Heparin prevents programmed cell death in human trophoblast

Frank A. Hills1,5, Vikki M. Abrahams2, Belen González-Timón3, Julia Francis1, Brianna Cloke1, Larry Hinkson1, Raj Rai4, Gil Mor2, Lesley Regan4, Mark Sullivan1, Eric W.-F. Lam3 and Jan J. Brosens1,6

1Institute of Reproductive and Developmental Biology, Wolfson & Weston Research Centre for Family Health, Imperial College London, Hammersmith Hospital, London, UK, 2Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, CT, USA, 3Cancer Research-UK Labs, Department of Oncology, Imperial College London, Hammersmith Hospital, London, 4Clinical Department of Obstetrics and Gynaecology, Imperial College London, St Mary’s Hospital, London and 5Biomedical Sciences, Institute of Health and Social Research, School of Health and Social Sciences, Middlesex University, London, UK 6To whom correspondence should be addressed at: Institute of Reproductive and Developmental Biology, Imperial College London, Hammersmith Campus, London W12 0NN, UK. E-mail: j.brosens@imperial.ac.uk

Heparin is used clinically for the prevention of pregnancy complications associated with prothrombotic disorders, especially antiphospholipid antibody syndrome. Recent studies have suggested that heparin may exert direct effects on placental trophoblast, independently of its anticoagulant activity. We now demonstrate that heparin abrogates apoptosis of primary first trimester villous trophoblast in response to treatment with the pro-inflammatory cytokines interferon (IFN)-γ and tumour necrosis factor (TNF)-α. This multifunctional glycosaminoglycan also inhibited apoptosis induced by other agents, including staurosporin, broad-spectrum kinase inhibitor and thrombin. Furthermore, heparin attenuated caspase-3 activity, a hallmark of apoptosis, in human first trimester villous and extravillous trophoblast cell lines treated with peptidoglycan, a Toll-like receptor-2 agonist isolated from Staphylococcus aureus. The ability of heparin to antagonize cell death induced by such diverse apoptotic signals suggested that it acts as a survival factor for human trophoblast. We demonstrate that heparin, like epidermal growth factor (EGF) and heparin-binding EGF (HB-EGF), elicits phosphorylation of the EGF receptor and activation of the phosphatidyl inositol 3-kinase (PI3K)-, the extracellular signal-related kinase 1/2 (ERK1/2)- and the c-Jun NH2 terminal kinase (JNK)-signal transduction pathways in primary villous trophoblast. In summary, we have demonstrated that heparin activates multiple anti-apoptotic pathways in human trophoblast. Our results suggest that heparin may be useful in the management of at-risk patients, even in the absence of an identifiable thrombophilic disorder.

Key words: heparin/apoptosis/trophoblast/survival

Introduction

Heparin is a glycosaminoglycan formed by sulphated oligosaccharides that vary in the length of polymeric units and, therefore, has different molecular weights. Low molecular weight heparin (LMWH) is made by partial hydrolysis or enzymatic degradation of unfractionated heparin (UFH; Shriver et al., 2000). Thrombo-prophylaxis with UFH or LMWH initiated early in pregnancy is highly effective in preventing recurrent pregnancy loss associated with primary antiphospholipid syndrome (PAPS; Rai et al., 1997; Rai and Regan, 2002). Heparin is also increasingly used to prevent both early and late pregnancy complications associated with other thrombophilias such as factor V Leiden mutation, activated protein C resistance, prothrombin G20210A and protein S deficiency (Rey et al., 2003; Krabbendam et al., 2005). Normal pregnancy is an acquired hypercoagulable state because of an increase in procoagulants and impaired fibrinolysis. Hence, it has been widely assumed that the beneficial effects of heparin on pregnancy outcome in women with prothrombotic disorders are mediated by its anticoagulant properties. However, intravascular or intervillous blood clots are rarely found on histological examination of miscarriage samples from patients with APS (Sebire et al., 2002). Furthermore, accumulating evidence strongly suggests that antiphospholipid antibodies (aPL) have direct effects on trophoblast function and survival. For instance, aPL have been shown to bind human trophoblast cell membranes in both a cofactor-dependent and a cofactor-independent manner (Chamley et al., 1998; Di Simone et al., 2001, 2002; Bose et al., 2005). In primary human trophoblast cultures, aPL inhibit the expression of HCG, induce apoptosis, decrease trophoblast fusion, alter expression of cell adhesion molecules and limit trophoblast invasiveness in a Matrigel assay (Di Simone et al., 2001, 2002; Bose et al., 2004; Meroni et al., 2004; Quenby et al., 2005).

