The presence of heparins during decidualization modulates the response of human endometrial stromal cells to IL-1 β in vitro

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Received: 15 December 2015 / Accepted: 14 March 2016 / Published online: 24 March 2016
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Abstract

Purpose The aim of this paper is to study the impact of heparin on the response of human endometrial stromal cells (ESCs) to interleukin (IL)-1 β during decidualization in vitro. **Methods** ESCs were isolated from hysterectomy specimens of premenopausal women undergoing hysterectomy for benign reasons; decidualized in vitro and incubated in parallel with unfractionated heparin or tinzaparin; and stimulated with IL-1 β at days 0, 3, 6, and 9 during decidualization. IL-6, IL-11, and leukemia inhibitory factor (LIF) were analyzed using ELISAs and real-time RT-PCR. Cell viability was determined by a fluorometric assay.

Results IL-1 β dose-dependently stimulated IL-6, IL-11, and LIF in distinct patterns in ESCs during decidualization. Unfractionated heparin as well as tinzaparin attenuated the IL-1 β -mediated induction of IL-6, IL-11, and LIF on protein and messenger RNA (mRNA) levels. The relative effects of heparin and tinzaparin were getting more pronounced during the time course of decidualization.

Conclusions Unfractionated heparin and the low molecular weight heparin tinzaparin have modulating effects on IL-1 β -induced endometrial cytokines of the IL-6 family during decidualization. These effects of heparins beyond their

classical anti-coagulatory properties might have implications on the regulation of endometrial receptivity and early implantation.

Keywords IL-1 β · Cytokines · Decidualization · Heparin

Introduction

The decidualization of the endometrial stroma in the second half of the menstrual cycle is an essential prerequisite for the successful implantation of an embryo [1]. The steroid hormones estrogen and progesterone play a critical role in preparing the endometrium for the narrow time frame of receptivity, which is also known as the “window of implantation,” but locally expressed and acting growth factors and cytokines are the main mediators during endometrial development [1–3].

Interleukin (IL)-6, IL-11, and leukemia inhibitory factor (LIF) belong to the so-called IL-6 family and have been shown to act in a pro- as well as anti-inflammatory manner [4]. These cytokines are produced in the human endometrium and play an essential role during decidualization and early implantation with a clear impact on the physiology and pathophysiology of the ongoing pregnancy [5, 6]. IL-1 β is a key regulator of inflammatory responses and is also present at the feto-maternal interface modulating the interaction of the implanting embryo with the maternal endometrium [7, 8]. IL-1 β derived from cytotrophoblast cells has been shown to regulate cellular differentiation in the human endometrium, thereby affecting the receptivity of this tissue [9].

Unfractionated heparin and low molecular weight heparins (LMWHs) are polysulfated glycosaminoglycans known to have pleiotropic effects beyond their classical anti-coagulatory properties, thereby showing, e.g., anti-inflammatory activity [10, 11].

Capsule There is an attenuated response of human ESCs to the stimulation by IL-1 β when heparins are present during the time course of decidualization in vitro.

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Table 1 Sequences of PCR primers

Gene	Forward	Reverse
IL-6	5'-TGGCAGAAAACAACCTGAACCT-3'	5'-ACCAGGCAAGTCTCCTCATTGA-3'
IL-11	5'-TCTCTCCTGGCGGACACG-3'	5'-AATCCAGGTTGTGGTCCCC-3'
LIF	5'-GCCAATGCCCTCTTTATTCTCT-3'	5'-CCACATAGCTGTCCAGGTTGT-3'
β -Actin	5'-CCTGGCACCCAGCACAAAT-3'	5'-GCCGATCCACACGGAGTACT-3'

For the prophylaxis and treatment of repeated implantation failure and habitual miscarriages, the efficacy of heparins is still a matter of debate [12, 13]. However, there is growing evidence that the risk of placenta-mediated complications of pregnancy like preeclampsia and intrauterine growth restriction (IUGR) can be reduced by the application of heparins in selected patients, even without suffering from a thrombophilia [14, 15]. Taking into account a disordered process of implantation as a basis of placenta-mediated diseases, this initial phase of pregnancy seems to be very interesting for the use of heparin independently of its anti-coagulatory properties in a prophylactic regime. Further supporting this hypothesis, we could recently observe in vitro direct impacts of heparins on the decidualization and the cytokine secretion of human endometrial stromal cells (ESCs) [16–19].

