Mechanical stretch upregulates IGFBP-1 secretion from decidualized endometrial stromal cells

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Harada, Miyuki, Yutaka Osuga, Yuri Takemura, Osamu Yoshino, Kaori Koga, Yasushi Hirota, Tetsuya Hirata, Chieko Morimoto, Tetsu Yano, and Yuji Taketani. Mechanical stretch upregulates IGFBP-1 secretion from decidualized endometrial stromal cells. Am J Physiol Endocrinol Metab 290: E268–E272, 2006. First published August 23, 2005; doi:10.1152/ajpendo.00334.2005.—Decidualization is an essential process of endometrial differentiation for embryo implantation and maintenance of pregnancy. Recently, uterine movement-induced mechanical stress was noticed to have possible effects on endometrial functions. In this study, we addressed the possible effect of mechanical stretch on the process of decidualization of endometrial stromal cells (ESC). ESC were cultured on flexible-bottomed culture plates. After decidualization was achieved with estradiol and progesterone for 12 days, cultures were continued for 24 h with or without cyclic stretch (25% elongation) in serum-free conditions at a rate of 2 cycles/min using a computer-operated cell tension system. Concentrations of insulin-like growth factor-binding protein-1 (IGFBP-1), a marker of decidualization, in the conditioned medium were measured by specific ELISA, and IGFBP-1 mRNA expression in the ESC was measured by RT-PCR. Cyclic stretch remarkably increased IGFBP-1 secretion from decidualized ESC. It also increased IGFBP-1 mRNA in decidualized ESC. The increase in IGFBP-1 secretion was inhibited by actinomycin D but not by indomethacin, PD-98059, or H-89. Conditioned medium of decidualized ESC cultured with cyclic stretch increased IGFBP-1 secretion from decidualized ESC cultured under stationary conditions. These findings imply that uterine movement modulates decidualization of the endometrium and has a regulatory effect on reproduction.

Insulin-like growth factor-binding protein-1; endometrium; decidualization; prostaglandin E2; indomethacin

Decidualization, a process of endometrial differentiation, is essential for embryo implantation and maintenance of pregnancy. During the process of decidualization, characteristics of endometrial cells change profoundly. This was demonstrated by marked alterations in the expression of numerous genes in decidualized human endometrial stromal cells (ESC) in vitro (17). Progesterone is a well-known inducer of decidualization, whereas prostaglandin E2 (PGE2) and relaxin enhance the process via activation of cAMP signaling (8, 11, 16, 20). However, the complex mechanisms that regulates decidualization remains to be elucidated.

Recently, uterine movement, a mechanical process, has been noticed to play important roles in fertility (4). Subendometrial myometrium exerts wave-like activity throughout the menstrual cycle (3, 13), and the movement is believed to support embryo and sperm transfer. The peristaltic uterine movement is also suggested to be involved in other uterus-related events such as endometriosis and menstruation. Recently, we (9) suggested that the subendometrial myometrial movement is transduced to a biochemical signal in the endometrium and may play roles in endometrium-related physiological and pathological events.

Given that the mechanical stress associated with uterine peristalsis alters biochemical characteristics of the endometrium, it is plausible that decidualization, a differentiation process of the endometrium, may be affected by the uterine movement. To address the issue, we conducted an in vitro study to see the effect of mechanical stretch on the production of IGFBP-1, a representative marker of decidualization, in ESC.

Materials and Methods

Reagents and materials. Type I collagenase and antibiotics (a mixture of penicillin, streptomycin, and amphotericin B) were purchased from Sigma (St. Louis, MO). DEMM-Ham’s F12 (DMEM-F12) medium was from Invitrogen (Carlsbad, CA). Indomethacin, a cyclooxygenase inhibitor, and actinomycin D, an inhibitor of RNA synthesis, were purchased from Sigma. PD-98059, an inhibitor of p42/44 MAPK, and H-89, an inhibitor of PKA, were from Calbiochem (La Jolla, CA). Charcoal-stripped fetal bovine serum was from HyClone (Logan, UT). Deoxyribonuclease I was from Takara (Kyoto, Japan).

Patients and samples. Endometrial tissues were obtained from 48 women without endometriosis undergoing hysterectomy for uterine fibroids (n = 43) or adenomyosis (n = 5). The mean age of women was 43.8 yr (SD 2.5). All the women had regular menstrual cycles, and none had received hormonal treatment for ≥6 mo before surgery. At the time of surgery, 20 women were in the proliferative phase and 28 were in the secretory phase. The tissues collected by curettage under sterile conditions were processed for primary cell cultures. The experimental procedures were approved by the institutional review board of the University of Tokyo, and signed informed consent for the use of the endometrium was obtained from each woman.