If doubt is cast on the prothrombotic mechanism of pregnancy loss associated with PAPS, then the therapeutic mechanism of heparin must also be questioned (Girardi, 2005). Bose and co-workers recently demonstrated that heparin prevents aPL-induced trophoblast apoptosis and that it restores cellular invasiveness in a placental villous explant model (Bose et al., 2004). Furthermore, an elegant study in mice demonstrated that heparin, but not other anticoagulants such as hirudin and fondaparinux sodium, prevents aPL-induced fetal loss, although these compounds achieved comparable levels of anticoagulation
In addition to its anticoagulant activity, heparin has biological properties that could be critical for the prevention of tissue injury at the feto–maternal interface. For instance, heparin suppresses natural killer cell cytotoxicity (Yamamoto et al., 1985; Johann et al., 1995), prevents leukocyte adhesion/influx (Christopherson et al., 2002; Manduteau et al., 2002; Wan et al., 2002), antagonizes interferon (IFN)-γ signalling (Fritchie et al., 2000) and inhibits complement activation (Girardi et al., 2004). In addition to these anti-inflammatory and immunomodulatory effects, heparin also enhances the ability of several growth factors, including hepatocyte growth factor, epidermal growth factor (EGF), heparin-binding EGF (HB-EGF) and fibroblast growth factors (FGFs), to activate their cognate cell surface receptors (Folkman and Shing, 1992; Li et al., 1999). Interestingly, these growth factors are critical for trophoblast survival and promote invasion (Johnstone et al., 2005).

In this study, we demonstrate that addition of heparin to primary human villous trophoblast cultures enhances cellular survival. We also show that heparin confers resistance to apoptosis triggered by a variety of pathological stimuli, including pro-inflammatory cytokines, thrombin and staurosporin. Furthermore, this cytoprotective effect of heparin was recapitulated in human trophoblast cell lines treated with peptidoglycan (PGD).

Methods and materials

Primary villous trophoblast cultures

First trimester placental samples were obtained from termination of pregnancies carried out between 8 and 12 weeks of gestation. The study was approved by Hammersmith Hospitals NHS trust Ethics Committee, and informed consent for the use of the tissues was obtained from patients. Villous trophoblast cells were isolated and purified as described previously (Hills et al., 2004). Briefly, tissue samples were thoroughly washed and digested with 0.0125% trypsin for 40 min at 37°C. The supernatant, containing isolated cells, was removed, and the remaining tissue underwent two more cycles of digestion. Villous trophoblasts were separated from other cell types by immunoselection using an antibody (W6/32) that recognizes epitopes common to human leukocyte antigen (HLA) class I antigens. Following incubation with W6/32 antibody, cells were then washed twice in PBS and stained in 50 mM phosphate-buffered saline (PBS) and then fixed in 90% ethanol. Fixed cells were then washed twice in PBS and stained in 50 μM PI containing 5 μg/ml DNase-free RNase (Sigma) for 1 h, then analysed by flow cytometry using a FACScalibur (Becton Dickinson, Cowley, Oxford, UK) and analysed using Cell Quest software (Becton Dickinson).

Caspase-3 activity assay

The effects of heparin on peptidoglycan (PGD)-induced apoptosis in HS and 3A cells were determined by measuring caspase-3 activity using the Caspase-Glo™ assay (Promega, Madison, WI, USA) as described previously (Abrahams et al., 2004). Briefly, 10 μg of whole cell lysates were incubated at room temperature in the dark for 1 h with the caspase-3 substrate. Following incubation, luminescence was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) in triplicate. The amount of luminescence detected as a multiple of control values. These experiments were repeated four times on different primary cultures.

Flow cytometry

Cell cycle analysis of first trimester villous trophoblast was performed using propidium iodide (PI) staining as described (Labiède et al., 2005). Briefly, isolated villous trophoblast cultures (n = 3) were trypsinized, washed in phosphate-buffered saline (PBS) and then fixed in 90% ethanol. Fixed cells were then washed twice in PBS and stained in 50 μM PI containing 5 μg/ml DNase-free RNase (Sigma) for 1 h, then analysed by flow cytometry using a FACScalibur (Becton Dickinson, Cowley, Oxford, UK) and analysed using Cell Quest software (Becton Dickinson).

