery. Term decidua/myometrial biopsies taken from nonplacental 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.^{16,17} In brief, unmodified spiral arteries were dissected from term decidua/myometrial biopsies under sterile conditions and mounted on glass cannulas in a pressure myography perfusion chamber (Living Systems Instrumentation, Burlington, VT). Arteries were denuded of endothelium by passing a column of air through the vessel and then perfused with the appropriate medium or cells (1×10^5 /ml), as indicated. Etoposide (100 μ mol/L), which complexes with topoisomerase II to enhance cleavage of DNA and induce apoptosis, was used as a positive control. The ends of each vessel were tied and the arteries incubated for up to 72 hours in 1:1 Dulbecco's modified Eagle's medium/Ham's F12 culture medium supplemented with 10% fetal bovine serum (FBS), glutamine (2 mmol/L), penicillin (100 IU/ml), and streptomycin (100 μ g/ml).

Umbilical artery segments were dissected under sterile conditions from umbilical cords and cannulated with a

needle and syringe. Sterile phosphate-buffered saline (PBS) was forced through the segments to remove the endothelium. Arteries were then perfused with the appropriate medium or cells, as indicated. The ends of each vessel were tied and the arteries incubated for up to 96 hours in 1:1 Dulbecco's modified Eagle's medium/Ham's F12 culture medium supplemented with 10% FBS, glutamine (2 mmol/L), penicillin (100 IU/ml), and streptomycin (100 μ g/ml).

Cell Culture and Labeling

SGHPL-4 cells (derived from primary human first trimester extravillous trophoblasts transfected with the early region of SV40, previously known as MC4¹⁸) were cultured in Ham's F10 medium supplemented with 10% FBS, L-glutamine (2 mmol/L), penicillin (100 IU/ml), and streptomycin (100 μ g/ml). First trimester primary CTBs were cultured in 1:1 Dulbecco's modified Eagle's medium/Ham's F12 culture medium supplemented with 10% FBS, L-glutamine (2 mmol/L), penicillin (100 IU/ml), and streptomycin (100 μ g/ml). Isolation of primary CTBs was performed using the method as previously described.^{17,19} Cell isolates were plated onto Matrigel-coated flasks; $91.1 \pm 4.2\%$ ($n = 5$) of cells stained positive for cytokeratin-7. Primary CTBs were cultured on Matrigel for up to 48 hours to promote a more advanced extravillous phenotype before introduction into vessel segments or cocultures. The human aortic SMC (HASMC) line was obtained by transfection of primary HASMCs with the plasmid pSV3neo using the method as previously described.²⁰ The cells were maintained in Kaighn's modification of Ham's F12 medium supplemented with 10% FBS, L-glutamine (2 mmol/L), penicillin (100 IU/ml), and streptomycin (100 μ g/ml). All cells were incubated with 95% air and 5% carbon dioxide at 37°C in a humidified incubator.

For all co-culture experiments, cells were stepped down to medium supplemented with 0.5% FBS at the commencement of the experiment. In noncontact co-culture experiments, SGHPL-4 cells (3×10^4) were seeded into transwell inserts that were positioned in dishes plated with HASMCs (3×10^4). For co-culture experiments using primary CTBs, HASMCs were plated on dishes coated with a thin layer of Matrigel (diluted 1:4 in serum-free medium). CTBs (3×10^4) were subsequently added 24 hours later and then allowed to adhere for a further 1 hour before commencement of the experiment. In all co-culture experiments, HASMCs were pre-labeled with 5 μ mol/L CellTracker Orange dye for 1 hour at 37°C before the addition of CTBs to the culture plate. This enabled identification of HASMCs in the cultures by fluorescence at the commencement of time-lapse microscopy.

Conditioned Medium and Blocking Antibody Treatments

Primary CTB-conditioned medium was produced in the presence or absence of serum by culturing first trimester

CTBs in a T₂₅ flask with 5 ml of medium for 24 hours. Conditioned medium was collected, centrifuged, and diluted 1:1 (v/v) with control medium before use. For flow cytometry experiments using the FasL blocking antibody NOK-2, serum-free unconditioned medium (control) or CTB-conditioned medium was incubated with NOK-2 (10 μ g/ml) or the isotype-matched control antibody IgG2a κ (10 μ g/ml) for 1 hour at room temperature. HASMCs (1×10^5) were then incubated with the depleted medium for 2 hours, also in the absence of serum. Likewise, for vessel experiments, serum-free medium was incubated with control or blocking antibody for 1 hour at room temperature. Depleted medium was then introduced into artery segments denuded of endothelium. Vessel segments were kept in culture for 24 hours (spiral arteries) or 72 hours (umbilical arteries) before analysis. For time-lapse experiments, control or blocking antibody was added 1 hour after the addition of trophoblasts.

