Low-molecular-weight heparins induce decidual heparin-binding epidermal growth factor–like growth factor expression and promote survival of decidual cells undergoing apoptosis

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Objective: To evaluate the effects of low-molecular-weight heparins (LMWHs) on decidual heparin-binding epidermal growth factor–like growth factor (HB-EGF) expression/secretion and on TNF-α–induced decidual apoptosis.
Design: Experimental study.
Setting: Department of Obstetrics and Gynecology, Università Cattolica del Sacro Cuore, Rome, Italy.
Patient(s): Cultures of primary decidual cells isolated from human term placenta.
Intervention(s): The effects of LMWHs (tinzaparin and enoxaparin) on decidual HB-EGF expression and secretion were investigated by Western blot analysis and ELISA, respectively. TNF-α–induced decidual apoptosis was evaluated by annexin V staining, terminal deoxynucleotide transferase–mediated dUTP nick-end labeling (TUNEL) assay, and caspase activities.
Main Outcome Measure(s): Decidual HB-EGF expression/secretion and apoptotic rate induced by TNF-α were investigated.
Result(s): Tinzaparin enhanced decidual HB-EGF expression and secretion. TNF-α reduced the number of viable cells by inducing apoptosis. Simultaneous addition of LMWHs (primarily tinzaparin) blocked the increase in annexin V– and TUNEL-positive cells and reduced the amount of caspase activities.
Conclusion(s): Both LMWHs induced a significant increase in decidual HB-EGF expression/secretion and reduced TNF-α–induced decidual apoptosis. Tinzaparin demonstrated higher efficacy. (Fertil Steril® 2012;97:169–77. ©2012 by American Society for Reproductive Medicine.)
Key Words: Decidua, LMWH, HB-EGF, apoptosis, cytokines, TNF-α, caspases

Heparin is widely used in obstetric practice for prophylaxis and treatment of venous thromboembolism in pregnancy and puerperium. Antithrombotic therapy has been shown to be beneficial in improving fetal outcome in women with antiphospholipid syndrome (1) and in previous second- and third-trimester placental dysfunction, although there has never been a randomized double-blind trial (2), which may now be unethical.

It has also been suggested that heparin exerts a beneficial effect in preventing pregnancy loss in women without thrombophilia (3). However, although low-molecular-weight heparin (LMWH) is generally considered to be be safe in pregnancy (4, 5), no univocal evidence arises from the international literature about its efficacy in preventing unexplained adverse pregnancy outcome not related to thrombophilias. In a multicenter, randomized, placebo-controlled trial, Kaandorp et al. (6) observed that neither aspirin combined with LMWH nor aspirin alone improved the live birth rate, compared with placebo, among women with unexplained recurrent miscarriages.

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Conclusive data are lacking regarding the use of LMWH alone in these patients.

In in vitro studies, the functional role of heparin on human placenta has not yet been completely clarified. Previously, we demonstrated that heparin plays a role on trophoblast cells obtained from unexplained spontaneous recurrent miscarriages, increasing cellular invasion along with an enhancement of the activity of specific proteases, such as matrix metalloproteinases (7). Hills et al. (8) demonstrated that heparin abrogates apoptosis of primary first-trimester villous trophoblast in response to treatment with proinflammatory cytokines such as interferon-γ and tumor necrosis factor (TNF) α. On the other hand, Ganapathy et al. (9) observed that heparin suppresses the invasiveness of hepatocyte growth factor–stimulated trophoblast cell line cultures.

Endometrial decidualization is essential for successful implantation. Decidualization begins around the blood vessels of the midsecretory-phase endometrium and amplifies and extends through the stroma in response to the continued influence of estrogens and progesteron during implantation (10). The regulation of endometrial functions involves a complex hierarchy of extracellular signals, including steroid hormones, growth factors and cytokines, and the extracellular matrix, the molecular details of which are poorly understood (11). Heparin-binding epidermal growth factor–like growth factor (HB-EGF) is now recognized as having a significant function in reproduction (12). HB-EGF is a member of the EGF family (13). It is synthesized as a transmembrane protein of 208 amino acids. A small part of the membrane-anchored HB-EGF form, or proHB-EGF, is cleaved from the cell surface to yield a soluble growth factor of 75–86 amino acids while most of the molecule remains uncleaved on the cell surface. ProHB-EGF is not merely a precursor of the soluble form; it is also a biologically active molecule that is complexed with both CD9 and $\alpha 3\beta 1$ integrin. In this form it has several biologic effects on different cell types (14). A heparin-binding domain in the N-terminal portion of the EGF-like domain was identified as a sequence able to modulate EGF-like biologic activity (15, 16).

