Heparin inhibits interferon-γ signaling in human endometrial stromal cells by interference with the cellular binding of interferon-γ

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Objective: To examine the impact of heparins on interferon-γ (IFN-γ) signaling in 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 P and 17β-E_2, and incubated with recombinant IFN-γ, unfractionated heparin, and low molecular weight heparins (LMWHs).
Main Outcome Measure(s): Interferon response factor 1 (IRF-1) and N-myc interactor (Nmi) messenger RNA (mRNA) were measured using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Phosphorylation of signal transducer and activator of transcription 1 (STAT-1) was detected by an in-cell Western assay, expression of the IFN-γ receptor by flow cytometry. Cell-bound IFN-γ was determined in lysates by an ELISA.
Result(s): Heparin and LMWHs inhibit the IFN-γ-mediated induction of IRF-1, but not Nmi in undifferentiated and decidualized ESCs. The phosphorylation of signal transducer and activator of transcription 1 STAT-1 upon IFN-γ stimulation is inhibited as well. Heparin has no effect on the IFN-γ receptor in ESCs, but inhibits the binding of IFN-γ to the cells.
Conclusion(s): Unfractionated heparin, as well as LMWHs, are able to inhibit IFN-γ signaling in human ESCs and therefore might be clinically interesting agents to modulate the actions of this proinflammatory cytokine at the implantation site. (Fertil Steril® 2011;95:1272–7. ©2011 by American Society for Reproductive Medicine.)

Key Words: Heparin, low molecular weight heparin, IFN-γ, STAT-1, IRF-1, Nmi, endometrium

Interferon-γ (IFN-γ) is a proinflammatory T helper 1 type cytokine and plays an important role during endometrial differentiation and implantation (1, 2). At the fetomaternal interface IFN-γ is secreted by decidual natural killer (NK) cells of the $CD56^{bright}$, $CD16^{negative}$ phenotype, which are essentially involved in the physiological as well as pathological aspects of implantation (3, 4).

Several immunomodulatory molecules such as interleukin (IL)-6, IL-8, IL-15, RANTES (regulated upon activation, normal T-cell expressed and secreted), and macrophage colony-stimulating factor are regulated by IFN-γ in the human endometrium (5–7). In addition, human endometrial stromal cells (ESCs) are sensitized to Fas-mediated apoptosis by IFN-γ in combination with tumor necrosis factor-α (TNF-α) (8). Furthermore, IFN-γ has a regulating influence on endometrial proliferation and differentiation during the menstrual cycle (9, 10). These observations suggest an essential impact of NK cell-derived IFN-γ on the milieu at the implantation site.

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Upon binding to the IFN-γ receptor (IFN-γ-R), IFN-γ activates signal transducers and activators of transcription (STAT)-1 by its phophorylation and subsequent nuclear translocation (11). We have recently reported an inverse regulation of the IFN-γ-R and its signaling response through STAT-1 in human ESCs during decidualization (12). In line with these observations, another study observed that decidualizing ESCs acquire a reduced responsiveness to IFN-γ due to a convergence of IFN-γ and P signaling (13).

Heparin and low molecular weight heparins (LMWHs) are widely used in the treatment of women with recurrent miscarriage without apparent causes or inherited thrombophilia (14, 15). However, there is evidence that the effectiveness of heparin in the treatment of implantation disorders might be due to effects beyond its classic anticoagulatory function (16–18). Due to its molecular properties, heparin not only has anticoagulant qualities but has been described as an anti-inflammatory and immunoregulatory agent, suppressing NK cell cytotoxicity (19) or inhibiting leukocyte recruitment and vascular adhesion (20, 21). Furthermore, we have recently observed a modulating effect of unfractionated heparin, as well as LMWHs, on the decidualization of human ESCs in vitro (22). However, there is no information about a possible influence of heparins on the action of inflammatory cytokines in the endometrium.

Based on these observations we investigated whether unfractionated heparin and LMWHs have an impact on IFN-γ signaling in human ESCs. In addition we wanted to characterize the

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observed effects, if any, to define the mechanism of this heparin action.

