

The molecular charge and size of heparins determine their impact on the decidualization of human endometrial stromal cells

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ABSTRACT: Heparin modulates the decidualization of human endometrial stromal cells (ESCs), but the molecular mechanisms behind these effects are still unknown. In the present study, we further specified this biological effect of heparin in human ESCs *in vitro*. ESCs were isolated from hysterectomy specimens, decidualized over 12 days using progesterone and 17 β -estradiol and incubated with thrombin, factor Xa (FXa), unfractionated heparin, dextran sulfate, danaparoid or different low-molecular-weight heparins (LMWHs). Production of insulin-like growth factor (IGF)-I, prolactin (PRL) and IGF-binding protein (IGFBP)-I by ESCs was measured using ELISAs. Like heparin, thrombin and FXa cause an increase in IGF-I in ESCs, suggesting an action of heparin independent from its anticoagulatory effects. This was supported by demonstrating the induction of the same effects on IGF-I, PRL and IGFBP-I as heparin by dextran sulfate, a polysaccharide of similar size and charge as heparin, but without anticoagulatory properties. LMWHs with the same anti-FXa activity as heparin showed less pronounced effects on ESCs than heparin, whereas the very short pentasaccharide fondaparinux (17 kDa) had barely any effect, further supporting the primary role of molecular size and charge mediating these biological effects of heparin on ESCs. In conclusion, the effects of heparin on the decidualization of human ESCs seem to be independent of its anticoagulatory function, but rather depend on the charge and the size of this polysulfated glycosaminoglycan. Therefore, highly sulfated polysaccharides with a molecular weight > 17 kDa might be an interesting pharmacological approach for the therapy of endometrial pathologies, e.g. the treatment of women suffering from recurrent miscarriage or repeated implantation failure.

Key words: decidualization / endometrium / heparin / IGF-I

Introduction

Unfractionated heparin is a mixture of polysulfated glycosaminoglycans (GAGs) with molecular weights ranging from 5 to 30 kDa, with an average molecular weight of 13 kDa (Linhardt *et al.*, 1988). Heparin is the GAG with the highest charge density. It interacts with proteins containing positively charged amino acids. This results in various biological activities beside the anticoagulatory activity of heparin, which is based on its binding to antithrombin thereby catalyzing inhibition of factor Xa (FXa) and thrombin (Linhardt *et al.*, 1992). Low-molecular-weight heparins (LMWHs) are derived from unfractionated heparin by enzymatic or chemical depolymerization and are mainly inhibiting FXa via antithrombin (Shriver *et al.*, 2000).

Heparin and LMWHs are increasingly used to improve the pregnancy outcome in women suffering from thrombophilia and recurrent miscarriage (Kutteh, 1996; Rai *et al.*, 1997). The theory of placental thrombosis and infarction as a cause for early pregnancy loss was the original rationale for this thromboprophylactic application of heparins (Nelson and Greer, 2008). However, intravascular or intervillous blood clots are rarely found in first trimester placenta and decidua samples from patients suffering from early miscarriage (Sebire *et al.*, 2003). Moreover, heparins have also been shown to be effective in the treatment of women with recurrent miscarriage without apparent causes or inherited thrombophilia (Badawy *et al.*, 2008). In addition, LMWHs given in the luteal phase of the menstrual cycle seem to be beneficial to improve the implantation rate as well as

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the live birth rate in women with repeated implantation failure treated in an *in vitro* fertilization program (Urman *et al.*, 2009). Taken together, these clinical observations suggest molecular effects of heparin beyond its classical anticoagulatory action.

