Effect of heparin and fractionated heparin on trophoblast invasion

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BACKGROUND: Heparin can significantly reduce pregnancy complications in women with certain thrombophilias, such as antiphospholipid syndrome. Recent reports suggest that heparin may act by mechanisms other than anticoagulation. However, the effect of heparin on trophoblast biology in the absence of thrombophilia has not been extensively investigated. Therefore, this study aimed to evaluate trophoblast invasion, using an established cell line and primary extravillous trophoblasts (EVTs), following exposure to heparin and fractionated heparin.

METHODS: An EVT cell line (SGHPL-4) was used to study invasion in the presence of hepatocyte growth factor (HGF) and varying concentrations of fractionated and unfractionated heparin. These experiments were repeated using first trimester primary EVTs. RESULTS: Both forms of heparin significantly reduced HGF-induced invasion in the SGHPL-4 cell line. This suppression of invasion appeared to be dose-dependent for fractionated heparin. In primary EVT cells, fractionated heparin also demonstrated significant suppression of invasion. CONCLUSIONS: Heparin has the potential to reduce trophoblast invasion in cell lines and first trimester EVT cells. This article highlights the need for further evaluation of these medications in vitro and in vivo, especially when used in the absence of thrombophilic disorders.

Keywords: heparin; fractionated heparin; trophoblast invasion

Introduction
Regulation of trophoblast invasion is integral to a successful pregnancy. Inadequate trophoblast invasion is associated with conditions such as spontaneous abortion, fetal growth restriction, stillbirth and pre-eclampsia (Khong et al., 1986; Burton and Jauniaux, 2004). Pregnancies in women with certain thrombophilic conditions, such as antiphospholipid syndrome (aPLs), activated protein C resistance, protein S deficiency and factor V Leiden mutation, are known to be at a higher risk for the latter complications (Kupferminc et al., 1999; Greer, 2003). Since the use of heparin can result in a significant reduction in pregnancy complications in women with aPLs, it has been suggested that the beneficial effects in pregnancy relate to anticoagulant actions (Woodhams et al., 1989; Chamley, 1998; Franklin and Kutteh 2003). More recent reports suggest that heparin may act by many different mechanisms, such as reducing aPL antibody binding, inhibiting complement binding, inhibiting placental apoptosis and stimulating placental proliferation (Bose et al., 2004; Hills et al., 2006). The relative success of heparin in improving pregnancy prognosis in women with previous aPLs-related pregnancy complications has led to the increased, unsupported use of heparin, and more recently fractionated heparin, in absence of confirmed thrombophilias (Tzafettas et al., 2002).

The effect of heparin on trophoblast biology in the absence of thrombophilia has not been extensively investigated. The aim of this article was to evaluate trophoblast cell invasion in an established cell line and primary first trimester trophoblasts when exposed to heparin and fractionated heparin.

Materials and Methods
Culture of SGHPL-4 cells
A human extravillous trophoblast (EVT) cell line, SGHPL-4, derived from primary first trimester EVT cells was used throughout this study (Choy et al., 2000; Shiverick et al., 2001). Invasion assays were carried out as previously detailed (Cartwright et al., 1999). In brief, SGHPL-4 cells were incubated with gelatin-coated microcarrier beads to allow the cells to adhere to the beads. Individual bead–cell complexes were added to fibrin gels which were then set and layered with Ham’s F-10 solution. Heparin (heparin sodium; CP Pharmaceuticals, UK) and fractionated heparin (Dalteparin; Fragmin®, Pharmacia, UK) was added to the plates in the doses of 50, 5 and 0.5 U/ml. The effect heparin and fractionated heparin had on SGHPL-4 cell invasion was determined in the presence or absence
of hepatocyte growth factor (HGF) at 10 ng/ml at 37°C in an atmosphere of 5% CO2 in air.

After incubation for 48 h, images of 20 bead–cell complexes per plate were taken at random from each plate using an Olympus IX50 inverted microscope (Middlesex, UK) and a digital camera (JVC TK-C1360E CCD). The researcher was blinded to the treatment until the analysis was completed. For each bead–cell complex, the length of the invasive processes formed was measured using Image Pro-Plus software (Media-Cybernetics, Silver Spring, MD, USA). Invasion was determined as any process greater than the average radius of a bead. All experiments were performed in duplicate and repeated three times.