Recent studies revealed that HB-EGF mRNA expression in isolated endometrial stromal and epithelial cells is regulated by estrogen and progesteron (17). HB-EGF mediates decidualization of endometrial cells and potentiates the survival of endometrial stromal cells exposed to apoptotic factors such as TNF-α or transforming growth factor β (12). However, little is known about the regulators of HB-EGF protein, and the effect of heparin on decidual cells has not yet been investigated.

The present study was designed to test the possibility of a direct effect of LMWHs on human decidual cells. We investigated: 1) the role of two LMWHs (tinzaparin and enoxaparin) on decidual HB-EGF secretion/expression; and 2) whether incubation with these LMWHs promotes survival of human decidual cells undergoing apoptosis in response to TNF-α.

MATERIALS AND METHODS

Primary Decidual Cell Isolation

Each of the patients provided informed consent before participating in this study, which was approved by the Ethics Committee of the Università Cattolica del Sacro Cuore. Isolation of primary decidual cells from human fetal membranes was performed by Percoll gradient methodology (18) with modifications. Briefly, fetal membranes from women undergoing elective cesarean section before labor were collected. The fetal membranes were dissected from the placenta and transported to the laboratory in sterile phosphate-buffered saline solution (PBS). The decidua was separated from the chorion by gentle scraping with a glass slide and digested with 0.02% collagenase A and 20 U/mL deoxyribonuclease-1 at 37°C for 60 minutes. The cells were collected by filtration and centrifugation, overlaid onto a discontinuous Percoll gradient (5%–60% Percoll), and centrifuged at 400 *g* for 20 minutes. Decidual cells migrating between 1.017 and 1.045 g/mL were collected, washed, and plated onto 2% gelatin-coated plates in RPMI 1640 containing 10% fetal bovine serum and antibiotics. Cultures were grown to confluence and subcultured three times before use to eliminate nonadherent cells.

Immunostaining

For immunostaining, decidual cells cultured on slides were fixed with acetone and labeled by an indirect immunoperoxidase method. Briefly, samples were rehydrated in PBS and incubated with hydrogen peroxide and human serum to block endogenous peroxidase. The samples were then incubated for 30 minutes at room temperature in a humid chamber with an appropriately diluted monoclonal antibody (mAb; antidesmin and anti–human prolactin; Dako Italia). Normal mouse serum or an irrelevant mAb for negative control samples were substituted for the first antibody. After three brief washes with PBS, samples were overlaid with peroxidase-conjugated goat antimouse IgG (BioRad) and diluted 1:100 in 1% PBS–bovine serum albumin (BSA), and the reaction was developed with 0.5 mg/mL diaminobenzidine (Sigma) containing 0.01% hydrogen peroxide. The reaction was stopped after 5–10 minutes by washing in excess PBS. Samples were counterstained with Mayer hematoxylin.

Western Blot Analysis

HB-EGF expression was investigated by Western blotting of human decidual cell extracts in the presence of serial concentrations of LMWHs [0.1, 1, and 10 IU/mL tinzaparin (Innohep; LEO Pharma) or enoxaparin (Clexane; Sanofi-Aventis S.p.A Milano) as previously described (19). Eighty micrograms of each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 12% gels. After electroblotting onto polyvinylidene fluoride, samples were incubated with 5% nonfat dry milk in 1 mol/L Trizma/base, 1.54 mol/L NaCl, and 0.05% Tween 20 (TBST; pH 7.4) and then exposed overnight at 4°C to TBST containing 0.4 μg/mL primary antibody (polyclonal goat IgG antihuman HB-EGF; Oncogene Sciences). After further washes, samples were incubated with peroxidase-conjugated rabbit antigoat IgG antiserum (Cappel/MP Biomedicals). Bands were analyzed with the use of a Gel Doc 200 Image Analysis System and quantified with the use of Quantity One Quantitation Software (both from BioRad). The level of HB-EGF was estimated versus the

constant level of a 42-kd protein present in the cytosolic extract (β-actin), which was identified with the use of a mouse monoclonal antihuman β-actin antibody (Sigma-Aldrich).

ELISA

Microtiter plates (Costar; Corning Life Sciences) were coated for 24 hours at 4°C with 100 μL of 0.5 μg/mL anti–HB-EGF mAb (R&D Systems) and blocked overnight at 4°C with 1% PBS-BSA. A standard curve was prepared by diluting recombinant human HB-EGF (R&D Systems) in culture medium (0–1,000 pg/mL). Samples (100 μL of cell culture supernatants in presence of serial concentration of tinzaparin or enoxaparin), were incubated for 2 hours at 25°C in duplicate. After four washes with 300 μL PBS–0.05% Tween-20, 100 μL biotin-labeled affinity-purified polyclonal antihuman HB-EGF (200 ng/mL; R&D Systems) was added to each well for 30 minutes at 25°C. The wells were washed again and incubated for 30 minutes at 25°C with 100 μL streptavidin-conjugated horseradish peroxidase (1:200 dilution; R&D Systems). The wells were washed and peroxidase activity detected. The optical density (OD) at 450 nm was determined with a spectrophotometer (Titertek Multiscan Plus; ICN Flow). The calculated interassay and intraassay coefficients of variation of the immunoassay were 8% and 6%, respectively. Standard curves using recombinant HB-EGF were generated for each run and used to extrapolate the concentration of detectable HB-EGF in cell-conditioned medium. No cross-reactivity was found with recombinant human EGF, recombinant fibroblast growth factor, or recombinant vascular endothelial growth factor, respectively.

