Heparin is a promising agent for the treatment of endometriosis-associated fibrosis

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Objective: To assess the use of heparin for the medical treatment of endometriosis-associated fibrosis.

Design: The effects of heparin on the endometriotic stromal cells (ECSCs)-mediated contractility were investigated.

Setting: Research laboratory at a medical school.

Patient(s): Endometriotic tissues from nine patients were used.

Intervention(s): Endometriotic stromal cells were cultured three dimensionally in the presence of heparin.

Main Outcome Measure(s): The contractility of ECSCs was assessed by collagen gel contraction assay. Heparin-induced morphological changes of ECSCs were evaluated by laser scanning microscopy. The expression of contractility-related molecules in ECSCs was examined by Western blot analysis.

Result(s): In the presence of 10% fetal bovine serum, treated ECSCs showed significant collagen gel contractility (75.9% decrease in surface area after 48 hour vs. 0 hour controls). Endometriotic stromal cell-mediated gel contraction was significantly attenuated in the presence of heparin in a dose-dependent manner (55.7% reduction of the gel contraction at a concentration of 100 μg/mL of heparin sodium versus untreated controls after 48 hours). Heparin suppressed the ECSC attachment to collagen fibers. The expression of α-smooth muscle actin, Ras homology (Rho) A, Rho-associated coiled-coil-forming protein kinase (ROCK)-I, and ROCK-II was down-regulated by heparin administration.

Conclusion(s): The present study suggests that heparin is a promising agent for the treatment of endometriosis-associated fibrosis. The inhibition of myofibroblastic differentiation, the attenuation of attachment to collagen fibers, and the suppression of Rho–ROCK-mediated pathway activation in ECSCs are involved in the action mechanisms of heparin. (Fertil Steril 2010;94:46–51. ©2010 by American Society for Reproductive Medicine.)

Key Words: Heparin, endometriosis, contractility, Rho–ROCK-mediated pathway, fibrosis

Endometriosis, a disease affecting 3% to 10% of women of reproductive age, is characterized by the ectopic growth of endometrial tissue (1). Histologically, this disease is characterized by dense fibrous tissue surrounding the endometrial gland and stroma (2–4). The presence of fibrosis has been demonstrated in superficial peritoneal lesions as well as in ovarian and deep infiltrating endometriosis (2, 4–6). During the development and progression of endometriotic lesions, excess fibrosis may lead to scarring, chronic pain, and the alterations of tissue function that are the characteristics of this disease (2–4, 5). α-Smooth muscle actin (SMA)-positive fibroblastic cells were frequently detected in the fibrotic areas associated with endometriosis of the peritoneum, ovary, rectovaginal septum, and uterosacral ligaments (2, 4, 6). Immunohistochemical analysis led Anaf et al. (6) to suggest that endometriotic stromal cells can differentiate to α-SMA-positive myofibroblasts. It has been suggested that type I collagen is a major contributor to endometriosis-associated fibrosis (5, 7).

We have established a three-dimensional (3D) collagen gel culture system with human endometriotic stromal cells (ECSCs) as a model of fibrosis formation in endometriosis (8, 9). Endometriotic stromal cells were cultured in floating collagen lattices to reorganize and compact the collagen fibers, resulting in contraction of the collagen gels. This culture system provided a model of mechanically relaxed tissue with low tensile strength comparable to the early stages of endometriotic lesions. Using this model, we have demonstrated that ECSCs cultured in floating 3D collagen gels have an enhanced contractile profile and a greater ability to differentiate into a myofibroblast phenotype compared than do normal endometrial stromal cells (8). Activation of the Ras homology (Rho)-associated coiled-coil-forming protein kinase (ROCK)-mediated pathway in ECSCs may be involved in this phenomenon (8, 9).