Immunohistochemistry

After culture, vessels were fixed with 2% (v/v) paraformaldehyde in PBS for 30 minutes and then incubated with 0.5 mol/L sucrose in PBS for at least 1 hour. The tissue was then placed in OCT embedding medium, frozen, and stored at -80°C . Transverse sections (10 μ m) of frozen vessels were prepared using a cryostat, transferred to poly-L-lysine-coated slides and stored at -80°C . For immunostaining, sections were warmed to room temperature, fixed with 4% (v/v) paraformaldehyde for 20 minutes, and washed in PBS. Tissue sections were permeabilized with 0.1% (v/v) Triton-X in PBS for 5 minutes, washed in PBS, and allowed to air-dry before the addition of the primary antibody. Primary and secondary antibodies were diluted in PBS and applied to the tissue (50 μ l/section) for 1 hour, during which time the slides were placed in a humidified chamber at room temperature. Slides were protected from light once the secondary antibody was applied. After each antibody incubation, the slides were washed with PBS. Antibody dilutions used were as follows: CD34 (1:10), cytokeratin-7 (1:40), cleaved PARP (1:100), active caspase-3 (0.25 μ g/ml), Fas (1:20), and FITC-conjugated rabbit anti-mouse and swine anti-rabbit antibodies (1:40). Sections were mounted using Vectashield mounting medium containing propidium iodide or 4,6-diamidino-2-phenylindole and stored at 4°C in the dark until viewed. Slides were analyzed at room temperature using either an Olympus IX70 inverted fluorescence microscope (Tokyo, Japan), a Bio-Rad Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA) with UPlanF1 $\times 10/0.30$ or UPlanApo $\times 40/1.00$ oil iris objective lenses, and LaserSharp 2000 image analysis software or a Zeiss 510 Meta confocal microscope with a Neo-FLUAR $\times 40/1.3$ oil objective lens and Zeiss 510 Meta analysis software (Fas staining on HASMCs only).

Terminal dUTP Nick-End Labeling (TUNEL) Staining

Tissue sections were fixed using 4% (v/v) paraformaldehyde in PBS for 20 minutes at room temperature, washed in PBS for 20 minutes, and allowed to air dry. Slides were then incubated with permeabilization solution [0.1% (v/v) Triton in 0.1% (w/v) sodium citrate in H₂O] for 8 minutes and washed in PBS. Slides were allowed to air dry before incubation with 16 μ l of TUNEL reagent per tissue section. The working TUNEL reagent solution was prepared according to the manufacturer's instructions, although the TUNEL enzyme provided was diluted 1:5 with PBS to reduce background fluorescence. Slides were incubated in a humidified chamber for 1 hour in the dark and then washed in PBS. Slides were mounted using Vectashield mounting medium containing propidium iodide and stored at 4°C in the dark. Quantification of the number of TUNEL-positive cells per vessel section was performed blind, using an Olympus IX70 inverted fluorescence microscope. TUNEL-positive cells present in the two layers of cells closest to the lumen were excluded to omit residual endothelial cells or adherent trophoblasts from the counts. A minimum of six sections were stained and counted per vessel.

Time-Lapse Microscopy

Apoptosis was monitored by time-lapse microscopy using an Olympus IX70 inverted fluorescence microscope with a motorized stage and cooled charge-coupled device camera (Hamamatsu model C4742-95) enclosed in a humidified chamber at 37°C with 5% CO₂ in air as described previously.¹⁶ Images were taken at 15-minute intervals, and time-lapse sequences were analyzed using ImagePro Plus (Media Cybernetics, Silver Spring, MD). Forty cells were scored in each field of view, and the time at which apoptotic morphology was first observed was recorded (characterized by membrane blebbing, cytoplasmic shrinkage, nuclear condensation, a phase bright appearance, and the formation of blisters). In co-culture experiments, one fluorescent picture was captured at the start of the experiment, which enabled SMCs to be identified for analysis. SMCs were individually tracked through every frame of the time-lapse sequence from the commencement to the end of the experiment, thus enabling the time point at which individual SMCs underwent apoptosis to be determined.

