Collagen gel contractility is enhanced in human endometriotic stromal cells: a possible mechanism underlying the pathogenesis of endometriosis-associated fibrosis

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BACKGROUND: Excessive fibrosis is frequently associated with endometriosis. To evaluate the involvement of the extracellular matrix contractility of endometriotic stromal cells (ECSCs) in the pathogenesis of endometriosis-associated fibrosis, we compared the collagen gel contractility of cultured ECSCs with that of normal endometrial stromal cells. To clarify the mechanism underlying collagen gel contraction by ECSCs, we also evaluated the effect of (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride, monohydrate (Y-27632), a selective Rho-associated coiled-coil-forming protein kinase (ROCK) inhibitor, on the collagen gel contraction by ECSCs. METHODS AND RESULTS: ECSCs showed enhanced collagen gel contractility in comparison with NESCs. Myofibroblastic differentiation and the increased expression of fibronectin, RhoA, ROCK-I and ROCK-II proteins were observed with ECSCs using the 3D culture. Y-27632 significantly inhibited the collagen gel contractility of ECSCs without cytotoxicity. CONCLUSIONS: The present findings suggest that the enhanced collagen contractility in ECSCs is associated with myofibroblastic differentiation, the increased expression of fibronectin and the activation of the Rho-ROCK-mediated signalling pathway, all of which may be involved in the pathogenesis of endometriosis-associated fibrosis. These results suggest that the inhibition of the Rho-ROCK-mediated signalling pathway may provide a novel strategy for the treatment of this disease. In addition, our experimental system of ECSCs using 3D collagen gel culture would be suitable for evaluating novel treatments for endometriosis.

Key words: collagen gel contractility/endometriosis/endometriotic stromal cells/fibrogenesis/Rho-ROCK pathway

Introduction

Endometriosis, a disease affecting 3–10% of women of reproductive age, is characterized by the ectopic growth of endometrial tissue (Olive and Schwartz, 1993). Histologically, this disease is characterized by endometrial gland and stroma with surrounding dense fibrous tissue (Nisolle and Donnez, 1997; Bonte et al., 2002; Itoga et al., 2003). During the development of endometriotic lesions, excess fibrosis may lead to scarring and alteration of tissue function (Matsuzaki et al., 1998). It has been suggested that type I collagen is a major contributor to endometriosis-associated fibrosis (Stovall et al., 1992; Matsuzaki et al., 1998). One approach to understanding the pathogenesis of endometriosis is to investigate the mechanisms underlying fibrogenesis associated with this disease.

During the course of granulation tissue formation, fibroblasts synthesize the connective tissue matrix, proliferate and differentiate into contractile phenotypes that are morphologically and immunohistochemically characterized by the appearance of cytoplasmic microfilaments and cytoskeletal proteins such as α-smooth muscle actin (α-SMA), actin stress fibers and vimentin (Darby et al., 1990; Sappino et al., 1990; Grinnell, 2000; Hinz et al., 2001). These fibroblasts, called myofibroblasts, play an important role in both wound contraction and healing by generating a contractile force (Tomasek et al., 2002; Hinz and Gabbiani, 2003). α-SMA is considered the most reliable marker of myofibroblastic differentiation (Tomasek et al., 2002), and a correlation between the expression of α-SMA and the contractile activity of fibroblastic cells has been suggested (Hinz et al., 2001). Myofibroblasts are present in certain normal tissues, in healing wounds and in fibrocontractive diseases (Grinnell, 1994; Ronnov-Jessen et al., 1996). α-SMA-positive fibroblastic cells are frequently detected in the fibrotic areas associated with endometriosis of the peritoneum, ovary, rectovaginal septum and uterosacral ligaments (Nisolle and Donnez, 1997; Anaf et al., 2000; Itoga et al., 2003). Immunohistochemical analyses have led Anaf et al. (2000) to suggest that endometriotic stromal cells (ECSCs) can differentiate to α-SMA-positive myofibroblasts.

