Heparin and low-molecular-weight heparins modulate the decidualization of human endometrial stromal cells

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Objective: To examine the impact of unfractionated heparin and low-molecular-weight heparins (LMWHs) on the decidualization of human endometrial stromal cells (ESCs) in vitro.
Design: In vitro experiment.
Setting: Research laboratory at a medical university center.
Patient(s): Premenopausal women undergoing hysterectomy for benign reasons.
Intervention(s): The ESCs were isolated from hysterectomy specimens, decidualized in vitro using progesterone and 17β-estradiol, and incubated with unfractionated heparin and three different LMWHs.
Main Outcome Measure(s): Insulin-like growth factor–binding protein (IGFBP) 1, PRL, and insulin-like growth factor (IGF) I were measured using ELISA and real-time reverse-transcription polymerase chain reaction. Cell viability was determined by a fluorometric assay. Intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) was measured using a luminescent assay.
Result(s): Heparin dose- and time-dependently delayed the production of IGFBP-1 and amplified the levels of PRL and IGF-I in ESCs during decidualization in vitro. Similar effects were seen under the influence of the three different LMWHs. Intracellular cAMP was increased in decidualizing ESCs under the influence of heparin and LMWHs.
Conclusion(s): Unfractionated heparin as well as LMWHs are able to modulate the decidualization of human ESCs in vitro and therefore might be useful to control endometrial differentiation and receptivity in assisted reproduction. (Fertil Steril® 2010;93:2581–7. ©2010 by American Society for Reproductive Medicine.)

Key Words: Heparin, low-molecular-weight heparin, endometrium, decidualization, implantation, IGFBP-1, PRL, IGF-I

Unfractionated heparin is a mixture of linear polysulfated glycosaminoglycans with different molecular weights. Low-molecular-weight heparins (LMWHs) are derived from heparin by enzymatic or chemical depolymerization and often replace heparin in daily clinical use (1, 2).

Heparin as well as LMWHs have been shown to improve the pregnancy outcome in women suffering from thrombophilia and pregnancy complications such as recurrent miscarriage, preeclampsia, and intrauterine growth restriction (3, 4). The rationale for their use was based on the theory that placental thrombosis and infarction might be a cause for miscarriage and that thomboprophylaxis could prevent this process (5). Surprisingly, intravascular or intervillous blood clots are rarely found in placentas and first-trimester decidua samples from patients suffering from miscarriage or complicated pregnancies (6). Furthermore, a recent meta-analysis showed, that only some, not all, thrombophilias were associated with fetal loss (7). Nevertheless, anticoagulants are widely used in the treatment of women with recurrent miscarriage without apparent causes or inherited thrombophilia and have been shown to be effective (8). These observations further underscore the concept that heparin acts not only as an anticoagulant, but also seems to be an antiinflammatory and immunoregulatory agent (9), which could be useful for the prevention of pathologic conditions at the fetomaternal interface.

Despite the substantial improvements in the field of artificial reproductive techniques, the live birth rates resulting from these techniques have plateaued and are still unsatisfactory (10). LMWHs are empirically used to improve implantation rates, but until today this practice lacks both molecular background and clinical evidence. However, there are some reports about an influence of heparin on apoptosis as well as on the invasiveness of trophoblast cells (11, 12). In addition, a recently published clinical trial describes a beneficial effect of LMWH administration in the luteal phase on the implantation rate as well as live birth rate in women with repeated implantation failure (13).

An essential prerequisite for successful implantation of the embryo is the decidualization of the endometrial stroma in the second half of the menstrual cycle (15). The steroid hormones estrogen and progesterone play a critical role in preparing the endometrium for the "window of implantation" (16). Furthermore, locally expressed and acting growth factors and cytokines are the main mediators during endometrial development (14, 15). Insulin-like growth factor

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(IGF)–binding protein (IGFBP) 1 is a major product of decidualized endometrial stromal cells (ESCs) and may regulate endometrial differentiation and implantation (17, 18). During the menstrual cycle, PRL is expressed in ESCs between the midsecretory phase and menstruation, coinciding with the first signs of decidualization. Its expression increases if implantation occurs (19) and it seems to have a role in preparing the endometrium for implantation as well as in maintaining pregnancy (20, 21). We have recently shown a temporary up-regulation of IGF-I in the early phase of decidualization, which seems to be necessary for endometrial receptivity (22).