Isolation and culture of extravillous trophoblasts

Preparation of first trimester placental explants \((n = 6)\) was performed as previously described (Aplin et al., 1999). Local ethical committee approved the study, and informed written consent was obtained from each patient. Prior to dissection, collagen gels were prepared: 0.9 ml of type-I collagen was mixed with 100 \(\mu\)l of Ham's F12/DMEM (1:1 mix, 10% FCS) and 80 \(\mu\)l of 7.5% sodium bicarbonate. Approximately, 100 \(\mu\)l of the collagen mix was placed in each well of a six well plate. The gel was formed following incubation of the plate for 30 min at 37°C. Placental villous tissue was separated from the decidua and rinsed extensively with PBS. Under a dissecting microscope, small pieces of tissue (3–4 mm) were removed from the periphery of the villous tree and placed on top of the collagen gel. Ham's F12/DMEM was then added around the collagen raft and the explant was incubated in 5% CO2 at 37°C to allow the explants to adhere. After 16 h of incubation, a further 2 ml of Ham's F12/DMEM was added to each well and non-adherent tissue gently removed. After a further 24 h, photographs of four areas of each explant were taken where migration was noted from the ends of the anchoring villi using an Olympus (Middlesex, UK) IX50 inverted microscope and digital camera (JVC TK-C1360E CCD). After the photographs were taken, the medium was changed to 2 ml of Ham's F12/DMEM with varying concentrations of heparin or unfractionated heparin (0.5–50 U/ml). The explants were then incubated for a further 48 h to allow the migration of EVTs from the ends of the anchoring villi. At the end of 48 h, the photographs were taken again from the same sites. The area of migration was measured using Image Pro-Plus software (Media-Cybernetics, Silver Spring, MD, USA) by drawing a line around the perimeter of the invasive area and calculating the area within (arbitrary units). The area of invasion at the end of the experiment was divided by the area of invasion at the start of the experiment to determine the relative change. The experiment was repeated using different tissue samples \((n = 6)\); hence, 24 area measurements were made for each experimental condition.

Statistical analysis

Statistical analysis was carried out using the Mann–Whitney U-test and comparison of proportions tests in view of the number of experiments undertaken.

Results

SGHPL-4 cells cultured on gelatin-coated microcarrier beads embedded in a fibrin gel for 48 h formed invasive processes into the fibrin gel (Fig. 1a). Explants of chorionic villous tips cultured on collagen showed outgrowths of invasive EVT after 3–4 days (Fig. 1b).

Figure 1: (a) SGHPL4 trophoblast cells forming invasive processes into a fibrin gel (b) primary EVT cells invading from chorionic tip explant cultures into a collagen matrix

Figure 2: Influence of increasing concentrations of unfractionated heparin (0.5–50 U/ml) on trophoblast cell line (SGHPL-4) invasion using gel-embedded bead–cell complexes

The length of the invasive processes formed was measured from 20 bead–cell complexes. All experiments were performed three times (in duplicate). Invasion was determined in the presence (black bars) or absence (white bars) of HGF at 10 ng/ml. Results are expressed as the mean percentage (± standard error of the mean) of length of SGHPL4 invasion compared with the control without HGF.

**\(P = 0.01\), ***\(P < 0.001\)
Heparin and fractionated heparin showed no significant effect on SGHPL-4 invasion in the absence of HGF stimulation. The addition of HGF had a stimulatory effect on SGHPL-4 invasion (Figs 2 and 3). Both heparin and fractionated heparin suppressed trophoblast invasion in the HGF-stimulated SGHPL-4 cell line (Figs 2 and 3). The suppression was uniform for heparin, but dose-dependent in the presence of fractionated heparin. Significant suppression was seen at the lowest dose (0.5 U/ml) for both heparin and fractionated heparin.

In the primary EVT cells, heparin had no discernable effect on EVT cell invasion (Fig. 4). Fractionated heparin demonstrated significant suppression of trophoblast invasion at a dose of 5 U/ml (Fig. 5). There was evidence of EVT suppression at 0.5 and 50 U/ml of fractionated heparin, but this did not reach statistical significance.