The Ras homology (Rho) family of small Guanosine triphosphatase (GTPase) is known to regulate cell shape, motility,
cell–substratum adhesion and cell contraction through the reorganization of actin cytoskeletons (Ridley and Hall, 1992; Van Aelst and D’Souza-Schorey, 1997; Hall, 1998; Amano et al., 2000). RhoA is a representative Rho protein expressed in most cells. The active form of Rho is GTP-bound (Van Aelst and D’Souza-Schorey, 1997; Hall, 1998), and many polypeptides have been reported as targets of activated Rho, including Rho-associated coiled-coil-forming protein kinase (ROCK)-I/p160ROCK (Nakagawa et al., 1996) and Rho-kinase/ROCK-II (Nakagawa et al., 1996). ROCKs phosphorylate the myosin regulatory light chain of myosins (Amano et al., 1996) and the myosin-binding subunit of myosin phosphatase (Kimura et al., 1996), and inhibit myosin phosphatase activity (Kimura et al., 1996) in cultured fibroblasts and epithelial cells. Thus, the activation of ROCKs by Rho can promote the assembly of focal adhesions, actin stress fiber formation and contraction of non-muscle cells (Kimura et al., 1996; Hall, 1998; Amano et al., 2000; Riento and Ridley, 2003) in which RhoA regulates α-SMA expression (Mack et al., 2001).

The culture of fibroblasts in 3D collagen gels has been used as a model of tissue contraction that characterizes both wound repair and tissue fibrosis (Grinnell, 1994). When cultured in 3D gels composed of native type I collagen, fibroblasts induce gel contraction, a process resembling both scar contraction in normal wound healing and tissue contraction characteristic of fibrosis (Bell et al., 1979; Grinnell, 1994). The mechanism by which fibroblasts regulate the contraction of 3D collagen matrices has been shown to vary according to growth factor stimulus, mechanical environment and the differentiation state of the cells.

We have demonstrated that normal endometrial stromal cells (NESC) can contract the collagen gel matrix in vitro (Matsumoto et al., 2005; Nasu et al., 2005a). In vitro collagen gel contraction is considered to be equivalent to the wound contraction process in vivo (Bell et al., 1979). In the presence of serum, the degree of collagen gel contraction was found to be influenced by cell spreading, cell movement and gel reorganization (Bell et al., 1979). Because of the similarities between the in vitro system and contracting wound tissue, Bell et al. (1979) proposed that fibroblast-contracted collagen gel represents a tissue-equivalent construct. The contractile activity of NESC may favour expulsion of the endometrium during menstruation as well as reduce the size of an endometrial wound defect for eventual wound closure and inhibit fibroplasia and angiogenesis; both of which are necessary for the re-establishment of organ integrity (Matsumoto et al., 2005; Nasu et al., 2005a).

In the present study, we investigated the formation of fibrosis in the development of endometriosis by applying this culture model for ECSC culture. The aims of this study were to evaluate: (i) the collagen gel contractility of ECSCs in comparison with NESC; (ii) the mechanism underlying the collagen gel contraction by ECSCs; and (iii) the effect of (+)-(R)-trans-4-[(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride, monohydrate (Y-27632), a selective ROCK inhibitor (Uehata et al., 1997), on collagen gel contraction by ECSCs. We also discuss herein the pathogenesis and a therapeutic strategy of endometriosis-associated fibrosis.

Materials and methods

**ECSC and NESC isolation procedure and cell culture conditions**

ECSCs were obtained from premenopausal patients who had undergone salpingo-oophorectomy or cystectomy for ovarian endometriotic cysts (n = 10, aged 26–37 years). NESC were obtained from premenopausal patients who had undergone hysterectomies for leioymoma and had no evidence of endometriosis (n = 8, aged 33–42 years). All patients were free of any hormonal treatments prior to the operation. All the specimens were diagnosed as being in the mid to late proliferative phase using a standard histological examination of endometrial tissues. This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Oita University.

ECSCs were isolated from ovarian endometriotic tissues by enzymatic digestion as previously described (Nishida et al., 2004). NESC were also isolated by digesting the endometrial tissue fragments with 0.5% collagenase as previously described (Nishida et al., 2004). Isolated ECSCs and NESC were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 IU ml⁻¹ of penicillin (Gibco-BRL, Gaithersburg, MD, USA), 50 mg ml⁻¹ of streptomycin (Gibco-BRL), and 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL) at 37°C in 5% CO₂ in air.

