Regulation of extravillous trophoblast invasion by uterine natural killer cells is dependent on gestational age

Gendie E. Lash*, Harry A. Otun, Barbara A. Innes, Kathryn Percival, Roger F. Searle, Stephen C. Robson, and Judith N. Bulmer

Uterine Cell Signalling Group, Institute of Cellular Medicine and School of Medical Development and Education, Newcastle University, 3rd Floor, William Leech Building, Newcastle upon Tyne NE2 4HH, UK

*Correspondence address. Tel: +44-191-222-8578; Fax: +44-191-222-5066; E-mail: g.e.lash@ncl.ac.uk

Submitted on August 27, 2009; resubmitted on January 14, 2010; accepted on January 25, 2010

BACKGROUND: Extravillous trophoblast (EVT) cell invasion of uterine decidua and the inner third of myometrium is critical for successful pregnancy. Many decidual factors are likely to play a role in regulating this process. We have previously shown that cytokines, known to be produced by uterine natural killer (uNK) cells, such as TNF-α, TGF-β1 and IFN-γ inhibit EVT invasion. We therefore hypothesized that supernatants from purified uNK cells would inhibit EVT invasion.

METHODS AND RESULTS: Total unfractionated decidual cell supernatants from 8 to 10 weeks gestation increased EVT invasion from placental villous explants, although uNK cell supernatants from 8 to 10 weeks gestation had no effect. In contrast, both total decidual and uNK cell supernatants from 12 to 14 weeks gestation stimulated EVT invasion. MMP-2, uPA, PAI-1 and PAI-2 levels did not differ under any of the conditions tested, whereas MMP-9 levels were increased in the presence of both total decidual and uNK cell supernatants from both gestational age groups. There was a decrease in the level of EVT apoptosis in the presence of uNK cell supernatant from 12 to 14 weeks, but not 8–10 weeks, gestation.

CONCLUSIONS: Decidual uNK cell supernatants from 12 to 14 weeks gestational age stimulated EVT invasion, potentially by increasing MMP9 levels and reducing apoptosis. Total decidual cell isolates stimulated EVT invasion at both gestational ages investigated, potentially reflecting the complex nature of these cell culture supernatants.

Key words: uterine natural killer cells / extravillous trophoblast / invasion / apoptosis / protease

Introduction

Invasion by placental-derived trophoblast into maternal uterine tissues is a key process in the establishment of successful pregnancy (Pijnenborg et al., 1980). During normal placental development, cytotrophoblast cells from the tips of chorionic villi proliferate to form cell columns and differentiate into extravillous trophoblast (EVT) cells which invade maternal uterine tissues via two routes: interstitial and endovascular paths. Interstitial EVT invade through decidualized endometrium to the inner third of the myometrium. Endovascular EVT invade within the lumen of the uterine spiral arteries in a retrograde direction against the blood flow (Brosens et al., 1967; Pijnenborg et al., 1980, 2006). Unlike uncontrolled invasion by neoplastic cells, the process of EVT invasion is tightly regulated (Lala and Chakraborty, 2003). Several cytokines and growth factors have been shown to regulate EVT invasion in vitro, including TGF-β1 (Lash et al., 2005), TNF-α (Otun et al., 2003), IFN-γ (Lash et al., 2006a), VEGF-A (Lash et al., 1999) and HGF (Cartwright et al., 1999). However, the mechanisms regulating trophoblast invasion in vivo remain to be established.

Uterine natural killer (uNK) cells are a distinct endometrial leucocyte population that are prominent in late secretory phase non-pregnant endometrium and early pregnancy decidua, where they account for up to 70% of the stromal leucocyte population (Bulmer and Lash, 2005). uNK cells are phenotypically distinct from the majority of peripheral blood NK cells in that they are CD56 bright and CD16 dim or negative. In vitro studies have shown that uNK cells secrete several cytokines and growth factors that may be involved in regulating trophoblast invasion, including TGF-β1, TNF-α and IFN-γ (Saito et al., 1993; Jokhi et al., 1997; Otun et al., 2003; Lash et al., 2005, 2006a, b).

