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Effect of tumour necrosis factor-α in combination with interferon-γ on first trimester extravillous trophoblast invasion

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ABSTRACT

Successful pregnancy is dependent upon invasion of the uterine tissues by extravillous trophoblast cells (EVT). The mechanisms that control trophoblast invasion are unclear, but several cytokines and growth factors appear to be involved. We have previously demonstrated that IFN-γ inhibits EVT invasion via a mechanism partially dependent on an increase in EVT apoptosis and decreased secretion of matrix metalloproteinase (MMP)-2. In the current study we show that TNF-α, both alone and in combination with IFN-γ, inhibits EVT invasion via a mechanism associated with increased trophoblast apoptosis, decreased trophoblast proliferation and/or altered production of active proteases. TNF-α and its receptors, TNF-αRI and TNF-αRII, were immunolocalised in the placental bed. Uterine natural killer (uNK) cells, EVT and villous cytotrophoblast were shown to all produce TNF-α, and TNF-α receptors were primarily immunolocalised to EVT in the placental bed. TNF-α increased EVT apoptosis, decreased villous cytotrophoblast proliferation and increased expression of pro-MMP-9 (but not active MMP-9), urokinase plasminogen activator (uPA) and plasminogen activator inhibitor (PAI)-1 by EVT. The combination of TNF-α and IFN-γ inhibited EVT via a mechanism associated with increased EVT apoptosis, reduced proliferation, reduced pro-MMP-2 secretion and increased secretion of uPA. TNF-α is one of several decidua-derived factors with the capacity to inhibit EVT invasion. The mode of activity of TNF-α was modified by the presence of IFN-γ, suggesting that the local cytokine milieu may be critical in determining spatial and/or temporal changes in EVT invasion.

1. Introduction

Extravillous trophoblast cell (EVT) invasion of the placental bed is a key process in the establishment of pregnancy. EVT invasion proceeds via an interstitial route (through the decidua to the inner third of the myometrium) and via an endovascular route (up the lumen of the spiral arteries) (Pijnenborg et al., 1980). Deficient invasion is associated with miscarriage (Ball et al., 2006), fetal growth restriction (Khong et al., 1986), pre-eclampsia (Pijnenborg et al., 1991) and pre-term birth (Kim et al., 2003). The mechanisms that control trophoblast invasion are incompletely understood, but several *in vitro* studies suggest that a number of cytokines and growth factors are involved (Meisser et al., 1999; Lash et al., 2005, 2006a).

Tumour necrosis factor (TNF)-α is a potent pro-inflammatory cytokine that elicits its effects via two membrane-bound receptors, TNF-αRI and TNF-αRII (Haider and Knofler, 2009). TNF-α has been localised to proliferating trophoblast cells in villous tips, interstitial and endovascular EVT in the placental bed (Chen et al., 1991; Pijnenborg et al., 1998), and to a number of decidual

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cell types including T cells, uterine natural killer (uNK) cells (Jokhi et al., 1994), stromal cells, macrophages (Vince et al., 1992) and endothelial cells (Tabibzadeh, 1991). TNF-α receptors are expressed in placenta (Yelavarthi and Hunt, 1993), although their expression in the placental bed is less clear. Elevated circulating levels of TNF-α have been reported in preterm labour and pre-eclampsia (Casey et al., 1989) and TNF-α has also been reported to induce spontaneous abortion in rodents (Chaouat et al., 1990).

TNF-α has been reported to inhibit trophoblast invasion (Bauer et al., 2004; Renaud et al., 2005; Huber et al., 2006); however, this is not consistent in all studies (Bass et al., 1994). The mechanism(s) by which TNF-α exerts this inhibitory effect are unclear, but likely include alteration of proteolytic enzymes such as the matrix metalloproteinases (MMPs) (Bischof et al., 1995) and the urokinase plasminogen activator system (Queenan et al., 1987) as well as altering EVT apoptosis or proliferation (von Rango et al., 2003).

We have previously demonstrated that exogenous IFN-γ inhibits EVT invasion *in vitro* via a mechanism involving increased apoptosis and decreased protease activity (Lash et al., 2006a). IFN-γ can inhibit the stimulatory effect of TNF-α on endothelial cell invasion (Niedbala and Picarella, 1992). As both TNF-α and IFN-γ are produced in the decidua these cytokines may act synergistically or antagonistically to regulate trophoblast invasion.