Immunoblotting

After treatments as indicated, HASMCs were washed with ice-cold PBS and incubated on ice in lysis buffer [1× PBS, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethyl sulfonyl fluoride, and 10 μ g/ml aprotinin]. Cells were then scraped from the dishes and centrifuged, and the lysate was retained for analysis. Medium from each plate was also centrifuged, and any

pelleted cells were lysed and added to the adherent cell lysates. Equal amounts of protein (100 μ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels and then transferred to polyvinylidene difluoride membranes overnight. Membranes were blocked in Tris-buffered saline containing 0.1% (v/v) Tween with 5% (w/v) milk powder added and probed with rabbit anti-human cleaved PARP (1:1000) prepared in Tris-buffered saline-Tween with 10% (w/v) bovine serum albumin added, followed by horseradish peroxidase-conjugated secondary antibody. After washing, proteins were detected by enhanced chemiluminescence.

Flow Cytometry

Phosphatidylserine externalization was quantified by flow cytometry, using a commercially available annexin V-FITC apoptosis detection kit following the manufacturer's guidelines (BD Pharmingen). After treatment, HASMCs were washed twice in PBS, trypsinized, and collected. The culture medium was also retained and pooled with the adherent cells. Cells were centrifuged, the supernatant discarded, and the cell pellets resuspended in kit binding buffer. The cells were centrifuged again, the supernatant discarded, and the pellet resuspended in kit buffer (100 μ l/pellet) containing annexin V solution (5 μ l/pellet) and propidium iodide (2.5 μ g/ml). Samples were incubated in the dark for 10 minutes and the percentage of annexin V-positive cells was analyzed using a Coulter Epics Elite flow cytometer.

Measurement of Fas Ligand

Lysates of primary CTBs were prepared as described in immunoblotting with the exception that any cells pelleted from the medium were not added to the cell lysate. Medium and lysates were analyzed for Fas ligand using the human Fas ligand immunoassay kit (R&D Systems) according to the manufacturer's instructions.

Statistics

Data were compared using either the Student's *t*-test for normally distributed data or a repeated measures analysis of variance with Bonferroni's posthoc test using GraphPad Prism software, version 4 (GraphPad Software, San Diego, CA). Significance was taken as *P* < 0.05. Data are presented as the mean $\pm$ SEM from at least three independent experiments.

Results

Trophoblasts Induce Apoptosis in Arterial Media SMCs in Situ

In previous experiments we have shown that the presence of trophoblasts in spiral arteries initiates endothelial cell apoptosis.¹⁶ The current study was designed to examine interactions between medial SMCs and invading

Figure 1. Trophoblasts induce apoptosis of arterial SMCs *in situ*. **A:** Spiral artery (minus endothelium) perfused with primary CTB for 48 hours and stained with an antibody against cytokeratin-7 (green). **B–D:** Spiral artery (minus endothelium) perfused with control medium, 24 hours (**B**); primary CTB, 24 hours (**C**); primary CTB-conditioned medium [50% (v/v)], 24 hours (**D**). TUNEL-positive cells are labeled with FITC (green). Nuclei are counterstained with propidium iodide (red). *Vessel lumen. Pictures are representative of $n \geq 3$ independent experiments, with each experiment performed on vessels from a different biopsy. Scale bars = 100 μ m.

trophoblasts, thus, all spiral artery segments were mechanically denuded of endothelium to facilitate rapid access of trophoblasts to the vessel media. Removal of the arterial endothelium was confirmed by CD34 staining (data not shown). Spiral arteries were perfused with control culture medium, primary CTBs, CTB-conditioned medium, or etoposide, and cultured for up to 72 hours. Umbilical arteries, which have many more layers of SMCs, were used for comparison. Trophoblast adherence and invasion into medial SMC layers has previously been documented in this model¹⁷ and was verified here

with cytokeratin-7 staining (Figure 1A). Figure 1, B–D, shows transverse sections of spiral artery, with apoptotic SMCs identified using TUNEL. Antibodies against active caspase-3 and cleaved PARP were used to confirm cell death by apoptosis (data not shown). Control arteries (Figure 1B) demonstrated negligible SMC apoptosis. In contrast, apoptosis and cell detachment were noted in the layers of SMCs closest to the vessel lumen, when spiral artery segments were perfused with primary CTBs (Figure 1C). Introduction of CTB-conditioned culture medium into vessels also led to SMC apoptosis after 24

hours (Figure 1D), indicating that CTBs secrete soluble apoptotic factor(s). CTB-conditioned medium induced more extensive SMC apoptosis than a CTB cell suspension, most likely because all of the cells lining the vessel were exposed to secreted apoptotic factor(s) present in the conditioned medium at the same time. When vessels were perfused with cells, it is likely that trophoblasts only secrete apoptotic factors after they have attached to the vessel wall. Consequently, only SMCs in close proximity to each adherent trophoblast would be subjected to these factors; thus, apoptosis would occur at a slower rate. Apoptosis of cells in the loose connective tissue surrounding the vessels was commonly observed in all arteries after 24 hours in culture and is evident in Figure 1, C and D. This is attributable to disruption of the tissue architecture on dissection and leads to generation of DNA fragments, which are labeled by TUNEL.