Trophoblast invasion is regulated by a large number of autocrine and paracrine factors, including cytokines, hormones and oxygen (Bischof et al., 2000; Lala and Chakraborty, 2003; Bischof and Irminger-Finger, 2005). Many of these have been shown to regulate
protease activity, especially matrix metalloproteinases (MMPs) and the urokinase plasminogen activator (uPA) system, which play pivotal roles in degradation of basement membranes and extracellular matrix (ECM; Bischof et al., 2000; Lala and Chakraborty, 2003; Bischof and Irminger-Finger, 2005). MMPs are proteolytic zinc-requiring enzymes which include the collagenases (MMP-1, -4, -8), the stromelysins (MMP-3, 10, -11) and the gelatinases (MMP-2, -9) (Nagase and Woessner, 1991). MMP-2 and MMP-9 (also known as gelatinase A and B) are regarded as key enzymes in degradation of the basement membrane, which consists mainly of type-IV collagen (Nagase and Woessner, 1991). Several tissue inhibitors for MMP, including TIMP-1, TIMP-2 and TIMP-3 regulate protease activity. The uPA system is mainly composed of uPA, uPA receptor (uPAR) and two major uPA inhibitors (PAI-1 and PAI-2; reviewed in Vassalli et al., 1991). On binding to uPAR on the leading cell edge, pro-uPA is cleaved to uPA which then binds plasminogen and converts it into the active protease plasmin. Plasmin acts by converting pro-metalloproteinases (pro-MMPs) to active MMPs as well as being a pro-angiogenic factor. Plasmin acts by converting pro-angiogenic factors.

In vivo trophoblast invasion is inhibited by binding of either PAI-1 or PAI-2 to the active protease plasmin. Plasmin acts by converting pro-MMPs to active MMPs as well as being a pro-angiogenic factor. Plasmin acts by converting pro-angiogenic factors.

Several groups have proposed that uNK cells are key regulators of EVT invasion in vivo (reviewed in Bulmer and Lash, 2005). Recently, Hu et al. (2006) reported that supernatants from interleukin (IL)-15-stimulated uNK cells inhibited migration of EVT from villous explants, an effect that was abrogated by IFN-γ neutralizing antibodies. In contrast, Hanna et al. (2006) demonstrated that uNK cells, also stimulated with IL-15, increased invasion of isolated cytotrophoblast cells in a Matrigel invasion assay; this effect was partially attenuated by the addition of neutralizing antibodies to IL-8 and IP-10. The gestational age groups used in the previous studies were not well defined. We have previously demonstrated that EVT are inherently more invasive in vitro at 8–10 weeks gestational age compared with 12–14 weeks gestational age (Lash et al., 2006c) and that uNK cells secrete varying levels of cytokines and growth factors (Lash et al., 2006b, d) depending on gestational age. However, uNK cells are not found in some reproductive tissues invaded by EVT, namely inner myometrium and fallopian tube in ectopic pregnancy (Bulmer and Lash, 2005). Further, uNK cells are present in species which lack trophoblast invasion (Bulmer and Lash, 2005). Hence, the role of uNK cells in regulating trophoblast invasion in vivo remains unclear. This study aimed to further explore this role.

Materials and Methods

Sample collection

Placental and decidual samples were obtained from women undergoing elective surgical termination of pregnancy at the Royal Victoria Infirmary, Newcastle upon Tyne, UK. The study was approved by the Joint Ethics Committee of Newcastle upon Tyne Health Authority and Newcastle University and all women gave informed written consent. Placenta and decidua 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 immediately prior to pregnancy termination). Following collection, placental and decidual tissues were immediately suspended in sterile saline, transported to the laboratory and washed two to three times in sterile PBS to remove excess blood.