We have recently shown that unfractionated heparin and LMWHs modulate the decidualization of human endometrial stromal cells (ESCs) *in vitro* (Fluhr *et al.*, 2010). Heparin dose- and time-dependently delayed the production of insulin-like growth factor-binding protein (IGFBP)-I and amplified the levels of prolactin (PRL) and IGF-I (Fluhr *et al.*, 2010). IGFBP-I and PRL are the major products of the decidualized endometrium and are known to play an important role in endometrial differentiation and implantation (Giudice *et al.*, 1998; Gleeson *et al.*, 2001; Jabbour and Critchley, 2001). They act locally and show typical expression patterns during the second half of the menstrual cycle determining the narrow time frame of endometrial receptivity, called 'window of implantation' (Giudice and Irwin, 1999; Wilcox *et al.*, 1999; Jabbour and Critchley, 2001; Fluhr *et al.*, 2008). However, the molecular mechanisms of these effects of heparin remain unclear.

To further clarify the molecular properties of heparin responsible for its effects on endometrial differentiation, we investigated the impact of heparin on the decidualization of human ESCs *in vitro* in comparison with related molecules with distinct differences in negative charge, molecular size and anticoagulatory potency.

Materials and Methods

Tissue collection and cell culture

Endometrial tissue samples were obtained after written informed consent from premenopausal women undergoing hysterectomy for benign reasons, which typically were intramural or subserous leiomyomata of the uterus. All patients had regular menstrual cycles, proven fertility and were considered to be healthy. Patients suffering from adenomyosis uteri or endometriosis were excluded. The study protocol was approved by the institutional ethical board of the University of Greifswald, Greifswald, Germany. ESCs were isolated, cultured and characterized as described previously (Fluhr *et al.*, 2008). Briefly, minced endometrial tissue was digested by incubation with 1000 IU/ml collagenase (Biochrom, Berlin, Germany) for 1 h. The dispersed endometrial cells were separated by filtration through a 40- μ m filter (BD Falcon, Heidelberg, Germany). ESCs were maintained in Dulbecco's modified Eagle's medium/F-12 cell culture medium without phenol red (Gibco/Invitrogen, Karlsruhe, Germany) containing 10% charcoal-stripped fetal bovine serum (Biochrom) and 50 μ g/ml of gentamicin (Ratiopharm, Ulm, Germany) and cultured until confluency in 75 cm² culture flasks (Sarstedt, Nürmbrecht, Germany). The purity of ESC cultures was proven by the standard immunofluorescent staining of vimentin. For experiments, ESCs were detached using 0.25% trypsin (Biochrom) and plated in 48- and 96-well plates (Greiner, Frickenhausen, Germany) in replicates of four.

Decidualization *in vitro* and experimental conditions

ESCs were decidualized *in vitro* by incubating the cells with 1 μ M progesterone and 30 nM 17 β -estradiol (both from Sigma-Aldrich, Taufkirchen, Germany) for 12 days with renewal of the medium every 3 days. Decidualization was proven by measuring a significantly increased secretion of IGF-I at Day 3 as well as IGFBP-I and PRL at Day 12 of the time course. In addition to progesterone and 17 β -estradiol, ESCs were incubated with the following

agents during decidualization in parallel: human thrombin (Sigma-Aldrich), human FXa (Enzo Life Sciences, Lörrach, Germany), unfractionated heparin (Ratiopharm), dextran sulfate (9–20 kDa, Sigma-Aldrich), danaparoid (Orgaran[®], Essex Pharma, Munich, Germany), dalteparin (Fragmin[®]P, Pharmacia, Berlin, Germany), enoxaparin (Clexane[®], Sanofi-Aventis, Frankfurt, Germany), reviparin (Clivarin[®], Abbott, Wiesbaden, Germany), fondaparinux (Arixtra[®], GlaxoSmithKline, Munich, Germany). The dosages of the mentioned agents are indicated in detail in the corresponding figures.

Cell viability assay

The relative number of viable ESCs under the influence of the indicated agents was measured during the time course of decidualization at Days 3 and 12 using the CellTiter-Blue[®] assay (Promega, Madison, WI, USA) following the manufacturer's instructions. Fluorescence was recorded using the FLUOstar OPTIMA system (BMG Labtech, Offenburg, Germany).