Based on these findings, we investigated the influence of unfractionated heparin and LMWHs on the decidualization of human ESCs in vitro and analyzed their impact on the expression pattern of IGFBP-1, PRL, and IGF-I in the time course.

MATERIALS AND METHODS

Isolation and Culture of Human ESCs

Endometrial tissue samples were obtained after written informed consent from premenopausal women undergoing hysterectomy for benign reasons. All of the patients had regular menstrual cycles and proven fertility and were considered to be healthy. The study protocol was approved by the institutional ethical board of the University of Greifswald, Greifswald, Germany.

The ESCs were isolated, cultured, and characterized as described previously (23, 24). Briefly, minced endometrial tissue was digested by incubation with 1,000 IU/mL collagenase (Biochrom, Berlin, Germany) and the dispersed endometrial cells were separated by filtration through a 40 μm filter (BD Falcon, Heidelberg, Germany). The ESCs were maintained in DMEM/F-12 cell culture medium without phenol red (Gibco/Invitrogen, Karlsruhe, Germany) containing 10% charcoal-stripped fetal bovine serum (Biochrom) and 50 μg/mL gentamycin (Ratiopharm, Ulm, Germany).The purity of ESC cultures was proven by flow-cytometric analysis of intracellular vimentin expression.

Dezidualization in Vitro and Experimental Conditions

Decidualization in vitro was induced by incubating the ESCs in culture medium containing 30 nmol/L 17β-estradiol and 1 μmol/L progesterone (both from Sigma-Aldrich, Taufkirchen, Germany) for 12 days. For control, ESCs were cultured in parallel over 12 days without hormonal treatment. To test the impact of heparin and LMWHs on decidualization, the cells were incubated in parallel to the hormonal treatment with unfractionated heparin (Ratiopharm), enoxaparin (Clexane; Sanofi-Aventis, Frankfurt, Germany), dalteparin (FragminP; Pharmacia, Berlin, Germany), or certoparin (Mono-Embolex; Novartis Pharma, Nuremberg, Germany).

Cell Viability Assay

Cell viability was measured during decidualization at days 0, 3, 6, 9, and 12 using the CellTiter-Blue assay (Promega, Madison, WI) 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 0, 3, 6, 9, and 12 during decidualization. IGFBP-1, PRL, and IGF-I were determined using ELISA kits (DuoSet,; R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. The sensitivity of the assays was 31.3 pg/mL (IGFBP-1), 15.6 pg/mL (PRL), and 31.3 pg/mL (IGF-I). There was no significant cross-reactivity, and intra- and interassay variabilities were <5%. Unfractionated heparin and LMWHs did not show any significant interference with the ELISA kits. Absorbance was measured using the Fluostar Optima system.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total ribonucleic acid (RNA) was isolated from ESCs using PeqGold TriFast reagent (PeqLab, Erlangen, Germany) following the manufacturer's standard protocol. Total RNA was reverse transcribed using the High Capacity Complementary DNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA) according to the manufacturer's instructions. Semiquantitative real-time polymerase chain reaction (PCR) was performed to quantify messenger RNA levels of IGFBP-1, PRL, and IGF-I relative to the housekeeping gene β-actin. cDNA samples were amplified using Sybr Green PCR Master Mix (Applied Biosystems). 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 primers used were:

β-actin forward 5′-CCTGGCACCCAGCACAAT-3′
β-actin reverse 5′-GCCGATCCACACGGAGTACT-3′
IGFBP-1 forward 5′-TACCTGCCAAACTGCAACAAGA-3′
IGFBP-1 reverse 5′-CCATGGATGTCTCACACTGTCTG-3′
PRL forward 5′-CACCCCCGAAGACAAGGAG-3′
PRL reverse 5′-CCAGGATCGCAATATGCTGAC-3′
IGF-I forward 5′-AGTCAGCTCGCTCTGTCCGT-3′
IGF-I reverse 5′-TGCGTTCTTCAAATGTACTTCCTT-3′

The PCR amplification was performed in duplicate in a 7300 Real-Time PCR System (Applied Biosystems) using a standard cycling program (22). The PCR products were analyzed by thermal dissociation to verify that a single specific PCR product had been amplified. Relative expression levels of IGFBP-1, PRL, and IGF-I in relation to the reference β-actin were calculated using the mathematical model ratio $= 2^{-\Delta\Delta CT}$ as described by Livak and Schmittgen (25).