After quantifying the number of TUNEL-positive SMCs in sections of the perfused vessels, it was evident that trophoblasts rapidly induced SMC apoptosis in spiral arteries (Figure 2A). Indeed, SMC apoptosis was significantly increased above levels observed in control vessels after treatment with primary CTB (24 hours, $P < 0.001$ versus control) and primary CTB-conditioned medium at 24 and 48 hours, ($P < 0.001$, both time points versus relevant control). Etoposide was used as a positive control. In contrast, SMC apoptosis in umbilical arteries increased more slowly throughout time (Figure 2B). All treatments resulted in elevation of SMC apoptosis compared to control vessels at the same time point, with apoptosis significantly increased on treatment with primary CTB at 24, 48, 72, and 96 hours ($P < 0.001$ all time points versus relevant control), primary CTB-conditioned medium at 24, 48, 72, and 96 hours ($P < 0.001$ all time points versus relevant control) and etoposide at 24, 48, 72, and 96 hours ($P < 0.001$ all time points versus relevant control). It is notable that CTB-conditioned culture medium also induced significant levels of SMC apoptosis in both spiral and umbilical arteries, suggesting a mechanism that does not require direct cell contact.

Trophoblasts Induce Apoptosis in Arterial SMCs in Vitro

To assess the ability of trophoblasts to induce SMC apoptosis *in vitro*, co-cultures of trophoblasts and human aortic SMCs (HASMCs) were monitored by time-lapse microscopy with images captured every 15 minutes throughout the course of 60 hours. This system has been well characterized and used previously to monitor apoptosis in live culture systems.^{16,21,22} The onset of apoptosis was determined as the time at which clear apoptotic morphology was first observed. Characteristic apoptotic morphology was easily identifiable in HASMCs; Figure 3A shows phase contrast images of a typical individual cell from a time-lapse sequence. Cytoplasmic and nuclear shrinkage (21 hours) precede a change to a phase-bright appearance (21.5 hours). Membrane blebs (23 hours) and blisters (24 hours) form as the cell begins to fragment into apoptotic bodies (26.5 hours). Although

Figure 2. Trophoblast-induced SMC apoptosis in an *ex vivo* vessel model. Spiral arteries or umbilical arteries were denuded of endothelium, perfused with control culture medium, primary CTB, primary CTB-conditioned medium [50% (v/v)], or control medium containing etoposide (10 μ mol/L) and tied off. Some vessels were frozen immediately (0 hours), and others were cultured and frozen after 24 hours (open bars), 48 hours (stippled bars), 72 hours (shaded bars), or 96 hours (umbilical arteries only, hatched bars). After cryosectioning, the number of TUNEL-positive SMCs per vessel section was quantified by fluorescence microscopy (excluding cells in the two tissue layers closest to the lumen). **A:** Spiral arteries (mean $\pm$ SEM, $n = 3$ vessels from individual biopsies (primary CTB-conditioned medium, etoposide); $n = 4$ vessels from individual biopsies (control, primary CTB)). Six sections were counted per vessel. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, versus control vessel at corresponding time point, paired Student's *t*-test. **B:** Umbilical arteries (mean $\pm$ SEM, $n = 5$ vessels from individual cords).

the timing of onset of these events varied between cells, the morphological changes observed occurred within the same time frame.

Using time-lapse microscopy, HASMC cultures were monitored for 60 hours after the addition of trophoblasts, and the percentage of apoptotic cells was scored. In contact co-culture, both primary CTBs and extravillous

Figure 3. Time course of HASMC death induced by trophoblasts in co-culture. **A:** A representative series of images captured from time-lapse microscopy of a single HASMC showing the morphological changes associated with apoptosis. HASMCs undergo cytoplasmic shrinkage and nuclear condensation associated with a change in the refractive index (phase bright appearance). Characteristic membrane blebs (23 hours) and blisters (24 hours) appear. **B and C:** Contact co-cultures of HASMCs alone ($\blacktriangle$, $\bullet$) or HASMCs and primary CTBs ($\triangle$) (**B**) or the extravillous trophoblast-derived cell line SGHPL-4 ($\circ$) (**C**), were monitored by time-lapse microscopy. HASMC apoptosis was determined by scoring 40 cells and recording the time point at which they underwent apoptosis. **B:** Time course of primary CTB-induced HASMC apoptosis. The **inset** bar graph shows the percentage of apoptotic HASMCs at 60 hours, $*P < 0.05$, paired *t*-test. **C:** Time course of SGHPL-4-induced HASMC apoptosis. The **inset** bar graph shows the percentage of apoptotic HASMCs at 60 hours, $*P < 0.005$, paired *t*-test (mean $\pm$ SEM, $n = 3$ independent primary CTB isolations; $n = 7$ independent SGHPL-4 cultures).

trophoblast-derived SGHPL-4 cells stimulated a significant increase in the rate of apoptosis of HASMCs (Figure 3, B and C). The inset bar graphs (Figure 3, B and C) show the percentage of apoptotic HASMCs observed at 60 hours. A significant increase was seen in the presence of primary CTB ($P < 0.05$, versus HASMCs alone) and SGHPL-4 cells ($P < 0.005$, versus HASMCs alone).