Enzyme-linked immunosorbent assays

Cell culture supernatants were collected at Days 3 and 12 and analyzed for IGF-I (Day 3) as well as IGFBP-I and PRL (Day 12) using commercially available ELISA kits from R&D Systems (Wiesbaden, Germany). These different days of measurements were chosen based on the previously described expression patterns of IGF-I, IGFBP-I and PRL during the time course of decidualization *in vitro* (Fluhr *et al.*, 2010). The sensitivities of the assays were 31.3 pg/ml (IGF-I and IGFBP-I) and 15.6 pg/ml (PRL). There was no significant cross-reactivity or interference. Intra- and inter-assay variabilities were lower than 5%. All assays were performed according to the manufacturer's instructions. Absorbance values were measured using the FLUOstar OPTIMA system and normalized to the relative number of viable ESCs.

Statistics

Each experiment was performed in quadruplicates on cell cultures derived from five to seven different patients. Statistical analysis was carried out with one-way analysis of variance, followed by the Dunnett and the Bonferroni multiple comparison tests using GraphPad PRISM version 5 software (GraphPad, San Diego, CA, USA). The results are expressed as the mean \pm standard error of the mean (SEM). Differences were considered to be significant if $P < 0.05$.

Results

Impact of thrombin and FXa on IGF-I in ESCs during decidualization

First of all, we investigated whether thrombin and FXa—the two clotting enzymes inhibited by heparin in its classical anticoagulatory function—have an impact on the decidualization of human ESCs *in vitro*. Therefore, the cells were incubated with the decidualizing stimuli progesterone plus 17 β -estradiol in combination with 1 IU/ml thrombin or 0.5 IU/ml FXa over 3 days. As shown in Fig. 1, the addition of thrombin as well as FXa led to a significantly higher increase in IGF-I secretion than the hormonal stimulation alone.

Differential effects of heparin, dextran sulfate and danaparoid on ESCs

To provide further evidence that the previously shown effect of heparin on human ESCs is independent of its anticoagulatory properties, we tested dextran sulfate. Dextran sulfate is also a linear

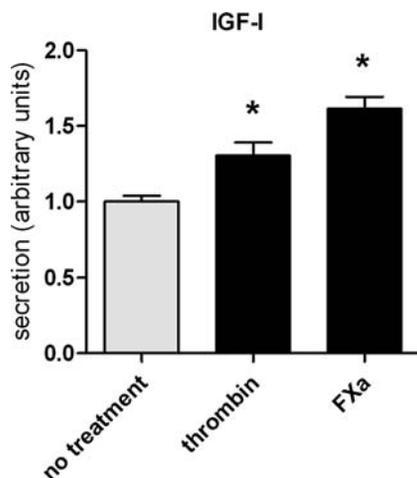


Figure 1 Thrombin and FXa stimulate IGF-I in decidualizing ESCs. ESCs were incubated with the decidualization stimuli 1 μ M progesterone and 30 nM 17 β -estradiol alone ('no treatment') and the addition of 1 IU/ml thrombin or 0.5 IU/ml FXa for 3 days. Thereafter, the levels of IGF-I in the cell culture supernatant were determined by ELISA. Results are standardized relative to the values of 'no treatment', which are set to 1 arbitrary unit. Bars represent the mean \pm SEM; * P < 0.05 compared with 'no treatment'.

polyanion with a strongly sulfated hexose backbone showing the same molecular weight distribution like heparin but having no anticoagulatory properties. As shown in Fig. 2, the effect of dextran sulfate on the secretion of IGF-I in decidualizing ESCs was similar to that caused by heparin (each 5 μ g/ml). In contrast, danaparoid, a mixture of polysulfated GAGs with anti-FXa activity but about only 50% of the negative charge of heparin or dextran sulfate, caused a significant stimulation of IGF-I (Fig. 2) but this effect was significantly less than that with heparin.