Total RNA Extraction

After treatment with tinzaparin or enoxaparin (0.1–10 IU/mL) for 8 hours, total cellular RNA was extracted from decidual cells with the use of the QuickPrep Total RNA Extraction kit (GE Healthcare) according to the manufacturer's protocol. Briefly, cell pellets were suspended in lithium chloride solution containing β-mercaptoethanol and extraction buffer. Samples were homogenized and incubated for 10 minutes on ice with cesium trifluoroacetate solution. After centrifugation at 14,000 rpm for 15 minutes, the RNA pellets were washed and dissolved in 50 μL diethyl pyrocarbonate–treated water. RNA quality was evaluated according to the absorbance ratio at 260–280 nm. The total RNA concentration was determined by measuring the absorbance at 260 nm.

Quantitative RT-PCR

Analysis of the quantitative expression of the HB-EGF gene was performed by real-time real-time reverse-transcription polymerase chain reaction (RT-PCR) using an iCycler iQ system (BioRad) as previously described (19). For the target gene and the endogenous housekeeping gene encoding for GAPDH (Supplemental Table 1, available online at www.fertstert.org), a primer pair and a TaqMan probe, which hybridizes to the region between primers, were designed with the use of Beacon Designer 2 version 3.00 software (Premier Biosoft) and synthesized by MWG Biotech.

Fluorescence-Activated Cell Sorting Analysis of Apoptosis

Decidual cells were grown to confluence. TNF-α (10 ng/mL; Sigma-Aldrich) with or without LMWHs (tinzaparin or enoxaparin; 0.1, 1, or 10 IU/mL) was added in Dulbecco modified Eagle medium for 24 hours. Apoptotic cells, exposing phosphatidylserine on the outer membrane, were analyzed by incubation with fluorescein isothiocyanate–conjugated annexin V (BD Pharmigen) (20). Labeling procedures were performed according to the manufacturer's instructions. Briefly, cells were resuspended in annexin labeling solution containing 10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 5 mmol/L CaCl, and fluorescein-conjugated annexin V for 15 minutes. After being washed twice with PBS, cell pellets were resuspended in propidium iodide (2 μg /mL) and analyzed by flow cytometry. At least 100,000 events were analyzed, and apoptosis was presented as the percentage of positive cells stained with annexin V.

TUNEL Staining

After 24 hours of incubation with TNF-α, with or without LMWHs, decidual cells were cytocentrifuged on coverslips and fixed with ethanol. The cells were processed with a deoxynucleotidyl transferase –mediated dUTP digoxigenin nick-end labeling (TUNEL) commercial kit, according to the manufacturer's instruction (Roche). This kit allowed the identification of fluorescein-labeled DNA fragments. The cells were visualized using a fluorescence microscope.

Caspase Fluorimetric Assay

The effect of LMWHs (tinzaparin and enoxaparin) on TNF-α–induced apoptosis in decidual cells were determined by measuring caspase-3 and -8 activities using the Caspase/FLICE Fluorimetric Assays kit (Biovision Research Products). The assay is based on detection of cleavage of specific substrates. Briefly, 10 μg cell lysates were incubated at 37°C for 1 hour with 1 mmol/L IETD-AFC substrate for caspase-8 and 1 mmol/L DEVD-AFC substrate for caspase-3. After incubation, fluorescence was measured with the use of a fluorometer (400 nm excitation filter and 505 nm emission filter). The amount of fluorescence detected as relative fluorescence unit (RFU) was proportional to the caspases' activity.

Statistical Analyses

The results are presented as the mean $\pm$ SE. Data were analyzed with the use of one-way analysis of variance followed by post hoc test (Bonferroni test). P values of $<.05$ were considered to be significant.

RESULTS

Effect of LMWHs on HB-EGF Expression

We performed experiments to demonstrate the effect of LMWHs (tinzaparin and enoxaparin) on decidual HB-EGF expression and secretion. HB-EGF protein levels were analyzed in cell extracts and in conditioned medium by Western blotting and ELISA, respectively. To rule out any variability

related to the donors' decidua, the experiments were performed wth samples from four different donors. After 24 hours of culture, tinzaparin (1–10 IU/mL) induced a significant increase ($P<.01$) in decidual HB-EGF expression compared with enoxaparin or no treatment (Fig. 1A and B).

