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Original article

Phosphatidylinositol-3 kinase-Akt-mammalian target of rapamycin signaling pathway mediates contractility of human endometriotic stromal cells: A promising new target for the treatment of endometriosis-associated fibrosis

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ABSTRACT

Objective: To assess the involvement of phosphatidylinositol-3 kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) on the extracellular matrix contractility of endometriotic cells.
Materials and methods: The effects of wortmannin, LY294002, Akt inhibitor IV, and Ku-0063794 on the contractility of endometriotic cyst stromal cells (ECSCs) were investigated using collagen gel contraction assay.
Results: All four inhibitors of PI3K-Akt-mTOR evaluated in the current study significantly inhibited the contractility of ECSCs.
Conclusion: The current findings suggest that the PI3K-Akt-mTOR signaling pathway is involved in the development of endometriosis-associated fibrosis. The PI3K-Akt-mTOR signaling pathway is a promising target for the treatment of endometriosis.
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Introduction

Endometriosis—a benign, estrogen-dependent, tumor-like disease characterized by chronic pelvic pain, dysmenorrhea, dyspareunia, and/or subfertility—is caused by the uncontrolled ectopic growth of proliferative phase endometrial tissue. Women of reproductive age are most commonly affected, with lesions usually occurring in the peritoneum, ovaries, and rectovaginal septum.[1] Symptoms of endometriosis may markedly reduce a woman's quality of life.

Histologically, this disease is characterized by dense fibrous tissue surrounding the endometrial glands and stroma.[2] During the development and progression of endometriotic lesions, excess fibrosis typically leads to scarring, chronic pain, and/or alteration of tissue function.[2] Fibroblastic cells positive for α-smooth muscle actin (SMA) are frequently detected in fibrotic areas associated with endometriosis of the peritoneum, ovary, rectovaginal septum, and uterosacral ligaments.[2,3] Immunohistochemical analysis led Anaf et al[3] to suggest that endometriotic stromal cells can differentiate into α-SMA-positive myofibroblasts.

We have established a three-dimensional (3-D) collagen gel culture system with human endometriotic cyst stromal cells (ECSCs) as a model of fibrosis formation in endometriosis.[4–7] In this system, ECSCs are cultured in floating collagen lattices to induce the reorganization and compaction of collagen fibers, resulting in the contraction of collagen gels. This culture system provides a model of mechanically relaxed tissue with low tensile strength comparable to that in the early developmental stages of endometriotic lesions. Research on endometriotic stromal cell biology in 3-D collagen matrices offers new opportunities to gain a better understanding of the reciprocal and adaptive interactions that take place between cells and the surrounding matrix in a tissue-like environment. Such interactions are integral to the regulation of endometriotic tissue morphogenesis and the dynamics that characterize endometriosis-associated fibrosis.[4–7]

The phosphatidylinositol-3 kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway is a key cellular signaling pathway that affects multiple cellular functions, including metabolism, growth, proliferation, and apoptosis.[8–10] The PI3K-Akt-

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mTOR pathway is activated by various stimuli via specific receptors, including antigen receptors, cytokine receptors, vascular endothelial growth factor receptors, and platelet-derived growth factor receptors, epidermal growth factor receptor, human epidermal growth factor receptor 2, insulin receptor, or the insulin-like growth factor I receptor.[11,12] Following the activation of receptor tyrosine kinases, class IA PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) generates phosphatidy linositol-3,4,5-trisphosphate (PIP3) as a second messenger to recruit and activate downstream targets, including Akt and PDK1 (phosphoinositide-dependent kinase-1), and then mTOR.[13] mTOR controls protein synthesis through phosphorylation and inactivation of the repressor of messenger RNA translation, eukaryotic initiation factor 4E-binding protein 1, and through phosphorylation and activation of S6 kinase.[14]

In the current study, we investigated the effects of PI3K-Akt-mTOR inhibitors on the contractility of ECSCs. We also discuss a novel 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 ($n = 6$, aged 27–32 years). None of the patients had undergone any hormonal treatments for at least 12 months prior to the operation. This study was approved by the Institutional Review Board of the Faculty of Medicine at Oita University, Oita, Japan. ECSCs were isolated from ovarian endometriotic tissues by enzymatic digestion as previously described.[4] Isolated ECSCs were cultured in Dulbecco modified eagle medium supplemented with 100 IU/mL of penicillin (Gibco-BRL, Gaithersburg, MD, USA), 50 mg/mL of streptomycin (Gibco-BRL), and 10% heat-inactivated charcoal-stripped fetal bovine serum (FBS) (Gibco-BRL) at 37°C in 5% CO_2 in air.