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 patients had regular menstrual cycles, proven fertility, and were considered to be healthy. Patients suffering from adenomyosis uteri or endometriosis were excluded from this study. 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) for 1 hour. The dispersed endometrial cells were separated by filtration through a 40-μm filter (BD Falcon, Heidelberg, Germany). The ESCs were maintained in Dulbecco's minimum essential medium (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) and cultured until confluency in 75-cm^2 culture flasks (Sarstedt, Nümbrecht, Germany). For experiments, ESCs were detached using 0.25% trypsin (GIBCO/Invitrogen) and plated in different multiwell microplates (Greiner, Frickenhausen, Germany). The purity of ESC cultures was proven by flow cytometric analysis of intracellular vimentin expression.

Decidualization In Vitro and Experimental Conditions

Human ESCs were decidualized in vitro by incubation with 30 nM 17β-E_2 and 1 μM P (both from Sigma-Aldrich, Taufkirchen, Germany) for 9 days with renewal of the medium every 3 days. For controls, ESCs were cultured in parallel during 9 days without hormonal treatment. Decidualization was proven by a significant increase of insulin-like growth factor-binding protein-1 (IGFBP-1) and PRL secretion measured by ELISAs, as described previously (23). On day 9 decidualized as well as undifferentiated ESCs were incubated with the following agents: human recombinant IFN-γ (R&D Systems, Wiesbaden, Germany), unfractionated heparin (Sigma-Aldrich), enoxaparin (Clexane; Sanofi-Aventis, Frankfurt, Germany), dalteparin (FragminP; Pharmacia, Berlin, Germany) or certoparin (Mono-Embolex; Novartis Pharma, Nuremberg, Germany). The different combinations, dosages, and incubation times of these agents are shown in detail in Figures 1–4.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from ESCs after the respective treatments using PeqGOLD TriFast reagent (PeqLab, Erlangen, Germany) following the manufacturer's standard protocol. Total RNA was reverse-transcribed using the High Capacity complementary DNA (cDNA) 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 (mRNA) levels of interferon response factor 1 (IRF-1) and N-myc interactor (Nmi) relative to the housekeeping gene β-actin. The cDNA samples were amplified using Power 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 are: β-actin forward 5′-CCTGGCACCCAGCACAAT-3′; β-actin reverse 5′-GCCGATCCACACGGAGTACT-3′; IRF-1 forward 5′-CATTCACACAGGCCGATACAAA-3′; IRF-1 reverse 5′-AGCGAAAGTTGGCCTTCCA-3′, Nmi forward 5′-ACCGCGTGGACTATGACAGAC-3′; and Nmi reverse 5′-TTGTCAGCCACTCCAATCTCC-3′.

The PCR amplification was performed in duplicates in a 7300 Real-Time PCR System (Applied Biosystems) using a standard cycling program (24). The PCR products were analyzed by thermal dissociation to verify that a single specific PCR product had been amplified. Relative expression levels of IRF-1 and Nmi 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).

In-Cell Western Assay

To quantify the levels of phosphorylated (Tyr701) STAT-1 in ESCs we used an in-cell Western assay, based on immunofluorescent staining performed in a 96-well microplate format. After treatment with the respective agents the cells were fixed with 3.7% formaldehyde and permeabilized with ice-cold methanol. Blocking with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) was followed by incubation with an anti-phospho (Tyr701) STAT-1 antibody (model no. 9171; Cell Signaling Technology, Danvers, MA) and an IRDye800CW conjugated secondary antibody (LI-COR). Normalization to the cell number of each sample was peformed by parallel DNA staining using DRAQ5 (Biostatus Limited, Shepshed, United Kingdom). The assays were visualized and analyzed using the LI-COR Odyssey imaging system (LI-COR).

Flow Cytometry

The IFN-γ-R expression on the surface of ESC was determined by extracellular staining with a specific monoclonal antibody (clone MMHGR-1; PBL Biomedical Laboratories, Piscataway, NJ) according to a previously described protocol (8). The secondary antibody conjugated with Alexa Fluor 647 was obtained from Molecular Probes (Eugene, OR). Unspecific isotype controls (BD Biosciences, Heidelberg, Germany) were used to detect nonspecific binding. A FACSCanto flow cytometer and FACSDiva software (BD Biosciences) were used for measurements and analysis.