SDS–PAGE and Western blot analysis

First trimester villous trophoblast was cultured in 6-well plates (approximately 500 000 cells/well) in RPMI 1640 medium containing 2 mM l-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO2. Following overnight incubation, the medium was replaced with serum-free RPMI supplemented with various concentrations of tumour necrosis factor (TNF)-α (R&D Systems, Oxon, UK) and IFN-γ (R&D Systems), staurosporin (Sigma, Poole, UK), CRM197 (Sigma) or thrombin (Sigma) in the presence or absence of UFH (Sigma) for an additional 96 h. At the end of this incubation, the medium was removed, and cell numbers in each well were assessed using a cell proliferation assay (MTS; Promega UK, Southampton, UK) according to manufacturers’ instructions. The absorbance change was converted to cell number based on a calibration with known cell number. All experiments were performed three times or more with fresh cultures.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin DNA-nick end labelling

To determine whether any effect of heparin is because of anti-apoptotic effects, we cultured first trimester villous trophoblast on glass slides (approximately 100 000 cells/well) in RPMI 1640 medium containing 2 mM l-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO2. Following overnight incubation, the medium was replaced with serum-free RPMI supplemented with various concentrations of TNF-α and IFN-γ, staurosporin or thrombin for 96 h in the presence or absence of UFH for an additional 96 h. Subsequently, cells were fixed in aceton/ methanol (1:1 v/v) for 5 min, washed and stained for DNA fragmentation using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin DNA-nick end labelling (TUNEL) apoptosis kit (Apoptaq, Chemicon, Chandlers Ford, Hampshire, UK). TUNEL-positive cells were visualized using fluorescence microscopy and quantified by determination of the luminosity of each cell present in a single field with a minimum of 10 fields analysed. Luminosity was expressed as a multiple of control values. These experiments were repeated three times or more with fresh cultures.

Cell lines

The human first trimester extravillous trophoblast cell line, HTR8 (referred to hereon as H8; Graham et al., 1993), was a kind gift from Dr Charles Graham (Queens University, Kingston, Ontario, Canada). The human first trimester-like villous trophoblast cell line, 3A, was purchased from ATCC. Both cell lines were cultured at 37°C/5% CO2 in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 10 mM HEPES, 0.1 mM minimum essential medium non-essential amino acids, 1 mM sodium pyruvate and 100 mM penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The cell lines were not used beyond passage number 8.

MTS assay

Primary villous trophoblast cells were plated out onto 96-well plates at a concentration of approximately 20 000 cells/well in RPMI 1640 medium containing 2 mM l-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO2. Following overnight incubation, the medium was replaced with serum-free RPMI supplemented with various concentrations of tumour necrosis factor (TNF)-α (R&D Systems, Oxon, UK) and IFN-γ (R&D Systems), staurosporin (Sigma, Poole, UK), CRM197 (Sigma) or thrombin (Sigma) in the presence or absence of UFH (Sigma) for an additional 96 h. At the end of this incubation, the medium was removed, and cell numbers in each well were assessed using a cell proliferation assay (MTS; Promega UK, Southampton, UK) according to manufacturers’ instructions. The absorbance change was converted to cell number based on a calibration with known cell number. All experiments were performed three times or more with fresh cultures.
phosphorylated at Thr183/Tyr185 (9251), INK1/2 (99252), p38 phosphorylated at Thr180/Tyr182 (9211), p38 (9212), Erk1/2 phosphorylated at Thr202/Tyr204 (9101) and total Erk1/2 (9102) were purchased from Cell Signalling Technologies (Hitchin, UK). The anti-phospho EGFR receptor (EGFR) (Tyr1173) antibody (9H2) was purchased from Upstate (Dundee, UK) and anti-EGFR antibody (SC-03) from Santa Cruz (La Jolla, CA, USA). Antibodies against Bcl-2 and Bax were from Dakocytomation (Cambridgeshire, UK), against Bim from Calbiochem (Nottingham, UK) and against XIAP from R&D Systems. The antibodies were detected using horseradish peroxidase-linked goat anti-mouse or anti-rabbit IgG (Dako) and visualized using the enhanced chemiluminescent detection system (Amersham Biosciences, Little Chalfont, UK). Western blot analysis was repeated three times using different primary cultures.