To further investigate the ability of trophoblasts to induce apoptosis, HASMCs were assayed for evidence of PARP cleavage after co-culture. SGHPL-4 cells were grown in transwells in a noncontact co-culture with HASMCs, and cleaved PARP was examined by Western blotting. After 24 hours, there was a significant increase in the expression of cleaved PARP in the presence of SGHPL-4, compared to HASMCs alone (Figure 4A). This confirmed that SGHPL-4 cells induced apoptosis in HASMCs, even in the absence of direct cell contact. Time-lapse microscopy demonstrated that HASMC death induced by both primary CTBs and SGHPL-4 cells was significantly inhibited in the presence of the broad spectrum caspase inhibitor zVAD-fmk (Figure 4B), confirming

that trophoblast-induced HASMC death was occurring via an apoptotic mechanism.

The Fas Pathway Is Functional in Arterial SMCs

We have previously shown by immunofluorescent staining that Fas is present on medial SMCs in spiral arteries at term.¹⁶ Using confocal analysis we now also demonstrate that Fas is expressed on cultured HASMCs (Figure 5A). Further examination of the Z-series of images reveals the membrane distribution of Fas (Figure 5B). Treatment of HASMCs with a Fas-activating antibody (anti-human CD95) caused a concentration-dependent increase in apoptosis, indicating that the Fas pathway is functional. The percentage of apoptotic cells after 60 hours is shown in Figure 5D. A significant increase in PARP cleavage in HASMCs was observed at 24 and 60 hours after treatment with the Fas-activating antibody (Figure 5E), confirming an apoptotic mechanism.

Figure 4. HASMC death induced by trophoblasts in co-culture occurs by apoptosis. **A:** Noncontact co-cultures of HASMCs alone or HASMCs and the extravillous trophoblast-derived cell line SGHPL-4 (added in transwells) were established. Apoptosis was assessed at 24 hours by immunoblot analysis of cleaved PARP expression. A representative autoradiograph from $n = 4$ experiments is shown. **B:** Contact co-cultures of HASMCs alone (open bars) or HASMCs and primary CTBs (light shading) or the extravillous trophoblast-derived cell line SGHPL-4 (dark shading), were treated with the pan-caspase inhibitor zVAD-fmk (50 μ M/L) and then monitored by time-lapse microscopy. HASMC apoptosis was determined by scoring 40 cells and recording the time point at which they underwent apoptosis. The percentage of apoptotic HASMCs at 60 hours is shown, * $P < 0.05$ and ** $P < 0.05$ (primary CTB) and * $P < 0.05$ and ** $P < 0.001$ (SGHPL-4), repeated measures analysis of variance (mean $\pm$ SEM, $n = 6$ independent primary CTB isolations; $n = 5$ independent SGHPL-4 cultures).

Trophoblasts Stimulate SMC Apoptosis Partly Through the Fas/FasL System

It has previously been shown that first trimester primary CTBs secrete FasL,²³ and there are other reports of FasL production by extravillous trophoblasts.^{24,25} We also found detectable amounts of FasL in cell lysates prepared from first trimester CTBs (average, 7.35 pg of FasL/mg of protein). Hence, we examined the contribution of the Fas/FasL system to SMC apoptosis in our model systems.

The presence of a FasL blocking antibody (NOK-2) significantly decreased both primary CTB- and SGHPL-4-induced HASMC apoptosis 60 hours after treatment (Figure 6A). In addition, pretreatment of primary CTB-conditioned medium with the FasL blocking antibody significantly decreased its proapoptotic potency toward HASMCs (Figure 6B). Treatment of HASMCs with primary CTB-conditioned medium containing control IgG for 2

hours resulted in a twofold increase in the number of cells externalizing phosphatidylserine. This again suggested that trophoblast release a soluble factor that is, at least in part, responsible for inducing SMC apoptosis *in vitro*. Pretreatment of CTB-conditioned medium with NOK-2 significantly reduced the percentage of cells externalizing phosphatidylserine [$P < 0.05$, CTB-conditioned medium (CM) + IgG versus CTB-CM + NOK-2], indicating that FasL secreted by trophoblasts induces SMC apoptosis.