Distinct effects of different LMWHs on IGF-I in ESCs

To further clarify the role of anticoagulatory properties, molecular size and the amount of negative charge for the heparin-mediated effects on endometrial decidualization, we tested LMWHs of different molecular weights. Dalteparin, enoxaparin, reviparin and fondaparinux were added to decidualizing ESCs during 3 days, and the secretion of IGF-I was measured and compared with cells treated with unfractionated heparin.

As shown in Fig. 3A, the addition of dalteparin, enoxaparin and reviparin at a dosage of the same anticoagulatory potency (1 IU/ml anti-FXa) caused a significantly less pronounced increase in IGF-I as seen for unfractionated heparin at the same dosage. In addition, fondaparinux also added at 1 IU/ml anti-FXa had no significant influence on IGF-I in decidualizing ESCs.

Similar results were obtained by the addition of LMWHs at a uniform dosage (5 μ g/ml) to obtain the same overall negative charge (Fig. 3B). Again, dalteparin, enoxaparin and reviparin caused a significantly less pronounced increase in IGF-I when compared

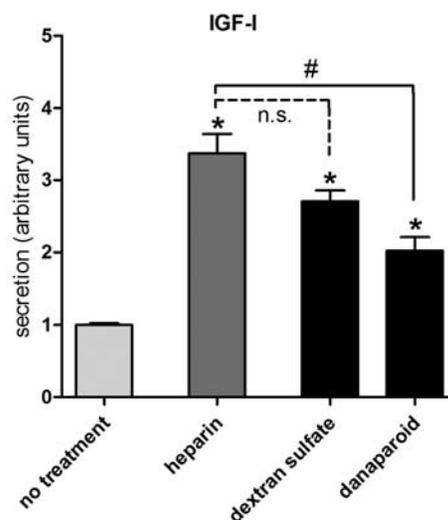


Figure 2 Heparin and dextran sulfate but not danaparoid stimulate IGF-I in decidualizing ESCs. ESCs were incubated with the decidualization stimuli 1 μ M progesterone and 30 nM 17 β -estradiol alone ('no treatment') and the addition of unfractionated heparin, dextran sulfate or danaparoid (each 5 μ g/ml) for 3 days. Thereafter, the levels of IGF-I in the cell culture supernatant were determined by ELISA. Results are standardized relative to the values of 'no treatment', which are set to 1 arbitrary unit. Bars represent the mean \pm SEM; * P < 0.05 compared with 'no treatment', # P < 0.05 compared with heparin-treated ESCs.

with unfractionated heparin, whereas fondaparinux as the smallest molecule had no significant impact on IGF-I (Fig. 3B).

Dose-dependent influence of heparin on decidualizing ESCs

We also tested whether the stimulating effect of unfractionated heparin on early decidualization of ESCs depends on its dosage. The cells were incubated with the decidualizing stimuli progesterone plus 17 β -estradiol in combination with increasing concentrations (0–102.4 IU/ml) of unfractionated heparin for 3 days, followed by the measurement of the IGF-I secretion. As shown in Fig. 4, heparin dose-dependently amplified the hormonal-driven increase in IGF-I, being statistically significant at 0.4 IU/ml and higher when compared with decidualizing ESCs without heparin. Doses higher than 0.4 IU/ml caused only little further increase.

Effects of dextran sulfate on markers of decidualized ESCs

Finally, we wanted to see, whether dextran sulfate has similar effects as heparin on PRL and IGFBP-1 as late markers of decidualization. After incubating ESCs for 12 days with the decidualizing stimuli progesterone plus 17 β -estradiol in combination with heparin or dextran sulfate (each 5 μ g/ml), we measured the secretion of PRL and IGFBP-1. Similar to heparin, dextran sulfate significantly increased the PRL levels in decidualized ESCs (Fig. 5A) and markedly reduced IGFBP-1 secretion (Fig. 5B).