uNK cell isolation

Total decidual cell isolates and purified CD56+ uNK cell isolates were prepared by enzymatic disaggregation and positive immunomagnetic selection (MACS) as previously described (Vassiliadou and Bulmer, 1998; Lash et al., 2006b). Briefly, decidua tissue was finely minced, incubated in DNase/collagenase, allowed to adhere overnight and either used as total decidual cell suspensions or subjected to positive immunomagnetic selection (MidMACS, Miltenyi Biotec., Surrey, UK) with anti-CD56 (Coulter, High Wycombe, UK) to obtain CD56+ cell suspensions. 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 (FBS; all from Sigma Chemical Co., Poole, UK)]) and incubated for 48 h. Cell-free conditioned medium was removed and stored at −20°C until required for analysis. Using this methodology the CD56+ cell enriched isolate has been shown to be consistently >95% pure by immunohistochemistry of cell smears and flow cytometry (Jones et al., 1997); purity was confirmed in representative samples from the present study. Cell viability was routinely tested by trypan blue exclusion and was 80–90% after 48 h cell culture. No gestational age differences in cell viability were observed. Approximately 25% of the total unfractionated decidial cell suspension were CD56+ cells.

Invasion assay

Placental explants were prepared as previously described (Lash et al., 2005). Briefly, chorionic villous tips were dissected, minced to ~0.5 mm^3 and resuspended in culture medium [DMEM:F12 containing 10% BCS, 1000 U/ml penicillin, 1 mg/ml streptomycin, 2 mM L-glutamine and 1.5 µg/ml amphotericin B (all from Sigma Chemical Co.)] such that 15 µl of the suspension constituted ~10 mg of tissue. Matrigel invasion assays were performed as previously described for 6 days (Lash et al., 2005).

In order to investigate the effects of total decidual cell isolates and purified CD56+ uNK cells on trophoblast invasion, 48 h cell culture supernatants (33% v/v) were added to the explant culture medium for the invasion assay. Two gestational age groups were tested: both placental explants and decidual cell culture supernatants came from the same gestational age group (8–10 or 12–14 weeks gestation). At the end of each experiment (Day 6), supernatants were harvested from the placental explant cultures and stored at −80°C until required for western blot analysis and zymography. In addition, at the end of the experimental period, the placental explants were fixed in 10% neutral buffered formalin for 24 h and routinely embedded in paraffin wax for immunohistochemical studies. Each experiment was performed in triplicate on 10 cases at both 8–10 and 12–14 weeks gestational age. Data are expressed as the mean invasion index (± SEM) where the level of invasion was normalized to the control within each experiment to account for placenta to placenta variation. The invasion index is the average number of invaded cells in test filters divided by the average number of invaded cells in control filters for any given experiment. The number of invaded cells on control filters ranged from 40 to 227.

Immunohistochemistry

At the end of the invasion assay, placental explants were fixed in 10% neutral-buffered formalin for 24 h and processed into paraffin wax (n = 3 each gestational age). Serial 3 µm sections were immunostained using an avidin–biotin peroxidase method (Vectastain Elite mouse kit, Vector Laboratories, Peterborough, UK or ImmPRESS kit, Vector Laboratories...
for uPA) for HLA-G (extravillous trophoblast) and cytokeratin 7 (villous and extravillous trophoblast) as previously described (Lash et al., 2005).

In addition, immunostaining was performed for members of the gelatinase family (MMP-2, MMP-9, TIMP-1, TIMP-2 and TIMP-3) and uPA system (uPA, uPAR, PAI-1 and PAI-2) in EVT, identified by immunopositivity for HLA-G, in the same samples. All antibodies were mouse monoclonal antibodies and dilutions, incubation times and pretreatments are detailed in Table I. The sections were scored for the percentage of immunopositive cells (1, <10% immunopositive; 2, 11–24% immuno-positive; 3, 25–74% immunopositive cells; 4, >75% immunopositive cells) by one investigator (K.P.) who was blinded to the identity of the sample (Adams et al., 1999). Data are shown as the mean ± SEM score for each antibody.