Preparation of Cellular Lysates

To determine IFN-γ bound to ESCs, cells were incubated with IFN-γ for the indicated times, followed by the removal of unbound IFN-γ by washing the cells three times with phosphate-buffered saline (PBS; GIBCO). After washing, cell monolayers were gently detached by the use of Accutase (PAA Laboratories, Pasching, Austria) and proteins were isolated by mild detergent lysis using M-PER (Pierce/Perbio, Bonn, Germany) according to the manufacturer's standard protocol.

Enzyme-Linked Immunosorbent Assay

The IFN-γ in cellular lysates was determined using a commercially available high sensitivity ELISA kit (BMS228HS; Bender MedSystems, Vienna, Austria) with a sensitivity of 0.06 pg/mL. There was no significant cross-reactivity and intra-assay or interassay variabilities were lower than 5%. Heparin did not show any significant interference with the ELISA kit. Absorbance was measured using the FLUOstar OPTIMA system (BMG Labtech, Offenburg, Germany).

Statistical Analysis

Statistical analysis was carried out with one-way analysis of variance (ANOVA), followed by Dunnett's and Bonferroni multiple comparison tests or unpaired Mann-Whitney t-tests using GraphPad PRISM v5 software (GraphPad, San Diego, CA). The results are expressed as mean $\pm$ SEM. Differences were considered to be significant if $P<.05$.

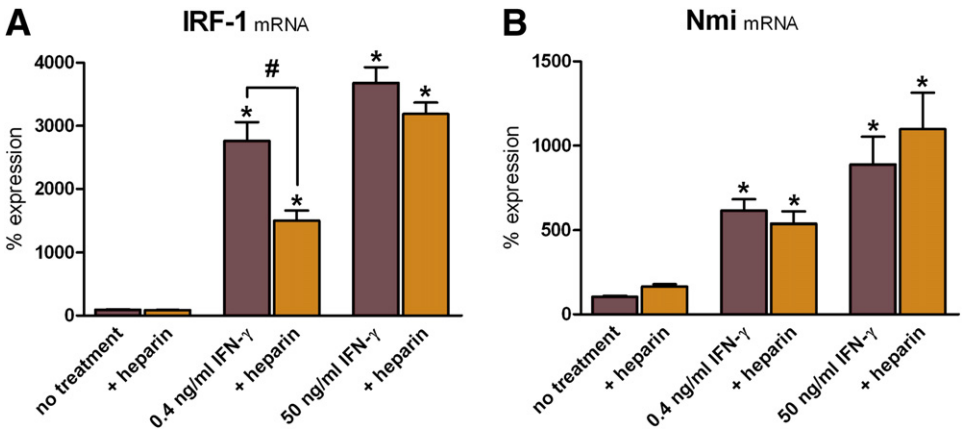
RESULTS

Heparins Inhibit the IFN-γ-Mediated Induction of IRF-1, but not Nmi, in ESCs

To investigate whether heparin has an influence on the two known IFN-γ-inducible genes IRF-1 and Nmi, human ESCs decidualized in vitro were incubated with recombinant IFN-γ alone or combined with unfractionated heparin. As shown in Figure 1A, heparin significantly inhibited the stimulating effect of 0.4 μg/mL IFN-γ on IRF-1 mRNA, but had no effect in the presence of 50 ng/mL IFN-γ. Interestingly, the expression of Nmi induced by IFN-γ was not significantly affected by heparin (Fig. 1B).

FIGURE 1

The interferon-γ (IFN-γ)-mediated induction of interferon response factor 1 (IRF-1), but not N-myc interactor (Nmi), in endometrial stromal cells is inhibited by heparin. Decidualized cells were incubated with the indicated concentrations of recombinant IFN-γ alone (*brown bars*) or combined with 5 μg/mL unfractionated heparin (*orange bars*) for 6 hours. The mRNA expression levels of (**A**) IRF-1 and (**B**) Nmi were determined using semiquantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Bars show the mean ± SEM of four different cell cultures performed in quadruplicates, values of untreated endometrial stromal cells were set to 100%. #$P < .05$ IFN-γ versus IFN-γ + heparin, *$P < .05$: each versus no treatment.