Consistent with the Western blot data, incubation of decidual cells with tinzaparin (1–10 IU/mL) significantly increased HB-EGF protein secretion ($P<.001$) in culture supernatants as determined by ELISA (Fig. 1C). This was not seen with enoxaparin.

The increased HB-EGF expression in decidual cells was confirmed at the mRNA level by real-time RT-PCR (data not shown).

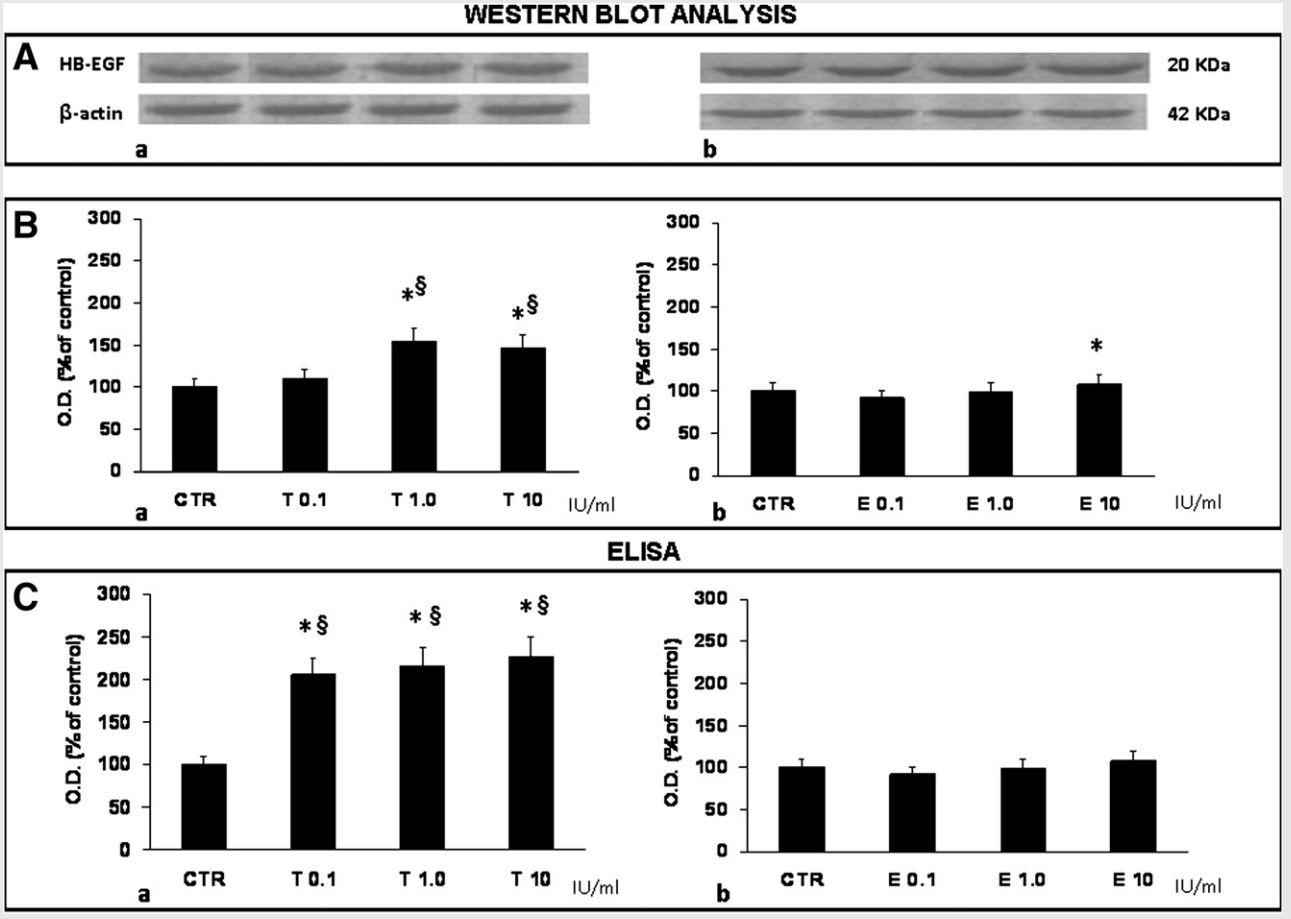
Effect of LMWHs on TNF-α–Induced Decidual Cell Apoptosis

Because TNF-α has been shown to induce apoptosis of some cell types, we further explored its possible function in human decidua. Cell apoptosis was assessed by staining with propidium iodide and fluorescein isothiocyanate–labeled annexin V and using flow cytometry and a TUNEL commercial kit, allowing the identification of fluorescein-labeled DNA fragments with the use of a fluorescence microscope.