After the third passage, the ECSCs in the monolayer culture were >99% pure, as determined 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). These ECSCs were used for the subsequent experiments.[4] Each experiment was performed in triplicate and repeated at least three times.

Collagen gel contraction assay

To assess the involvement of the PI3K-Akt-mTOR pathway on the contractility of ECSCs, cellular collagen gel contraction assays were performed as previously described.[4,6] Wortmannin and LY294002 were chosen as representative PI3K inhibitors, Akt inhibitor IV as a representative Akt inhibitor, and Ku-0063794 as a representative mTOR inhibitor.

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 were embedded in collagen gel and cultured three-dimensionally. Briefly, the ECSCs were suspended in collagen solution (3.0×10^5 cells/mL), and this collagen/cell mixture (2 mL/plate) was dispensed into 35-mm culture plates (Corning, Corning Incorporated, Corning, NY, U.S.A) coated with 0.2% BSA (bovine serum albumin), after which the mixture was allowed to polymerize at 37°C for 30 minutes. Immediately after polymerization, 1 mL of culture medium containing wortmannin (final concentration: 1μM), LY294002 (final concentration: 50μM), Akt inhibitor IV (final concentration: 25μM), or Ku-0063794 (final concentration: 10μM) was added to each plate. All of these PI3K-Akt-mTOR inhibitors were purchased from Merck (Darmstadt, Germany). After incubation for 48 hours, the collagen gels were photographed, and the area of the gel surface was measured using the public domain image program ImageJ 1.44 developed at the National Institutes of Health (Bethesda, MD, USA).

Statistical analysis

The gel surface area at 0 hours that is equal to the surface area of the 35-mm culture plate was defined as 100%, which is equal to the surface area of the 35-mm culture dish. Data were calculated as percentages relative to the untreated controls at 0 hours, presented as means ± standard deviations, and appropriately analyzed using the t test with Bonferroni correction using Sigmaplot 11.2 (Systat Software, Inc., San Jose, CA, USA). Values of $p < 0.05$ were considered to be statistically significant.

Results

The effect of inhibitors of the PI3K-Akt-mTOR pathway on the contractility of ECSCs was evaluated using a collagen gel contraction assay. As shown in Fig. 1, in the presence of 10% FBS, untreated ECSCs showed significant collagen gel contractility after 48 hours of 3-D culture (relative surface area was 19.8 ± 2.6%). The contractility of ECSCs was significantly attenuated by the addition of wortmannin (1μM), LY294002 (μM), Akt inhibitor IV (μM), and Ku-0063794 (10μM) ($p < 0.0001$).

Discussion

In an effort to clarify the pathological fibrosis in endometriosis and to establish novel therapeutic strategies for this disease, our laboratory has been conducting an ongoing investigation into the contractile profiles of endometriotic cells. In the current study, we demonstrated that the inhibitors of the PI3K-Akt-mTOR pathway significantly attenuated the contractility of ECSCs *in vitro*. This finding suggests that the PI3K-Akt-mTOR signaling pathway is involved in the pathogenesis of endometriosis-associated fibrosis. It also suggests that the PI3K-Akt-mTOR signaling pathway could serve as a target for the treatment and prevention of endometriosis-associated fibrosis.