Trophoblast-Derived FasL Induces Vascular SMC Apoptosis in Situ

To determine whether trophoblast-derived FasL is capable of inducing SMC apoptosis in our *ex vivo* vessel model, spiral arteries were denuded of endothelium and perfused with primary CTB-conditioned medium pretreated with NOK-2 or control IgG. After 24 hours in culture, SMC apoptosis was assessed by TUNEL. SMC apoptosis was evident throughout the vessel wall in arteries treated with conditioned medium and IgG; however, TUNEL-positive SMCs were observed with far less frequency in vessels treated with conditioned medium containing NOK-2 (Figure 7), with apoptosis limited to the layers of cells closest to the vessel lumen. Minimal apoptosis was observed in spiral arteries perfused with unconditioned medium containing either NOK-2 or IgG, indicating that the resident cells are viable under these conditions. These results demonstrate that trophoblast-derived FasL induces SMC apoptosis in explanted vessels. Similar findings were made in umbilical arteries after 72 hours.

Discussion

Loss of smooth muscle from spiral arteries in the placental bed was first noted in classical studies of pregnancy hysterectomy.⁹ The phenomenon appears to be specific for arteries, which are colonized by invading trophoblasts in preference to veins or lymphatic vessels. The transformation of arterial walls that follows trophoblast invasion is associated with profound changes including the partial or complete loss of endothelium, SMCs and the associated extracellular matrix, and its replacement with a fibrinoid matrix containing embedded trophoblasts. Later in pregnancy, endothelium sometimes returns to cover the transformed media. Although incomplete remodeling of spiral arteries has long been associated with complications of pregnancy such as pre-eclampsia, and a trophoblast defect as an underlying cause is much debated, the process itself and the precise role of trophoblasts are mostly unexplored.

We have previously shown that uterine arteries are more receptive to trophoblast invasion than arteries from adult omentum, and that the arteries from pregnant uterus are more receptive than arteries from nonpregnant uterus.²⁶ Indeed, before trophoblast colonization, histological changes evident in uterine arteries might imply

Figure 5. Activation of Fas on HASMCs induces apoptosis. **A–C:** Confocal images of cultured HASMCs stained for Fas expression. **A:** Composite Z-stack. **B:** One Z-slice from the center of the Z-stack. **C:** Negative control (IgG). **D:** HASMCs were treated with a Fas-activating antibody (anti-human CD95) and then monitored by time-lapse microscopy. HASMC apoptosis was determined by scoring 40 cells and recording the time point at which they underwent apoptosis. The percentage of apoptotic HASMCs at 60 hours is shown, $*P < 0.05$ and $**P < 0.001$, repeated measures analysis of variance (mean $\pm$ SEM, $n = 5$ independent SMC cultures). **E:** HASMCs were treated with a Fas-activating antibody (1 $\mu\text{g/ml}$). Apoptosis was assessed at 24 and 60 hours by immunoblot analysis of the expression of cleaved PARP. The dividing line represents where unrelated lanes have been cropped from the image. A representative autoradiograph from $n = 4$ experiments is shown. Scale bar = 20 μm .

conversion to a state of receptivity for invasion by trophoblasts.²⁷ As pregnancy proceeds, invading cells appear within the vessel media, close to or at the surface of arterial walls, in the surrounding adventitia and more generally in the decidual and myometrial stroma. There is evidence that interstitially invading trophoblasts tend to cluster in the vicinity of these vessels, and although it has not so far been possible to track the pathway of invasion of these cells, it has been hypothesized that transformation of the arterial walls occurs as a combined result of the action on sensitized vessels of trophoblasts of interstitial and endovascular origin.^{6,7} Another study from our group has shown that introduction of first trimester trophoblasts into segments of spiral artery leads to endothelial cell apoptosis.¹⁶ A limited number of apoptotic SMCs were observed in the vessel media during these experiments, although the arteries were not incubated for long enough time periods to follow the fate of the SMCs after the endothelium had been lost. We have addressed this question in the present study, in which we have developed *in vitro* and *ex vivo* co-culture models to examine trophoblast/SMC interactions as a function of time.

This study and our previous work have begun to address the questions of how normal spiral arteries are remodeled, what factors are critical, and how trophoblasts might contribute.