**Statistical analysis**

Data are expressed as mean ± SD. Statistical significance (P < 0.05) was determined using one-way ANOVA with the Bonferroni correction.

**Results**

**Heparin inhibits apoptosis in primary human villous trophoblast cultures**

We used purified first trimester villous trophoblast cultures to determine the effect of heparin on cell viability. Cultures were maintained in serum-free medium for 96 h in the absence or presence of different concentrations of UFH. The number of viable cells in each culture was determined after 96 h using an MTS colorimetric assay. As shown in Figure 1A, UFH significantly increased the number of viable cells in a dose-dependent manner, and its effect was maximal at a concentration of 100 ng/ml. Next, we used the same cell system to examine the effect of heparin on villous trophoblast apoptosis. TUNEL assay revealed that UFH, even at concentrations as low as 1 ng/ml, reduced the apoptotic index by 50% or more. These results demonstrate that heparin exerts strong anti-apoptotic activity in human placental cells.

**Heparin inhibits IFN-γ/TNF-α-induced apoptosis in primary human trophoblast cultures**

The pro-inflammatory cytokines IFN-γ and TNF-α have been widely implicated in the pathogenesis of both early pregnancy loss and late pregnancy complications such as pre-eclampsia and fetal growth restriction. In trophoblast culture, TNF-α and IFN-γ reduce the number of viable cells (Figure 2A) by inducing apoptosis (Figure 2B). However, simultaneous addition of UFH, at a concentration between 10 and 100 ng/ml, completely abrogated the increase in TUNEL-positive cells in cultures treated with IFN-γ (10 ng/ml) plus TNF-α (10 ng/ml). To confirm these observations, we examined the cell-cycle phase distributions in the different cultures using flow cytometry analysis following PI staining of cellular DNA. As shown in Figure 2C, heparin completely abolished the increase in the number of dead or dying cells containing <2N DNA content in villous trophoblast cultures treated with TNF-α and IFN-γ. The ability of UFH to inhibit cell death induced by IFN-γ and TNF-α was maintained.

![Figure 1. Heparin enhances cell viability in primary villous trophoblast cultures. (A) First trimester villous trophoblast cultures were maintained in serum-free medium in the absence or presence of unfractionated heparin of different concentrations as indicated. The number of viable cells was determined using MTS assay 96 h later. (B) In parallel cultures, the apoptotic cell fraction was determined using terminal deoxynucleotidyl transferase-mediated dUTP-biotin DNA-nick end labelling (TUNEL). *Denotes P < 0.05 versus untreated cells. The results represent the mean (±SD) of triplicate measurements of one representative experiment.](https://academic.oup.com/molehr/article-abstract/12/4/237/1071220)

![Figure 2. Heparin inhibits villous trophoblast apoptosis in response to pro-inflammatory cytokines. (A) First trimester villous trophoblast cultures were maintained in serum-free medium and treated with a combination of tumour necrosis factor (TNF-α) and interferon (IFN-γ) for 96 h at the doses shown. Subsequently, the number of viable villous trophoblasts was determined using MTS assay. *Denotes significant difference compared with untreated cells (P < 0.01). The results are the mean (±SD) of triplicate measurements. (B) Dose-dependent effect of unfractuated heparin on the apoptotic index, as determined using terminal deoxynucleotidyl transferase-mediated dUTP-biotin DNA-nick end labelling (TUNEL), of primary cultures treated with a combination of TNF-α and IFN-γ (10 ng/ml each) for 96 h. Open bar denotes control (no treatment), whereas the solid bars denote treatment with TNF-α and IFN-γ. *Denotes a significant increase (P < 0.05) in apoptotic cells when compared with control, whereas *denotes significant decrease (P < 0.05) in cell death when compared with treatment with TNF-α plus IFN-γ only. The results are the mean (±SD) of triplicate measurements of one representative experiment. (C) Cell cycle analysis of first trimester villous trophoblast maintained in serum-free medium for 96 h in the presence of TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) with or without unfractionated heparin (100 ng/ml) is indicated. A representative experiment of four similar experiments is shown.](https://academic.oup.com/molehr/article-abstract/12/4/237/1071220)
when cells were cultured under hypoxic conditions (5% oxygen, data not shown).