Cyclic Adenosine 3′,5′-Monophosphate Assay

Intracellular cAMP levels were measured at days 3 and 12 of decidualization using the cAMP-Glo assay (Promega) according to the manufacturer's instructions. Luminescence was recorded using the Fluostar Optima system.

Statistical Analysis

Each experiment was performed in quadruplicate on cell cultures derived from at least three different patients. Statistical analysis was carried out with one-way analysis of variance, followed by Dunnett and Bonferroni multiple comparison tests using GraphPad Prism version 5 software (GraphPad, San Diego, CA). The results are expressed as mean $\pm$ standard error of the mean (SEM). Differences were considered to be significant if $P<.05$.

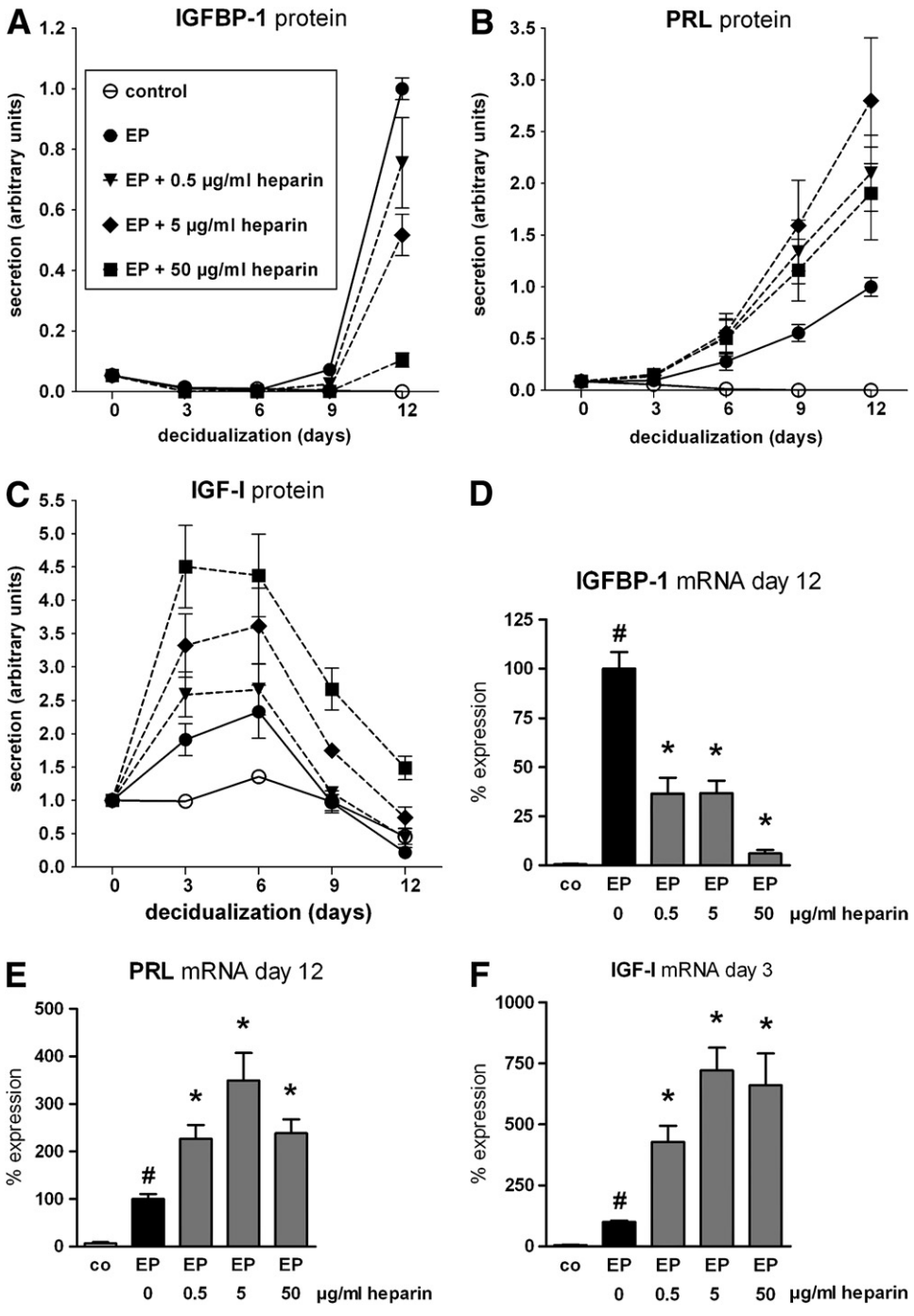
RESULTS

Heparin Dose-Dependently Modulates IGFBP-1, PRL, and IGF-I in Dezidualizing ESCs in Vitro

First of all we investigated whether heparin has an influence on relevant markers of decidualization in human ESCs. Therefore the cells were incubated with 0.5, 5, and 50 μg/mL unfractionated heparin in combination with the decidualizing stimuli 17β-estradiol plus progesterone (EP). As shown in Figure 1A, heparin dose-dependently inhibited the onset of IGFBP-1 secretion in a significant manner ($P<.05$: days 9 and 12, EP vs. EP + 0.5, 5, and 50 μg/mL heparin). In contrast, the secretion of PRL was increased significantly by heparin during decidualization, with 5 μg/mL heparin being the most effective concentration (Fig. 1B; $P<.05$: days 9 and 12, EP vs. EP + 0.5, 5, and 50 μg/mL heparin). The typical secretion pattern of IGF-I, with an increase from day 0 to day 6 followed by a decline toward day 12, was significantly pronounced by the addition of heparin dose-dependently (Fig. 1C; $P<.05$: day 3, EP vs. EP + 0.5, 5, and 50 μg/mL heparin; $P<.05$: day 6, EP versus EP + 5 and 50 μg/mL heparin). Determination of mRNA expression showed corresponding results of IGFBP-1 and PRL levels at day 12 (Figs. 1D and 1E) as well as the IGF-I level at day 3 (Fig. 1F). There were no significant changes in cell viability regardless of whether they were incubated with hormones alone or in addition to heparin during the whole time course of 12 days (data not shown).