Isolation and culture of human ESC. The isolation and culture of human ESC were conducted as previously reported (23). Briefly, the tissues were minced into small pieces and incubated in DEMM-Ham’s F-12 containing 0.25% type I collagenase and 15 U/ml deoxyribonuclease I for 60 min at 37°C with agitation. The resultant dispersed endometrial cells were separated by filtration through a 40-μm nylon mesh (Becton-Dickinson, Franklin Lakes, NJ). Stromal cells in the filtrate were collected and plated in a 100-mm culture plate and kept at 37°C in a humidified 5% CO2-95% air environment. At first passage, the cells were seeded onto six-well flexible-bottomed culture plates coated with collagen I (Flexcell International, Hillsborough, NC) at a plating density of 4 × 105 cells/well. The purity of stromal...
cell preparations was >95%, as judged by positive cellular staining for vimentin. ESC from individual patients, not a mixture of multiple patients, were used in each experiment.

**In vitro decidualization.** In vitro decidualization was achieved as described previously (23). Briefly, after 70% confluence in the six-well plate, the cells were rinsed and treated with 2.5% charcoal-stripped FBS in the presence of 10 ng/ml (36.7 nM) estradiol (E) and 100 ng/ml (318 nM) progesterone (P) or 0.1% ethanol vehicle (control). Culture media were collected, and wells were replenished every 3 days. After 12 days of this E+P treatment, ESC achieved decidualization and were used for subsequent experiments as decidualized ESC.

**Application of stretch.** Stretch was applied to cells as previously reported (9). Briefly, plated cells were stretched in a cyclic fashion at a rate of 2 cycles/min (23 s stretch and 7 s release) using a Flexcell tension system FX-4000T (Flexcell International), a computer-operated, vacuum-driven stretch device. We chose the cycles in light of the fact that elongation levels of endometrial cells in the physiological environment are unknown, we set the levels with reference to our previous study (9). Control cells were cultured under identical conditions but remained stationary.

**Treatments of decidualized ESC.** To examine whether autocrine/paracrine factor(s) induced IGFBP-1 production, conditioned media had been prepared by collecting the culture media of decidualized ESC treated with and without cyclic stretch for 24 h. In an independent setting, ESC had been plated onto plastic-bottomed six-well plates at first passage and underwent in vitro decidualization. On day 12, total culture media were removed, and 40% of the volume of the media was replaced with the prepared conditioned media, the rest of its volume was filled with 2.5% charcoal-stripped FBS containing E+P, and the incubation was continued for 24 h to examine the secretion of IGFBP-1.

To evaluate the effect of inhibitors such as indomethacin, actinomycin D, PD-98059, and H-89 on decidualized ESC, the complete media were removed and replaced with 2.5% charcoal-stripped FBS containing E+P plus each inhibitor. After 1 h of preincubation, stretch was applied to the cells for 24 h. At the end of each experiment, the conditioned media were collected, centrifuged, and stored at −80°C. At the same time, the cells were harvested for total protein measurement.

**RNA extraction, RT, and real-time quantitative PCR.** Total RNA was extracted from ESC, by use of an RNeasy minikit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse transcribed in a 20-μl volume using Rever Tra Ace-α (Toyobo, Tokyo, Japan) according to the manufacturer’s instructions. To assess IGFBP-1 mRNA expression, 0.15 μg of cDNA in 20-μl volume was applied to real-time quantitative PCR and data analysis using Light Cycler (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Expression of IGFBP-1 mRNA was normalized to RNA loading for each sample, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. IGFBP-1 primers (sense, 5′-GAGAGCAGGGAGATAACTGG-3′; antisense, 5′-TTGGTGACATGGAGAGCCTTCG-3′) were chosen to amplify a 131-bp fragment. GAPDH primers were purchased from Toyobo. The PCR conditions for amplification of IGFBP-1 were 30 cycles at 95°C for 15 s, 67°C for 8 s, and 72°C for 5 s, followed by a melting-curve analysis.

**Measurement of IGFBP-1.** Concentrations of IGFBP-1 in the media were measured using a specific ELISA kit (R&D Systems, Minneapolis, MN). The concentrations were normalized to total protein of cell lysates from each well of the culture plates.

**Measurement of PGE2.** Concentrations of PGE2 in the media were measured using a specific EIA kit (Cayman, Ann Arbor, MI). The concentrations were normalized to total protein of cell lysates from each well.