ECSC and NESC in monolayer culture after the third passage were >99% pure as analysed by immunocytochemical staining with antibodies to vimentin (V9; Dako, Copenhagen, Denmark), CD10 (SS2/36; Dako), cytokeratin (Dako), factor VIII (Dako) and leukocyte common antigen (2B11 + PD7/26, Dako) and were used for the following experiments (Nasu et al., 2005b). 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 (Matsumoto et al., 2005; Nasu et al., 2005a). 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. ECSCs and NESC were embedded in collagen gel and cultured three-dimensionally. Briefly, ECSCs and NESC were suspended in the collagen solution (3.0 × 10⁵ cells ml⁻¹). The collagen/cell mixture (2 ml plate⁻¹) was dispensed into 35-mm culture plates (Corning, New York, NY, USA) coated with 0.2% bovine serum albumin (BSA) (Sigma–Aldrich, St Louis, MO, USA) and the mixture was allowed to polymerize at 37°C for 30 min. Immediately after polymerization, 1 ml of culture medium was added to each plate. After incubation for 36 h, 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, USA). The incubation time was determined by background experiments.

The supernatant of ECSC and NESC cultures were collected after 36 h incubation and stored at −70°C for the enzyme-linked immunosorbent assay (ELISA) for fibronectin.

A ROCK inhibitor, Y-27632 (Merck Biosciences, Tokyo, Japan) (final concentration: 0.1–100 μM) was also added to each plate as an inhibition assay for collagen gel contraction.

**Assessment of actins and contraction-associated proteins in ECSCs and NESC**

The expression of α-SMA, β-actin, RhoA, ROCK-I and ROCK-II in ECSCs and NESC in the abovementioned 3D culture was investigated by western blotting analysis. The contracted collagen gels were minced and incubated in 0.02% collagenase type I (Sigma–Aldrich) in PBS for 40 min at 37°C. ECSCs and NESC 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% phenylmethylsulfonyl-fluoride). The suspension was centrifuged at 15 000 rpm for 15 min at 4°C, and the supernatant was collected. The total protein concentration was quantified using the Coomassie protein assay reagent (Pierce, Rockford, IL, USA). 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 a Immobilon-P transfer membrane (Millipore, Bedford, MA, USA), 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, USA) 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, USA), β-actin (AC-15, Sigma–Aldrich), RhoA, ROCK-I (Sigma–Aldrich), ROCK-II (Santa Cruz Biotecnology, Santa Cruz, CA, USA) and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) (Ambion, Austin, TX, USA)] at appropriate dilutions for 1 h at room temperature. The membrane was washed three times with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the membrane was washed three times with TBS-T and analysed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Chicago, IL, USA).

The relative expression of actins, contraction-associated proteins, and GAPDH protein images were analyzed using the public domain Image program 1.61.

**ELISA for quantification of fibronectin production**

The levels of fibronectin in the supernatants of ECSCs and NESCs in the abovementioned 3D culture were determined with a commercially available ELISA (Bioxys, Brussels, Belgium). The sensitivity of the ELISA was 40 ng ml⁻¹.

**Modified methylthiazol tetrazolium assay**

The cytotoxic effects of Y-27632 on ECSCs and NESCs were determined in 96-well plates by a modified methyl thiazol tetrazolium (MTT) assay using WST-1 (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer’s protocol. A total of 5 × 10⁵ cells of ECSCs and NESCs in DMEM supplemented with 0.1% BSA were distributed into each well of a 96-well flat-bottomed microplate (Corning) and incubated overnight. The medium was then removed and the cells were incubated for 36 h in 200 μl of experimental medium (DMEM + 0.1% BSA) containing various concentrations of Y-27632 (0.1–100 μM). Thereafter, 20 μl of WST-1 dye was added to each well and further incubated for 4 h. Cell proliferation was evaluated by measuring the absorbance at 540 nm.

**Statistical analysis**

Data are presented as means ± SD of representative experiments and were appropriately analysed by the Bonferroni/Dunn test, Student’s t-test or Mann–Whitney U test with StatView 4.5 (Abacus Concepts, Berkeley, CA, USA). P < 0.05 was accepted as statistically significant.

**Results**

**Collagen gel contractility of ECSCs and NESCs**

Collagen gel contractility of ECSCs and NESCs was evaluated using the collagen gel contraction assay. As shown in Figure 1, in the presence of 10% FBS, ECSCs showed significant collagen gel contractility (83.7% decrease in surface area after 36 h versus 0 h controls). NESCs also showed collagen gel contractility (50.8% decrease in surface area after 36 h versus 0 h controls). ECSCs had significantly higher contractility than NESCs (P < 0.0001, Student’s t-test). Similar results were obtained from seven repeated experiments.