Based on these facts, we investigated in vitro whether the presence of unfractionated heparin as well as the LMWH tinzaparin during the time course of decidualization has an impact on the response of human ESCs to IL-1 β . Thereby, we focused on the associated molecules IL-6, IL-11, and LIF as physiologically relevant cytokines in the endometrial milieu.

Materials and methods

Tissue collection and cell culture

Endometrial tissue samples were obtained from premenopausal women undergoing hysterectomy for benign reasons after written informed consent. The study protocol was approved by the institutional ethical board of the University of Greifswald, Germany (No. BB 23/08). ESCs were isolated, cultured, and characterized as described previously [20]. Briefly, minced endometrial tissue was digested by incubation with collagenase (Biochrom, Berlin, Germany), and the dispersed endometrial cells were separated by filtration. ESCs were maintained in a DMEM/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 gentamicin (Ratiopharm, Ulm, Germany). The purity of stromal cells obtained by this method was >90 %, as determined by immunofluorescent staining of the stromal marker vimentin following standard procedures.

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 9 days. Decidualization was proven by measuring a significantly increased secretion of insulin-like growth factor-binding protein (IGFBP)-1 and prolactin as described previously [20]. Additionally, ESCs were incubated with the following agents: human IL-1 β (BioSource, Camarillo, CA, USA), unfractionated heparin (Ratiopharm), tinzaparin (Innohep[®], LEO Pharma, Neu-Isenburg, Germany). The different combinations, dosages, and incubation times of the mentioned agents are indicated in detail in the corresponding figures as well as in the results part.

Cell viability assay

The relative number of viable ESCs under the influence of the indicated agents was measured at the end of the respective experiments 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

Human ESCs were decidualized in vitro using 1 μ M progesterone and 30 nM 17 β -estradiol (EP) plus/minus 5 μ g/mL unfractionated heparin or tinzaparin. At days 0, 3, 6, and 9, cells were stimulated with 50 ng/mL or 50 pg/mL IL-1 β for 48 h. Cell culture supernatants were collected

Fig. 1 Heparins attenuate the stimulating effect of IL-1 β on IL-6 in decidualizing human ESCs. Human ESCs were decidualized in vitro (EP) plus/minus 5 μ g/mL unfractionated heparin or tinzaparin. At days 0, 3, 6, and 9, cells were stimulated with 50 ng/mL (a) or 50 pg/mL (b) IL-1 β for 48 h. Thereafter, the levels of IL-6 in the cell culture supernatants were determined by ELISAs. Results are shown in concentration pg/mL per 10,000 viable cells. Bars represent mean $\pm$ SEM; * p < 0.05 compared to EP at days 0, 3, 6, and 9; ** p < 0.05 compared to EP + heparin at days 0, 3, 6, and 9; *** p < 0.05 compared to EP + tinzaparin at days 0, 3, 6, and 9; # p < 0.05 compared to EP + IL-1 β 50 ng/mL (a) or 50 pg/mL (b) at days 0, 3, 6, and 9; §value under the detection level