**Statistical analysis.** Data were analyzed using ANOVA with post hoc analysis (Fisher’s protected least significance) for multiple comparisons. *P < 0.05 was considered to be significant.

**RESULTS**

Stretch increased secretion of IGFBP-1 from decidualized ESC but not from nondecidualized ESC. We first conducted time course experiments to study effects of stretch on the secretion of IGFBP-1 from decidualized ESC treated with E+P.
for 12 days and nondecidualized ESC treated with control vehicle for 12 days (Fig. 1). Under the stationary condition, decidualized ESC secreted a remarkably greater amount of IGFBP-1 than nondecidualized ESC (9.187 ± 0.736 and 0.0819 ± 0.0098 ng/ml, respectively). Cyclic stretch significantly increased the secretion of IGFBP-1 from decidualized ESC from 8 h compared with that from decidualized ESC kept stationary. At 24 h, the IGFBP-1 secretion was 25-fold over control. On the other hand, stretch of nondecidualized ESC did not increase IGFBP-1 secretion compared with the stationary control (data not shown).

**Effect of conditioned media of stretch-treated decidualized ESC on IGFBP-1 secretion from decidualized ESC.** We addressed whether the stretch-induced IGFBP-1 secretion from decidualized ESC is regulated by autocrine/paracrine factors. As shown in Fig. 2, the conditioned media of stretch-treated decidualized ESC induced a significant increase (15-fold over control) in IGFBP-1 secretion from decidualized ESC under stationary condition compared with that of decidualized ESC without stretch.

**Effect of indomethacin on stretch-induced secretion of PGE2 and IGFBP-1 from decidualized ESC.** Considering that PGE2 enhances decidualization of E+P-treated ESC (5), we examined whether stretch-induced secretion of IGFBP-1 is mediated by PGE2 in an autocrine or paracrine fashion. As shown in Fig. 3A, stretch induced PGE2 production from decidualized ESC. The PGE2 secretion was inhibited by indomethacin. On the other hand, indomethacin did not alter stretch-induced IGFBP-1 secretion (Fig. 3B).

**Stretch induced IGFBP-1 expression by activating IGFBP-1 transcription, not altering IGFBP-1 mRNA stability.** Stretch induced IGFBP-1 mRNA expression in decidualized ESC from 6 h compared with the control (Fig. 4A). Preincubation of

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**Fig. 3.** Effects of indomethacin, a cyclooxygenase inhibitor, on stretch-induced secretion of prostaglandin E2 (PGE2; A) and IGFBP-1 (B). Treatment of decidualized ESC with indomethacin of indicated concentrations or vehicle (0.1% ethanol) was started 1 h before cyclic stretch was applied. After being stimulated by cyclic stretch or kept stationary for 24 h, conditioned media were collected and assayed for PGE2 concentrations by EIA and IGFBP-1 concentrations by ELISA. Values are means ± SE of quadruplicate cultures. Both PGE2 and IGFBP-1 concentrations were normalized to total protein of cell lysates from each well of the culture plates. *P < 0.001 vs. without stretch. **P < 0.001 vs. stretch without indomethacin.

**Fig. 4.** Effects of cyclic stretch on IGFBP-1 mRNA levels in decidualized ESC. A: time course effects of stretch on expression of IGFBP-1 mRNA in decidualized ESC. Total RNA isolated from ESC stimulated by stretch for the indicated hours were reverse transcribed and amplified by real-time PCR using primers of IGFBP-1. Data were calculated by subtracting signal threshold cycles (Ct) of the internal standard (GAPDH) from Ct of IGFBP-1. Values are means ± SE of quadruplicate cultures. *P < 0.05, **P < 0.01, ***P < 0.001 vs. without stretch. B: effects of actinomycin D, an inhibitor of RNA synthesis, on stretch-induced secretion of IGFBP-1 from decidualized ESC. Treatment of decidualized ESC with actinomycin D (5 μg/ml) or vehicle (0.1% DMSO) was started 1 h before cyclic stretch was applied. After being stimulated by cyclic stretch or kept stationary for 24 h, conditioned media were collected and assayed for IGFBP-1 concentrations by ELISA. Values are means ± SE of quadruplicate cultures. IGFBP-1 concentrations were normalized to total protein of cell lysates from each well of the culture plates. *P < 0.001 vs. stretch without actinomycin D.
 decidualized ESC with actinomycin D for 1h before application of stretch almost completely abrogated the stretch-induced IGFBP-1 secretion (Fig. 4B).