FIGURE 1

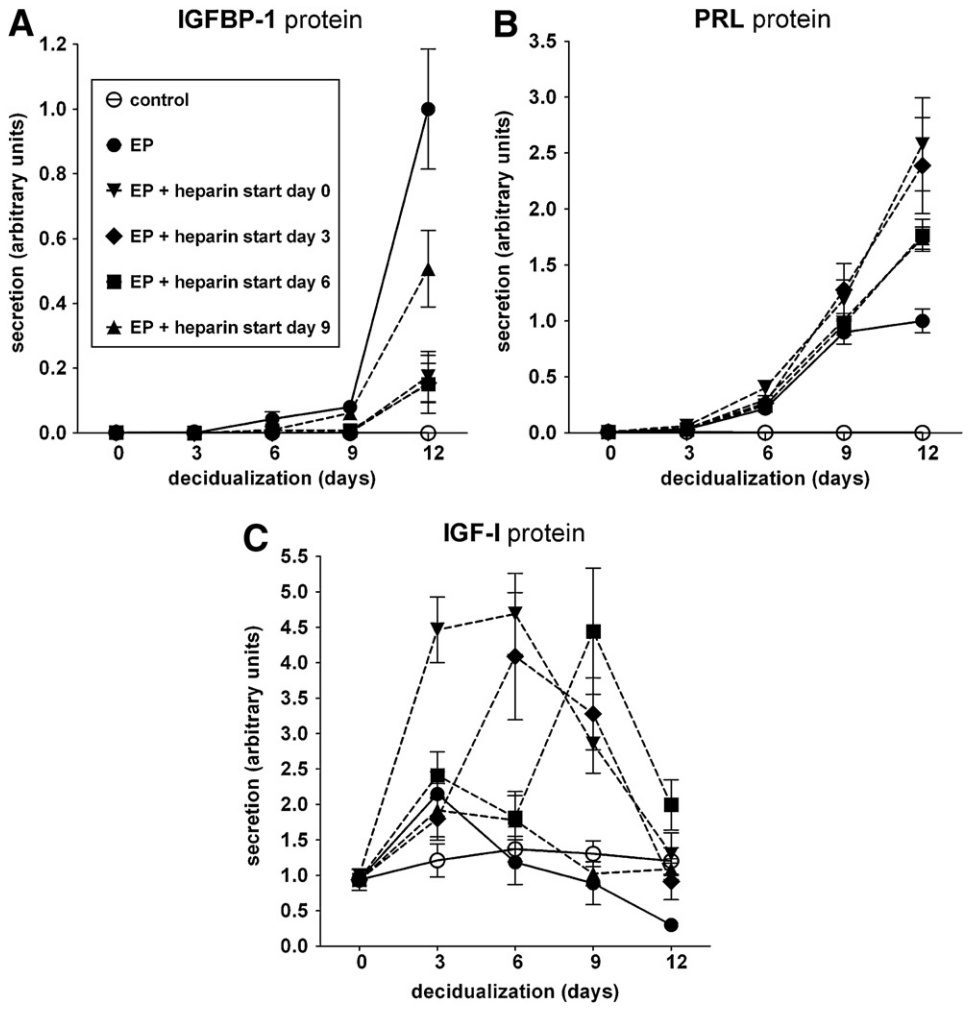
Unfractionated heparin dose-dependently regulates Insulin-like growth factor–binding protein (IGFBP) 1, PRL, and insulin-like growth factor (IGF) I protein and mRNA during decidualization of human endometrial stromal cells (ESCs) in vitro. ESCs were incubated with 30 nmol/L 17β-estradiol plus 1 μmol/L progesterone (EP) alone or in combination with 0.5, 5, or 50 μg/mL unfractionated heparin over a time course of 12 days. For comparison, cells were incubated without hormones or heparin in parallel (control). The protein levels of (**A**) IGFBP-1, (**B**) PRL, and (**C**) IGF-I in the cell culture supernatant were determined at days 0, 3, 6, 9, and 12 using ELISA. Values of day 12 (IGFBP-1 and PRL) and day 0 (IGF-I) were set to 1 arbitrary unit. Each value represents the mean ± SEM of three different cell cultures performed in quadruplicate. The mRNA expression of (**D**) IGFBP-1, (**E**) PRL, and (**F**) IGF-I was determined at day 9 (IGFBP-1 and PRL) and day 3 (IGF-I) using semiquantitative real-time reverse-transcription polymerase chain reaction. Bars show the mean ± SEM of four different cell cultures performed in quadruplicate, and values of decidualized ESCs (EP) were set to 100%. #$P<.05$ vs. control (co), *$P<.05$ vs. EP.



Fluhr. Heparin modulates decidualization. Fertil Steril 2010.

FIGURE 2

The impact of unfractionated heparin on IGFBP-1, PRL, and IGF-I secretion during decidualization of human ESCs depends on the time when heparin is added. ESCs were incubated with 30 nmol/L 17β-estradiol plus 1 μmol/L progesterone (EP) alone or in combination with 5 μg/mL unfractionated heparin starting at days 0, 3, 6, or 9 over a total time course of 12 days. For comparison, cells were incubated without hormones or heparin in parallel (control). The protein levels of (**A**) IGFBP-1, (**B**) PRL, and (**C**) IGF-I in the cell culture supernatant were determined at days 0, 3, 6, 9, and 12 using ELISA. Values of day 12 (IGFBP-1 and PRL) and day 0 (IGF-I) were set to 1 arbitrary unit. Each value represents the mean ± SEM of four different cell cultures performed in quadruplicate. Abbreviations as in Figure 1.