In decidual cells, TNF-α (10 ng/mL) reduced the number of viable cells by inducing apoptosis (Fig. 2A; $P<.001$). Simultaneous addition of tinzaparin or enoxaparin (1 and 10 IU/mL) completely blocked the increase in annexin V–positive cells. As shown in Figure 2B, tinzaparin ($P<.05$) induced a significant decrease in apoptotic cell number compared with enoxaparin (10.5% vs. 17.9% and 13.72% vs. 19.3%, respectively).

We also noticed that incubation of decidual cells with TNF-α induced TUNEL positivity with an intense nuclear fluorescence (Fig. 3A). Quantitative analysis showed that after 24 hours of exposure, the number of TUNEL-positive nuclei induced by TNF-α was 55 (100%), significantly greater than that observed in presence of LMWHs (30%–18%; Fig. 3B).

FIGURE 1



Heparin-binding epidermal growth factor–like growth factor (HB-EGF) expression and secretion in decidual cells in presence of serial concentrations (0.1–10 IU/mL) of tinzaparin or enoxaparin. (A) Representative results of Western blot analysis. β-Actin was used as a loading control. (B) Densitometric analysis: The levels of HB-EGF in trophoblast cells were estimated in comparison with a constant level of β-actin and are expressed as a percentage of the values obtained in untreated cells. (C) Secretion of HB-EGF protein in trophoblast culture supernatants, as quantified by ELISA assay. HB-EGF levels are expressed as optical density (OD), means ± SE of four independent experiments. *Statistical significance versus untreated cells (CTR): $P<.01$. §Statistical significance between tinzaparin (T) and enoxaparin (E): $P<.03$.

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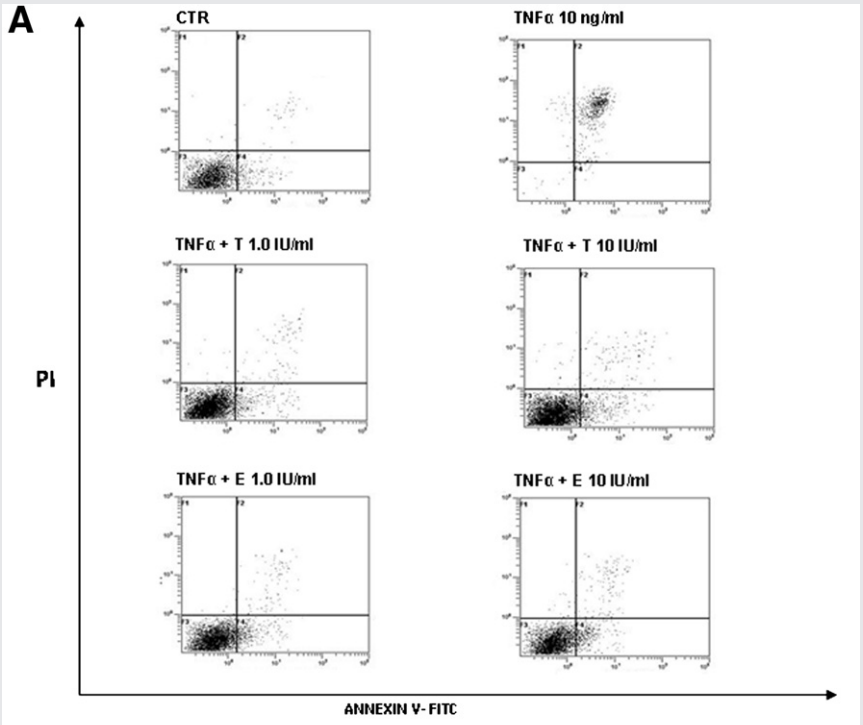
FIGURE 2



B

	ANN-/IP+	ANN+/IP+	ANN+/IP-	TOTAL	%
CTR	0,43 ± 0,02	2,29 ± 0,5	7 ± 1	9,29 ± 1.5	15
TNFα 10 ng/ml	2,13 ± 0,1	60,05 ± 7,9	1,5 ± 0,4	61,55 ± 8.3 ∞	100 ∞
TNFα 10 ng/ml + E 1.0 IU/ml	0,16 ± 0,01	2,63 ± 0,2	8,41 ± 2	11,04 ± 2,2 *	17,9 *
TNFα 10 ng/ml + E 10 IU/ml	0,31 ± 0,03	2,88 ± 0,02	9 ± 0,8	11,88 ± 0,82 *	19,3 *
TNFα 10 ng/ml + T 1.0 IU/ml	0,20 ± 0,03	2,19 ± 0,01	4,27 ± 1	6,46 ± 1,1 *§	10,5 *§
TNFα 10 ng/ml + T 10 IU/ml	0,36 ± 0,01	2,45 ± 0,5	6 ± 0,6	8,45 ± 1,2 *§	13,72 *§

Tumor necrosis factor (TNF) α–induced decidual cell apoptosis. (A) Apoptosis assessed by propidium iodide (PI) and fluorescein isothiocyanate–labeled annexin V staining and flow cytometry. The figure shows one of the four experiments which gave similar results. (B) Results are expressed as means ± SE of four independent experiments. Data were analyzed using one-way analysis of variance followed by post hoc test (Bonferroni test). ANN−/IP+ = necrosis; ANN+/IP+ = late apoptosis; ANN+/IP− = early apoptosis; TOTAL = early + late apoptosis. ∞Statistical significance versus untreated cells (CTR): $P<.0001$. *Statistical significance versus TNF-α: $P<.01$. §Statistical significance between tinzaparin (T) and enoxaparin (E): $P<.05$.