We also examined the effect of heparin on the expression of pro-apoptotic (Bim and Bax) and anti-apoptotic (Bcl-2 and Xiap) effector molecules in cultures treated with these pro-inflammatory cytokines. In keeping with the TUNEL and flow cytometry results, heparin reversed the inhibition of Bcl-2 expression and up-regulation of Bim in primary cultures treated with TNF-α and IFN-γ (Figure 3). The expression levels of Xiap and Bax remained unaltered, suggesting that they are not implicated in TNF-α/IFN-γ-induced cell death (Figure 3).

**Effect of heparin on apoptosis of human villous trophoblast in response to different stimuli**

To determine whether the cytoprotective action of heparin is restricted to cell death triggered by pro-inflammatory cytokines, we tested other pro-apoptotic stimuli. Incubation of primary cultures with either recombinant thrombin or the broad-spectrum kinase inhibitor staurosporin reduced the number of viable cells in a dose-dependent manner (Figure 4A and B). As shown in Figure 4C, apoptosis induced by either thrombin or staurosporin was significantly inhibited by heparin.

Recently, it has been demonstrated that the exposure of first trimester trophoblast cells to the gram-positive bacterial component, PDG, induces apoptosis through activation of Toll-like receptor 2 (TLR-2) and Toll-like receptor 6 (TLR-6) (Abrahams et al., 2004). Therefore, the anti-apoptotic properties of heparin were tested on human first trimester villous (3A) and extravillous (H8) trophoblast cell lines treated with PDG. As shown in Figure 4D, heparin significantly reduced the amount of caspase-3 activity triggered through TLR-2 in first trimester trophoblast cells (69 and 56% inhibition for 3A and H8 cells, respectively).

**Heparin activates survival signal transduction pathways in human villous trophoblast cells**

The ability of heparin to antagonize diverse apoptotic signals suggested that it confers cytoprotection by promoting trophoblast survival rather than by interference with a specific pro-apoptotic signalling pathway. Members of the EGF family of growth factors, including EGF and HB-EGF, play an important role in cytotrophoblast survival and proliferation through activation of PI3K and MAPK pathways. Consequently, we examined whether heparin modulated the phosphorylation (activation) status of the EGFR and key downstream-signalling intermediates in human villous trophoblast cells. Whole cell lysates from primary cytrophoblast cultures treated for 30 min with heparin, EGF or HB-EGF were immunoblotted with antibodies that either recognize total or phospho-specific EGFR, Akt, ERK1/2, p38 and JNK. As shown in Figure 5, treatment of cells with heparin triggered the phosphorylation of the EGFR, as did treatment with EGF or HB-EGF. This resulted in phosphorylation of the PI3K intermediate Akt (also termed PKB) as well as phosphorylation of ERK1/2 and JNK. Somewhat surprisingly, we found no evidence that heparin, EGF or HB-EGF

![Figure 3](https://example.com/image3.png)

**Figure 3.** Heparin antagonizes the effect of tumour necrosis factor (TNF)-α/interferon (IFN)-γ Bcl-2 and Bim expression. Whole cell lysates from first trimester villous trophoblast maintained in serum-free medium for 96 h in the presence of TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) with or without unfractionated heparin (100 ng/ml) were immunoblotted for the expression of anti- (Bcl-2, Xiap) and pro- (Bax, Bim) apoptotic proteins. β-actin served as a loading control.

![Figure 4](https://example.com/image4.png)

**Figure 4.** Heparin inhibits apoptosis of human trophoblast in response to different pathological stimuli. (A) First trimester villous trophoblast cultures were maintained in serum-free medium in the absence or presence of thrombin at different concentrations. The number of viable cells was determined using MTS assay 96 h later. *Denotes significant difference (P < 0.05). (B) Parallel cultures were treated with different concentrations of the broad-spectrum kinase inhibitor staurosporin, and cell viability was determined using MTS assay 96 h later. *Denotes significant difference (P < 0.05). (C) Effect of unfractionated heparin (UFH) (100 ng/ml) on trophoblast cell death upon treatment of primary cultures with tumour necrosis factor (TNF)-α plus interferon (IFN)-γ (at 10 ng/ml each), staurosporin (50 μM) or thrombin (100 IU/ml) for 96 h. Apoptosis was assessed using terminal deoxynucleotidyl transferase-mediated dUTP-biotin DNA-nick end labelling (TUNEL) assay 96 h later. *Denotes significant difference (P < 0.05). (D) First trimester trophoblast cell lines (H8 and 3A) were incubated with either no treatment or peptidoglycan (PDG) (40 μg/ml) in the presence (+) or absence (−) of UFH (100 ng/ml) for 48 h. Subsequently, cell lysates were prepared and caspase-3 activity was determined using the Caspase-Glo assay. The results show PDG-induced caspase-3 activity relative to the no treatment controls. The presence of UFH significantly decreased PDG-induced caspase-3 activity in first trimester trophoblast cells (*P < 0.001). The results represent the mean ±SD of triplicate measurements of one representative experiment.
Heparin promotes trophoblast survival