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The Effects of Heparin on IGFBP-1, PRL, and IGF-I in ESCs are Time Dependent

To see whether the observed effects of heparin on IGFBP-1, PRL, and IGF-I are time dependent, we varied the time of addition during the time course of decidualization. As shown in Figure 2A, the earlier heparin was added, the more pronounced was the inhibiting effect on the IGFBP-1 secretion in human ESCs. In addition to this, heparin effects on PRL secretion during decidualization were also more pronounced when heparin was added at days 0 or 3 compared with days 6 or 9 (Fig. 2B). Furthermore, the delayed addition of heparin to decidualizing ESCs caused a delayed increase of IGF-I secretion, clearly dependent on the starting time of heparin (Fig. 2C). This time-dependent (significant) influence of heparin on IGFBP-1, PRL, and IGF-I was reflected in the respective mRNA levels (data not shown).

LMWHs modulate IGFBP-1, PRL, and IGF-I in ESCs during decidualization

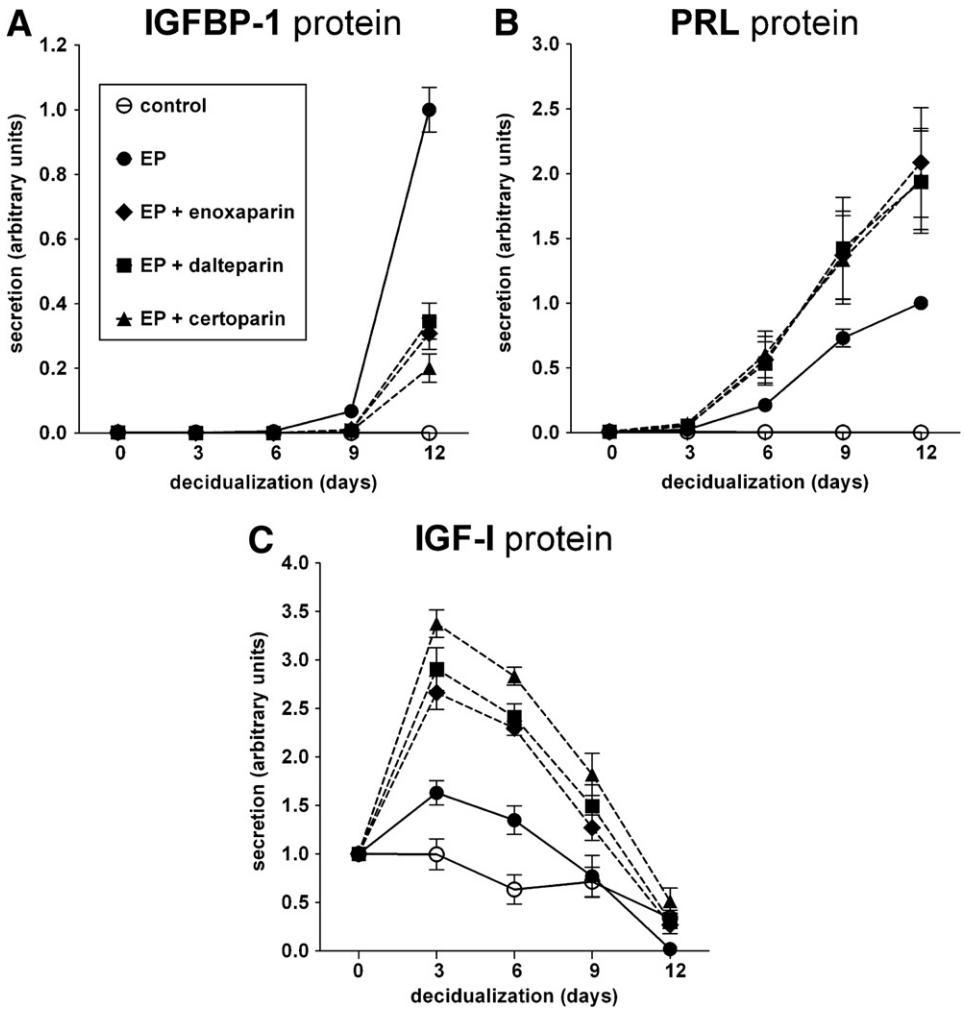
We also tested the influence of enoxaparin, dalteparin, and certoparin on decidualization of human ESCs in vitro. All three LMWHs added to ESCs decidualized over a time course of 12 days caused a significant delay of IGFBP-1 secretion (Fig. 3A) but a significant increase of PRL as well as IGF-I secretion (Figs. 3B and 3C; $P<.05$: IGFBP-1 and PRL days 9 and 12, EP vs. EP + LMWHs; $P<.05$: IGF-I days 3 and 6, EP vs. EP + LMWHs).