The PI3K-Akt-mTOR signaling pathway has been found to be activated in ovarian endometriosis.[15–17] Akt activity is higher in ovarian endometriosis than in the normal endometrium,[15] and it has been postulated that estrogens might be one of the factors responsible for the high Akt activation in endometriotic cells.[17] Leconte et al[18] found that Akt is hyperactivated in endometriotic lesions from patients with deep infiltrative endometriosis, as in ovarian endometriosis; however, ERK (extracellular signal-regulated kinase) and Akt activation predominates in endometriotic stromal cells in deep infiltrative endometriosis. Leconte et al[18] also suggested that the constitutive activation of the AKT pathway in endometriotic cells could be explained by the overproduction of endogenous ROS (reactive oxygen species). Similarly, Laschke et al[19] reported that rapamycin induced the regression of endometriosis by inhibiting neovascularization and cell proliferation. Various phosphoproteins that interact with the PI3K-Akt-mTOR pathway show increased expression in a subtype-specific manner, which might lead to the development of endometriosis. Expression profiling of genes involved in the PI3K-Akt-mTOR pathway has shown high expression and/or activation of phosphatase and tensin homolog deleted from chromosome 10 (PTEN),[20] p21-activated kinase (PAK1),[21] X chromosome-linked IAP (inhibitor of apoptosis protein) (XIAP),[22] and NFkB1[23,24] in endometriosis.