Our data conclusively show that trophoblasts can induce apoptosis of SMCs by a mechanism involving the Fas/FasL pathway. Such a mechanism is well supported by other studies. Apoptosis of SMCs can be instigated by FasL derived from endothelial cells and monocytes/macrophages,^{28,29} and vessel remodeling during atherosclerosis involves SMC apoptosis mediated by Fas/FasL.³⁰ Expression of FasL by normal trophoblasts has been demonstrated^{25,31,32} and a role for the Fas/FasL pathway in the loss of endothelial cells from spiral arteries,¹⁶ as well as in maternal immunotolerance has been defined.^{23,24} Our data are further reinforced by the recent finding that a maternal polymorphism leading to decreased Fas expression increased the risk of pre-eclampsia and pre-eclampsia-associated intrauterine growth restriction in women delivering preterm babies.³³ A reduction in Fas expression on maternal spiral artery SMCs would be expected to lead to a reduction in FasL-

Figure 6. Trophoblast-induced HASMC apoptosis is inhibited by a Fas ligand-blocking antibody. **A:** Contact co-cultures of HASMCs alone (open bars) or HASMCs and primary CTBs (light shading) or the extravillous trophoblast-derived cell line SGHPL-4 (dark shading) were treated with a Fas ligand-blocking antibody (NOK-2) and monitored by time-lapse microscopy. HASMC apoptosis was determined by scoring 40 cells and recording the time point at which they underwent apoptosis. The percentage of apoptotic HASMCs at 60 hours is shown. * $P < 0.001$ and ** $P < 0.001$ (primary CTB) and * $P < 0.05$ and ** $P < 0.001$ (SGHPL-4), repeated measures analysis of variance (mean $\pm$ SEM, $n = 7$ independent primary CTB isolations; $n = 5$ independent SGHPL-4 cultures). **B:** HASMCs were incubated with serum-free unconditioned trophoblast medium (control) or serum-free primary CTB-conditioned medium [CM; 50% (v/v); both pretreated with NOK-2 or an IgG isotype control (10 μ g/ml, 1 hour)] for 2 hours. The percentage of cells externalizing phosphatidylserine was quantified using flow cytometry. * $P < 0.05$, paired Student's t -test (mean $\pm$ SEM, $n = 4$ independent SMC cultures).

mediated, trophoblast-induced SMC apoptosis during early pregnancy. This may result in inadequate spiral artery remodeling and defective placentation, phenomena that go hand in hand with pre-eclampsia and intra-uterine growth restriction.²⁻⁵ Animal models are of limited use in the analysis of this phenomenon; in particular, trophoblast invasion is much more limited in the mouse uterus, and pre-eclampsia does not occur. The Fas knockout mouse appears to be normally fertile, perhaps because it is mostly uterine natural killer (NK) cells³⁴ and

Figure 7. SMC apoptosis in vessel segments perfused with primary CTB-conditioned medium is inhibited with a Fas ligand blocking antibody. Spiral arteries or umbilical arteries were denuded of endothelium and perfused with serum-free unconditioned culture medium (control), or serum-free primary CTB-conditioned medium [CM, 50% (v/v)], that had been pretreated with the anti-FasL antibody NOK-2 or a control IgG (10 μ g/ml, 1 hour). The number of TUNEL-positive SMCs per vessel section was quantified by fluorescence microscopy. Nine sections were counted per vessel. Spiral arteries (open bars) were analyzed after 24 hours in culture. Umbilical arteries (shaded bars) were analyzed after 72 hours in culture. *** $P < 0.001$, repeated measures analysis of variance (mean $\pm$ SEM, $n = 4$ vessels obtained from individual biopsies or umbilical cords).

not trophoblasts³⁵ that mediate spiral artery transformation in this species.

We have demonstrated here that a FasL-blocking antibody significantly inhibited trophoblast-induced SMC apoptosis; however, it did not completely abrogate the effect. A higher concentration of antibody may be required to achieve maximum inhibition of apoptosis, or alternatively, other factors may be necessary to bring about the required level of SMC apoptosis. A mix of apoptotic cytokines may work together, with individual SMCs exhibiting varying activation thresholds based on receptor expression. TNF- α is produced by primary CTBs³⁶ and has been shown to cause SMC apoptosis.³⁷ In addition, mRNA for TNF-related weak inducer of apoptosis (TWEAK) and lymphotoxin α (LT- α) are produced by primary CTBs,³⁶ and TRAIL mRNA has been detected in trophoblast cell lines after interferon- γ stimulation.³⁸ The effects of these apoptotic cytokines on SMCs are not documented, although they represent a potentially complex system that trophoblasts could use to initiate SMC apoptosis. Trophoblasts also synthesize and secrete matrix metalloproteinases (MMPs), and trophoblast invasion is dependent on MMP production.^{39,40} MMP-mediated tissue disruption may lead to SMC detachment and subsequent anoikis. Indeed, we have observed TUNEL-positive SMCs detaching from the vessel wall and accumulating in the lumen. The presence of MMP in the trophoblast-conditioned medium may explain why SMC apoptosis is not completely inhibited by blocking FasL.