◀ **Fig. 2** Heparins attenuate the stimulating effect of IL-1 β on IL-11 in decidualizing human ESCs. Human ESCs were decidualized in vitro (EP) plus/minus 5 μ g/mL unfractionated heparin or tinzaparin. At days 0, 3, 6, and 9, cells were stimulated with 50 ng/mL (a) or 50 pg/mL (b) IL-1 β for 48 h. Thereafter, the levels of IL-11 in the cell culture supernatants were determined by ELISAs. Results are shown in concentration pg/mL per 10,000 viable cells. Bars represent mean $\pm$ SEM; * p < 0.05 compared to EP at days 0, 3, 6, and 9; ** p < 0.05 compared to EP + heparin at days 0, 3, 6, and 9; *** p < 0.05 compared to EP + tinzaparin at days 0, 3, 6, and 9; # p < 0.05 compared to EP + IL-1 β 50 ng/mL (a) or 50 pg/mL (b) at days 0, 3, 6, and 9; §value under the detection level

after the respective treatments of the cells and analyzed for IL-6, IL-11, and LIF using commercially available DuoSet[®] ELISA kits from R&D Systems (Wiesbaden, Germany). The sensitivities of the assays were 9.4 pg/mL (IL-6), 31.3 pg/mL (IL-11), and 15.6 pg/mL (LIF), respectively. 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.

Real-time reverse transcriptase-polymerase chain reaction

Human ESCs were decidualized in vitro using 1 μ M progesterone and 30 nM 17 β -estradiol (EP) plus/minus 5 μ g/mL unfractionated heparin or tinzaparin. At day 9, cells were stimulated with 50 ng/mL IL-1 β for 6 h. Thereafter, the levels of IL-6, IL-11, and LIF were determined by semiquantitative real-time RT-PCR. Total ribonucleic acid (RNA) was isolated from ESCs using PeqGOLD TriFast[™] (PeqLab, Erlangen, Germany) and reverse-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA). Semiquantitative real-time polymerase chain reaction (PCR) was performed to quantify the messenger RNA (mRNA) levels of IL-6, IL-11, and LIF in relation to the housekeeping gene β -actin. Complementary DNA (cDNA) samples were amplified using the Power SYBR[®] Green PCR-Master Mix (Applied Biosystems) and the respective forward and reverse primers. The primers (Invitrogen) were designed using Primer Express[®] Primer Design Software v2.0 (Applied Biosystems) with the resulting amplicons having an intron-overlapping sequence. The sequences of the primer are shown in Table 1. PCR amplification was performed in duplicate in an ABI Prism[™] 7300 sequence detector (Applied Biosystems) using a standard cycling program [20]. PCR products were analyzed by thermal dissociation to verify that a single specific PCR product had been amplified. Relative expression levels of IL-6, IL-11, and LIF in relation to β -actin were determined using the mathematical model: ratio = $2^{-\Delta\Delta CT}$ [21].

Statistics

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

Results

IL-1 β stimulates the production of IL-6, IL-11, and LIF in human ESCs

To investigate the influence of IL-1 β on endometrial IL-6, IL-11, and LIF, undifferentiated and decidualized human ESCs were incubated in a first step with increasing concentrations (ranging from 0.05 pg/mL to 50 ng/mL) of IL-1 β in vitro. IL-1 β dose-dependently induced the secretion of all the three cytokines tested beginning at a dosage of 50 pg/mL IL-1 β and reaching a plateau at 50 ng/mL IL-1 β (data not shown). Based on these observations, the concentrations 50 pg/mL and 50 ng/mL of IL-1 β were chosen for the following experiments.

Heparins attenuate the IL-1 β -induced secretion of IL-6, IL-11, and LIF in human ESCs during decidualization