Heparin is an analogue of heparan sulfate, a unique class of macromolecule that is widely expressed on the cell surface and in the extracellular matrix (10). It is a commonly used anticoagulant drug (11). Heparin achieves its anticoagulant
activity by interacting with antithrombin III (12–14). Recently, heparin has been shown to inhibit the gel contraction mediated by dermal fibroblasts (15, 16), endothelial cells (16), and vascular smooth muscle cells (17). In the present study, we investigated the effect of heparin on the contractility of ECSCs and clarified the underlying mechanisms of ECSC-mediated collagen gel contraction. We also discuss herein the therapeutic strategy for endometriosis-associated fibrosis.

MATERIALS AND METHODS

ECSC Isolation Procedure and Cell Culture Conditions

Endometriotic tissues were obtained from premenopausal patients in the mid to late proliferative phase who had undergone salpingo-oophorectomy or cystectomy for ovarian endometriotic cysts (n = 9, aged 27–39 years). All patients were free of any hormonal treatments at least for 12 months prior to the operation. This study was approved by the institutional review board of the Faculty of Medicine, Oita University. Endometriotic stromal cells were isolated from ovarian endometriotic tissues by enzymatic digestion as previously described (18). Isolated ECSCs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 IU/mL of penicillin (Gibco-BRL, Gaithersburg, MD), 50 mg/mL of streptomycin (Gibco-BRL), and 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL) at 37°C in 5% CO2 in air.

Endometriotic stromal cells in monolayer culture after the third passage were >99% pure, as analyzed by immunocytochemical staining with antibodies to vimentin (V9; Dako, Copenhagen, Denmark), CD10 (SS2/36; Dako), cytokeratin (C226, Dako), and leukocyte common antigen (CD45) (PD7/26, Dako), and were used for the following experiments (18). Cells isolated from each individual patient were used for one experiment at a time. Each experiment was performed in triplicate and conducted at least four times.

Collagen Gel Contraction Assay

Cellular collagen gel contraction assays were performed as previously described (8, 19). A sterile solution of acid-soluble collagen type I purified from porcine tendons (Cellmatrix type I-A; Nitta Gelatin Inc., Osaka, Japan) was prepared according to the manufacturer’s instructions. Endometriotic stromal cells were embedded in collagen gel and cultured three dimensionally. Briefly, ECSCs were suspended in the collagen solution (3.0 × 10^5 cells/mL). The collagen/cell mixture (2 mL/plate) was dispensed into 35-mm culture plates (Corning, New York, NY) coated with 0.2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO); the mixture was allowed to polymerize at 37°C for 30 minutes. Immediately after polymerization, 1 mL of culture medium containing heparin sodium (Sigma-Aldrich) (final concentration: 1–100 μg/mL) was added to each plate. After incubation for 48 hours, the collagen gels were photographed, and the area of the gel surface was measured with the public domain Image program 1.61 developed at the National Institutes of Health (Bethesda, MD). The incubation time was determined by background experiments.

Assessment of the Morphology of ECSCs in 3D Collagen Gel Culture

At the cessation of the 3D culture, the contracted collagen gels were fixed and incubated in 0.02% collagenase type I (Sigma-Aldrich) in PBS for 40 minutes at 37°C. ECSCs were isolated from collagen gels by centrifugation, and whole-cell extracts were prepared by lysing the cells in lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediamine tetraacetic acid, 50 mM NaF, and 0.1% phenylmethylsulfonylfluoride). The suspension was centrifuged at 15,000 rpm for 15 minutes at 4°C, and the supernatant was collected. The total protein concentration was quantified using the Coomassie protein assay reagent (Pierce, Rockford, IL). The whole cell protein extract was resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel under reduced conditions. After transfer to Immobilon-P transfer membrane (Millipore, Bedford, MA), the protein was stained with Ponceau S (Sigma-Aldrich) to verify uniform loading and transfer. Membranes were blocked with 5% skim milk (Becton-Dickinson, Sunnyvale, CA) in Tris-buffered saline with Tween 20 (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) (TBS-T) overnight and subsequently incubated with primary antibodies (α-SMA [1A4, R&D Systems, Minneapolis, MN], Rho A, ROCK-I [Sigma-Aldrich], ROCK-II [Santa Cruz Biotechnology, Santa Cruz, CA, USA], and glyceraldehydes-3-phosphate-dehydrogenase [GAPDH; Ambion, Austin, TX]) at appropriate dilutions for 1 hour at room temperature. The membrane was washed three times with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Subsequently, the membrane was washed three times with TBS-T and analyzed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Chicago, IL).