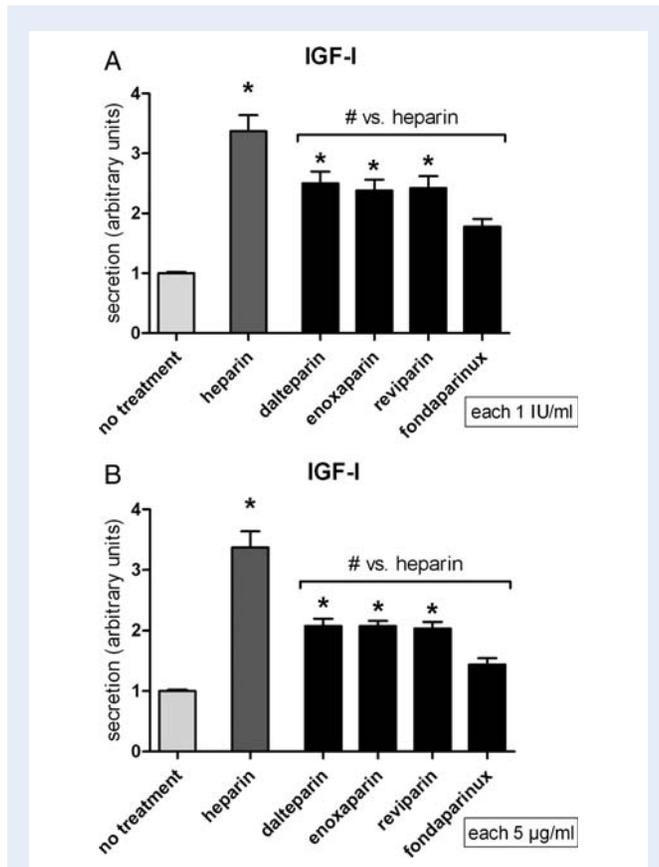


Figure 3 In decidualizing ESCs, stimulation of IGF-I by LMWHs is independent of their anticoagulatory properties, but dependent on their molecular size. ESCs were incubated with the decidualization stimuli 1 μ M progesterone and 30 nM 17 β -estradiol alone ('no treatment') and the addition of unfractionated heparin or the LMWHs dalteparin, enoxaparin, reviparin and fondaparinux for 3 days. The dosages used were each 1 IU/ml (representing uniform anticoagulatory potency) (A) or 5 μ g/ml (representing uniform overall negative charge) (B), respectively. Thereafter, the levels of IGF-I in the cell culture supernatant were determined by ELISA. Results are standardized relative to the values of 'no treatment', which are set to 1 arbitrary unit. Bars represent the mean \pm SEM; * P < 0.05 compared with 'no treatment', # P < 0.05 compared with heparin-treated ESCs.

Discussion

In the present study, we characterized the molecular mechanisms of the effects of heparins on endometrial differentiation. By comparing the effect of heparin and various heparin-related agents with different characteristics in molecular size, charge or anticoagulatory activity, we found the effects of heparins on endometrial differentiation to be independent of the anticoagulatory effects of heparin but dependent on the molecular size and the negative charge of a linear polyanion.

The two molecules typically inhibited by heparin, thrombin and FXa, have been shown to mediate direct effects on cellular biology independently of the coagulation cascade. In human vascular smooth muscle cells, thrombin and FXa induce DNA synthesis via transactivation of fibroblast growth factor receptor-1, which can be inhibited by heparin (Rauch *et al.*, 2004). The production of vascular endothelial