### EVT isolation for assessment of apoptosis

EVT were isolated from placenta at 8–10 and 12–14 weeks gestation (n = 3 each gestational age group) using a modification of the method reported by Tarrade et al. (2001). Briefly, placental chorionic villi were washed thoroughly in Hank's balanced salt solution and were carefully dissected to contain only villous material. The dissected chorionic villi were enzymatically digested in 0.125% trypsin (Roche Diagnostics Ltd, Burgess Hill, UK) and 0.5 mg DNase I (Sigma Chemical Co.) for 25 min at 37°C without agitation. Supernatant containing a single cell suspension was removed and the remaining chorionic villi were digested for a second time. As above. The single cell suspension was combined with newborn calf serum (Sigma Chemical Co.) to inhibit any residual enzymes and centrifuged. The cell pellet was then resuspended in incomplete culture medium (DMEM:F12) and layered on to a percoll gradient (10–70% percoll, Sigma Chemical Co.). The percoll gradient was centrifuged (1200g, 30 min, no brake) and the layer containing the EVT cells was removed, supplemented with 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), then centrifuged (300g, 10 min) and the pellet was resuspended and plated in two wells of a 24 well plate coated with growth factor reduced Matrigel® (Becton Dickinson, Franklin Lakes, NJ, USA). Isolated EVT cells were cultured overnight before 20% uNK cell supernatant of the same gestational age group was added to one of the wells and cells were cultured for a further 24 h. At the end of the culture period, protein was extracted from the cells directly in the culture plate with protein extraction buffer (20 mM HEPES buffer, pH 7.4; 150 mM NaCl; 2 mM CaCl2; 10 mM CHAPS). Protein extracts were stored at −80°C until required for western blot analysis of M30, a neo-epitope of cytokeratin 18 exposed after cleavage by active caspase 3.

### Substrate gel zymography

Zymographic analysis was performed on conditioned medium collected from placental explant cultures as described previously (Graham and McCrae, 1996; Lash et al., 2005). Briefly, 20 μg total protein was resolved in a 12% SDS—PAGE containing either 2 mg/ml gelatin (for MMP-2 and MMP-9) or 2 mg/ml casein and 0.025 units/ml plasminogen (for uPA) (American Diagnostica Inc., Greenwich, CT, USA). The gels were washed to remove SDS and incubated overnight at 37°C to allow digestion of substrate. The gels were stained with Coomassie Brilliant Blue R250, destained, preserved and dried. Dried gels were then scanned and densitometry performed (UnScan-It, Silk Scientific Co., Orem, UT, USA). In order to remove inter-subject variability, all results were normalized to their respective controls.

### Western blot analysis

Western blot analysis was performed as previously described (Naruse et al., 2009a). Thirty microgram cell culture supernatant (PAI-1 and PAI-2) or 20 μg EVT total protein extract (M30) (determined by BioRad DC protein assay) was mixed with reducing dye, denatured and resolved in a 12% SDS—PAGE. Proteins were transferred to Hybond-P membrane (Amersham Biosciences, Buckinghamshire, UK) and blocked overnight in TBS containing 5% skimmed milk and 0.05% Tween 20 at 4°C. Membranes were probed with rabbit anti-human PAI-1 antibody (AbCam Ltd., Cambridge, UK; 1:500, 2 h), mouse anti-human PAI-2 antibody