Heparin, in either form, had no effect on SGHPL-4 cell invasion in the absence of HGF. Introduction of HGF, a heparin-binding protein and growth factor, resulted in a significant increase in invasion in the SGHPL-4 cell line. Abrogation of the HGF response was elicited by heparin and fractionated heparin at all doses tested. HGF is naturally occurring protein which is important in placental function, so these findings may be relevant in a clinical setting. HGF increases human trophoblast motility and invasion in vitro models (Cartwright et al., 1999; Kauma et al., 1999; Nasu et al., 2000; Tse et al., 2002). HGF is abundantly expressed in the placenta and reduced levels have been implicated in the pathogenesis of pre-eclampsia (Furugori et al., 1997). Disruption of the genes for both HGF and its receptor results in small placentae with poor trophoblastic development (Uehara et al., 2000). In contrast to our study, most previous authors have used...
trophoblast-derived cell lines from neoplastic choriocarcinoma and also examined the effect of heparin on apoptosis rather than invasion. They have demonstrated that co-culture with serum from women with aPLs, pregnancy failure or pro-inflammatory cytokines results in increased trophoblast apoptosis, and that this effect is abrogated by the addition of heparin (Bose et al., 2004, 2005; Hills et al., 2006). This discrepancy may be explained by either the different origin of the trophoblast cell lines, co-culture in an adverse biological environment or the use of supra-physiological levels of heparin in the latter studies.

The suppression of HGF-stimulated invasion in our study was not dose-dependent in the case of heparin and was dose-dependent for fractionated heparin. The latter finding suggests that heparin and fractionated heparin may have different mechanisms of action. The dose-dependent nature of the fractionated heparin suppression may be mediated by competing with HGF for cell binding (Rubin et al., 2001), although heparin may have a different mechanism independent of HGF. Heparin was originally thought to have mainly anticoagulant effects, but subsequent studies have demonstrated that heparin is capable of modifying trophoblast proliferation by antibody binding, and altering trophoblast apoptosis and cell division (Hills et al., 2006).

The effect of heparin on EVT invasion in normal placenta is unclear from the published evidence. One previous study examined trophoblast invasion in primary cultures from term placenta, showing reduced differentiation into giant cells in response to heparin (Quenby et al., 2004). The current study shows that fractionated heparin (5 U) suppresses first trimester EVT invasion. Bose et al. (2005) investigated the effects of heparin on trophoblast motility rather than invasion in a matrigel assay and also showed equivocal results (Bose et al., 2004). In contrast, Di Simone et al. (2007) studied first trimester EVT cells and demonstrated increased cellular invasion with the addition of fractionated heparin. This discrepancy may be a consequence of sourcing the trophoblast from early first trimester spontaneous abortions in the latter study.

Different, as yet uncharacterized invasive processes (adhesion/loss of adhesion, cell migration and digestion of matrix proteins) will operate in each of the in vitro models. It is interesting to note that some inhibition of trophoblast invasion occurs in the presence of heparin in both models and the precise mechanisms of this inhibition will require further studies. These in vitro methods attempt to represent in vivo EVT invasion in simple, robust models allowing experimental interventions. Our studies have used only one matrix protein whereas there will be a complex extracellular matrix environment in the decidua; therefore, we must be cautious when extrapolating to the in vivo situation.

Conclusions

Heparin, but not other anticoagulants, has been shown in clinical studies to prevent aPL-induced fetal loss by promoting trophoblast survival and proliferation (Girardi et al., 2004). Other proposed mechanisms for the pregnancy-protecting effects of heparin include suppression of natural killer cell cytotoxicity, prevention of leukocyte adhesion, blockage of interferon-γ signaling, inhibition of complement activation and promotion of growth factors (Johann et al., 1995; Fritchley et al., 2000; Christopherson et al., 2002; Girardi et al., 2004). These findings have been interpreted as implying that heparin could be clinically useful for the prevention of unexplained recurrent fetal loss or pre-eclampsia and has led to the increased, unsupported use of these therapeutic agents in normal pregnancy. Although this assertion is supported by a single small observational study (Tzafettas et al., 2002), it is refuted by a small randomized controlled study (Farquharson et al., 2002). Our data demonstrate that heparin, in both the fractionated and unfractionated form, significantly suppresses in vitro trophoblast invasion. Until studies demonstrate that the use of heparin in low-risk pregnancy is not associated with deleterious clinical effects, the unrestricted use of heparin in uncomplicated pregnancy should be tempered.

References


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