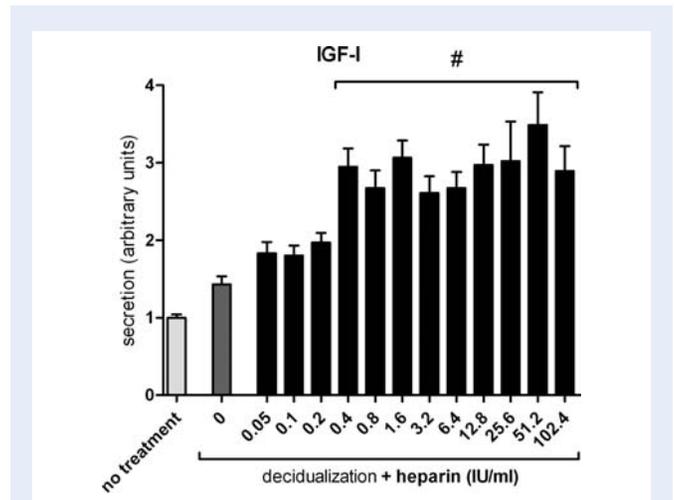


Figure 4 Unfractionated heparin dose-dependently stimulates IGF-I in decidualizing ESCs. ESCs were incubated without anything ('no treatment') or with 1 μ M progesterone and 30 nM 17 β -estradiol alone ('decidualization') and the addition of increasing dosages of unfractionated heparin for 3 days. Thereafter, the levels of IGF-I in the cell culture supernatant were determined by ELISA. Results are standardized relative to the values of 'no treatment', which are set to 1 arbitrary unit. Bars represent the mean \pm SEM; # P < 0.05 compared with decidualizing ESCs without heparin.

growth factor and matrix metalloproteinases has been shown to be stimulated by thrombin in human ESCs, involving the mitogen-activated protein kinase system (Furukawa *et al.*, 2009).

In the present study, we observed an increase in IGF-I under the influence of thrombin and FXa during early decidualization in human ESCs *in vitro*. Heparins, the typical counterparts of thrombin and FXa, however, also stimulate IGF-I secretion during endometrial differentiation (Fluhr *et al.*, 2010). Being aware of these observations, the impact of heparin on endometrial differentiation seems not to be related to its classical anticoagulatory function and especially seems not to be just an inhibiting effect on thrombin or FXa.

Dextran sulfate is a well-defined synthetic linear polyanion with a strongly sulfated hexose backbone, being quite similar to unfractionated heparin regarding molecular size and negative charge, but lacking its specific anticoagulatory properties (Greinacher *et al.*, 1992). In our experiments, dextran sulfate stimulated IGF-I and PRL and inhibited IGFBP-1 during decidualization *in vitro*, revealing the same regulatory pattern as seen for heparin. This suggests that the modulating effects of heparin on endometrial differentiation are independent of its anticoagulatory function. In addition, Eyal *et al.* (2007) described an autocrine inhibitory effect of PRL on endometrial decidualization as measured by a reduction in IGFBP-1. Being aware of the observed reducing effect of heparin on IGFBP-1 and a stimulating impact on PRL in the present study, heparin might boost an autocrine regulatory circle within the decidualizing endometrium.

Since the endometrial effects of heparin can be mimicked by dextran sulfate, the negative charge of both molecules might play a role for their impact on human ESCs. To test this concept, we examined LMWHs, fondaparinux and danaparoid, having decreasing molecular weight (LMWHs, fondaparinux) or lower molecular