### Table I Primary antibodies for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone or Cat#</th>
<th>Dilution</th>
<th>Incubation time</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin 7&lt;sup&gt;1&lt;/sup&gt;</td>
<td>OV-TL 12/30</td>
<td>1:20</td>
<td>30 min, RT</td>
<td>Citrate buffer, pH 6.0&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>HLA-G&lt;sup&gt;2&lt;/sup&gt;</td>
<td>MEM-G/1</td>
<td>1:300</td>
<td>2 h, RT</td>
<td>Citrate buffer, pH 6.0&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>uPA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>ab24 12 I</td>
<td>1:100</td>
<td>60 min, RT</td>
<td>Citrate buffer, pH 6.0&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>uPAR&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3937</td>
<td>1:50</td>
<td>Overnight, 4°C</td>
<td>Nil</td>
</tr>
<tr>
<td>PAI-1&lt;sup&gt;5&lt;/sup&gt;</td>
<td>TJ46</td>
<td>1:10</td>
<td>60 min, RT</td>
<td>Citrate buffer, pH 6.0&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>PAI-2&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3750</td>
<td>1:75</td>
<td>60 min, RT</td>
<td>Nil</td>
</tr>
<tr>
<td>MMP-2&lt;sup&gt;4&lt;/sup&gt;</td>
<td>42 SD11</td>
<td>1:100</td>
<td>60 min, RT</td>
<td>Nil</td>
</tr>
<tr>
<td>MMP-9&lt;sup&gt;6&lt;/sup&gt;</td>
<td>56 2A4</td>
<td>1:50</td>
<td>Overnight, 4°C</td>
<td>Tryptsin, 10 min, 37°C</td>
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<tr>
<td>TIMP-1&lt;sup&gt;5&lt;/sup&gt;</td>
<td>102D1</td>
<td>1:20</td>
<td>Overnight, 4°C</td>
<td>Nil</td>
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<tr>
<td>TIMP-2&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3A4</td>
<td>1:200</td>
<td>60 min, RT</td>
<td>Nil</td>
</tr>
<tr>
<td>TIMP-3&lt;sup&gt;5&lt;/sup&gt;</td>
<td>136-13H4</td>
<td>1:100</td>
<td>30 min, RT</td>
<td>Citrate buffer, pH 6.0&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Novocastra, Newcastle upon Tyne, UK; <sup>2</sup>Serotec, Oxford, UK; <sup>3</sup>Abcam, Cambridge, UK; <sup>4</sup>American Diagnostica Inc., Stanford, CT, USA; <sup>5</sup>Chemicon, Hampshire, UK; <sup>6</sup>Citrate pretreatment is pressure cooked for 1 min.

All primary antibodies were monoclonal except for anti-uPA which was rabbit polyclonal.
Effect of total decidual cell isolate and uNK cell culture supernatants on EVT invasion depends on gestational age

When placental explants from 8 to 10 weeks’ gestation were cultured in the presence of total decidual cell isolates (8–10 weeks gestational age), there was an increase in EVT invasion (*P < 0.05, Fig. 1). In contrast, uNK cell culture supernatants at 8–10 weeks gestational age, there was an increase in EVT invasion (*P < 0.05, uNK cell *P < 0.003; Fig. 1). 

Effect of total decidual cell isolate and uNK cell culture supernatants on EVT protease production

MMP family

MMP-2, MMP-9, TIMP-1, TIMP-2 and TIMP-3 were examined in explants at the end of the invasion assay using immunohistochemistry. All trophoblasts were identified by immunostaining for cytokeratin 7 (Fig. 2A) whereas EVT were identified by immunostaining for HLA-G (Fig. 2B). MMP-2 (Fig. 2C) and MMP-9 (Fig. 2D) were localized with medium and strong intensity, respectively, to EVT. There was no detectable TIMP-1 in any of the samples (Fig. 2E), TIMP-2 (Fig. 2F) and TIMP-3 (Fig. 2G) localized with medium and strong intensity, respectively, to EVT. Semicontinuous analysis did not reveal any difference in the proportion of EVT cells that were immunopositive for any of the antibodies studied under any of the experimental conditions cultured (data not shown).

At 8–10 weeks gestational age, secretion of MMP-9 by explants cultured in the presence of both total decidual cell isolate (P = 0.0007; Fig. 3A and B) and uNK cell (P = 0.03; Fig. 3A and B) supernatants was increased compared with controls. For 12–14 week gestational age samples, MMP-9 secretion by explants cultured in the presence of both total decidual cell isolate (P = 0.0002; Fig. 3A and B) and uNK cell (P = 0.02; Fig. 3A and B) supernatants was also increased compared with controls. Secretion of MMP-2 by explants from 8–10 and 12–14 weeks gestational age was not altered by any of the culture conditions tested (Fig. 3A and C).

uPA system

After the invasion assay, placental explants were immunostained for uPA, uPAR, PAI-1 or PAI-2. EVT showed weak staining for uPA (Fig. 2H) and PAI-2 (Fig. 2K), and moderate staining for uPAR (Fig. 2I) and PAI-1 (Fig. 2J). Semicontinuous analysis did not reveal any difference in the proportion of EVT cells that were immunopositive for any of the antibodies studied under any of the experimental conditions (data not shown).