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Involvement of Caspase-3 and -8

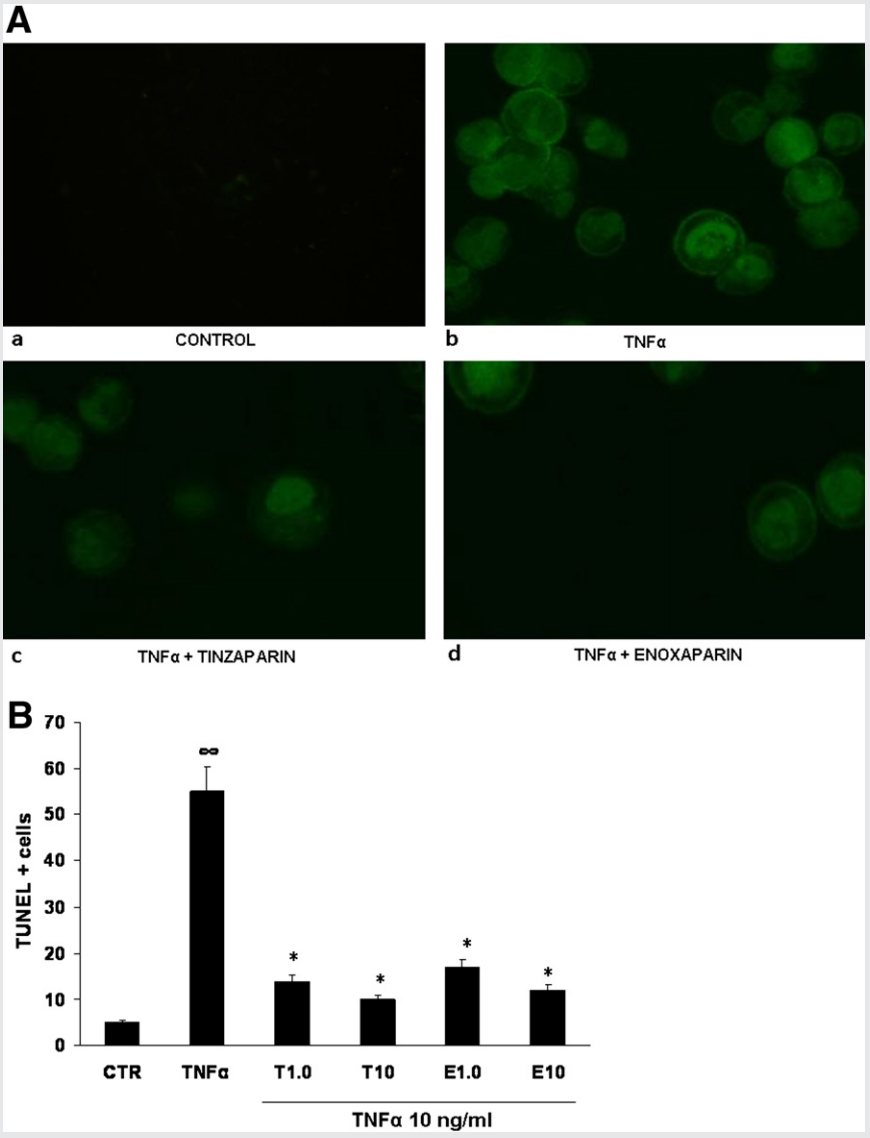
Because the family of aspartate-specific cysteinyl proteases (caspases) plays a pivotal role in the execution of programmed cell death (21, 22), we tested whether treatment of decidual cells with TNF-α resulted in activation of upstream caspase-8 and of the downstream caspase-3. In particular, we evaluated caspase-8 activity because it represents the apical caspase in the death receptor pathway (21) and caspase-3 because it has been shown to be one of the most important cell executioners for apoptosis (22).

As shown in Figure 4, LMWHs significantly reduced the amount of caspase-3 (Fig. 4A) and -8 (Fig. 4B) activity triggered by TNF-α (TNF-α = 100%; caspase-3: 75% and 78% in presence of enoxaparin (10 IU/mL) or tinzaparin (10 IU/mL), respectively; caspase-8: 50% and 41% enoxaparin (10 IU/mL) and tinzaparin (10 IU/mL), respectively).