**Assessment of actins and contraction-associated proteins in ECSCs and NESCs**

To analyse the underlying mechanisms of the above findings, we evaluated the expression of α-SMA, β-actin, RhoA, ROCK-I and ROCK-II proteins in ECSCs and NESCs. As shown in Figure 2, the levels of α-SMA, RhoA, ROCK-I and ROCK-II protein in ECSCs were significantly higher than those in NESCs (P < 0.0001, Mann–Whitney U test), whereas the levels of β-actin protein in these cells were similar. GAPDH protein was detected in all samples at almost equal quantities. Similar results were obtained from four repeated experiments.

**Figure 1.** Collagen gel contractility of ECSCs and NESCs as assessed by cellular collagen gel contraction assay. ECSCs and NESCs were cultured three-dimensionally for 36 h. The collagen gels were then photographed (A, B) and the collagen gel contractility was assessed by measuring the gel surface area with the image analysis program (C). Data are presented as means ± SD of a representative experiment. *P < 0.0001 versus NESCs (Student’s t-test).
Quantification of fibronectin production by ELISA

The concentrations of fibronectin in the culture medium without cells were below the level of detection (\(<40\) ng ml\(^{-1}\)). As shown in Figure 3, fibronectin was detected in the supernatant of both ECSCs and NESCs cultured for 36 h (927.1 \(\pm\) 40.2 and 589.2 \(\pm\) 42.2 ng ml\(^{-1}\), respectively). Production of fibronectin in ECSCs was significantly higher than in NESCs (\(P < 0.001\), Student’s \(t\)-test). Similar results were obtained from five repeated experiments.

The levels of fibronectin extracted from collagen gels were also evaluated by ELISA as a background experiment. Fibronectin levels in the supernatant correlated with those in the collagen gels (data not shown).

**Effects of Y-27632**

To confirm the involvement of the Rho-ROCK-mediated pathway in the contractility of ECSCs, the inhibitory effect of Y-27632 was evaluated using the collagen gel contraction assay. As shown in Figure 4, Y-27632 significantly inhibited the collagen gel contractility of ECSCs in a dose-dependent manner (65.3% increase in surface area at the concentration of 100 \(\mu\)M versus untreated controls, \(P < 0.0001\), Bonferroni/Dunn test). The collagen gel contractility of NESCs was
also inhibited by the addition of increasing amounts of Y-27632 (26.5% increase in surface area at the concentration of 100 μM versus untreated controls, P < 0.0001, Bonferroni/Dunn test). Y-27632 showed stronger effects on ECSCs when compared with NESCs. Similar results were obtained from five repeated experiments.

The cytotoxic effect of Y-27632 was assessed by a modified MTT assay using WST-1. The administration of Y-27632 did not affect the cell number of viable ECSCs or NESCs (data not shown). Similar results were obtained from four repeated experiments.

Discussion

Although histologically similar, distinct molecular differences have been noted between endometriosis and normal eutopic endometrium. These include a variety of anomalies in structure and proliferation, as well as altered expression and responsiveness of various proteins including immune components, adhesion molecules, proteolytic enzymes and their inhibitors, steroids and cytokines. Such anomalies include, for example, the deficiency expression of 17-hydroxysteroid dehydrogenase type 2 (Zeitoun et al., 1998) and interleukin-1 receptor type I (Nishida et al., 2004), decreased expression of HOXA10 (Browne and Taylor, 2006), aberrant expression of aromatase P450 (Noble et al., 1996) and enhanced expression of interleukin-6 (Tseng et al., 1996), B-cell lymphoma/leukemia-2 (Bcl-2) (Nishida et al., 2005), urinary-type plasminogen activator, and plasminogen activator inhibitor-1 (Guan et al., 2003). All of these differences may promote the development and progression of endometriosis. During the development and progression, excess fibrosis may lead to the scarring, chronic pain and alteration of tissue function that are the characteristics of this disease (Nisolle and Donnez, 1997; Anaf et al., 2000; Itoga et al., 2003). All of these differences may promote the development and progression of endometriosis. Our in vitro findings are consistent with these previous reports and may explain these findings.