There were no differences in levels of secreted uPA (Fig. 4A and B) or PAI-1 (Fig. 4A) under any of the experimental conditions tested at either gestational age. Since we have previously demonstrated that altered PAI-2 levels secreted by EVT correlate with their invasiveness (Lash et al., 2006c), in the absence of changes in uPA levels as determined by histochemistry, secreted PAI-2 levels were determined in placental explant culture supernatants by western blot analysis. The antibody reacts with both the high molecular weight (60 kDa) glycosylated PAI-2 found in the plasma during pregnancy, and the low molecular weight (46 kDa) non-glycosylated intracellular PAI-2 detected in the placenta (Lecander and Astedt, 1987). The predominant band identified by western blotting had a MW of 46 kDa molecular weight (Fig. 4A) and densitometry was performed on this band only. There was no difference in levels of the 46 kDa PAI-2 band (Fig. 4A and C) under any of the experimental conditions tested at either gestational age. Other bands at a higher molecular weight of...
~50 kDa were identified in all lanes but were not measured (Fig. 4A); this band may represent a glycosylated form of PAI-2 (Massaro-Giordano et al., 2005). A band of ~43 kDa molecular weight (Fig. 4A), was also identified only in lanes corresponding to explants cultured in total decidual cell isolate conditioned medium. A band of this molecular weight has been reported previously (Mikus et al., 1993) and is thought to represent a proteolytic fragment of the 46 kDa PAI-2 molecule.

**Effect of uNK cell culture supernatants on EVT apoptosis**

There was no difference in the level of M30 protein detected in EVT cells from 8 to 10 weeks gestation cultured in uNK cell supernatants from the same gestational age (Fig. 5) compared with the control. In contrast, when EVT cells from 12 to 14 weeks gestation were cultured in uNK cell supernatants from the same gestational age, there was a reduction in M30 protein levels as detected by western blot analysis of the 20 kDa M30 cleavage product \( P = 0.04; \) Fig. 5).

**Discussion**

In the current study, we have demonstrated that supernatants prepared by culture of purified uNK cells from 12 to 14 weeks gestation, but not from 8 to 10 weeks gestation, stimulate the invasiveness of EVT derived from explants of the same gestational age. This increased invasion was associated with an increase in secreted MMP-9 and a reduction in EVT apoptosis.

We have previously demonstrated that three key cytokines produced by uNK cells, namely TNF-α, TGF-β1 and IFN-γ, inhibit EVT invasion in the matrigel invasion assay by various mechanisms, including down-regulation of MMP-2, MMP-9 and uPA secretion, up-regulation of PAI-1 and increased apoptosis/decreased proliferation (Otun et al., 2003; Lash et al., 2005, 2006a). We therefore hypothesized that uNK cell supernatants would also inhibit EVT invasion, potentially by one or all of these cytokines. However, our findings indicated that soluble products of uNK cells either had no effect or stimulated EVT invasion dependent on the gestational age of the sample, suggesting that the stimulating effect of other cytokines...
and/or growth factors in the uNK cell supernatants overrides the inhibiting effect on invasion of these three uNK cell cytokine products.

uNK stimulation of EVT invasion at 12–14 weeks gestation was associated with a reduction in EVT apoptosis. Although uNK cells have reduced cytotoxic ability compared with peripheral blood NK cells they do retain low cytotoxic activity and also express perforin and granzyme cytoplasmic granules (King et al., 1989; Ritson and Bulmer, 1989). However, the ability of uNK cells to kill EVT remains unclear, with some evidence that expression of HLA-G by EVT protects them from uNK attack (Rouas-Freiss et al., 1997). It is interesting to note therefore that in the current study uNK cell supernatants reduced the level of EVT apoptosis in vitro as determined by western blot analysis of M30. This protection from apoptosis would increase the number of cells available to invade and suggests that uNK cells do not kill EVT. Whether this result is physiologically relevant is not clear since there appears to be a high level of interstitial EVT apoptosis in vivo (von Rango et al., 2003).