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In addition we tested the influence of three typical LMWHs on IFN-γ-mediated gene induction. As shown in Figure 2A,B enoxaparin, dalteparin, and certoparin also inhibited the up-regulation of IRF-1 in the presence of low-dose IFN-γ without any effect on Nmi. Incubation of the cells with LMWHs alone had no significant effect on IRF-1 or Nmi (data not shown). Similar effects of unfractionated heparin as well as LMWHs on IRF-1 were obtained with undifferentiated ESCs (data not shown).

The IFN-γ-Induced Phosphorylation of STAT-1 in ESCs Is Inhibited by Heparin

In the next step we analyzed the impact of heparin on the phosphorylation and thus the activation of STAT-1. Undifferentiated and decidualized ESCs were incubated with increasing concentrations of IFN-γ with or without unfractionated heparin. As shown in Figure 3A,B, heparin significantly inhibited the phosphorylation of STAT-1 induced by 0.4 and 2 ng/mL. No effect was seen when

FIGURE 2

The interferon-γ (IFN-γ)-mediated induction of interferon response factor 1 (IRF-1), but not N-myc interactor (Nmi) in endometrial stromal cells is inhibited by low molecular weight heparins. Decidualized cells were incubated with the indicated concentrations of recombinant IFN-γ alone (*brown bars*) or combined with 1 IU antifactor Xa/mL enoxaparin, dalteparin, or certoparin (*orange bars*) for 6 hours. The mRNA expression levels of (**A**) IRF-1 and (**B**) Nmi were determined using semiquantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Bars show the mean ± SEM of three different cell cultures performed in quadruplicates, values of untreated endometrial stromal cells were set to 100% (*open bars*). *$P < .05$: each 0.4 ng/mL IFN-γ versus 0.4 ng/mL IFN-γ + enoxaparin, dalteparin, or certoparin.

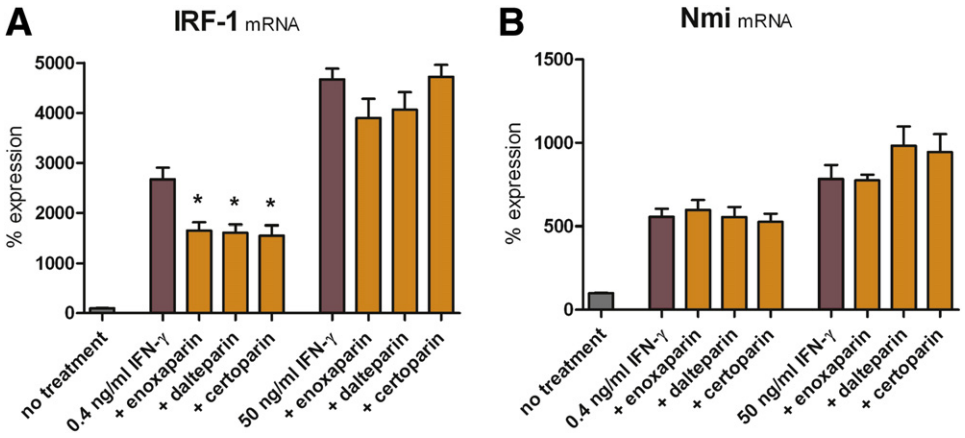

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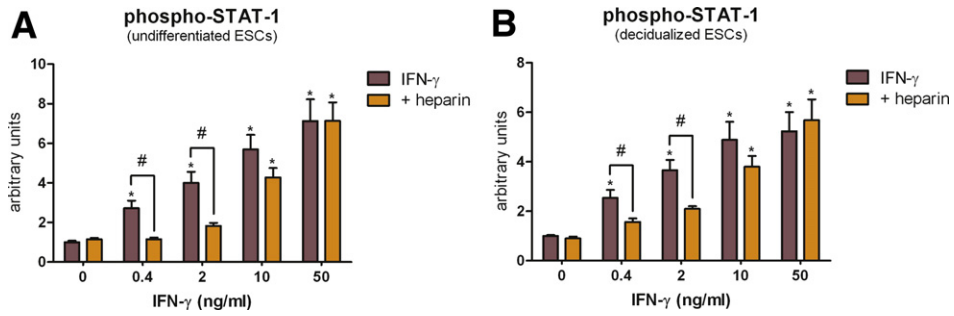
FIGURE 3