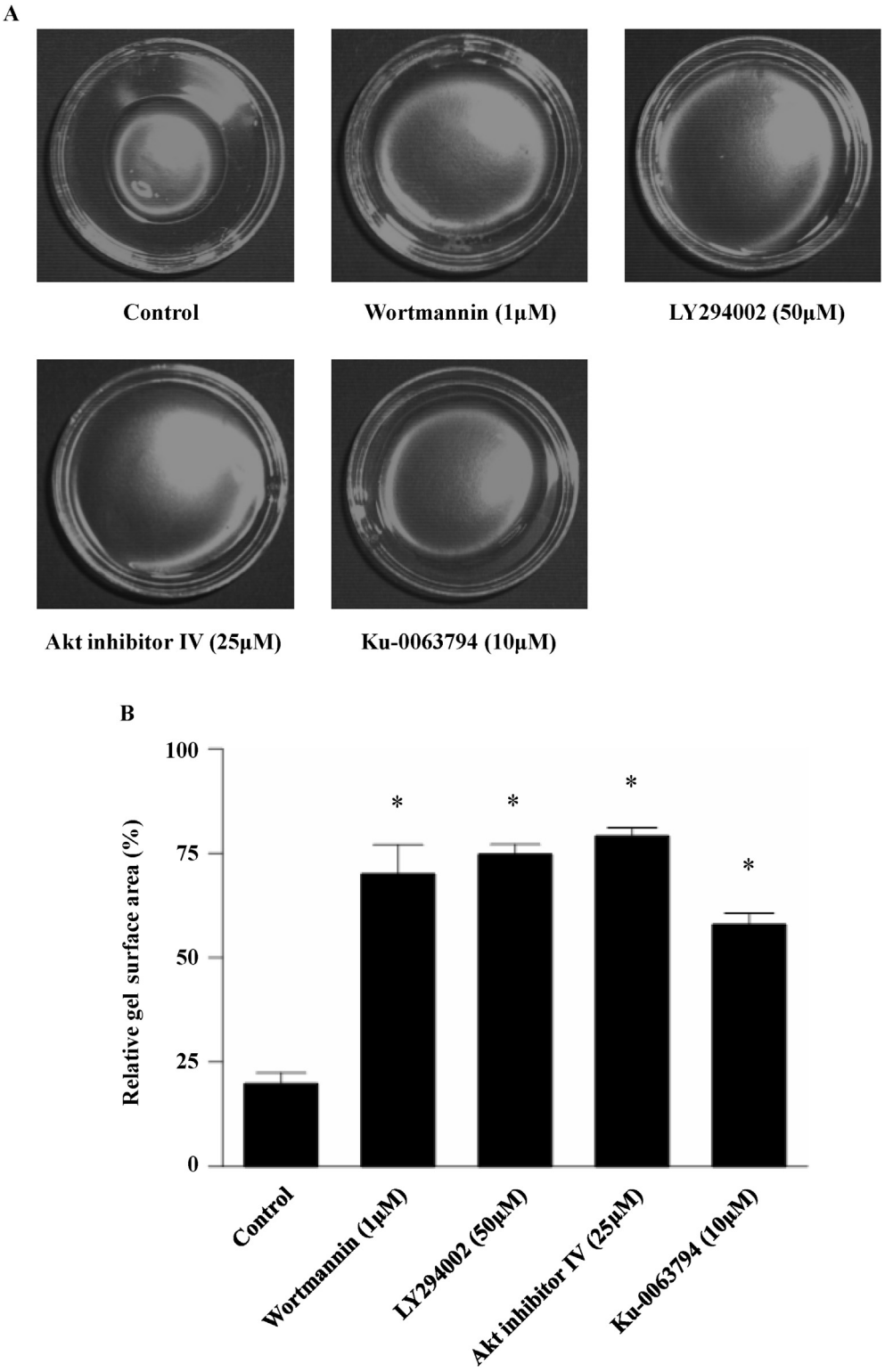

Fig. 1. Effects of PI3K-Akt-mTOR inhibitors on the contractility of endometriotic cyst stromal cells (ECSCs) as assessed by collagen gel contraction assay. ECSCs were cultured in three-dimensional collagen gels for 48 hours in the presence of wortmannin (1μM), LY294002 (50μM), Akt inhibitor IV (25μM), or Ku-0063794 (10μM). (A) Photograph of the collagen gels. The ECSC-mediated collagen gel contraction was assessed by measuring the gel surface area using an image analysis program. The contractility of untreated ECSCs at 0 hours that is equal to the surface area of a 35-mm culture plate was defined as 100%. (B) The relative gel surface areas of the ECSCs treated with PI3K-Akt-mTOR inhibitors were calculated. Data are presented as means ± standard deviations of representative experiments. $^{*}p < 0.0001$ versus untreated controls (Bonferroni test). PI3K-Akt-mTOR = phosphatidylinositol-3 kinase -Akt-mammalian target of rapamycin.

It is considered that the PI3K signaling pathway plays an important role in activation of focal adhesion kinase and α-SMA and stress fiber formation.[25] Recently, Leconte et al[18] demonstrated that Akt-mTOR inhibition by temsirolimus decreased endometriotic cell proliferation both *in vitro* and in a mouse model of deep infiltrating endometriosis. Rapamycin (sirolimus) is a bacterial macrolide, which is currently widely used as an antifungal, immunosuppressant, and antitumor drug that blocks mTOR.[26]

After forming a complex with the FK506 binding protein-12, rapamycin blocks mTOR, which is known to be a central controller of multiple mitogenic signaling pathways, regulating cell growth under physiological and pathological conditions.[27] The mTOR pathway drives a feedback loop and keeps the PI3K/Akt activity under tight control. One consequence of mTOR inhibition is alleviation of this negative feedback loop, resulting in activation of PI3K and subsequent activation of Akt. Therefore, simultaneous targeting of both PI3K/Akt and mTOR has the potential to inhibit both upstream and downstream signaling in the pathway. Dual PI3K/mTOR inhibitors are now under preclinical evaluation.[28]

It has been suggested that the mevalonate-Rho/ROCK pathway plays important roles in the formation of endometriosis-associated fibrosis. We previously demonstrated that simvastatin, which acts as a Rho inhibitor by inhibiting 3-hydroxy-3-methylglutary lcoenzyme A reductase, and fasudil and Y27632, representative ROCK (Rho-associated coiled-coil-forming protein kinase) inhibitors in clinical and preclinical use, reduced the proliferation and contractility of ECSCs.[4,6] The PI3K-Akt-mTOR signaling pathway should be recognized as an additional regulatory mechanism of endometriosis-associated fibrosis in addition to the mevalonate-Rho/ROCK pathway. It is also speculated that targeting of the PI3K-Akt-mTOR signaling pathway offers new prospects for the treatment and prevention of endometriosis-associated fibrosis.

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