A key aspect of cellular invasion is breakdown of the ECM by proteases with movement of the invading cell into the cleared space (Cohen et al., 2005). EVT employ several different protease systems including the gelatinases MMP-2 and MMP-9 (Cohen et al., 2005) and the uPA system (Chakraborty et al., 2002). In the current study there was no alteration in levels of secreted MMP-2, uPA, PAI-1 or...
PAI-2. However, levels of MMP-9 produced by placental explants were increased in response to both total decidual and uNK cell supernatants at both 8–10 and 12–14 weeks gestational age. The contribution of the total decidual cell or uNK cell supernatants to the protease activity observed with zymography is not clear. We have previously reported that uNK cells are a major source of MMP-2; however, it was MMP-9 that was altered in the current study. We conclude that EVT protease secretion has been altered in response to the decidual and uNK cell culture supernatants. Similar changes were not confirmed by immunohistochemistry but it is acknowledged this is, at best, a semiquantitative technique. It is possible that a subset of EVT increased production of MMP-9 after exposure to total decidual and uNK cell supernatants which was not detected by the immunohistochemical assessment of the whole EVT cell population. Why the increased protease level after exposure to uNK cell supernatants at 8–10 weeks gestation does not correspond with an increase in EVT invasion is not clear. EVT may also secrete other proteases or inhibitors not examined in the present study that may influence invasion early in the first trimester.

We have previously reported MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3, uPA, uPAR, PAI-1 and PAI-2 expression in the placental bed (Naruse et al., 2009a, b). Although those studies concentrated on uNK expression of the proteases, EVT immunostaining was also observed. In the current study, MMP-2, MMP-9, TIMP-2 and TIMP-3 were also immunolocalized to the placenta, including EVT in the cell columns. Interestingly, only weak minimal immunostaining for TIMP-1 was observed in the cytotrophoblast cell columns of the placenta. Previous studies using in situ hybridization or immunohistochemistry have reported expression of both MMP-2 and MMP-9 by EVT in cell columns, but only expression of MMP-9 in villosus cytotrophoblast (Hurskainen et al., 1996; Vegh et al., 1999; Isaka et al., 2003).

In addition, mRNA for TIMP-1, TIMP-2 and TIMP-3 has been reported in trophoblast cell columns (Hurskainen et al., 1996). In the current study, uPA, uPAR, PAI-1 and PAI-2 were all immunolocalized to trophoblast cell columns. This is in agreement with previous reports of uPA system expression in the placenta (Liu et al., 1998; Floridon et al., 1999).

The present study highlights the importance of examining the biological effects of the whole cellular environment rather than of one or more growth factors/cytokines in isolation. Invading EVT within the placental bed are exposed to a wide range of growth factors and cytokines produced by a variety of different cell types and these exhibit considerable cross-talk which may alter or abrogate individual protein effects. Alternatively, cytokines and growth factors may have different actions when acting in combination compared with their activity in isolation. Therefore, it may be impossible to fully determine the key growth factors and cytokines within the uNK cell supernatants responsible for the stimulation of EVT invasiveness, due to redundancy of activity.