In the next step, we tested whether the presence of unfractionated heparin or the LMWH tinzaparin during the time course of decidualization has an impact on the response of human ESCs to IL-1 β . An incubation of decidualizing ESCs at days 0, 3, 6, or 9 with IL-1 β stimulated the secretion of IL-6 (Fig. 1), IL-11 (Fig. 2), and LIF (Fig. 3) in a time- and dose-dependent manner. The presence of either unfractionated heparin or tinzaparin during the whole time course of decidualization attenuated the stimulating effect of IL-1 β on the secretion of IL-6 (Fig. 1), IL-11 (Fig. 2), and LIF (Fig. 3) at the different days. The modulating impact of the two heparins tested on ESC response to IL-1 β was more profound as the period of cell decidualization increased; being more pronounced in decidualized ESCs treated on day 9, as shown in Figs. 1, 2, and 3. No influence upon the relative number of viable ESCs was seen under the treatment of the indicated agents which was tested at the end of the experiments using the CellTiter-Blue[®] assay, and the results were normalized to the number of viable ESCs.

Fig. 3 Heparins attenuate the stimulating effect of IL-1 β on LIF in decidualizing human ESCs. Human ESCs were decidualized in vitro (EP) plus/minus 5 μ g/mL unfractionated heparin or tinzaparin. At days 0, 3, 6, and 9, cells were stimulated with 50 ng/mL (a) or 50 pg/mL (b) IL-1 β for 48 h. Thereafter, the levels of LIF in the cell culture supernatants were determined by ELISAs. Results are shown in concentration pg/mL per 10,000 viable cells. Bars represent mean $\pm$ SEM; * p < 0.05 compared to EP at days 0, 3, 6, and 9; ** p < 0.05 compared to EP + heparin at days 0, 3, 6, and 9; *** p < 0.05 compared to EP + tinzaparin at days 0, 3, 6, and 9; # p < 0.05 compared to EP + IL-1 β 50 ng/mL (a) or 50 pg/mL (b) at days 0, 3, 6, and 9; §value under the detection level

The modulating effects of heparins on the response of human ESCs to IL-1 β are also seen on the mRNA level

Finally, we investigated whether the observed effects of heparins on the response of decidualized human ESCs to IL-1 β are also seen on the mRNA level. As shown in Fig. 4, the stimulation of IL-6 (Fig. 4a), IL-11 (Fig. 4b), and LIF (Fig. 4c) by IL-1 β was also significantly attenuated on the mRNA level in human ESCs decidualized in the presence of unfractionated heparin or tinzaparin.

Discussion

In the present study, we observed an attenuated response of human ESCs to the stimulation by IL-1 β when heparins were present during the time course of decidualization in vitro.

Fig. 4 Heparins attenuate the stimulating effect of IL-1 β on IL-6, IL-11, and LIF in decidualized human ESCs on the mRNA level. Human ESCs were decidualized in vitro (EP) plus/minus 5 μ g/mL unfractionated heparin or tinzaparin. At day 9, cells were stimulated with 50 ng/mL IL-1 β for 6 h. Thereafter, the levels of IL-6 (a), IL-11 (b), and LIF (c) were determined by semiquantitative real-time RT-PCR. Results are standardized relative to the values of IL-1 β -stimulated ESCs which are set to 1 arb. Bars represent mean $\pm$ SEM; * p < 0.05 compared to EP; # p < 0.05 compared to EP + 50 ng/mL IL-1 β ; §value under the detection level

Thereby, heparins reduced the IL-1 β -induced production of IL-6, IL-11, and LIF in decidualizing ESCs in a time-dependent manner.

Unfractionated heparin is a mixture of linear polysulfated glycosaminoglycans with different molecular weights, whereas LMWHs (e.g., tinzaparin) are derived from heparin by enzymatic or chemical depolymerization and often replace heparin in daily clinical use [22]. However, the clinical data in this field is still limited, and the existing studies are heterogeneous and difficult to compare heparins which might have been suggested to improve the pregnancy outcome in women with as well as without thrombophilia suffering from pregnancy complications such as recurrent miscarriage, preeclampsia, or IUGR [14].

An essential prerequisite for successful implantation of the embryo is the decidualization of the endometrial stroma in the second half of the menstrual cycle [1]. The steroid hormones estrogen and progesterone play a critical role in preparing the endometrium for the “window of implantation” [2]. Additionally, locally expressed and acting growth factors and cytokines are the main mediators during endometrial development and at the fetomaternal interface during implantation and placentation [3].