We hypothesised that TNF-α, both alone and in combination with IFN-γ inhibits EVT invasion via a mechanism associated with either increased trophoblast apoptosis, decreased trophoblast proliferation or by altering protease production.

2. Materials and methods

2.1. Sample collection

Women undergoing elective surgical termination of pregnancy were recruited at the Newcastle upon Tyne Hospitals NHS Foundation Trust. The study received ethical approval and all patients gave informed written consent. Decidua and placenta were obtained from pregnancies at 8–10 and 12–14 weeks' gestational age (as determined by ultrasound measurement of crown rump length or biparietal diameter) ($n = 12$ in each group). Following collection, placental or decidual tissues were immediately suspended in sterile saline, transported to the laboratory and washed 2–3 times in sterile PBS to remove excess blood.

Placental bed biopsies at 8–10 ($n = 5$), 12–14 ($n = 5$) and 16–20 ($n = 5$) weeks' gestational age were obtained as described previously (Robson et al., 2002). All biopsies were fixed in 10% neutral-buffered formalin for 24 h and routinely processed into paraffin wax. All placental bed biopsies included in the study included decidua, myometrium, interstitial EVT and at least one spiral artery (Robson et al., 2002).

2.2. Uterine natural killer cell isolation

Total decidual cell and CD56+ cell enriched isolates were prepared as previously described (Vassiliadou and Bulmer, 1998; Lash et al., 2006b). The CD56+ cell enriched isolate was consistently >95% pure, as assessed by immunohistochemistry of cell smears. Approximately 25% of the total decidual cell suspension comprises CD56+ cells. Total decidual cell suspensions or CD56+ uNK cells were plated in a 96-well plate at a concentration of 1×10^5 cells/well in 100 μl RPMI 1640 (containing 1000 U/ml penicillin, 1 mg/ml streptomycin, 2 mM L-glutamine and 10% fetal bovine serum [all from Sigma Chemical Co., Poole, UK]) and incubated for 24 h in a standard 5% CO_2 in air incubator at 37 °C. Conditioned medium was removed and stored at −80 °C until required for analysis.

2.3. EVT and villous cytotrophoblast isolation

Extravillous trophoblast and villous cytotrophoblast were isolated from placenta at 8–10 and 12–14 weeks' gestation ($n = 12$ each group) as described previously (Lash et al., 2010a). Isolated cells were resuspended in complete culture medium (DMEM:F12, 10% FBS, 1000 U/ml penicillin, 1 mg/ml streptomycin, 2 mM L-glutamine and 1.5 μg/ml amphotericin B). EVT cells (2×10^5 cells/600 μl/well) were plated in a 24-well plate coated with growth factor-reduced Matrigel® (Becton Dickinson, Franklin Lakes, NJ, USA). Villous cytotrophoblast cells (2×10^5 cells/600 μl/well) were plated in a 24-well plate coated with fibronectin (Sigma). Isolated trophoblast cells were cultured overnight in a standard 5% CO_2 in air incubator at 37 °C. Non-adherent cells were washed off, cell culture medium replaced, cells cultured for a further 24 h before cell culture supernatants were harvested and stored at −80 °C until required for analysis. The isolated EVT and CTB were consistently >95% pure, as assessed by immunohistochemistry of cell smears for HLA-G and cytokeratin 7 (Lash et al., 2010a).

2.4. FAST Quant® multiplex array for TNF-α

To determine levels of TNF-α secreted from CD56+ uNK cells, total decidual cell isolates, EVT and villous cytotrophoblast at 8–10 and 12–14 weeks' gestational age ($n = 12$ each group) a FAST Quant® human TH1/TH2 array was performed according to the manufacturer's instructions (GE Healthcare) and as previously described (Lash et al., 2006b). The slides were imaged using a GenePix scanner and data analysis was performed by the ArrayVision™FAST® software.

2.5. Trophoblast invasion assay

Placental explants (8–10 weeks' gestational age) were prepared and used in Matrigel invasion assays as previously described (Lash et al., 2005). Briefly, chorionic villous tips were dissected, minced to approximately 0.5 mm^3 and resuspended in culture medium (DMEM:F12 containing 10% fetal bovine serum [FBS], penicillin/streptomycin and amphotericin B) such that 15 μl of the suspension constituted approximately 10 mg of tissue. Matrigel invasion assays were performed as previously described (Lash et al., 2005). Confirmation that the invaded cells on the underside of the filter were EVT was obtained by immunos-