Figure 5. Heparin activates epidermal growth factor (EGF receptor (EGFR)) signal transduction in human trophoblast. Primary first trimester villous trophoblast cultures were maintained in serum-free medium and treated with either recombinant EGF (100 ng/ml), recombinant heparin binding-epidermal growth factor (HB-EGF) (100 ng/ml) or unfractionated heparin (100 ng/ml). Whole cell protein lysates were prepared after 30 min, and protein expression was analysed using western blotting. β-Actin served as a loading control.

Discussion

Programmed cell death of trophoblast cells is a physiological process important for appropriate tissue remodelling at the maternal–fetal interface. Apoptosis is implicated in embryo attachment, trophoblast invasion, spiral artery remodelling and trophoblast differentiation and turnover (reviewed in Straszewski-Chavez et al., 2005). The apoptotic index in the placenta increases during gestation, which has led to the suggestion that it may also be important for the onset of parturition (Smith et al., 1997a). Furthermore, experimental data suggest that apoptosis may promote immune tolerance towards the fetal semi-allograft (Straszewski-Chavez et al., 2005). On the contrary, aberrant and excessive trophoblast apoptosis is a common pathological process associated with a spectrum of pregnancy disorders, including recurrent pregnancy loss, pre-eclampsia, intrauterine growth restriction (IUGR) and preterm birth (Smith et al., 1997b; Allaire et al., 2000; Leung et al., 2001; Ishihara et al., 2002; Choi et al., 2003).

In this study, we demonstrate that UFH protects human villous trophoblast against apoptosis in response to a variety of pathological stimuli. A wealth of data indicate that a preponderance of pro-inflammatory Th1 cytokines, such as TNF-α and IFN-γ, predisposes for early fetal loss as well as late pregnancy complications such as pre-eclampsia (Choi et al., 2000; Raghuopathy et al., 2000; Banerjee et al., 2005; Lee et al., 2005). Although trophoblast is highly sensitive to these pro-inflammatory cytokines (Yui et al., 1994), we demonstrate that IFN-γ/TNF-α-mediated cell death can be inhibited by heparin in a dose-dependent manner. This was reflected at a molecular level by a reversal of IFN-γ/TNF-α-mediated down-regulation of Bcl-2 and up-regulation of Bim in the presence of UFH. Thrombophilias, including factor V Leiden mutation, activated protein C resistance, prothrombin G20210A and protein S deficiency, have been reported to be more prevalent in women with recurrent fetal loss and late pregnancy complications when compared with normal fertile controls (Rey et al., 2003; Krabbendam et al., 2005). In several cell types, such as neurons, vascular smooth muscle cells and tumour cells, thrombin has been shown to exert a dual effect, characterized by induction of cell proliferation at low concentrations and apoptosis at high concentrations (Smirnova et al., 1998; Rossignol et al., 2004). Although we did not observe an increase in the number of viable primary trophoblast cells when exposed to low levels of thrombin, prolonged exposure to a high dose of thrombin elicited apoptosis and again heparin conferred cytoprotection. Another factor that may cause increased trophoblast cell death during pregnancy is infection. Intrauterine infections have been associated with a number of pregnancy complications such as pre-eclampsia, IUGR and preterm delivery (Arehchavaleta-Velasco et al., 2002; von Dadelszen and Magee, 2002; Goncalves et al., 2002). Recently, it has been demonstrated that, in response to gram-positive bacterial PDG, first trimester trophoblast cells undergo apoptosis through a TLR-2-mediated pathway (Abrahams et al., 2004). A terminal step in the apoptotic cascade is the cleavage of caspase-3. We show here that heparin blocks cleaved caspase-3 production induced by the activation of the TLR-2.