DISCUSSION

The involvement of HB-EGF in decidualization and endometrial maturation is suggested by a number of observations. The local application of HB-EGF–soaked beads promotes decidualization in the murine uterus (23); the expression of HB-EGF mRNA in endometrial cells in vitro is regulated by estrogen and P (17), and recently it has been shown that exogenous HB-EGF stimulates expression of interleukin-11, a cytokine known to play a role in the decidualization process in mice (24). Furthermore, Chobotova et al. (12) presented evidence for the role of HB-EGF in the decidualization of human

FIGURE 3



Effect of low-molecular-weight heparins on tumor necrosis factor (TNF) α–induced apoptosis: TUNEL assay. (A) Representative microphotographs of TUNEL staining. Images obtained by fluorescence microscopy (×400) showed apoptotic changes in nuclei of decidual cells treated with TNF-α: irregular chromatin, margination, and shrinking into blocks (pycnotic nuclei). (a) Untreated cells); (b) TNF-α (10 ng/mL)–treated cells; (c) TNF-α plus tinzaparin (T; 10 IU/mL)–treated cells; (d) TNF-α plus enoxaparin (E; 10 IU/mL)–treated cells. (B) Quantitative analysis of TUNEL positive decidual cells. Results are expressed as number of TUNEL-positive cells, means ± SE of four independent experiments. $^{\infty}$Statistical significance versus untreated cells (CTR): $P<.001$. *Statistical significance versus TNF-α: $P<.01$.

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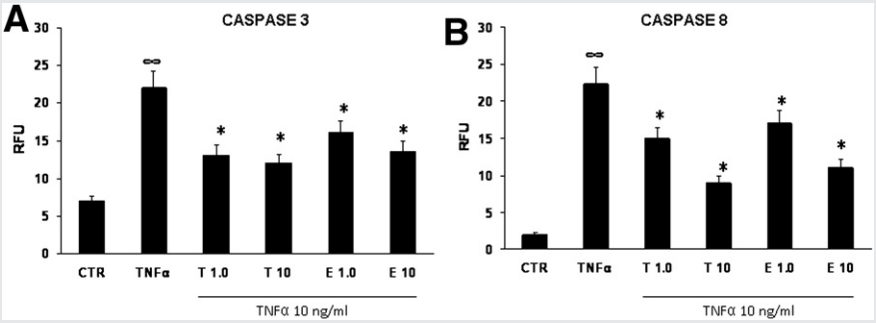
endometrial stromal cells. The use of two different inhibitors of HB-EGF activity, the diphtheria toxin analogue CRM197 and neutralizing HB-EGF antibodies, results in decreased levels of prolactin and insulin-like growth factor–binding protein 1 (17).

It has been suggested that accumulation of HB-EGF at sites of implantation may activate the downstream signaling of its receptors, human epidermal growth factor receptors 1 and 4, for cytotrophoblast survival (25). Consistently, HB-EGF down-regulation was reported in patients with preeclampsia, in association with impaired extravillous trophoblast invasion, spiral artery remodeling, and increased apoptosis (25).

In the present study, we attempt to clarify the effect of two LMWHs (tinzaparin and enoxaparin) on HB-EGF expression in human decidua. LMWHs are increasingly used to improve pregnancy outcome in women suffering from thrombophilia and recurrent miscarriages (26, 27). The theory of placental thrombosis and infarction as a cause for early pregnancy loss was the original rationale for this thromboprophylactic application of heparins (28). However, intravascular or intervillous blood clots are rarely found in

FIGURE 4



Fluorimetric assays of caspase 3 (A) and caspase 8 (B). Data are expressed as relative fluorescence units (RFU). Results are means ± SE of four independent experiments. CTR = untreated cells; E = enoxaparin; T = tinzaparin; TNF-α = tumor necrosis factor α. ∞Statistical significance versus untreated cells: $P<.001$. *Statistical significance versus TNF-α: $P<.05$.

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first-trimester placenta and decidua samples from patients suffering early miscarriage (29). Heparins have also been shown to be effective in preventing adverse pregnancy outcome in women with recurrent miscarriage without apparent cause or with inherited thrombophilia (30). Furthermore, LMWHs given in the luteal phase of the menstrual cycle seem to be beneficial in improving the implantation rate, as well as the live birth rate, in women with repeated implantation failure treated in an in vitro fertilization program (31). Taken together, these clinical observations suggest molecular effects of heparin beyond its classic anticoagulatory activity.

The present study is the first to show increased decidual expression of HB-EGF in the presence of LMWHs. We demonstrated that LMWHs, particularly tinzaparin, induced a significant increase in decidual HB-EGF expression and secretion in a dose-dependent manner at concentrations that are reached in vivo at therapeutic doses (1 and 10 IU/mL).