In this study we have found that SMC apoptosis occurs throughout an extended time frame. After 60 hours of co-culture with primary CTBs, only $62.5 \pm 5.1\%$ of the 40 cells tracked had undergone apoptosis. This could be considered a reflection of the way in which remodeling of

vessels is likely to occur *in vivo*. Invading trophoblasts would make their way along the vessels, removing SMCs on an individual basis with mechanisms in place to ensure that vessel integrity is maintained. Rapid and complete apoptosis would not be commensurate with sustaining the circulation or with the timing of spiral artery remodeling, which is known to occur throughout a period of several weeks. Although the events of apoptosis have been examined extensively in isolation, little work has been done to establish these in any time frame.⁴¹ Work on rat ventricular cardiomyocytes has addressed the issue of an apoptotic time scale.⁴² Our data correlates well with the proposed time course of apoptosis; early events such as annexin V expression were detectable at 2 hours, morphological changes such as nuclear condensation occurred after 12 to 20 hours, and caspase activation, assessed by expression of cleaved PARP, was seen at later time points. Analysis of the time lapse sequences also demonstrated that cells undergo apoptosis in an asynchronous manner and in a cumulative rather than all-or-none process, counterbalanced by cell proliferation and proceeding throughout many days.

The results presented do not exclude the possibility that trophoblasts stimulate resident vascular cells to produce apoptotic factors. Indeed, trophoblasts may trigger the expression/release of FasL by neighboring SMCs, so that SMC loss is regulated in a paracrine manner. Nor do the data exclude the possibility that other cells that express FasL, such as macrophages, are involved in the remodeling process. Macrophages constitute ~20% of the immune cells present in the placental bed during normal pregnancy and are observed in close contact with the invasive trophoblasts in decidual segments, yet their presence is inversely correlated with successful vessel remodeling,⁴³ so it is likely that their contribution to SMC apoptosis is minimal.

It has been suggested⁴⁴ that a gradient of decidualization may exist from the decidua to the inner myometrium, suggesting a mechanism to limit arterial remodeling. Anatomical studies have shown that myometrial vessels have thicker walls and contain a greater proportion of elastin than decidual vessels,⁴⁵ which may render them more resistant to remodeling. In addition, spiral arteries in the endometrial functionalis are renewed with each menstrual cycle; thus, these SMCs may exhibit a greater plasticity than those in myometrial segments. SMC proliferation within endometrial spiral arteries is an asynchronous process; vessels are composed of a mixture of proliferating and nonproliferating SMCs, with 2 to 2.5%, 3.6%, and 4.3% proliferating cell nuclear antigen-positive cells observed during the early, mid, and late phases of the menstrual cycle, respectively.⁴⁶ This suggests that individual SMCs may exhibit different phenotypes *in vivo*, correlating with our observations of asynchronous SMC apoptosis. However, the rate of SMC proliferation in myometrial spiral arteries of pregnant or nonpregnant women is yet to be documented. Taken together, these findings would suggest that a degree of variation in vessel structure and SMC phenotype may exist along the length of the spiral arteries, which could exert spatial and temporal control over SMC apoptosis

and the remodeling process. This would explain our observation that a peak of SMC apoptosis occurred in spiral artery segments perfused with CTBs at 24 hours, with a reduced but steady rate of cell death occurring after this time. If a subset of SMCs possessed a phenotype rendering them more sensitive to FasL stimulation, they may be responsible for the initial wave of death observed at 24 hours. Other SMCs, with a phenotype that conferred protection against FasL-induced apoptosis, may survive or die at a later point.

Vascular remodeling occurs during normal maintenance of the cardiovascular system, in response to exercise or pregnancy as well as in pathological states such as atherosclerosis. The outcome relies on a balance between apoptotic and proliferative events triggered by growth factors, cytokines, and mechanical stresses. During remodeling of the uterine spiral arteries in pregnancy, mural disruption results from a combination of maternal conditioning and invasion of CTB. Using novel experimental approaches we have provided evidence that one part of this process is SMC apoptosis instigated by CTBs via the Fas/FasL system. This may suggest novel therapeutic approaches for the incomplete vascular adaptation seen in pregnancy pathology. However, mechanisms other than apoptosis may also be in play, and further progress will be required, including detailed studies of the placental bed in early pregnancy, before we have a complete account of the processes that contribute to physiological remodeling of maternal spiral arteries and can define the factors responsible for its failure in disease.

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