Placenta 33 (2012) 188–194

Contents lists available at SciVerse ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Pro-inflammatory cytokine-stimulated first trimester decidual cells enhance macrophage-induced apoptosis of extravillous trophoblasts

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ARTICLE INFO

Article history:
Accepted 9 December 2011

Keywords:
First trimester decidual cells
Macrophage
Extravillous trophoblast
Apoptosis
GM-CSF
M-CSF

ABSTRACT

Objective: As human blastocyst-derived extravillous trophoblasts (EVTs) invade the early decidua, they are positioned to interact with immune cells and resident decidual cells, and remodel spiral arteries into high capacity vessels that increase blood flow to the developing fetal-placental unit. Shallow EVT invasion elicits incomplete vascular transformation and reduces uteroplacental blood flow that presages adverse pregnancy outcomes. Excess macrophages in the decidua induce EVT apoptosis via tumor necrosis factor-alpha (TNF-α) secretion. Our previous observation that pro-inflammatory cytokines enhance neutrophil and macrophage activator granulocyte-macrophage colony-stimulating factor (GM-CSF) expression in first trimester decidual cells is now extended to include: 1) the specific macrophage activator M-CSF; 2) macrophage activation and subsequent enhancement of EVT apoptosis by both GM-CSF and M-CSF.

Study design: Quantitative reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay assessed M-CSF expression in first trimester decidual cells incubated with interleukin-1 beta (IL-1β) or TNF-α. Peripheral monocyte-derived macrophages pre-incubated with conditioned media from decidual cell cultures were co-cultured with a first trimester EVT cell line, HTR-8/SVneo cells. Macrophage activation was examined and EVT apoptosis evaluated by DNA fragmentation, caspase activation and cell membrane asymmetry.

Results: IL-1β or TNF-α significantly enhanced M-CSF expression in first trimester decidual cells. The conditioned media from these cultures activates macrophages, which promote caspase 3/7-dependent EVT apoptosis with antibodies against GM-CSF or M-CSF blocking this effect.

Conclusions: Pro-inflammatory cytokines increases synthesis of M-CSF in first trimester decidual cells. Both GM-CSF and M-CSF activate macrophages, which initiate caspase-dependent EVT apoptosis.

1. Introduction

Blastocyst-derived extravillous trophoblasts (EVTs) invade an underlying decidua comprised primarily of resident decidual cells (50%) and immune cells (40%) [1]. Invasion of the decidua and inner third of the myometrium is accompanied by conversion of uterine spiral arteries into high capacity vessels that increase blood flow to the developing fetal-placental unit [2]. By contrast, shallow decidual invasion is associated with incomplete vascular remodeling. The resulting impaired uteroplacental blood flow is inadequate to support the developing fetal-placental unit [2] and is associated with such disorders of pregnancy as preeclampsia, intrauterine growth restriction (IUGR), miscarriage, preterm birth and placental abruption [3].

Shallow EVT invasion of the decidua reflects decreased EVT invasiveness and/or exaggerated apoptosis that produces lower numbers of invading EVTs [4,5]. Aberrant augmentation of EVT apoptosis has been reported in pregnancies complicated by gestational trophoblast disease [6], preeclampsia [7], IUGR [8] and preterm birth [9]. The consequent increased shedding of EVT debris into the maternal circulation is associated with excess inflammation [10] that may lead to rejection of fetus. Conversely, over invasion of the decidua with insufficient EVT apoptosis is associated

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doi:10.1016/j.placenta.2011.12.007

with blastocyst implantation on impaired decidua and frequently leads to placenta creta, which carries life threatening risks [11].

At the maternal interface of first trimester human decidua, invading EVTs interact with resident decidual cells and such immune cells as natural killer cells, dendritic cells and Mϕs [12]. Comprising 20–25% of first trimester human decidual leukocytes, Mϕs are major antigen-presenting cells that confer immune tolerance of the semi-allogeneic fetal transplant [13]. They act as major scavengers of apoptotic cells thereby reducing local inflammation [14]. Close apposition of significant number of Mϕs to invading EVTs positions decidual Mϕs to mediate normal EVT invasion by remodeling the decidua secondary to phagocytosis of apoptotic cells. Conversely, an excess of Mϕs in the preeclamptic decidua reported by our [15] and other laboratories has been shown to induce excess apoptosis in invading EVTs via the synthesis and release of TNF-α [16], thereby contributing to shallow preeclampsia-related EVT invasion.