Heparin and LMWHs Increase Intracellular cAMP in Decidualized Human ESCs

We finally measured intracellular cAMP levels in ESCs during the early phase (day 3) and late phase (day 12) of decidualization under

FIGURE 3

Low-molecular-weight heparins (LMWHs) modulate IGFBP-1, PRL, and IGF-I secretion during decidualization of human ESCs similarly to unfractionated heparin. ESCs were incubated with 30 nmol/L 17β-estradiol plus 1 μmol/L progesterone (EP) alone or with the addition of 1 IU antifactor Xa/mL enoxaparin, dalteparin, or certoparin over a time course of 12 days. For comparison, cells were incubated without hormones or LMWHs in parallel (control). The protein levels of (**A**) IGFBP-1, (**B**) PRL, and (**C**) IGF-I in the cell culture supernatant were determined at days 0, 3, 6, 9, and 12 using ELISA. Values of day 12 (IGFBP-1 and PRL) and day 0 (IGF-I) were set to 1 arbitrary unit. Each value represents the mean ± SEM of three different cell cultures performed in quadruplicate. Abbreviations as in Figure 1.



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the influence of unfractionated heparin and LMWHs. At day 3 there were still low levels of cAMP under control as well as under decidualizing conditions without any significant differences (data not shown). At day 12, however, there was a significant increase of cellular cAMP in decidualizing ESCs, which was further significantly augmented by the addition of heparin and LMWHs (Fig. 4).

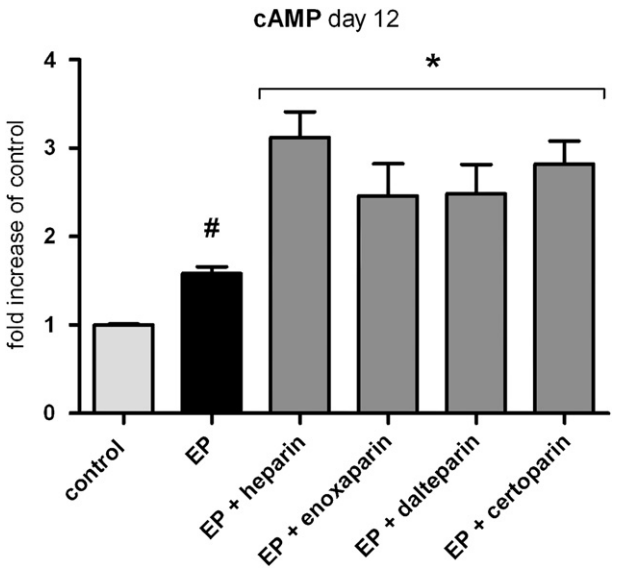
DISCUSSION

In the present study, we examined the influence of unfractionated heparin and LMWHs on the decidualization of human ESCs in vitro and showed for the first time a regulatory impact of these classic anticoagulants on endometrial IGFBP-1, PRL, and IGF-I.

An increasing number of cytokines and growth factors is known to bind to glycosaminoglycans of the heparin and heparan-sulfate family, thereby regulating their bioavalibility. Interleukin (IL) 11 is produced by human ESCs and has been shown to be an important autocrine factor promoting progesterone-induced decidualization (26). Interestingly, heparin acts synergistically with IL-11 to induce signal transducer and activator of transcription 3 in osteoclasts (27, 28). A similar mechanism in the endometrium might explain the observed increase of PRL in decidualizing ESCs under the influence of heparin and LMWHs. In contrast, the increase of IGFBP-1 during decidualization is delayed by the addition of heparin, although IGBP-1 also has been shown to be augmented by IL-11 (26). Therefore it is intriguing to speculate about other (or at least additional) factors interacting with heparin to modulate the process of decidualization. Furthermore, the decidualization marker PRL itself has heparin-binding capabilities affecting its biologic activity as a growth factor (29). Recently, Eyal et al. (30) described an autocrine