These results complement previous reports demonstrating that heparin also reverses the adverse effects of lupus anticoagulant sera on trophoblast viability, proliferation and invasiveness (Di Simone et al., 1997; Bose et al., 2004). Together, the data strongly suggest that heparin exerts its cytoprotective effect by promoting trophoblast survival and proliferation rather than through interference with a specific pro-apoptotic signal transduction pathway. Heparin has been shown to bind and enhance the actions of several growth factors including HB-EGF, EGF and basic FGF (bFGF). Binding of EGF or HB-EGF to their cognate receptors on cell surface of trophoblast activates a variety of signal transduction pathways, including the PI3K/Akt, ERK1/2 and JNK pathways, involved in cell survival (Johnstone et al., 2005). We demonstrate that the addition of heparin to primary cultures mimics the effects of EGF and HB-EGF and triggers rapid EGFR phosphorylation and activation of PI3K/Akt and MAPK signal intermediates. The ability of heparin to elicit EGFR signalling is of direct relevance to pathological pregnancies. For example, Leach et al. (2002) demonstrated a five-fold reduction in the expression of HB-EGF in placenta of pre-eclamptic women when compared with disease-free controls. Notably, the cytoprotective effect of heparin is not confined to human trophoblast cells. Ishikawa and Kitaamura (1999) demonstrated that heparin prevents glomerular cell death in response to different stimuli, including oxidative stress, staurosporin and ultraviolet light. In a rat model for gastric ulcer, heparin has been shown to increase bFGF and EGF levels and constitutive nitric oxide synthase activity, thereby promoting angiogenesis and ulcer healing (Li et al., 2000).

It has been widely assumed that heparin prevents pregnancy complications because of its anticoagulant properties. Hence, its use has been largely restricted to patients with a poor obstetrical history associated with an identifiable hereditary or acquired prothrombotic disorder (Greer and Hunt, 2005). However, this and other in vitro and in vivo studies demonstrate that heparin, in addition to its anti-inflammatory
and immunomodulatory properties, acts as a potent survival factor for human trophoblast (Girardi et al., 2004; Bose et al., 2005). The corollary of these observations implies that heparin could be useful clinically for the prevention of unexplained recurrent fetal loss or pre-eclampsia. This is further supported by a number of recent observational studies. A small prospective preliminary study suggested that treatment with LMWH and aspirin is as effective for the prevention of recurrent pregnancy loss in patients with or without a positive thrombophilia screen (Tzafettas et al., 2002). More recently, Mello and co-workers reported a dramatic reduction in the incidence of pre-eclampsia and fetal growth restriction in women with an angiotensin-converting enzyme insertion/deletion (I/D) polymorphism treated with LMWH from early on in pregnancy (Mello et al., 2005).

LMWHs have largely replaced UFH for the prevention of obstetric complications in women with thrombophilic disorders because of simpler dosing, more predictable anticoagulant activity and improved safety profile (Greer and Hunt, 2005). Different LMWH preparations, such as enoxaparin, dalteparin and tinzaparin, differ in their molecular weight, half-life and anticoagulant activity. Hence, additional in vitro and in vivo studies are needed to evaluate the efficacy of various LMWH preparations in preventing trophoblast apoptosis in response to pathological signals (Quenby et al., 2004). The outcome of these studies should underpin large, randomized, placebo-controlled trials assessing the efficacy of heparin for the prevention of pregnancy complications associated with or without an underlying identifiable thrombophilia.

Acknowledgements

This work was supported by project grants from Save the Baby charity and SPARKS. We thank Ms Josephine Goodchild for her technical support.

References

Abrahams VM, Bole-Aldo P, Kim YM, Straszewski-Chavez SL, Chaiworapongsa SPARKS. We thank Ms Josephine Goodchild for her technical support.

References

Abrahams VM, Bole-Aldo P, Kim YM, Straszewski-Chavez SL, Chaiworapongsa SPARKS. We thank Ms Josephine Goodchild for her technical support.
endothelial cells by a mechanism involving cell adhesion molecules. Pharmacology 65,32–37.


Submitted on December 16, 2005; resubmitted on February 8, 2006; accepted on February 12, 2006