CSFs are myeloid growth factors secreted by various cell types and exhibit pleiotropic functions. Specifically, GM-CSF is a potent differentiation-inducer and activator of Mϕs [17]. Previously, our laboratory found that pro-inflammatory cytokines associated with preeclampsia, miscarriage and preterm birth [18–20], IL-1β and TNF-α, markedly up-regulated GM-CSF production in cultured first trimester decidual cells. Complementing these *in vitro* observations, immunostaining revealed aberrantly high GM-CSF levels in preeclamptic versus gestational-age matched decidual cells [23]. In view of the established link between Mϕ-induced apoptosis of EVTs in preeclampsia [16] taken together with several reports indicating that macrophage-CSF (M-CSF) is a highly specific and potent inducer of differentiation and activation of Mϕs [21] and mediates Mϕ infiltration in the normal decidua [22], the current study: 1) evaluated the effects of IL-1β and TNF-α on M-CSF expression in first trimester decidual cells; 2) determined whether Mϕs can be activated by excess GM-CSF and M-CSF secreted by first trimester decidual cells; 3) assessed whether CSF mediated the enhancement of Mϕ-induced EVT apoptosis.

2. Materials and methods

2.1. Cell culture

2.1.1. First trimester decidual cell isolation

First trimester decidual cells were isolated as previously described [15]. Briefly, decidual specimens from elective terminations between 6 and 12 weeks of gestation were obtained under Yale University Human Investigation Committee approval. After digestion with 0.1% collagenase type IV and 0.01% DNase in Ham's F-10, the digestate was purified on 60/50/40% Percoll gradient. Cells were then cultured in basal medium, a phenol red-free 1:1 v/v mixture of DMEM and Ham's F-12 (Sigma–Aldrich, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 ng/ml fungizone (Invitrogen, Carlsbad, CA) and 10% charcoal-stripped calf serum (Sigma–Aldrich). Cell purity was determined by flow cytometric analysis of anti-CD14 and anti-CD45 (BD Pharmingen, San Diego, CA) to monitor the presence of leukocytes. Cultured decidual cells were vimentin-positive and cytokeratin 7-negative and displayed decidualization-related morphological and biochemical changes during incubation with progestin, including enhanced prolactin and plasminogen activator inhibitor-1 and inhibited interstitial collagenase and stromelysin-1 expression (results not shown). After 6 passages, confluent leukocyte-free decidual cells were primed with estradiol (10^{-8} M) + medroxyprogesterone acetate (10^{-7} M) for 7d, then stimulated in serum-free fresh medium ± 10 ng/ml IL-1β or TNF-α (R&D Systems, Minneapolis, MN) for 24 h. Conditioned media (CM) were stored at −80 °C.

2.1.2. Isolation of peripheral blood monocytes and development of macrophages

Peripheral blood mononuclear cells were isolated from healthy reproductive age female donors by gradient Ficoll-Hypaque (GE Healthcare, Piscataway, NJ) centrifugation. The monocytes (MOs) were purified with anti-CD14 paramagnetic beads from Miltenyi Biotec (Auburn, CA) according to the manufacturer's instructions. Mϕs were developed from MOs as previously described [22]. Briefly, MOs were cultured in AIM V serum-free medium (Invitrogen) for 5d. The purity of MOs and attached Mϕs was determined by flow cytometric analysis of CD14 and CD11b expression, respectively.

2.1.3. Co-culture

HTR-8 cells, a generous gift from Dr. Charles Graham [23], were labeled with PKH67 (green fluorescence) or PKH26 (red fluorescence) according to the manufacturer's instructions (Sigma–Aldrich). MO-derived Mϕs were pre-incubated in CM from first trimester decidual cells ± IL-1β or TNF-α ± anti-GM-CSF or anti-M-CSF neutralizing antibody for 48h. After pre-incubation, Mϕs were harvested and co-cultured with HTR-8 cells (HTR-8: Mϕ = 2:1) for 6, 12 or 24 h. After pre-incubation with CM, Mϕs were stained with anti-human CD16-PerCP-Cy5.5, CD18-APC, CD86-FITC and HLA-DR-PE antibodies to examine the expression of activation markers [24–26] on an LSRII flow cytometer using FACSDiva (BD Biosciences, San Jose, CA) and FlowJo software (FreeStar, Ashland, OR).