It has been suggested for some time that uNK cells regulate EVT invasion into the uterus in normal pregnancy (Lash et al., 2005), although only recently have studies directly examined this important putative role for uNK cells. Hanna et al. (2006) demonstrated that uNK cells grown in the presence of IL-15 stimulated Matrigel invasion of cytotrophoblast cells that were isolated from 8 to 10 week placentas. However, the gestational age of the uNK cells was not reported. In the same study, the cytokine mRNA profile of uNK cells (compared with peripheral blood NK cells) was determined by microarray analysis and this demonstrated IL-8 and IP10 as two of the major cytokines secreted (Hanna et al., 2006). Furthermore, blocking of IL-8 and IP10 in the uNK cell supernatants with neutralizing antibodies abrogated the stimulation of EVT invasion (Hanna et al., 2006). We have recently demonstrated that IL-8 can increase EVT invasion and that blocking of IL-8 in uNK cell supernatants partially abrogates their stimulatory effect on EVT invasion which is only observed at 12–14 weeks gestational age (Oliveira et al., 2007). In contrast, Hu et al. (2006) studied the role of uNK cells in the control of migration and outgrowth of EVT from placental villous explants grown on collagen gels (6–12 weeks gestational age). Uterine NK cells, via IFN-γ, inhibited the differentiation and outgrowth of EVT from placental explants and this was associated with a decrease in active MMP-2 and MMP-9 and an increase in active uPA and PAI-1 protein levels as determined by activity assay or ELISA (Hu et al., 2006). However, the physiological relevance of the latter study to EVT invasion is unclear since uNK cells are only in contact with trophoblast when they are within uterine decidua and therefore do not have the ability to directly affect cytotrophoblast cell column proliferation, differentiation and migration.

In the current study, we have used a placental explant invasion assay model which we have previously characterized for time course of invasion and phenotype of invaded cells (Lash et al., 2005). We have also used this model to explore the effects of TGF-β1, TGF-β2, TGF-β3, IFN-γ and oxygen on EVT invasion (Lash et al., 2005, 2006a, c). In this model the placental villous explants, containing cytotrophoblast cell columns, proliferate, differentiate into EVT, attach to the Matrigel and invade through the transwell filter. Differences in invasion rates seen using this model may reflect alterations in any of those steps. Although it is possible to isolate pure EVT populations, insufficient cell numbers are obtained to perform the experiments described in

**Figure 5** (A) Western blot showing M30 expression in EVT incubated in uNK cell supernatant for 24 h. (B) Densitometric analysis of M30 (n = 3, 8–10 weeks; n = 3, 12–14 weeks). Data are expressed as mean ± SEM relative light units compared with control.
the current study without pooling isolates from different placenta. Isolation of villous cytotrophoblast would give higher cell numbers that can then be artificially differentiated into EVT by culturing on Matrigel, but this approach is also not ideal.

In the current study, we demonstrate a gestational age difference in the effect of uNK cell but not total decidual supernatants on the invasiveness of EVT. We have previously demonstrated that EVT derived from placent al explants at 8–10 weeks gestation are approximately twice as invasive as those from any later stage of pregnancy (12–14 weeks, 16–20 weeks and term; Lash et al., 2006c). One could speculate that early in gestation, when EVT are inherently highly invasive, they are less susceptible to decidua-derived factors. However, as gestation progresses and EVT are less naturally invasive they may acquire an increased ability to respond to decidual/uNK cell derived factors to ensure maintenance of trophoblast cell numbers and sustained invasion into the inner third of myometrium (von Rango et al., 2003). Alternatively the balance of decidua/uNK-derived proteins that influence EVT invasion may alter with gestational age, such that later in gestational age an overall stimulatory effect is observed.

In summary, we have demonstrated that uNK cell supernatants can stimulate EVT invasion at 12–14 weeks’ but not at 8–10 weeks’ gestation. Increased invasion was associated with an increase in MMP-9 secretion and a reduction in EVT apoptosis. Gestational age-dependent differences in invasion may reflect the balance between the inherent invasiveness of EVT and the cytokine/growth factor milieu within the uterine decidua.

Acknowledgements

The authors wish to acknowledge the staff at the Royal Victoria Infirmary, Newcastle upon Tyne for their assistance in sample collection.

Funding

This project was supported by funding from BBSRC (S19967).

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