Statistical Analysis

Data are presented as means ± SD of representative experiments and analyzed using the Bonferroni/Dunn test with StatView 4.5 (Abacus Concepts, Berkeley, CA). A value of P < .05 was accepted as statistically significant.
RESULTS
Effects of Heparin on ECSC-Mediated Collagen Gel Contraction
As a preliminary experiment, we evaluated the effects of heparin on the collagen gel contraction without the presence of ECSCs. Heparin did not induce the gel contraction in the absence of ECSCs.

The effect of heparin on the contractility of ECSCs was evaluated using the collagen gel contraction assay. In the presence of 10% FBS, untreated ECSCs showed significant collagen gel contractility (75.9% decrease in surface area after 48 hour vs. 0 hour controls). The areas of gel surface after 48 hours under untreated conditions were defined as 100%. As shown in Figure 1, ECSC-mediated gel contraction was significantly attenuated in the presence of heparin in a dose-dependent manner (55.7% reduction of the gel contraction at a concentration of 100 \( \mu \text{g/mL} \) of heparin sodium, \( P < .0001 \) vs. untreated controls, Bonferroni/Dunn test). Similar results were obtained from seven repeated experiments.

Morphology of ECSCs in 3D Collagen Gel Culture
When untreated ECSCs were cultured in 3D collagen gels, the cells adhered to the collagen fibers and contracted the initially loose network to a dense tissue-like structure (Fig. 2). Their morphology was dendritic to stellate. In contrast, the contractile force of the heparin-treated ECSCs was weak. Their morphology remained round to polygonal, and they did not adhere to the collagen fibers in comparison to the untreated ECSCs. Similar results were obtained from four repeated experiments.

Expression of \( \alpha\)-SMA, Rho A, ROCK-I, and ROCK-II Proteins in ECSCs
To analyze the underlying mechanisms of the action of heparin on the ECSC-mediated contractility, the expression of \( \alpha\)-SMA, RhoA, ROCK-I, and ROCK-II proteins in ECSCs was evaluated. As shown in Figure 3, the levels of \( \alpha\)-SMA, RhoA, ROCK-I, and ROCK-II protein in 3D cultured ECSCs were strongly inhibited by the addition of heparin sodium (100 \( \mu \text{g/mL} \)). Glyceraldehydes-3-phosphate-dehydrogenase protein was detected in all samples at almost equal levels. Similar results were obtained from five repeated experiments.

DISCUSSION
We have established a 3D collagen gel culture system with ECSCs as a model of fibrosis formation in endometriosis (8). Research on endometriotic stromal cell biology in 3D collagen matrices offers new opportunities to understand the reciprocal and adaptive interactions that occur between cells and the surrounding matrix in a tissue-like environment (9). Such interactions are integrated with the regulation of endometriotic tissue morphogenesis and the dynamics that characterize endometriosis-associated fibrosis. We used the stromal cells from ovarian endometriomas in the present
study, because of the following two reasons: [1] a significant amount of fibrosis has been demonstrated in the superficial peritoneal lesions and ovarian endometriomas as well as in the deeply infiltrating endometriotic lesions. [2] Isolation of endometriotic stromal cells from the superficial peritoneal lesions or deeply infiltrating lesions is very difficult, whereas the isolation procedure from ovarian endometriomas is well established. Endometriotic stromal cells in 3D culture have an enhanced contractile profile and a greater ability to differentiate into a myofibroblast phenotype compared with normal endometrial stromal cells. Activation of the Rho–ROCK-mediated pathway in ECSCs may be involved in this phenomenon (8). Agents that suppress fibrosis formation by targeting the Rho–ROCK-mediated pathway as well as myofibroblast differentiation may be promising drugs for the treatment of endometriosis (9). Using this model, we demonstrated for the first time that heparin inhibited the collagen gel contraction mediated by ECSCs. Because heparin has been clinically used for a long time as an anticoagulant drug (11), it is suggested that it can be applied to the medical treatment and prevention of endometriosis-associated fibrosis. Treatment options of heparin may include intraoperative and postoperative intraperitoneal injection and postoperative subcutaneous injection.