FIGURE 4

Unfractionated heparin and LMWHs increase the cAMP levels in decidualizing ESCs. ESCs were incubated with 30 nmol/L 17β-estradiol plus 1 μmol/L progesterone (EP) over a time course of 12 days. In addition to the hormones, cells were incubated with 5 μg/mL unfractionated heparin, 1 IU antifactor Xa/mL enoxaparin, dalteparin, or certoparin. For comparison cells were incubated without hormones or heparin in parallel (control). The intracellular levels of cAMP were determined at day 12 using a luminescence-based assay. Bars show the mean ± SEM of five different cell cultures performed in sextuplicate, expressed as fold increase of untreated ESCs (control). #$P<.05$ vs. control, *$P<.05$ vs. EP. Abbreviations as in Figures 1 and 4.



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inhibitory effect of PRL on endometrial decidualization, as measured by a reduction of IGFBP-1. Considering 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 (30). Progesterone is an essential hormone for the decidualization of human ESCs (32). Emerging evidence suggests that locally expressed growth factors in combination with the cAMP second messenger pathway are necessary to integrate hormonal effects leading to successful decidualization (31, 33). Because heparin as well as LMWHs induce an increase of cAMP in decidualizing ESCs, this might be a central mechanism of the heparin-driven modulation of endometrial differentiation.

During the time course of decidualization in vitro we observed an expression pattern of IGFBP-1 that is very similar to the situation in vivo, with the beginning of endometrial IGFBP-1 secretion on day 10 after the LH peak (34). Interestingly, this time point is associated with a rapidly increasing risk of implantation failure and early loss of pregnancy (16). Therefore, it is intriguing to speculate about a possible direct role for IGFBP-1 in restricting uterine receptivity. The observed inhibition of IGFBP-1 by heparin and LMWHs may therefore be interpreted as a pharmacologic possibility to modulate the period of endometrial receptivity. Interestingly, similar effects on IGFBP-1 are seen for the early embryonic signal hCG (23). However, it is not clear to date whether IGFBP-1 stimulates or inhibits the implantation process of the invading trophoblast. On the one hand, IGFBP-1 stimulates migration of the first-trimester trophoblast through its binding to $\alpha5\beta1$-integrin (35, 36). On the other hand, an inhibition of cytotrophoblast invasion into decidualized endometrial stromal cells secreting large amounts of IGFBP-1 has been shown, and this effect also was due to the binding to the trophoblast's $\alpha5\beta1$-integrin (18).

The strong expression of PRL in the secretory endometrium suggests a role of PRL in regulating paracrine actions between decidualized cells, as discussed above. Additionally, the finding that PRL has direct effects on decidual natural killer cells demonstrates its immunoregulatory role in the endometrium (37). Recently, Garzia et al. (20) reported a lack of expression of endometrial PRL during the implantation window in some patients affected by unexplained infertility and recurrent miscarriages, giving further evidence that prolactin may be directly involved in implantation. The stimulating effect of heparin and LMWHs on endometrial PRL might therefore not only play a role in decidualization (see above), but also directly influence early implantation.

We confirmed our previous observation of a rapid and significant increase of IGF-I during decidualization in vitro between days 0, 3, and 6, followed by a decline toward day 12 (22). The function of IGF-I as growth factor finally preparing the endometrium for the window of implantation seems to be augmented by the pronounced expression pattern caused by heparin and LMWHs. The following down-regulation of IGF-I after prolonged incubation with progesterone may be necessary during early pregnancy and is not disturbed in the presence of heparins.

In conclusion, heparin modulates the process of decidualization in a way that may improve endometrial receptivity and support early implantation. Because the same effects can be achieved by the use of LMWHs, which are preferable for several reasons, these agents which are primarily used as anticoagulants might come to play an interesting and important role in assisted reproduction in the future.

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