To further elucidate the mechanisms underlying the enhanced contractility in ECSCs, we also evaluated the expression of RhoA, ROCK-I and ROCK-II proteins in these cells. RhoA, ROCK-I and ROCK-II expression was up-regulated in ECSCs in comparison with NESCs. To further explore whether or not the activation of the Rho-ROCK pathway plays a role in controlling contractility in endometriosis-associated fibrosis, the effect of Y-27632 on the collagen gel contractility of ECSCs and NESCs was also evaluated. Y-27632 is a new pyridine derivative that acts as a specific inhibitor of the ROCK family protein kinases, ROCK-I and ROCK-II (Uehata et al., 1997; Ishizaki et al., 2000). Y-27632 has been developed for the treatment of hypertension, vasospasm caused by subarachnoid haemorrhage (Uehata et al., 1997), chronic inflammatory bowel disease (Segain et al., 2003), hepatic fibrosis (Tada et al., 2001), glaucoma (Honjo et al., 2001) and malignant neoplasms (Nakagawa et al., 2005). Y-27632 has been shown to inhibit smooth muscle contraction both in vitro and in vivo as well as the formation of stress fibers and focal adhesions induced by ROCK-I in cultured cells (Uehata et al., 1997). This compound blocks the Rho-ROCK-mediated pathway without severe cytotoxicity in vitro and in vivo (Kuwahara et al., 1999; Nakagawa et al., 2005). Y-27632 has been reported to decrease α-SMA expression and the contractile force generated by retinal pigment epithelial cells (Zheng et al., 2004). In the present study, the blocking of the Rho-ROCK pathway by Y-27632 strongly inhibited the gel contraction of ECSCs, suggesting that this pathway plays a major role in collagen matrix contraction in these cells. Y-27632 showed a stronger effect on the contractility of ECSCs when compared with that of NESCs. We speculate that the enhanced expression of RhoA, ROCK-I and ROCK-II in ECSCs is responsible for these findings. We thus consider that the RhoA-ROCK-mediated pathway may be partly responsible for the progression of fibrosis in endometriotic lesions. In addition, the present findings suggest that inhibitors of the
Rho-ROCK signalling pathway, such as Y-27632, may have therapeutic potential for endometriosis-associated fibrosis. It has also been reported that α-SMA expression is regulated by RhoA (Mack et al., 2001). The previous results, taken together with the present findings, suggest that myofibroblastic differentiation of ECSCs may be regulated by Rho-ROCK-mediated pathways.

In addition to Rho-ROCK-mediated pathways, it has been demonstrated that intermediate filaments (Lee et al., 1993), myosin and its related proteins (Katoh et al., 2001), tyrosine kinases (Ravanti et al., 1999; Rosenfeldt and Grinnell, 2000; Grundstrom et al., 2003), cell cycle-regulatory proteins (Fringer and Grinnell, 2001), integrins (Lee et al., 1993; Carnevali et al., 2003; Galois et al., 2006), matrix metalloproteinases (Xu et al., 1998; Galois et al., 2006) and proteoglycans (Carnevali et al., 2003) all play important roles in the gel contractility of the fibroblasts in other tissues. Further experiments are necessary to elucidate the involvement of these molecules in the gel contractility of ECSCs.

Fibronectin is a multifunctional extracellular matrix (ECM) glycoprotein that participates in cell adhesion (Adams and Watt, 1990), migration (Shoji et al., 1989), phagocytosis (Czop et al., 1981) and ECM formation (Hynes and Yamada, 1982). It has been demonstrated that fibronectin enhances fibroblast-mediated collagen gel contraction (Asaga et al., 1991; Carnevali et al., 1998). Adachi et al. (1998) demonstrated that fibroblasts cultured in 3D collagen gel produce higher amounts of fibronectin in comparison with those cultured in monolayer. With this in mind, we evaluated the production of fibronectin in ECSCs and NESCs. ECSCs in 3D collagen gel culture produced more fibronectin than NESCs did. This suggested that the increase in the ECSC-secreted fibronectin may be involved in fibrogenesis during the development of endometriosis.

In conclusion, we demonstrated that ECSCs have enhanced collagen gel contractility in comparison with NESCs. During myofibroblastic differentiation, the increased expression of fibronectin, RhoA, ROCK-I and ROCK-II proteins in ECSCs may be involved in this phenomenon. Y-27632, a selective ROCK inhibitor, significantly inhibited the collagen gel contractility of ECSCs. This suggests that the inhibition of the Rho-ROCK-mediated signalling pathway may provide a novel therapeutic strategy for the treatment of endometriosis. In addition, our experimental system of ECSCs in 3D collagen gel culture may be suitable for testing new compounds in the treatment of endometriosis.

Acknowledgements
This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (no. 16591672 to K.N.).

References


Submitted on August 25, 2006; resubmitted on November 13, 2006; accepted on November 29, 2006