Heparin inhibits the interferon-γ (IFN-γ)-induced phosphorylation of signal transducers and activators of transcription-1 (STAT-1) in endometrial stromal cells. (**A**) Undifferentiated and (**B**) decidualized endometrial stromal cells were incubated with the indicated concentrations of recombinant IFN-γ alone (*brown bars*) or combined with 5 μg/mL unfractionated heparin (*orange bars*) for 20 minutes. Phopho-STAT-1 was measured using an in-cell Western assay. Bars show the mean ± SEM of three different cell cultures performed in quadruplicate, values of untreated endometrial stromal cells were set to 1 arbitrary unit (integrated fluorescence intensity). #$P<.05$ IFN-γ versus IFN-γ + heparin, *$P<.05$: each versus no treatment (0).



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high doses of IFN-γ (10 and 50 ng/mL) were used. Similar effects were seen in undifferentiated (Fig. 3A) as well as decidualized ESCs (Fig. 3B).

The IFN-γ-R on the Surface of ESCs Is not Regulated by Heparin

To determine whether heparin regulates the IFN-γ-R in ESCs, we measured its expression on the cell surface. Neither heparin nor IFN-γ alone, nor the combination of both agents had any significant influence on the IFN-γ-R in ESCs (data not shown). However, the cell surface expression of this receptor is higher in decidualized cells compared with undifferentiated cells (data not shown).

Heparin Interferes With the Binding of IFN-γ to ESCs

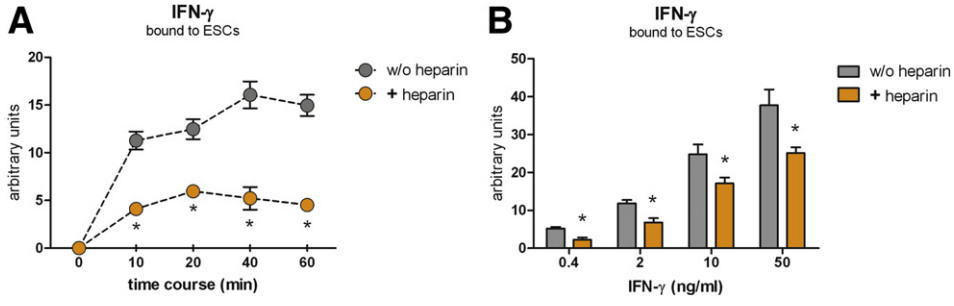
Finally we tested the influence of heparin on the binding of IFN-γ to ESCs. Undifferentiated cells were incubated with IFN-γ during a time course or with increasing concentrations of IFN-γ, each in the absence or presence of heparin. As shown in Figure 4A, binding of IFN-γ to ESCs increased during the time course, reaching a plateau after 40 minutes. The addition of heparin significantly inhibited this binding kinetic of IFN-γ to the cells (Fig. 4A). Figure 4B shows that heparin was able to significantly inhibit the binding of IFN-γ to ESCs, even in the presence of high concentrations of IFN-γ.

DISCUSSION

A complex network of cytokines with subtle interactions is the prerequisite for endometrial differentiation and successful early implantation (26). The IFN-γ, one of the cytokines expressed by decidual NK cells, has been shown to influence endometrial vascular remodeling as well as decidual differentiation (27, 28). In addition to these effects IFN-γ might be involved in the pathophysiology of implantation failure and early miscarriage (3, 4, 29).