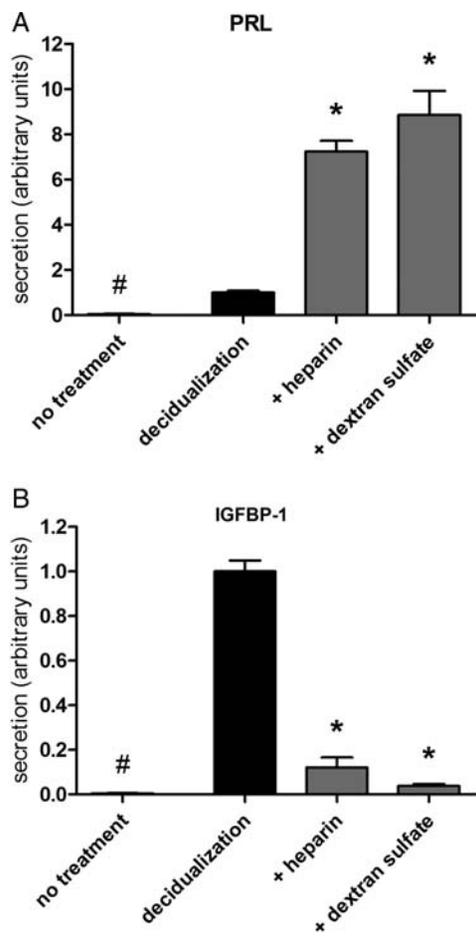


Figure 5 Heparin and dextran sulfate have similar effects on PRL and IGFBP-1 in decidualized ESCs. Cells were incubated without anything ('no treatment') or with 1 μ M progesterone and 30 nM 17 β -estradiol alone ('decidualization') and the addition of unfractionated heparin or dextran sulfate (each 5 μ g/ml) for 12 days. Thereafter, the levels of PRL (A) and IGFBP-1 (B) in the cell culture supernatant were determined by ELISAs. Results are standardized relative to the values of 'decidualization', which are set to 1 arbitrary unit. Bars represent mean \pm SEM; # and * P < 0.05 compared with 'decidualization'.

weight and lower negative charge (danaparoid) in comparison with unfractionated heparin. Incubating decidualizing ESCs with these compounds, we observed significantly less pronounced effects of LMWHs on endometrial IGF-I when compared with unfractionated heparin. In addition, fondaparinux and danaparoid having the lowest amount of negative charge had no significant effect on endometrial IGF-I. Surprisingly, the pentasaccharide fondaparinux had also no significant effect on the secretion of IGF-I when applied in the same concentration (weight per volume) and thereby the same overall charge as dalteparin, enoxaparin or reviparin. Therefore, it is intriguing to speculate about a role of the amount of negative charge of the molecules for the effects of heparins on endometrial differentiation.

In addition, the anti-FXa effect of these compounds is not causing the effect on ESCs. Even more interestingly, the effect on ESCs is

also not only dependent on the total amount of negatively charged polyanions. Otherwise, fondaparinux should have had the same effect as the LMWHs when added on the same weight basis and not on the molecular concentration, as the molecular concentration of fondaparinux was much higher than the one of LMWHs when added on a weight basis. Therefore, another key factor for their effects on human ESCs seems to be the molecular weight of the polyanions.

A huge number of cytokines, chemokines and growth factors are known to bind to GAGs of the heparin and heparan sulfate family, thereby regulating their bioavailability (Proudfoot et al., 2003). Additionally, heparin has been shown to be able to stimulate essential signaling pathways like the extracellular signal-related kinase 1/2 in human first trimester trophoblasts and primary B cell progenitors (Hills et al., 2006; Milne et al., 2008) or the Wnt signaling system in neuroblastoma cells (Colombres et al., 2008). Further investigations are necessary to find possible interaction partners of negatively charged polysaccharides or inducible signaling pathways in the human endometrium which are involved in mediating the modulatory effects of heparin on decidualization. Interestingly, we observed a dose-dependent effect of heparin on endometrial IGF-I, being constant even at supraphysiological dosages of heparin. This observation indicates that heparin might work on human ESCs by a direct binding effect rather than by forming complexes with interacting molecules, as these complexes typically dissociate at high heparin concentrations resulting in bell-shaped response curves (Greinacher et al., 1994, 1995).

Taken together, distinct molecular properties of heparin seem to be necessary to enable this GAG to modulate endometrial differentiation independently of its classical anticoagulatory function. This observation provides the intriguing possibility of developing new therapeutic strategies for women suffering from recurrent miscarriages or repeated implantation failure based on non-anticoagulatory polyanions without the risk of increased bleeding.

Authors' roles

H.F. initiated and coordinated this study, interpreted the data and wrote the manuscript. J.S. designed and performed experiments, analyzed the results and revised the manuscript. S.H. and J.E. performed experiments. A.G. contributed to the design of the study, the interpretation of the results and the writing of the manuscript. M.Z. supervised this study and revised the manuscript.

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