2.2. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Reverse transcription was carried out using Omniscript kit (Qiagen, Valencia, CA). Each RT reaction contained 2 μg of total RNA, 2 μl 1x buffer RT, 0.5 mM dNTPs, 1 μM T7-(dT)24 oligo-primer and 4 units of Omniscript reverse transcriptase. Specific primer pairs (Table 1) were designed and synthesized at the Yale DNA Synthesis Laboratory (Critical Technologies) for qPCR. A standard curve was created utilizing various concentrations of RT products by monitoring the increasing fluorescence of PCR products during amplification. Quantification of unknowns was determined and adjusted to the quantitative expression of β-actin from each unknown. Melting curve analysis determined the specificity of the amplified products and the absence of primer-dimer formation.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Total cell protein levels of whole decidual cell lysates were measured by the Bio-Rad Assay (Bio-Rad, Hercules, CA). A commercial ELISA kit was used to measure the levels of M-CSF in CM according to manufacturer's instructions (R&D Systems). The ELISA has a sensitivity of <11.5 pg/ml. The intra-assay and inter-assay coefficients of variation vary from 2.1 to 7.8 and from 5.4 to 10.7, respectively.

2.4. DNA content analysis

After co-culture, both adherent and floating cells were harvested by 0.5%Tripsin/EDTA and fixed in 70% ice-cold ethanol for >1 h at −20 °C. Permeablized cells were washed and resuspended in 1x PBS and incubated with propidium iodide (PI)/RNase staining buffer (BD Pharmingen) at room temperature for 30min prior to flow cytometric analysis using CellQuest Pro (BD Biosciences) and FlowJo software. An individual population of cells was selected by gating on an area versus width dot plot to exclude cell debris and for doublet discrimination. Difference in DNA degradation was examined by gating on subdiploid peaks of PKH67-labeled HTR-8 cells then analyzing the mean value obtained from 4 experiments.

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

DeadEnd™ Fluorometric TUNEL System from Promega (Madison, MI) was used to examine DNA fragmentation. After co-culture of PKH26 (red)-labeled HTR-8 cells with Mϕs in CM for 24 h, cells were fixed in 4% formaldehyde for 25 min at 4 °C. After permeablization in 0.2% Triton X-100, addition of equilibration buffer was followed by incubation with the TdT reaction mix for 1 h at 37 °C. Finally, the cover slips were mounted with VECTASHIELD® Mounting Medium containing 4,6-diamidino-2-phenylindole (Vector Lab, Burlingame, CA). The numbers of TUNEL-positive and -negative nuclei were counted in 3 randomly selected microscopic fields at 200 × magnification. The DNA fragmentation index was calculated as the number of cells with green fluorescence divided by the number of total red PKH26-stained cells.

2.6. Western blotting analysis

After co-culture with Mϕs for 24 h, HTR-8 cells were harvested in whole cell lysis buffer containing Complete Lysis-M (Roche, Indianapolis, IN). Protein concentration was determined by DC Protein Assay II (Bio-Rad). Fifty microgram of each sample were electrophoresed on 4–20% Mini-Protean TGX Ready Gels (Bio-Rad) and transferred to a nitrocellulose membrane. Membranes were then blocked in Odyssey blocker (LI-COR Biosciences, Lincoln, NE) and incubated with rabbit anti-human caspase 3 or anti-human caspase 7 (Cell Signaling, Beverly, MA) and mouse anti-human hsp90 overnight at 4 °C. After 1 h incubation in secondary IRDye 800-conjugated donkey anti-rabbit or anti-mouse (1:8000) antibody (Rockland,

Table 1
Sequences of primer sets for M-CSF.

Gene	Forward primers	Reverse primers
β-actin	5′-GCTTCTTTGCAGCTCCTTCGTT-3′	5′-GTTGTCGACGACCAGCGC-3′
M-CSF	5′-GTAGCCACATGATTGG-3′	5′-GTTATCTCTGAAGCGC-3′