Effects of PD-98059 or H-89 on stretch-induced production of IGFBP-1. Neither PD-98059 (25 μM), a specific inhibitor of p42/44 MAPK, nor H-89 (25 μM), a specific inhibitor of PKA, affected the stretch-induced secretion of IGFBP-1 from decidualized ESC (data not shown).

DISCUSSION

In the present study, we demonstrated that cyclic stretch increased the secretion of IGFBP-1 from E+P-decidualized ESC. The stimulatory effect was not observed in nondecidualized ESC. Autocrine/paracrine factor(s) and gene transcription are suggested to mediate the stimulatory effect.

We (9) have previously reported that mechanical stretch induced IL-8 production from ESC and that the effect might be involved in menstruation and endometriosis. Similarly, mechanical stretch has been shown to increase IL-8 secretion from alveolar epithelial cells (21, 22) and myometrial cells (12, 18). Mechanical stretch also upregulates the expression of cyclooxygenase-2 in ESC (our unpublished data) as well as in myometrial cells (19). These findings imply that mechanical stretch can be transduced into inflammation-related chemical mediators. In contrast, our present finding is novel in that mechanical stretch stimulates the production of IGFBP-1, a marker of decidualization, suggesting that mechanical stretch stimulates differentiation of ESC.

Decidualization is a prerequisite for the establishment of pregnancy. Inversely, impairment of decidualization may cause infertility. Thus the present findings imply that mechanical stretch may affect implantation and pregnancy via regulation of decidualization. Viewed in this way, it is tempting to notice that uterine leiomyoma is a possible cause of infertility (2). A recent study using MRI clearly demonstrated that uterine leiomyoma dysregulates peristalsis with submucosal uterine leiomyoma, a typical leiomyoma closely related to infertility (14). Given that disturbed uterine movement impairs regular mechanical stress on ESC, it is conceivable that uterine leiomyoma dysregulates the proper decidualization process and induces subsequent failure of implantation or pregnancy.

Interestingly, mechanical stretch did not stimulate IGFBP-1 secretion in nondecidualized ESC. In this aspect, mechanical stretch appears similar to PGE2, because PGE2 enhances decidualization of ESC with E+P, but not without E+P (5). It has been reported that PGE2 production is increased by mechanical stretch in human myometrial cells (19) and amnion cells (10). In the present study, mechanical stretch also increased PGE2 secretion from decidualized ESC. Taking these together, we suspected that PGE2 might mediate the stimulatory effect of cyclic stretch on IGFBP-1 secretion from decidualized ESC. However, indomethacin, which obviously inhibited stretch-induced PGE2 production, did not suppress the stretch-induced secretion of IGFBP-1. Accordingly, it is unlikely that PGE2 is involved in the stretch-induced IGFBP-1 secretion.

We (9) have previously reported that extracellular signal related kinase-1/2 (ERK1/2) inhibitor PD-98059 inhibited stretch-induced IL-8 secretion from ESC. On the other hand, PD-98059 did not suppress the stretch-induced increase in IGFBP-1 secretion from decidualized ESC. It is well known that activation of the PKA pathway is involved in decidualization (6, 23, 24). H-89, a PKA inhibitor, did not, however, inhibit the stretch-induced IGFBP-1 secretion. These findings imply that (an)other pathway(s) than ERK1/2 and PKA mediates the stretch-induced IGFBP-1 secretion. Further study is needed to elucidate the precise mechanism.

The present study suggests that paracrine/autocrine factors are involved in the stretch-induced IGFBP-1 secretion. Paracrine/autocrine mechanisms have also been suggested in the stretch-induced IL-8 secretion from ESC. It seems that stretch exerts multiple effects on ESC through paracrine/autocrine factors.

Another important finding in the present study is that actinomycin D, an inhibitor of gene transcription, almost completely inhibited the stimulatory effect of stretch on IGFBP-1 secretion from decidualized ESC. This finding indicates that a stretch-induced gene transcription is required for the increase of IGFBP-1 secretion. Therefore, it is unlikely that mechanical stress directly stimulates the release of intracellularly stored IGFBP-1 via physical mechanisms. It has been reported that IGFBP-1 protein production was induced by a transcriptional activation and mRNA stabilization (1, 15). Our finding that stretch increased the amount of IGFBP-1 mRNA as well as IGFBP-1 protein in decidualized ESC seems consistent with these reports.

In summary, the present study demonstrated that mechanical stretch induced the secretion of IGFBP-1 from decidualized ESC. Our findings imply that uterine movement has possible roles in implantation and pregnancy via regulation of decidualization of the endometrium.

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