IL-1 β is a key regulator of inflammatory responses and is assumed to play an essential role for the immunotolerance at the fetomaternal interface modulating the interplay of the implanting embryo with the maternal endometrium and

immune cells [7, 8]. Even more, IL-1 β derived from cytotrophoblast cells has been shown to regulate cellular differentiation in the human endometrium, thereby affecting the receptivity of this tissue [9]. In the present work, we could observe a diminished response of human ESCs to IL-1 β stimulation when the cells were decidualized in the presence of heparins in vitro. This impact of heparins seems to be time-dependent, as the attenuation of the response to IL-1 β was getting more pronounced during the time course of decidualization. We could recently observe that unfractionated heparin inhibits the tumor necrosis factor (TNF)- α -stimulated expression of IL-6 and IL-8 in human ESCs, caused by an interference with the transcriptional activity of nuclear factor (NF)- κ B [17]. As IL-1 β is also a typical stimulus of the NF- κ B pathway and endometrial IL-6 and LIF are known to be regulated by this transcription factor, the observations of the present study might be explained by an inhibitory effect of heparins on the activation of NF- κ B [9, 23]. In addition, the observed time dependency of the effect of heparins led us to speculate that heparins have a continuous desensitizing effect on the signaling pathways involved, rather than an acute interaction with the IL-1 β stimulus.

Various cytokines are expressed in the human endometrium, and most of them show specific cycle-dependent expression patterns due to their temporarily defined functions in endometrial differentiation and receptivity [3]. IL-6, IL-11, and LIF belong to the so-called IL-6 family and have pivotal roles in the female reproductive tract. LIF is known to play an important role during implantation which has been discovered in LIF knockout mice, being infertile due to defective implantation [24]. In humans, LIF gene mutations have been described in infertile women, and women suffering from idiopathic infertility show lower intrauterine LIF levels as compared to controls [25, 26]. During the decidualization of the endometrial stroma in the second half of the cycle, IL-11 has been shown to be an important autocrine promoter of progesterone-induced differentiation [27]. However, it has also been shown that decidual IL-11 is increased by IL-1 β in preeclampsia, suggesting a role for IL-11 in the pathogenesis of preeclampsia [28]. The maximal expression of IL-6 in the human endometrium is seen in the mid-secretory phase and at menstruation [6]. Elevated IL-6 levels are often reported in altered cytokine profiles of women suffering from unexplained infertility, recurrent miscarriage, preeclampsia, or preterm delivery [6]. In the present study, we observed an inhibiting effect of heparins on the IL-1 β -induced production of IL-6, IL-11, and LIF, further supporting the role of heparins beyond their classical anti-coagulatory role. Based on the abovementioned possible pathophysiological role of IL-6 and IL-11 in the pathogenesis of placenta-mediated pregnancy disorders, the attenuating effect of heparins might be beneficial for affected pregnancies. As these effects are seen in vitro, they have to be carefully interpreted, and direct conclusions on

the situation in vivo are not possible. Therefore, it remains to be determined whether this impact of heparins on endometrial cytokines positively modulates the process of implantation.

Taken together, heparins have a modulating impact on the cytokine network in the human endometrium in vitro, further underlining the view on heparins as immunomodulatory agents beyond their classical anti-coagulatory properties. A better understanding of the molecular and cellular mechanisms of the pleiotropic effects of heparin will enable a more targeted use as well as the development of heparin-derived substances with selected properties beyond anticoagulation.

Compliance with ethical standards

Author's roles JS and FB performed the experiments, analyzed the results, and revised the manuscript. FS and MZ supervised this study and revised the manuscript. HF initiated and coordinated this study, designed the experiments, interpreted the data, and wrote the manuscript.

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