The mechanisms of heparin action on the contractility of fibroblastic cells have been studied in other tissues (15, 16). Heparin has been shown to inhibit the gel contraction mediated by dermal fibroblasts (15, 16). Several mitogens have been identified recently as members of the fibroblast growth factor (FGF) family, all of which bind to heparin-affinity resins, presumably reflecting their physiologically significant partitioning into cellular and basement membrane heparan proteoglycans in vivo (20–22). Despite the fact that, as mitogens, they increase the number of fibroblasts, both acidic FGF and basic FGF decrease the ability of fibroblasts to contract collagen fibers (23, 24). Stabilizing these FGFs, heparin enhances their activity in vitro and in vivo (24–29). In our experimental model, 10% heat-inactivated FBS that may contain various kinds of FGFs was added in the culture medium. It is suggested that similar mechanisms may be present in the ECSC-mediated collagen gel contraction.

ECSCs were cultured in floating collagen lattices to reorganize and compact the collagen fibers, resulting in contraction of the collagen gels. The present study is the first to evaluate the effects of heparin on ECSC-mediated contractility. Morphologic observation of heparin-treated ECSCs suggested that heparin attenuated the attachment of ECSCs to collagen fibers, resulting in the inhibition of the contraction of collagen gels. However, heparin suppressed the expression of α-SMA in ECSCs. Because α-SMA is the most reliable marker for myofibroblast differentiation (30, 31), it is considered that heparin may inhibit ECSCs from differentiating into myofibroblast phenotypes. In the present study, we have also demonstrated that heparin inhibited the expression of RhoA, ROCK-I, and ROCK-II protein in ECSCs. Because the activation of the Rho–ROCK-mediated pathway is involved in

FIGURE 2

Morphological features of ECSCs observed by laser scanning microscopy. Untreated ECSCs cultured in 3D collagen gels adhered to the collagen fibers and contracted the initially loose network to a dense tissue-like structure. Their morphology was dendritic to stellate. In contrast, the contractile force of the ECSCs treated with 100 μg/mL of heparin sodium was weak. Their morphology remained round to polygonal, and they did not adhere to the collagen fibers in comparison to the untreated ECSCs.

the ECSC-mediated contractility (8), it is suggested that heparin may attenuate ECSC-mediated contractility by inhibiting the Rho–ROCK-mediated pathway in addition to influencing the ECSC differentiation. Interestingly, heparin has been demonstrated to activate the Rho–ROCK-mediated signaling pathway in vascular smooth muscle cells (17), suggesting the presence of distinct action mechanisms in ECSCs. Further studies are necessary to fully elucidate the action mechanisms of heparin on the ECSC-mediated contractility.

In conclusion, we demonstrated for the first time that heparin attenuated the collagen gel contractility mediated by ECSCs. The inhibition of myofibroblastic differentiation, the attenuation of attachment to collagen fibers, and the suppression of Rho–ROCK-mediated pathway activation in ECSCs are involved in the action mechanisms of heparin-induced collagen gel contractility, all of which could be involved in the pathogenesis of endometriosis-associated fibrosis. Although further in vitro studies and in vivo studies using animal models are necessary, it is considered from the present study that heparin is a promising agent for the treatment of endometriosis-associated fibrosis, one of the major pathogeneses of endometriosis. Furthermore, medical treatment that modulates the Rho–ROCK-mediated pathway as well as myofibroblast differentiation may provide a novel therapeutic strategy for the treatment of endometriosis.

In addition, our experimental system of ECSCs in 3D collagen gel culture proved to be suitable for testing new compounds for the treatment of endometriosis-associated fibrosis.

REFERENCES