It is known that tinzaparin differs from other LMWHs, because it is manufactured by enzymatic degradation, rather than chemical depolymerization, of porcine unfractionated heparin (32). In addition, it has a higher mean molecular weight (6,500 Da compared with 4,300 Da for enoxaparin) and is more heavily sulphated than other LMWHs (33). Based on our results, we can hypothesize that the effects of LMWHs on the HB-EGF decidual production are independent of their anticoagulant functions, but rather depend on the charge and the size of these polysulphated glycosaminoglycans (34).

A wealth of data indicate that a preponderance of proinflammatory T_H1 cytokines, such as TNF-α and interferon-γ, predisposes for early fetal loss as well as late pregnancy complications such as preeclampsia (35). In vitro studies have shown that TNF-α induces apoptosis of cytotrophoblasts, suggesting that aberrant expression of TNF-α may have harmful effects on placental development and function (36, 37). Furthermore, TNF-α stimulates neutrophil and monocyte release of inflammatory mediators, including reactive oxidants, proteolytic enzymes, and complement components, that directly damage decidual tissue and accelerate C5 cleavage, triggering the release of more TNF-α, which further amplifies local inflammation (38).

A large body of evidence supports the concept that heparin has antiinflammatory activities. In a mouse model of antiphospholipid syndrome, Girardi et al. (39) showed the essential and causative role of complement activation in pregnancy loss and fetal growth restriction. They demonstrated that heparin prevents placental damage as a result of its capacity to block complement activation, rather than of its anticoagulant activity. Hochart et al. (40) examined the regulation of lipopolysaccharide (LPS)–induced proinflammatory cytokine release by LMWH-pretreated monocytes and observed the ability of heparin to inhibit LPS-induced proinflammatory cytokines and nuclear translocation of nuclear factor κB in human monocytes. Other LMWH antiinflammatory effects have been postulated to be specifically TNF-α mediated (41). Salas et al. (42) showed that systemic administration of heparin markedly reduces the inflammatory response elicited by TNF-α in vivo.

Consistent with the findings of earlier studies (17), we demonstrated that TNF-α induced apoptosis of decidual cells in a concentration- and time-dependent manner (data not shown). The morphologic features of apoptosis were detectable early during the treatment, and the proapoptotic effect of TNF-α was confirmed by the cytofluorimetric analysis of annexin V binding. Some of the molecular pathways involved in decidual apoptosis were investigated. We found that TNF-α induced apoptosis by the activation of upstream caspase-8 and caspase-3, which have been shown to be necessary in determining the nuclear alteration of apoptosis (22). Analyzing the effect on TNF-α–induced apoptosis of decidual cell cultures, we observed that both these LMWHs greatly reduced the apoptotic rate.

On TNF-α–induced apoptosis of esophageal epithelial cells, Rahman et al. (43) demonstrated that cells became resistant to apoptosis when HB-EGF was added to the cultures. They noticed significant up-regulation of the BCL2 family of genes after treatment with HB-EGF. Therefore, bearing in mind these observations and the findings of our study regarding the effects of tinzaparin on HB-EGF expression, we might suggest that one of the possible mechanisms by which LMWH promotes survival of decidual cells undergoing apoptosis in

response to inflammatory cytokines such as TNF-α could be related to its ability to induce HB-EGF secretion/production.

In conclusion, although LMWHs represent a treatment for prevention of some pregnancy complications, little is known about the cellular mechanisms by which heparin exerts its effect on trophoblast and decidual functions.

LMWH preparations differ in their molecular weight, half-life, and anticoagulant activity. Therefore, additional in vitro and in vivo studies are needed to evaluate the efficacy of various LMWH preparations in preventing trophoblast/decidual apoptosis in response to pathologic signals. The outcomes of these studies should support large randomized placebo-controlled trials assessing the efficacy of LMWHs in the prevention of pregnancy complications associated or not with an identifiable thrombophilic condition.

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SUPPLEMENTAL TABLE 1

Primers and fluorescent probes used in real-time polymerase chain reaction.

Gene	Primer or probe	Sequence
HB-EGF	HB-EGFa	TACCTATGACCACACAACCATCCT
	HB-EGFb	CCCACGATGACCAGCAGACA
	HB-EGFpr	6FAM-AGATGACAGCACCACAGCCACCACGG-TAMRA
GAPDH	GAPDHa	GGACCTGACCTGCCGTCTAG
	GAPDHb	TAGCCCAGGATGCCCTTGAG
	GAPDHpr	TexasRed-CCTCCGACGCCTGCTTCACCACCT-BHQ2

Note: 6FAM = 6-carboxyfluorescein; BHQ2 = Black Hole Quencer 2; HB-EGF = heparin-binding epidermal growth factor–like growth factor; TAMRA = 6-carboxy-N,N,N′,N′-tetramethylrhodamine; Texas Red = trademarked product from Molecular Probes.

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