FIGURE 4

Heparin inhibits the binding of interferon-γ (IFN-γ) to endometrial stromal cells. (**A**) Cells were incubated with 2 ng/mL recombinant IFN-γ with (*orange dots*) or without (*grey dots*) 5 μg/mL unfractionated heparin for 10, 20, 40, and 60 minutes. (**B**) Endometrial stromal cells were incubated with 0.4, 2, 10, and 50 ng/mL recombinant IFN-γ with (*orange bars*) or without (*gray bars*) 5 μg/mL unfractionated heparin for 10 minutes. The IFN-γ bound to the cells was measured in lysates using a high sensitivity ELISA. Dots and bars show the mean ± SEM of four different cell cultures performed in triplicates. Values are given in arbitrary units representing the relative amount of bound IFN-γ normalized to the cell number. (**A**) *$P<.05$: each IFN-γ versus IFN-γ + heparin; (**B**) *$P<.05$: each IFN-γ versus IFN-γ + heparin.



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In the present study we demonstrate that heparin inhibits the binding of IFN-γ to human ESCs in the presence of a broad range of IFN-γ concentrations. However, only the subsequent activation of STAT-1 signaling as well as the induction of IRF-1 induced by low concentrations of IFN-γ can be inhibited by heparin. A possible explanation for this phenomenon might be the saturation of the available IFN-γ-R with a limitation of the signaling capacity. The inhibitory effect of heparin is therefore only effective if the concentration of active IFN-γ decreases below a critical threshold. The inhibitory effect of heparin on IFN-γ binding at high concentrations has no impact on the signaling, as there is still enough IFN-γ for maximum stimulation of the IFN-γ-R. The underlying effect might be a direct binding of heparin to IFN-γ, as shown by Hatakeyama and colleagues (30).

This concept is based on the model that IFN-γ is bound by membrane-associated heparan sulfate (HS) before its binding with the high-affinity receptor and signal transduction (31). In endothelial cells IFN-γ has been shown to initially bind to the cells by interaction between basic amino acids located within the carboxy-terminal region of the molecule and HS-containing proteoglycans on the cell surface (31, 32). This interaction seems to be necessary for the optimal binding of IFN-γ to its specific receptor, as shown by experiments in cells with reduced expression of cell surface HS (31, 32). In line with these reports, heparin might compete with cell surface HS for the binding of IFN-γ to human ESCs, thereby disturbing the interaction with its receptor and subsequently blocking the signal transduction.

Interestingly, heparin did not have the same inhibitory effect on the IFN-γ inducible gene Nmi as on IRF-1. A possible dose-dependency or the involvement of other signaling pathways might be an explanation for this observation. Nevertheless, the lack of effects of heparin on Nmi probably does not have many functional consequences as Nmi does not enhance IFN-γ-dependent transcription in decidualized ESCs (13).

The expression of the IFN-γ-R on human ESCs is not influenced by heparin and therefore does not seem to be the direct target of heparin action in our experiments. It must be said that conclusions concerning the responsiveness of ESCs to IFN-γ cannot be directly drawn from the expression level of the IFN-γ-R (e.g., an inverse regulation of receptor and signaling has previously been described) (12).

Unfractionated heparin is a mixture of polysulfated glycosaminoglycans having anticoagulatory effects due to its interaction with antithrombin III and factor Xa. Beyond its classic function, heparin has been shown to have effects outside of hemostasis based on its interactions with different growth factors, cell adhesion molecules, and matrix metalloproteinases (33). We could recently observe a modulating effect of unfractionated heparin as well as different LMWHs on the decidualization of human ESCs, further underlining its potential to improve implantation rates independently of its anticoagulant function (22). The modulating effect of heparins on IFN-γ signaling might be an additional mechanism improving implantation in patients with repeated implantation failure or miscarriages.

In summary, heparin modulates the signaling of the proinflammatory cytokine IFN-γ in human ESCs by interference with its binding to the cells. Because similar effects are seen with clinically well-established LMWHs, the molecular data presented here further underline the concept of these classic anticoagulants as anti-inflammatory and immunoregulatory agents. The effects of heparins beyond anticoagulation might prove to be interesting for the development of new therapeutic strategies for women suffering from implantation disorders.

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